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SCIENCE AT THE SERVICE OF PUBLIC HEALTH, FOOD CHAIN
SAFETY AND ENVIRONMENT

The Belgian Scientific Institute of Public Health (known as WIV-ISP) provides support for public health policy through scientific research, expert opinions and divisional tasks.

On the basis of scientific research, WIV-ISP formulates recommendations and solutions in respect of priorities for a proactive health policy at the Belgian, European and international levels. WIV-ISP assesses the status of health and health indicators on the basis of scientific methods which it approves, develops and analyses within a certified quality framework. WIV-ISP develops advanced solutions for the diagnosis, prevention and treatment of current and emerging diseases, as well as the identification and prevention of health risks, including those resulting from the environment.

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PREFACE

by the chairman of the board

2008 and 2009 meant a new milestone in the history of the Belgian Scientific Institute of Public Health (WIV-ISP). A new vision and mission, which are a truer reflection of what is now expected of a public health institute were developed. I would like to thank the members of our Scientific Board as well as our scientific committees, stakeholders and partners for their advice in adapting our objectives more effectively to a rapidly changing environment. In this way the institute is seeking to characterize itself as an inter-federal institute that provides support not only to the FPS Public Health but also to all federal and federalized stakeholders and partners who play a role in developing a public health policy.

To be able to perform this role effectively, the long drawn-out merger between the former Institute for Hygiene and Epidemiology and the Pasteur Institute of Brabant has been established. The Pasteur Institute and parts of the Microbiology department were merged into a newly established operational entity (OD), "Transmissible and Infectious Diseases", which, by consolidating all the scientific microbiological expertise, should provide a significant competitive advantage.

In addition, three other operational entities were set up for which the same strategy was followed to group together all the scientific expertise needed within the

same operational management: epidemiological research is now operating under the umbrella of the OD "Public Health and Monitoring" and chemical research under the OD "Food, Medicines and Consumer Safety". Services such as clinical biology, biological standardization and biosafety & biotechnology have been brought together in the OD "Expertise, Services and Customer Relations".

In 2009, each OD was given a new manager and, in line with the conclusions of the Jenner Project, a business process re-engineering project, they each drew up a suitable management plan and an organisation chart for their OD. These plans will now gradually be put into practice in 2010. The following actions were selected as overall target objectives for all the ODs in the coming years:

- Focus on expertise to become a centre of excellence in public health domains.
- Achieve equilibrium between routine demands and research-oriented incentives to fit with the institute's future innovative research strategy.
- Make the best possible use of resources.
- Keep pace with novel trends.
- Stimulate cooperation with universities through posts for doctoral research and provide incentives to increase productivity of scientists (e.g. promotion matrix).
- Increase communication and visibility of activities (image building).

Based on the procedures proposed in the Jenner Project, the support services for the former departments were centralized at corporate level and new transversal processes rolled out, which also included changes to the quality management system (ISO 17025, ISO 17020 and ISO 9001). Furthermore, a scientific coordination service was also set up to support researchers attracting and performing their scientific projects, as well as making them more accessible to their target publics.

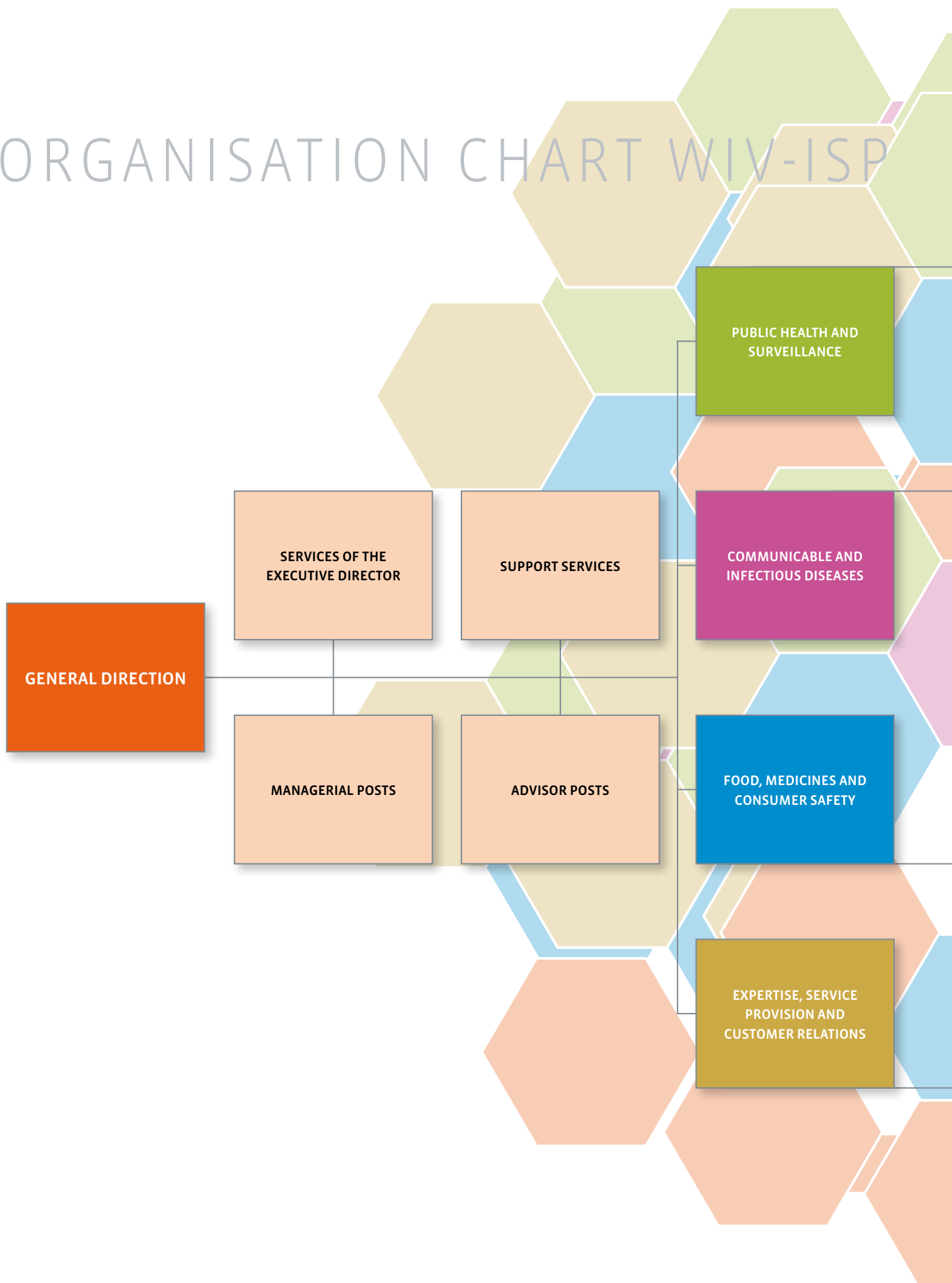
This report therefore already provides a glimpse of just a few of the WIV-ISP's key scientific achievements of the past two years. In the future, these will be increasingly grouped together around a number of priority areas and actions.

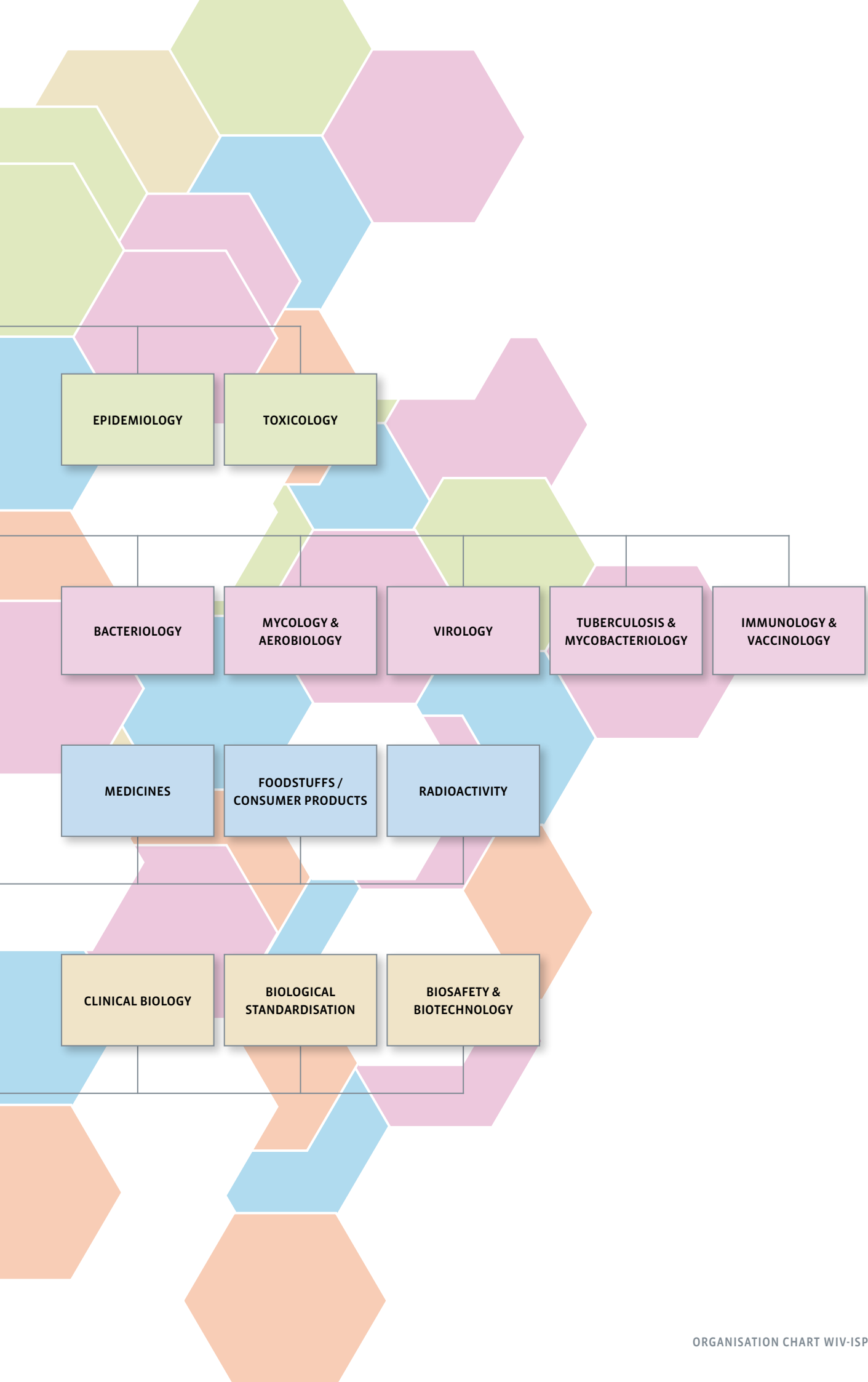
We hope you will enjoy your reading!



Dr Johan E. Peeters
General Director

ORGANISATION CHART WIV-ISP







PUBLIC HEALTH AND SURVEILLANCE DIRECTORATE

Introduction by Prof. Dr Herman Van Oyen

Population health and the distribution of health within societies is a dynamic phenomenon. Never has the rate of improving population health been as great as during the last century. There has been a shift, called the epidemiologic transition, in both causes of mortality and causes of morbidity from infectious toward non-infectious diseases and mainly chronic diseases. The current phase of the evolution is often called the era of “man-made diseases”. This refers to the fact that a substantial part of current population ill-health is a result of the way society is organised, the way individuals within society make choices about their lifestyles and the healthcare system which contributes to the survival of the sick.

As there are many determinants which affect status and change in population health, including the social environment, physical environment, genetic endowment, healthcare system, individual response (biological, lifestyle) etc., understanding the underlying mechanisms of population health is essential for determining appropriate, evidence-based public policy and action. To achieve a better understanding, it is necessary to initiate new research axes such as research into the role of knowledge of the human genome on public health, the application of more complex epidemiologic methods allowing research into different scenarios, and health outcome research. Moreover, it is becoming increasingly important to evaluate systematically through health impact assessments the effects of public policies and decisions on population health, such as the initiation of cancer screening programmes, vaccination policies, anti-smoking initiatives etc. The complexity of chronic diseases calls not only for a strengthening of the interaction between exposure measures as determined by laboratory examinations (in food, by way of bio-monitoring) and toxicological risk evaluations in order to reach a comprehensive evaluation of the population health risk, but it is also important to strengthen the support to the quality of available health services through research into health services. This is not just the task of the Cancer Centre set up in 2008, but is also addressed in various front-line projects, by general practitioners and by focusing on a number of specific target groups such as those suffering from diabetes, cystic fibrosis, AIDS and HIV.

Considerable attention is also paid to supporting institutional care, hospitals and nursing and care homes, mainly by following up care-associated infections and microorganisms' resistance to antibiotics.

Cooperation with health care workers is therefore very important and has materialised in a number of networks, including a general practitioners' network (celebrating its 30th anniversary in 2009) and a laboratories' network.

The Public Health and Surveillance Directorate is currently working on a network of officially recognised reference laboratories.

The Public Health and Surveillance Directorate assesses public health risks with a unique combination of expertise in epidemiology and biostatistics, chronic diseases, infectious

diseases, the relationship between food, the environment and health and toxicology. During such risk assessments, the Directorate works closely with international partners thanks to the establishment of European institutions on issues such as illegal substances and drugs, chemical substances and infectious diseases.

To counter any acute and/or accidental threats, the Public Health and Surveillance Directorate has a 24-hour on-call system providing scientific support to the Belgian authorities involved.

The Public Health and Surveillance Directorate aims to be a Centre of Excellence for epidemiological research and an important player in the network of Belgian, European and international knowledge centres. Many of the Public Health and Surveillance Directorate's research programmes have therefore been ISO 9001 certified, which allows us to solve health problems and offer support and solutions to Belgian, European and international governments at times of health crises.

To adequately inform the policymakers and population on new evolutions relating to health, food chain safety, food and the environment, the Public Health and Surveillance Directorate runs a number of programmes and activities that allow us:

- To gain an insight into population health and well-being and understand the relationship with the determinants;
- To monitor trends in health status and to organise surveillance systems for a series of diseases and health determinants;
- To evaluate the impact on the population health of specific health determinants such as lifestyle, population interventions and public policies;
- To promote quality of care through health service research;
- To evaluate toxicological risks;
- To provide information to help public health actors make decisions;
- To coordinate public health information in Belgium.

The Public Health and Surveillance Public Service has two divisions: Epidemiology and Toxicology.

The **Epidemiology** division's programmes and activities are:

- Quality of care in various healthcare settings;
- Health inequality;
- Health and the environment;
- Surveys: Health Survey 2008;
- Healthy food;
- Drugs and illegal substances;
- Care-associated infections;
- Infectious diseases in the population;
- The development of health indicators;
- The Cancer Centre;
- Climate change and health;
- Public health genomics;
- Epidemiological support in case of acute public health risks.

The **Toxicology** division's programmes and activities are:

- Toxicology risk analysis and providing toxicological advice via national and international expert groups;
- Risk evaluations for new and existing chemicals focusing specifically on pesticides;
- Toxicological evaluations of aromas, additives, contaminants and novel foods;
- Research and development projects (for example to optimise and adjust the strategy of *in vitro* studies to predict nanoparticles' toxicity to humans).

Cardiovascular risk and its treatment in Belgian secondary care recognised diabetes centres

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INTRODUCTION

Glycemic control is no longer the single key focus of diabetes treatment. The prevention of cardiovascular risk in diabetes and its timely and correct treatment has gained in significance over the last decade. Several studies have shown the importance of multifactorial intervention to prevent cardiovascular complications, both in primary and secondary prevention (1-4). The Steno-2-study in particular has shown the importance of a multifactorial approach in type 2 diabetes patients presenting microalbuminuria, which is a well-established marker of increased cardiovascular disease risk (1, 2). In these at-risk patients with type 2 diabetes, an intensive intervention with multiple drug combinations and behaviour modification, had sustained beneficial effect with respect to vascular complications and on mortality rates from any cause and from cardiovascular cause (1, 2).

IQED, the Initiative for Quality improvement and Epidemiology for Diabetes, has been organised since 2001 in all Belgian multidisciplinary secondary care diabetes centres that have been recognised by the National Institute for Sickness and Invalidity Insurance. It aims at assessing and improving the quality of diabetes care by a continuous system of audit and feedback using benchmarking (5). The latter allows the diabetes centres to compare its results, not only with the recommendations, but also with their peers, i.e. other diabetes centres. Initially the feedback used in IQED focused mainly on the quality indicators that are generally used, such as the degree to which necessary examinations are carried out (process indicators) and the degree to which treatment goals are attained (outcome indicators). Due to the increas-

ing importance of correct cardiovascular risk treatment in diabetes, in 2008, we also began to use benchmarking to inform centres on the cardiovascular risk of their patients on the one hand and their treatment on the other hand.

The present paper describes the prevalence of cardiovascular risk in type 1 and type 2 diabetes patients treated in these secondary care diabetes centres, the treatment of the patients with increased cardiovascular risk and the degree to which these patients reach their treatment goals with respect to LDL-cholesterol and blood pressure.

METHODS

Data collection

During the last quarter of 2007 all diabetes centres (n = 120) collected data from at least 10% of their patients (at least 50 patients included for each centre). Patients were selected through systematic sampling from an alphabetical patient list starting from the letter "N". If the obtained sample contained less than 25 type 1 diabetes patients, the centre needed to add type 1 diabetes patients until the number of 25 was reached. Collected data covered a wide range of diabetes care aspects (demographics, diabetes type and duration, frequency of blood glucose selfmonitoring, hypoglycemic treatment, additional treatment, glycemic control (HbA1c), cardiovascular risk (blood pressure, blood lipids, smoking status), anthropometry, follow-up examinations and presence of micro- and macrovascular complications). It concerned retrospective data originating from the most recent consultation during the period of 15 months preceding the data collection.

Patients with a history of at least one of the following events or interventions, percutaneous transluminal coronary angioplasty (PTCA) or coronary artery bypass grafting (CABG), myocardial infarction, stroke, transient ischemic attack, and percutaneous transluminal angioplasty (PTA) of the inferior members, were considered as **patients with a cardiovascular history** (Table 1). Patients over 40 years of age with microalbuminuria or manifest nephropathy [(macroalbuminuria or increased creatinin (≥ 1.5 mg/dl)], were considered as **patients with a Steno-2-like-profile** (i.e. strongly increased cardiovascular risk as in the Steno-2-study (Table 1) (1, 2). Finally patients over 40 years of age with at least one of the following cardiovascular risk factors: obesity, antihypertensive treatment, lipid-lowering treatment, blood pressure $\geq 140/90$ mm Hg, LDL-cholesterol ≥ 100 mg/dl, smoking frequency > 5 cigarettes a day were considered as **patients with ≥ 1 cardiovascular risk factor** (Table 1).

Statistical analysis

Data were analysed using Stata 9.2. Logistical regression, adjusting for age, diabetes duration and gender, was used to compare the proportions between different groups. All presented results were weighted for centre size. A p-value below 0.05 was considered statistically significant.

RESULTS

The sample consisted of 3,494 type 1 diabetes patients (32%) and 7,382 type 2 diabetes patients (68%). Median age and diabetes duration were respectively 46 and 17 years in type 1 and 68 and 13 years in type 2 diabetes. Almost all type 1 diabetes patients

Table 1: Definition of the cardiovascular risk.

Cardiovascular risk category	Definition
Cardiovascular history	Myocardial infarction and/or coronary surgery (PTCA/CABG) and/or stroke and/or transient ischemic attack and/or PTA of the inferior members
Steno-2-like-profile	Over 40 years of age with microalbuminuria or manifest nephropathy [macroalbuminuria or increased creatinin (≥ 1.5 mg/dl)]
≥ 1 cardiovascular risk factor	Over 40 years of age with at least one of the following cardiovascular risk factors: obesity, antihypertensive treatment, lipid-lowering treatment, blood pressure $\geq 140/90$ mm Hg, LDL-cholesterol ≥ 100 mg/dl, smoking frequency > 5 cigarettes a day

(94%) were treated with at least 3 daily insulin injections, while this was 41% in type 2 diabetes. Median HbA_{1c} was 7.8% in type 1 and 7.4% in type 2 diabetes.

In this population of patients treated in secondary care diabetes centres 11% of type 1 and 33% of type 2 diabetes patients presented with a cardiovascular history. Next, 10% of type 1 and 20% of type 2 diabetes patients had a Steno-2-like profile. Finally 34% of type 1 and 43% of type 2 diabetes patients were over 40 years of age and presented at least one cardiovascular risk factor. The proportion of patients “without cardiovascular risk” as defined by the present study reached 45% in type 1 and 4% in type 2 diabetes.

Table 2 shows the treatment for the different cardiovascular risk groups.

1. Of the patients with a **cardiovascular history**, about 90% were treated with antihypertensive drugs and about as much with antiplatelets. About three quarters received a lipid-lowering treatment. Almost all patients with a cardiovascular history (98%) were treated with at least one of these 3 types of drugs.
2. Of the patients with a Steno-2-like profile, 87% were treated with antihypertensive drugs. Treatment with antiplatelets and lipid-lowering drugs was seen in 60% of the patients. Almost all patients with a **Steno-2-like profile** (95%) were treated with at least one of these 3 types of drugs.
3. Of the patients **older than 40 years** with at least one cardiovascular risk factor, 71% were treated with antihypertensive drugs, 58% with lipid-lowering drugs and 51% with antiplatelets. Eighty eight percent were treated with at least one of these 3 types of drugs.



Table 2: Proportion of treated patients by cardiovascular risk (number and weighted proportion) – total and per diabetes type.

	Cardiovascular history	Steno-2-like profile	> 40 yrs and ≥ 1 CV risk factor	No cardiovascular risk
	n (%)	n (%)	n (%)	n (%)
Antiplatelets				
Total	2,447 (89)	1,040 (60)	2,107 (51)	150 (9)
Type 1	305 (88)	146 (46)	434 (38)	97 (7)
Type 2	2,142 (89)	894 (62)	1,673 (54)	53 (20)
Lipid-lowering drugs				
Total	2,074 (76)	1,039 (60)	2,436 (58)	161 (9)
Type 1	261 (73)	158 (48)	564 (48)	132 (8)
Type 2	1,813 (76)	881 (61)	1,872 (60)	29 (12)
Antihypertensive drugs				
Total	2,514 (92)	1,525 (87)	3,014 (71)	192 (11)
Type 1	303 (85)	247 (75)	619 (51)	164 (12)
Type 2	2,211 (93)	1,278 (89)	2,395 (77)	28 (11)
≥ 1 of these drugs				
Total	2,734 (98)	1,687 (95)	3,782 (88)	392 (23)
Type 1	351 (97)	287 (85)	904 (76)	306 (21)
Type 2	2,383 (98)	1,400 (96)	2,878 (91)	86 (33)

As cardiovascular risk lowered, the proportion of treated patients decreased significantly, except for the lipid-lowering treatment, where no difference was observed between the group with a Steno-2-like profile and the group over 40 years of age with at least one cardiovascular risk factor. Treatment was significantly higher among type 2 than among type 1 diabetes patients. However, treatment with antiplatelets and lipid-lowering drugs was more or less equal among type 1 and type 2 diabetes patients in the group with a cardiovascular history. In the group “without cardiovascular risk”, treatment with lipid-lowering and anti-hypertensive drugs was also more or less equal among type 1 and type 2 diabetes patients.

LDL-cholesterol results

In total, we observed 60% of the patients with an LDL-cholesterol below 100 mg/dl (55% of type 1 and 61% of type 2 diabetes patients). Among type 1 diabetes patients, the proportion of patients reaching this goal was significantly higher among the patients with a cardiovascular history

(61%) than in the 2 groups with a lower cardiovascular risk (44% among patients with a Steno-2-like profile and 47% among the patients over 40 years with ≥ 1 cardiovascular risk factor). This difference was also observed in type 2 diabetes, but it was only significant between the group with a cardiovascular history and the group older than 40 years with ≥ 1 cardiovascular risk factor (66% versus 57% respectively). Among each different cardiovascular risk group, the proportion of patients reaching the LDL goal was significantly higher among type 2 than among type 1 diabetes patients. Generally we observed a higher proportion of patients reaching the target among those receiving lipid-lowering drugs than among those without this treatment. One exception is observed among type 1 diabetes patients with a Steno-2-like profile, where the proportion of patients reaching the LDL target was similar in both the treated and non-treated groups.

Blood pressure results

In total, 23% of the patients presented with blood pressure below 130/80 mm Hg (33% of type 1 and 20% of type 2 diabetes patients). The number of patients reaching this goal was significantly higher in the group “without cardiovascular risk” than in the 3 risk groups. In type 2 diabetes, the proportion of patients reaching the blood pressure target was significantly higher among patients with a cardiovascular history than in the two other risk groups. In type 1 diabetes, no difference was observed between the 3 different risk groups. The proportion of patients reaching blood pressure targets was significantly higher among type 1 than among type 2 diabetes patients, except for the group with a cardiovascular history and the group “without cardiovascular risk”, where the proportions were comparable. Both in type 1 and type 2 diabetes patients, the group treated with blood pressure-lowering drugs was less likely to reach the blood pressure target than the group without treatment (respectively 23% versus 40% in type 1 and 19% versus 24% in type 2 diabetes).

CONCLUSION

Cardiovascular risk in these diabetes patients treated in secondary care diabetes centres is considerable. As expected, the cardiovascular risk profile is higher among type 2 diabetes patients than among type 1 diabetes patients. Given that the studied population concerns insulin treated patients only (≥ 2 daily insulin injections), these results are not representative for the entire Belgian diabetes population, but they can be considered representative for the adult type 1 diabetes patients and for the insulin treated (≥ 2 inj./day) type 2 diabetes patients.

The present results have shown that recommendations on treatment of patients with cardiovascular history and of patients with a Steno-2-like profile are closely followed. Although recent guidelines and several studies indicate the importance of multifactorial treatment of patients with a strongly increased cardiovascular risk (1, 2, 6), it is difficult to reach 100% treatment, because patients may



IMPACT ON PUBLIC HEALTH

Since the start of IQED, the quality of care has improved mainly regarding process and, to a lesser extent, outcome. Although it is impossible to attribute these changes to IQED alone, the monitoring aspect as such is important in the diabetes care setting.

have contra-indications or may suffer unpleasant side effects to drugs. Indeed, no studies regarding secondary cardiovascular prevention show maximum treatment. Compared to international studies, the present Belgian results are comparable or even better (7-12).

Although patients were adequately treated, the results show that it remains difficult to reach treatment goals, particularly for blood pressure. However, without the information on treatment duration and dose and individual follow-up, it is difficult

to assess the treatment effect.

So although the observed results are positive compared to other health care settings, there remains room for improvement (5). By using feedback that is specifically directed towards cardiovascular risk and its treatment, we aim to bring this to the attention of the diabetes teams, because due to high morbidity and mortality of cardiovascular complications, it is of utmost importance to screen for these risk factors and to act in a timely and correct manner, for both type 1 and type 2 diabetes.

SOURCE

Debacker N, Van Crombrugge P, Nobels F, Mathieu C. Vlaams tijdschrift voor Diabetologie 2009;2:7-10.

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Compliance and use of alcohol based hand rubs after three national hand hygiene campaigns in Belgian hospitals

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and the national working group for hand hygiene

INTRODUCTION

The use of an alcoholic solution for hand hygiene is the most important measure in preventing the transfer of microorganisms between patients, health care workers (HCW) and hospital surroundings (1). Unfortunately we see that compliance with hand hygiene recommendations among health workers is generally lacking (2, 3). As one approach to health improvement, there is a considerable body of evidence to support campaigning on health-related behaviours (4). Moreover, it is found in several studies that this kind of campaign needs to be repeated at regular intervals (5-7). In this article we firstly would like to describe the evolution of the results of three national campaigns to improve hand hygiene practices in Belgian hospitals over the last five years. The second aim is to evaluate the short term and long term impact on adherence to hand hygiene guidelines and on the consumption of alcohol based hand rubs as a result of these campaigns.

METHODOLOGY

In 2005, 2006-2007 and 2008-2009, a multi-disciplinary working group with representatives of the Federal Public Service Health, Food Safety and Environment, the Belgian Antibiotic Policy Coordination Committee, the Federal Platform for Hospital Hygiene, the Institute of Public Health, the *Association Belge pour l'Hygiène Hospitalière*, the Belgian Infection Control Society and the *Nationale Vereniging van Katholieke Vlaamse Verpleegkundigen*, organised a national campaign to promote hand hygiene in hospitals under the name "You are in good hands". These campaigns were financially, logistically, and politically supported by the Ministry of Public Health.

Acute care and chronic care hospitals were invited to participate on a voluntary basis. For the second and third campaigns, participation was extended to psychiatric hospitals.

The national campaigns addressed both HCW and hospitalised patients. The campaign was organised into two parts: (a) the actual awareness campaign of HCW and patients by the provided material, and (b) an impact assessment pre- and post-campaign. For the latter, four variables were examined: (i) the hand hygiene compliance of HCW by direct observation; (ii) the alcohol consumption and (iii) the compliance with the ground rules for good hand hygiene (optional).

The awareness part consisted of a wide range of campaign material inspired by several sources (3, 8, 9): posters highlighting five hand hygiene indications, the right use of hand alcohol and gloves, standardised training sessions for HCW, a web-

based quiz, an information leaflet with gadget for the patient, and an information leaflet for HCW.

Measuring the degree of hand hygiene compliance by observation (= percentage of observed hand hygiene with soap and/or hand alcohol divided by the number of observed hand hygiene opportunities) was carried out by the staff of the hospital hygiene team. The measurements were collected through a standardised observation grid with a minimum of 150 observations per unit and included at least the intensive care unit. For each hand hygiene opportunity, the observer recorded whether hand hygiene measures were taken: decontamination with alcohol, washing with water with or without soap, or no action. The data were then entered into NSIHWin4.08 (software on a MS Access database) and sent back for analysis and feedback to the Institute of Public Health. Feedback to the HCW was considered to be a substantial part of the awareness.

All data were processed and analysed using Stata 9.2. Institutional results were returned to the hospital with 95% confidence intervals, individual percentiles (position of the hospital in the national distribution), national results, and indicators stratified by professional group and hospital unit. National results were given as weighted mean, thus adjusting for varying numbers of observations between hospitals. One-way analysis of variance or Kruskal-wallis tests were performed where appropriate.



RESULTS

Participation

The number of hospital sites participating is shown in Table 1. Some hospitals did not send data concerning post-campaign observations.

Compliance

• General evolution

A total of 594,150 opportunities for hand hygiene were observed during the three campaigns (317,232 pre- and 276,918 post-campaign). Figure 1 shows the evolution of compliance rates pre- and post-campaign for the 3 campaigns for all HCW ("General") and stratified by professional category.

The overall compliance increased after each campaign: from 49.6% to 68.6% ($p < 0.05$), from 53.2% to 69.5% ($p < 0.05$) and from 58.0% to 69.0% ($p < 0.05$) after the first, second and third campaigns, respectively. This indicates a positive short term impact of each campaign. However, differences between pre- and post-campaign compliance rates tended to decrease (19.0%, 16.3% and 11.0% respectively for the first, second and third campaign). This can be explained by the fact that post-campaign compliance seemed to reach a maximum limit of 70%. By contrast the increase of pre-campaign compliance rates over time was significant ($p < 0.05$). This evolution indicates a positive long term impact of successive campaigns.

• By hospital type

The same positive short and long term evolution of compliance rates was observed by hospital type (acute, chronic and psy-

chiatric hospitals). In psychiatric hospitals pre-campaign compliance rates were low during the second campaign (45.2%), although they rapidly increased (+21.7%) reaching post-campaign compliance rates similar to other hospital types (66.9% and 64.6% for the second and third campaigns, respectively).

• By profession

The compliance rates by profession were evolving in a similar way. As stated in the literature, nurses had the best final results (73.2% post-campaign) whereas medical doctors had lower levels of final compliance rates (54.1%) and the campaign seemed to have a smaller effect on their hand hygiene practices (+16.5%, +12.2%, +8.3%) compared to the other professional categories. Nurse's aids improved their compliance rates especially during the first campaign (+22.9%) and reached 68.3% at the end of the third campaign.

• By indication

If we considered the compliance rates by type of contact, we noticed that efforts should be made on contacts protecting patients. Indeed compliance rates before a contact with patients and before a personal/invasive action were the lowest (56.8% and 60.0%, respectively, at the end of the third campaign), whereas compliance rates after the third campaign for after contact with patient and after biological liquids exposure were 76.6% and 78.8%, respectively.

Use of hydro-alcoholic hand rubs

The campaign promoted the use of alcohol-based hand rub to disinfect hands. Results

showed that the proportion of hydro-alcoholic hand rub used increased after each campaign: from 65.3% to 76.4%, from 67.6% to 76.1% and from 73.1% to 77.7% after the first, second and third campaigns, respectively. A significant long-term increase of hydro-alcoholic hand rub was noticed ($p = 0.002$).

CONCLUSIONS

The three multifaceted campaigns were successful, not only in terms of participation rate, but also in terms of results. The number of participating hospitals remained very high during the consecutive campaigns despite the high work load that goes hand in hand with the organisation of this kind of campaign. We saw an improvement in compliance when comparing the results within the same phase of all three campaigns. However, we observed a drop in compliance at the beginning of each new campaign compared to the compliance of the post-campaign measurements from the last previous campaign. This is a known phenomenon (10) and indicates the importance of repeating campaigns such as this one in order to achieve a long-lasting result. Therefore, the working group plans to repeat these national campaigns every two years from now on with a fourth campaign already scheduled for November 2010.

The use of alcohol-based hand rubs seems to have achieved a maximum score after the third campaign. The message seemed to be accepted and the resistance against the use of alcohol-based solutions conquered.

Table 1: Number of hospitals types participating before or after the 3 national hand hygiene campaigns in Belgium, 2005-2009.

Hospital type	Campaign 2005		Campaign 2006-2007		Campaign 2008-2009	
	Before	After	Before	After	Before	After
Acute hospital sites	143	124	143	128	135	121
Chronic hospital sites	5	3	3	3	5	4
Psychiatric hospital sites	0	0	32	27	26	20
TOTAL	148	127	178	158	166	145

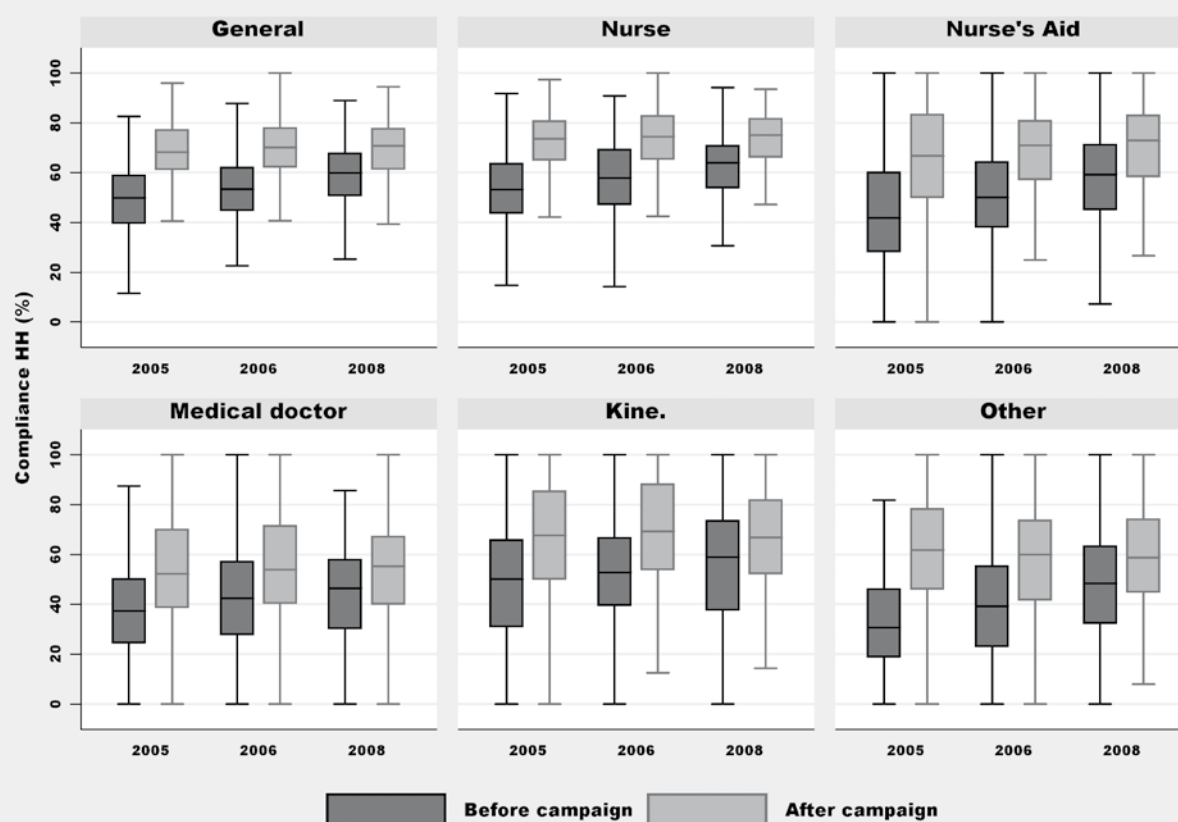


Figure 1: Evolution of the general hand hygiene compliance and the compliance by professional category, pre- and post-campaign in Belgium, 2005-2009.

Despite the general results of compliance and alcohol-based hand rub use being very good, a more detailed look shows possible tracks for future campaigns.

Although compliance rates increased for all types of HCW, it was remarkable that compliance was clearly lower among physicians than among other HCWs. This confirms reports of other authors (6, 7, 11-15), but the study by Salemi *et al.* (12) clearly showed that improvement of hand hygiene compliance among physicians is feasible. Influencing the compliance amongst physicians could have an effect on the compliance among other HCW and could even increase the overall compliance post-campaign. The working group is already brainstorming on interventions that specifically target physicians in the next campaign. Furthermore, compliance increased for all indications but was clearly higher

after than before patient contact. This observation has also been reported by other authors (6, 7, 14-16). Apparently, HCW are more inclined to protect themselves than to protect their patients.

As most of the studies in this research field, our study had several limitations (10, 17). Firstly, we used an uncontrolled before and after design because we wanted to implement the campaign in a maximum number of institutions (no control group at the hospital level) and to limit the workload of the IC teams, inclusion of control units was not required (no control group at the hospital unit level). Secondly, although direct observation is considered to be the most appropriate method for measuring hand hygiene compliance rates (3), it still has several drawbacks including the 'Hawthorne effect' concerns with inter-observer

reliability, and the fact that it only represents a sample of all hand hygiene opportunities (18, 19). Thirdly, rates of healthcare associated infections were not evaluated but the linking of our data with data from the national surveillance of nosocomial infections remains a possible future option. Finally, hand hygiene technique was not used as an outcome measure since standardised evaluation of this qualitative measure is extremely difficult, especially when so many observers are involved (19).

On the other hand, our study has several unique strengths. Until recently, national hand hygiene improvement programs in health-care settings were not widely organised (3). In 2005, Belgium was one of the first countries organising an intervention to improve hand hygiene on such a large countrywide scale, with a grand total

of 594,150 opportunities observed over a 5-year period. Furthermore, the scope was unprecedented with inclusion of acute care, chronic care and psychiatric hospitals and observation of all types of HCW on a broad range of different hospital units. Furthermore, we also provide data after 20 and 40 months of follow-up, and since our efforts are ongoing we are able to evaluate the long-term sustainability of our results. We conclude that countrywide campaigns to promote hand hygiene are feasible and can have positive results. However, such campaigns need to be repeated regularly to obtain lasting effects.



IMPACT ON PUBLIC HEALTH

Hand hygiene is the key factor in preventing the spread of microorganisms in hospitals causing healthcare-associated infections (HAIs). Each year 107,500 patients hospitalised in Belgian acute care hospitals suffer from HAIs, resulting in 2,500 to 3,000 deaths, and generating excess costs of at least 116 million euros.

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The Cytome test as an instrument in human biomonitoring and genetic toxicity testing

A. Maes

R. Anthonissen

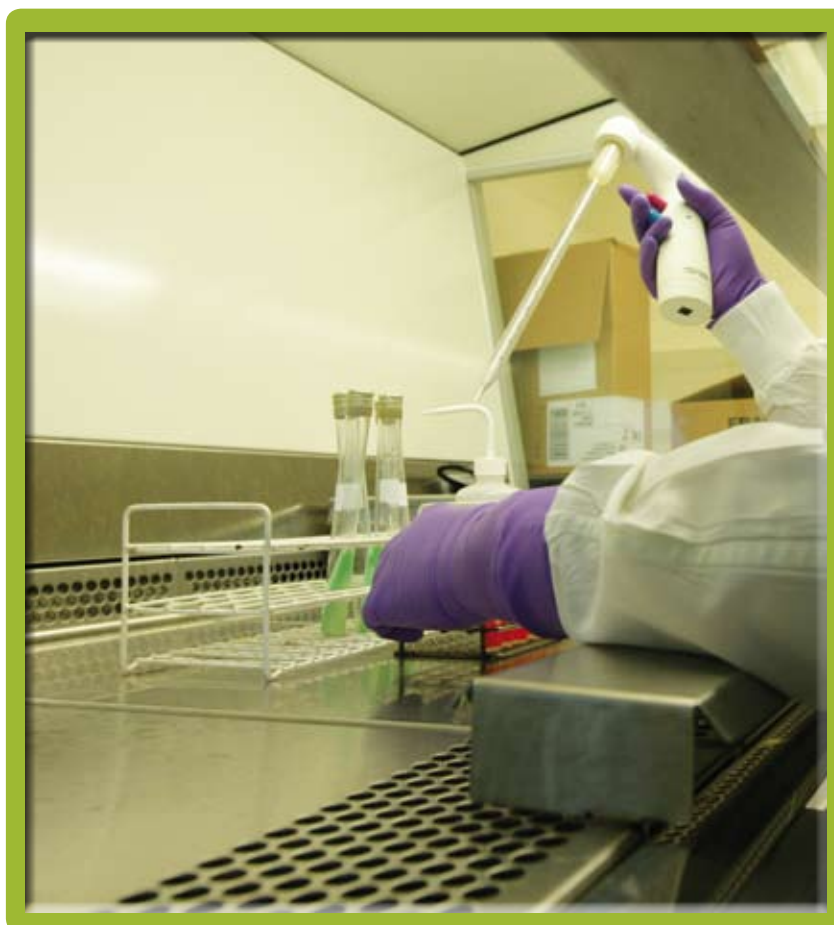
L. Verschaeve

INTRODUCTION

Human Biomonitoring has been defined as “monitoring activities in human beings, using biomarkers that focus on environmental exposures, diseases and/or disorders and genetic susceptibility, and their potential relationships”. It is an effective tool to assess human exposure to environmental pollutants and potential health effects of such pollutants and it is an essential element in a strategy aiming to integrate health and environment. Biomarker data are considered more relevant for risk assessment than extrapolations from chemical concentrations in soil, water, air or food. Human biomonitoring may involve the measurement of chemicals in human tissues for assessing people’s exposure to pollution (1) but also the cytogenetic survey of subjects which consists of determining the frequency of, for example, chromosomal aberrations in white blood cells of people from a test population compared to control subjects. Assessing chromosomal damage in cells from an “exposed population” has proved to be useful in biological dosimetry for dose assessment following ionising radiation exposure [e.g.(2, 3)] and in the prediction of cancer risk within a population (4-6). The same holds true for the so-called micronucleus assay which detects micronuclei in cells that reflect the presence of structural and/or numerical chromosome aberrations (7). Looking at genetic damage in cells from a study population is therefore considered an important tool in biomonitoring studies, which can be used as a biomarker of exposure as well as a biomarker of effect [see also (8)]. Performing chromosome aberration studies is labour intensive and time consuming and that is why the micronucleus test, which allows a faster evaluation, is often preferred.

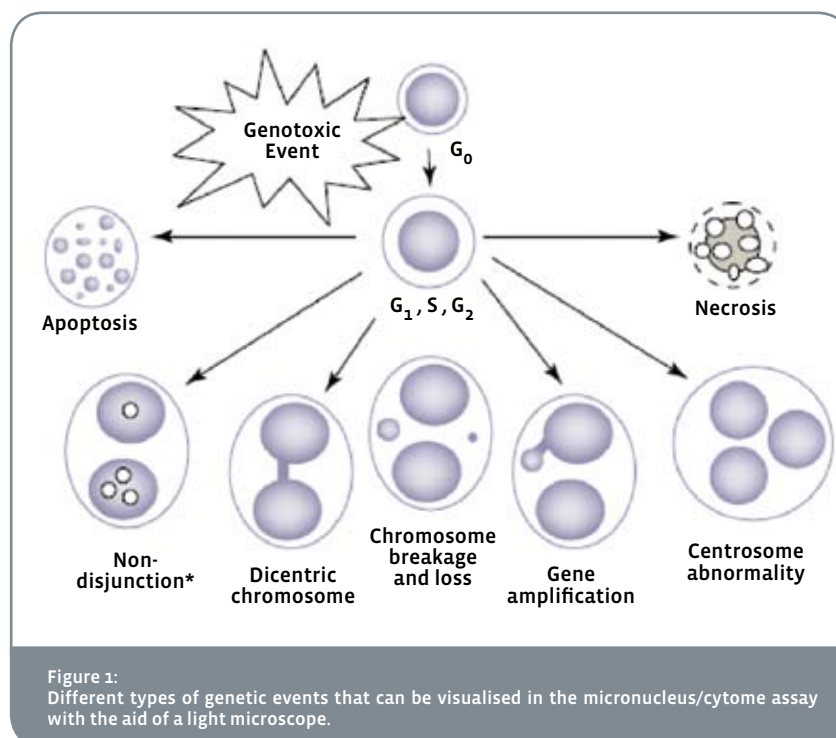
It is used regularly in human biomonitoring studies, e.g. on pesticide workers (9), subjects exposed to radiofrequency radiation (10), tannery workers (11) and many other occupationally or environmentally exposed subjects [e.g.(12, 13)]. A couple of years ago, an extension of the ‘classical’ micronucleus test was presented, which is now known as the *cytome assay* (14-16). In this assay, not only the frequency of cells with micronuclei are scored but also other morphological features that allow a better insight into (genetic) effects of pollutants and their mode of action. It is thought that this cytome assay can be very useful in human biomonitoring studies. So far this

test has already been applied in research on Alzheimer’s disease (17), the interactive effect of alcohol and folic acid on genome stability (18), the identification of lung cancer cases amongst smokers (19), effects of food supplements on biomarkers of cancer risk, oxidative stress and immune function (20) and radiation biodosimetry (21). Because of the anticipated importance of the cytome assay in future biomonitoring studies and genetic toxicity testing, we considered it worthwhile to introduce this test in our laboratory and to evaluate its applicability in determining mechanisms by which environmental mutagens induce genetic effects.



MATERIALS AND METHODS

We investigated different chemicals with different modes of action in the cytome assay. For this, C3A cells were exposed to subtoxic doses of the chemical for a period of 24 hours according to the protocol of Fenech (15). Doses were determined following a preliminary toxicity evaluation using the neutral red uptake test. C3A cells are derived from the human HepG2 hepatoma cell line which has already been used for several years in *in vitro* toxicity studies. C3A cells have conserved both phase I and phase II metabolic capacities and are therefore to be preferred over the HepG2 cells that have partially lost their metabolising power, or other cell lines that have often completely lost their metabolic potency. Cells were blocked in their (binucleated) telophase stage of cell division, fixed and spread onto microscope slides. After staining, 2,000 cells were investigated using a normal transmitted light microscope for the presence of micronuclei, nuclear buds (indicating gene amplification), nuclear bridges (dicentric chromosomes), apoptosis, necrosis and cell division disturbances (non disjunction or centrosome abnormalities) as indicated in Figure 1. As micronuclei (due to chromosome breakage or chromosome loss) are formed at cell division, we should investigate dividing cells only; this is why binucleated telophase cells are studied. Non disjunction (= abnormal cell division) results in daughter nuclei with an abnormal chromosome number; the distinction can eventually be made



using particular probes (not done here). Structural chromosome rearrangements such as dicentric chromosomes form bridges between both daughter nuclei whereas gene amplification (presence of multiple copies of a gene) is visualised by 'nuclear buds' (the nucleus is trying to expel the supernumerary DNA content). Centrosomes are organelles that serve as cell division organising centres responsible for the distribution of daughter chromosomes over two daughter nuclei. Abnormalities in centrosomes result in an abnormal chromosome distribution and hence in an abnormal number of daughter nuclei.

Apoptosis (programmed cell death) and necrosis (cell death) were not particularly induced by the subtoxic doses that we applied and are therefore not discussed here. The same holds true for the cell proliferation rate that was also studied for additional toxicity evaluation. Due to the absence of any sign of toxicity, results of this investigation will not be presented here.

Table 1: Results of the micronucleus/cytome assay for a few selected chemicals. Results are given per 1,000 analysed cells.

Test compound	Mechanism of action	Cells with micronuclei (/1,000)	Cells with nuclear bridge (/1,000)	Cells with bud(s) (/1,000)	Complex alterations (/1,000)
Negative control	-	6.0	0.5	1.5	0.5
D-mannitol (5 mg/mL)	-	4.5	1.5	5.0	0.0
Colcemid (0.01 µg/mL) Colcemid (0.025 µg/mL)	Aneugen	23.5 27.5	2.0 1.0	5.5 12.5	0.5 1.0
CdCl ₂ (73 µg/mL) CdCl ₂ (183.5 µg/mL)	Clastogen	18.0 22.2	6.0 11.1	13.5 44.4	1.5 20.0
Chloramphenicol (250 g/mL) Chloramphenicol (625 g/mL)	Antimicrobial agent and enzyme inhibitor	22.5 27.0	6.5 15.0	15.5 40.0	2.0 13.0

RESULTS

Table 1 and Figure 2 give an example of the main results found in 5 of the investigated chemicals, including a negative (unexposed) control. It can be seen that the frequency of micronucleated cells in unexposed C3A cells is 6/1000 (0.6%) which is in accordance with literature data and our own historical control values. There were, as expected, only a few cells with other aberrations. D-mannitol is known to be non-mutagenic and therefore the results should be comparable to those of the negative control. This was indeed the case, although there were somewhat more cells with nuclear buds. It is not clear so far if this is artefactual or meaningful.

Colcemide is a mitotic spindle inhibitor and therefore a well known aneugen (= compound inducing numerical chromosome aberrations). As micronuclei reflect structural as well as numerical chromosome aberrations, we expect increased micro-

nucleus frequencies with this compound. Such micronuclei contain predominantly whole chromosomes and therefore the micronuclei are larger than those that are induced by clastogens (= chromosome breaking agents). Although we did not measure micronucleus diameters or assessed aneuploidy using other methods (Fluorescence In Situ Hybridisation) we indeed undoubtedly found many more larger micronuclei than with the other treatments (*Figure 2b*). Our results are therefore in accordance with our expectations. There were also somewhat more buds compared to the controls but it is not clear whether this increased gene amplification is related to the aneugenic nature of the compound. CdCl₂ is a known clastogen and this is confirmed by the larger number of small micronucleated cells compared to the control cultures (*Figure 2a*). The presence of nuclear bridges indicates that, whereas most chromosome aberrations were chromatid type aberrations, some chromosome type aberrations (dicentric chromosomes and/or transloca-

tions) were also induced in this unsynchronised cell line where exposures may have been over more than one cell cycle. There were quite a lot of nuclear buds indicating that this compound is an inducer of gene amplification as well.

Chloramphenicol was shown to induce micronuclei as well as nuclear bridges and this is also in accordance with the literature. It was indeed shown that this widely used antimicrobial agent appears to cause chromosomal effects in somatic cells although it does not induce other genetic effects (22). As for CdCl₂ this compound is also an important inducer of gene amplification as indicated by the high number of nuclear buds. There were also many complex buds and alterations with a degree of complexity far above that of other compounds (*Figure 2f*). The significance of this is not clear at the present time but it is certainly worthwhile further investigating this compound and its mechanisms of action.

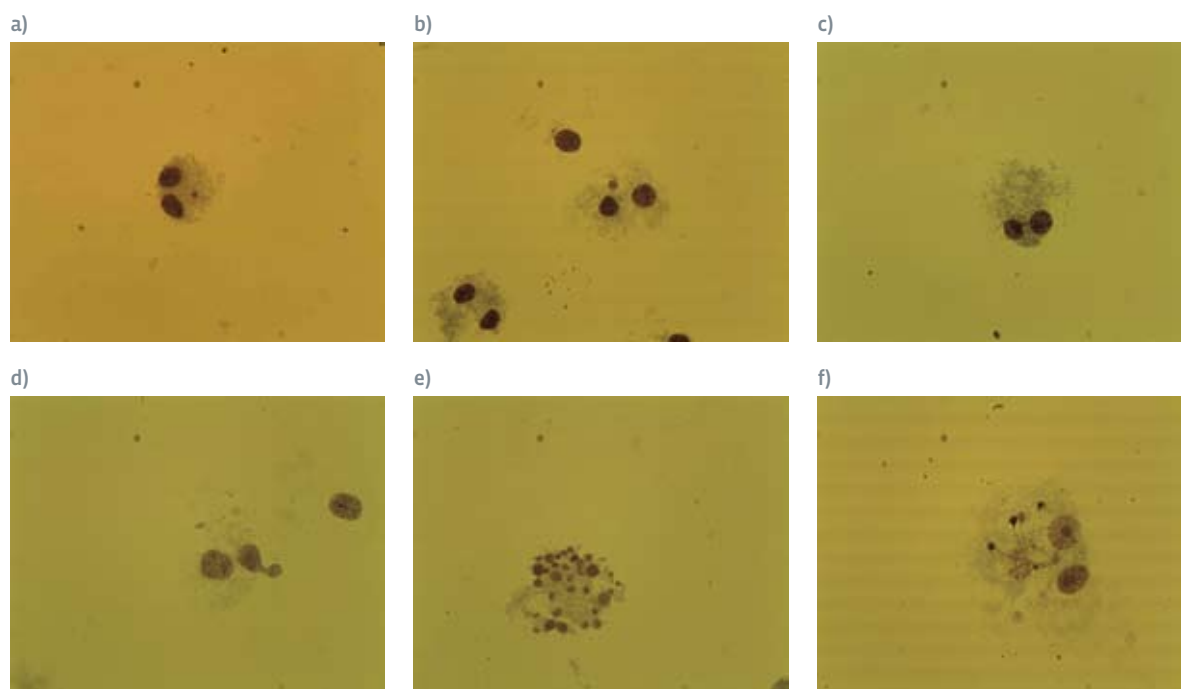


Figure 2:
Some examples of characteristic cell alterations indicating particular types of genetic effects as shown in the micronucleus/cytome assay:
a) cell with small micronucleus (clastogenic effect); b) cell with large micronucleus (aneugenic effect);
c) cell with nuclear bridge; d) cell with nuclear bud; e) apoptotic cell; f) cell with complex alteration (multiple buds?).

The above results only give a few examples that were obtained with a range of chemicals. In all cases, the results were in accordance with their known mode of action.

tal agents and to provide insight in their working mechanisms. It is anticipated that this assay will also be useful in human biomonitoring studies. For example, preliminary results indicate that nuclear bridges

might be a better predictor for cancer risk in a human population than the frequency of micronucleated cells or structural chromosome aberrations (Fenech, personal communication).

CONCLUSION

This investigation showed that agents with known properties regarding their genotoxic potency (or absence of genotoxicity) do show the expected response in the micronucleus/cytome assay. Some of these agents furthermore seem to induce gene amplification and sometimes exhibit complex aberrations that are less expected and merit further attention. The cytome assay proved to be suitable as a tool to investigate genetic effects of environmen-

IMPACT ON PUBLIC HEALTH

The micronucleus test is generally considered to be a very valuable tool in human biomonitoring studies. Extension of this test into the “cytome assay” provides an interesting tool to add information on working mechanisms of environmental genotoxicants and may increase the predictivity of diseases such as cancer.

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Eco-, geno- and human toxicology of bio-active nanoparticles for biomedical applications

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INTRODUCTION

Nanoparticles have attracted huge interest in recent decades covering a wide variety of applications and industries. One research field, in which these particles have been widely applied and studied, is the field of biomedical applications. One of the main advantages of nanoparticles within this field includes their very limited size: nanoparticles are typically smaller than 100 nm and can thus be easily taken up by cells. This opens up a huge range of interesting applications including the development of drug and gene delivery systems. Gene delivery has become an increasingly important strategy for treating a variety of human diseases. To avoid the difficulties of using viral carriers, non-viral gene delivery nanoparticles are being developed. The polyethylene imine (PEI) is considered to be one of the most effective polymer based solutions. PEI have been used for delivering DNA into various cell lines and *in vivo* applications (1-4, 5-7). PEI was, for example, successfully used as a carrier for the delivery in a tumour of the suicidal thymidine kinase gene, and the subsequent suppression of the tumour (8). Polymer-based gene delivery systems provide unlimited DNA packaging capacity with well-defined physico-chemical properties. The interaction of the positively charged polymer backbone and negatively charged DNA leads to the spontaneous formation of nano-size complexes of polymer:DNA (polyplexes) in aqueous milieu (9, 10). Nevertheless, an important concern with PEI is its significant cytotoxicity (11, 12). The toxicity observed immediately after transfection is due to the membrane destabilising activity of free PEI which can constitute an important part of the PEI:DNA mixture after the complexation (13), while the de-

layed cell death observed after transfection may be the result of the perturbation of nuclear function by PEI molecules that reached the nucleus. Therefore some alternative polymers have been investigated, such as PDMAEMA. These methacrylate polymers have excellent biocompatibility; they have been therefore used for medical purposes, such as contact lenses and artificial organs (14-16).

In this paper, we present the cytotoxic properties of two polymers (PEI and PDMAEMA) and their polyplexes (DNA covered nanoparticles) on human hepatocytes. The results obtained demonstrate the necessity to use a large variety of different tests for a comprehensive risk assessment.

MATERIAL AND METHODS

Cell culture

We used HepG2 hepatocytes (ECACC cat n° 85011430) from passage 5 to 15. The cells were grown in DMEM (Gibco), 10% FBS. The cells were tested in 96-well plates (6.4x10⁴ cells/well for NRU delayed tests, 4x10⁴ cells for acute cytotox tests, 8x10⁴ cells/well for ROS tests) or 24-well plates (4x10⁵ cells /well for the IL-8 secretion) 24 hours after seeding the plates.

DNA, polymers and polyplexes

The PEI and PDMAEMA were kept at -20°C in 1 ml aliquots of 10 mg/ml, thawed and diluted when required. The polymer solutions were sterilized by filtration (Millex – GS, 0.22 µm filters units, Milipore) and a loss of 4.3% was taken in account. The polyplexes with different charge ratios (2 (+) for 1 (-) [2:1] and 4 (+) for 1 (-) [4:1]) were prepared in sterile deionised water

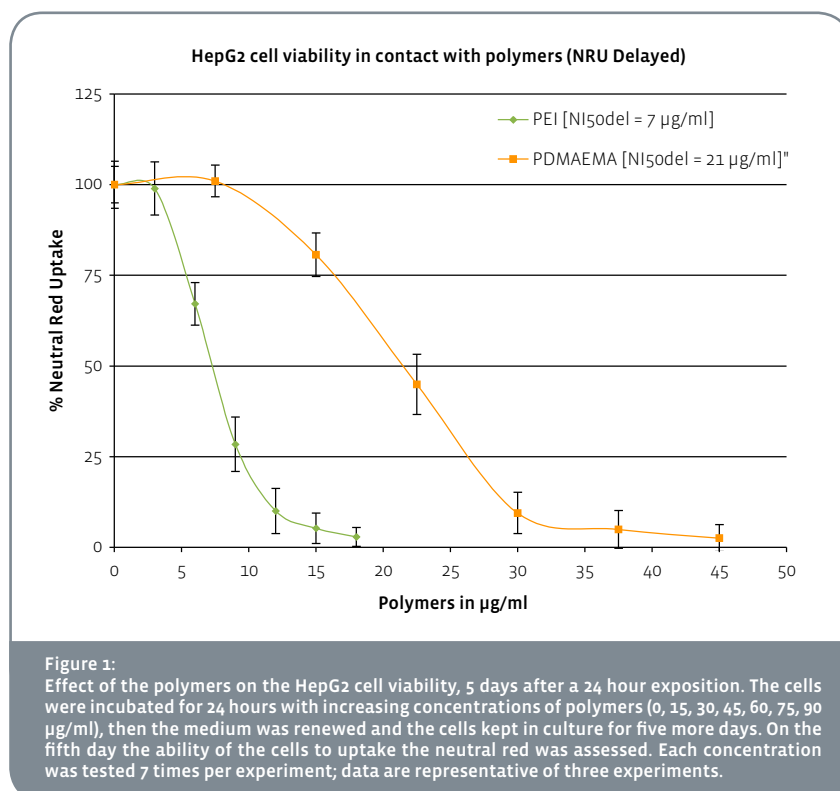
with a final concentration of DNA of 40 µg/ml. The charge ratio of the polyplexes is determined by the polymer/DNA ratio. 5 µl, 10 µl, 15 µl of the polyplex preparation were added to the test plate 30 minutes after the complexation. The DNA used is a calf thymus extract (Fluka). It was precipitated with ethanol and then resuspended in sterile water, a stock solution of 0.1 mg/ml was used.

Cell viability

The CBQCA assay assesses the survival/viability and is based on the capacity of the cells to incorporate and bind a fluorescent dye (ATTO-TAG CBQCA, MolecularProbes) to the protein amines. Each condition was tested in 7 wells and repeated 3 times. NRU acute and delayed cytotoxicity test: the cells were treated for 24 hours with the tested nanoparticles. The cytotoxicity is assessed from the uptake of neutral red and the results quantified by the NI₅₀ (the concentration of nanoparticles required to reduce the neutral red uptake [NRU] by 50%). In the case of delayed tests, the culture is maintained for 5 more days in normal growth medium after one washing step. Each condition was tested in 7 wells and repeated 3 times.

Reactive oxygen species production

The ROS production was evaluated with the fluorescent molecule dichlorofluorescein diacetate (H₂-DCFDA, Molecular probes), which become fluorescent upon cleavage by intracellular esterase in oxidative conditions. The ROS production was evaluated after 1 hour or 4 hours of exposition to the materials in 8 wells of a 96-well plate/condition and the experiment was repeated three times.



Pro-inflammatory response

The secretion of IL-8 was evaluated with the anti HU IL-8 EASIA kit from Biosource. 100 µl of supernatant from a 24-hour culture was used for the test. Each condition and the controls were tested in duplicate and repeated twice.

RESULTS

We worked with HepG2. These cells, isolated from a hepatocellular carcinoma (17), are easy to manipulate and commonly used in *in vitro* pharmaco-toxicity assays. The liver is the main site for biotransformation and defence against the xenobiotics. It has already been shown that nanoparticles (PEG coated quantum dots, polystyrene beads, metallo-fullerene) can translocate from the circulatory system to the liver (18, 19).

Cell viability

Preliminary shallow testing was performed in order to gain an idea of the concentrations and exposure time to use. The

concentration ranges and the charge ratio tested were determined to avoid precipitation of the nanopolyplexes.

Using CBQCA to monitor the decrease in cell viability after 24 hours of contact with increasing concentrations of polymers, we obtained with the PDMAEMA exposition a curve that was quite irregular (not shown here). This unusual figure showed that there are interactions between the polymer and the test system. It seems that CBQCA reacts quantitatively with the free amines of the proteins; it is probable that the positively charged PDMAEMA interacts with CBQCA and disturbs the test. On the other hand, the acute NRU assays showed a steady decrease of cell viability with the increasing concentration of polymers. The calculated NI_{50} were close to the ones determined during the shallow testing: PEI had a NI_{50} of 28 µg/ml after 24 hours of exposition with HepG2 cells, and PDMAEMA had a NI_{50} of 33 µg/ml after 24 hours of exposition with HepG2 cells.

Given the perturbations of the test observed with the CBQCA, the cytotoxicity

of the polyplexes with a charge ratio of 2:1 and 4:1 was tested only with NRU. We observed a decrease of 20% of the HepG2 cell viability when the cells were in contact with the highest amount of 4:1 PEI:DNA polyplexes, but also when the cells were in contact with 15 µl of a solution with the same amount of polymers than the amount used to prepare the 4:1 polyplexes. Although the 4:1 PEI:DNA polyplexes harbours fewer charges than the PEI alone, it induces the same cell mortality in these test conditions.

With the NRU delayed tests, we observed that, after 5 days, the effect of 24 hours exposition to the polymers is quite strong (Figure 1). The delayed NI_{50} of PEI is at 7 µg/ml, while the delayed NI_{50} of PDMAEMA is at 21 µg/ml. The dramatic effect of the PEI is not only due to the decrease of cell viability from the first 24 hours of exposition to the polymer, as we could monitor a loss of cells day after day following the medium replacement (observation with an inverted microscope).

Figure 2 shows that 4:1 PEI:DNA polyplexes also have a strong delayed effect on HepG2 cells. We could determine that the addition of 8 µl of a suspension of 4:1 PEI:DNA complexes made from a mix with 40 µg/ml DNA reduces the capacity of the NRU of HepG2 cells by 50%, five days after 24 hours exposition. However the effect of the control with the polymer alone is quite close ($NI_{50del} = 8.6$ µl).

Oxidative stress

An imbalance in the redox state of the cell is often linked to particles' cell toxicity. Here we looked at the oxidative activity in HepG2 cells in contact with the PEI or the PDMAEMA polymers and their respective polyplexes. The cells were grown for 24 hours in 96-well plates, then loaded with H_2DCFDA and exposed to the polymers/polyplexes for 1 hour or 4 hours in PBS. After this exposition, the fluorescence was measured and compared to the fluorescence of unexposed cells. We did not observe any

increase in fluorescence but rather a decrease in certain cases that were not concentration or time dependant (not shown here). With this single endpoint tested (oxidisation of a quite unspecific fluorescent probe), it is hard to explain this decrease in comparison to the negative control.

IL-8 secretion

The measurement of the pro-inflammatory response of the cell is interesting in order to assess the stress that the cell is undergoing when in contact with nanoparticles. *In vivo*, unbalanced pro-inflammatory response may lead to more damage than the event at the origin of the response. The capacity of the polymers and the polyplexes to elicit a pro-inflammatory response was investigated through the level of IL-8 secreted by the HepG2 cells in contact with the material for 24 hours. We made two assays with the different polymers and

polyplexes. We clearly did not observe any relevant increase in the IL-8 secretion, rather, our results suggest a decrease in the secretion of IL-8. Only a few tested conditions were significantly different from the controls: 20 µg/ml of PDMAEMA, 150 µl/ml 2:1 and 4:1 PDMAEMA:DNA polyplexes.

CONCLUSIONS

We were able to test the effects of the polymers and their relative polyplexes on different cellular processes. The cell viability assays showed that both cationic polymers are quite toxic for the cells. PEI is slightly more cytotoxic than PDMAEMA in the acute cytotoxicity testing. The PEI polyplex with a 4:1 charge ratio was also the only one to cause a reduction in cell viability at the highest concentration tested. An even bigger difference between PEI and PDMAEMA was visible with the delayed cy-

totoxicity tests. With PEI and the PEI:DNA polyplexes, some elements promoting cytotoxicity remain after the medium shift. It is probable that some PEI was still present in the cell culture, or even more likely in the cells, and continued to impair cell viability for five days after the medium shift, as observed with the delayed NRU tests.

The CBQCA assay was shown to be unfit to assess the effect of these positively charged polymers on cell viability.

The testing of the oxidative stress and the pro-inflammatory response were less conclusive. To reach further conclusions, analysis of the oxidative activity of the cells in contact with polymers and polyplexes is required using other means such as the use of another probe like APF or HPF or the measuring of the glutathione (GSH) activity of the cell. Our results suggest a decrease in the secretion of IL-8 in the presence of the highest concentrations of PDMAEMA but we cannot exclude that polymers/polyplexes interact with a part of the secreted IL-8. This should be further tested in order to draw any firm conclusions.

We did not distinguish a sharp difference in cytotoxicity between the polymer and the corresponding polyplexes (nanoparticles). This may be due to the fact that, after the complexation, a large amount of free polymer still remains in the mixture added to cells (23) or that the 4:1 polyplexes present still have enough charges to destabilise cell functions in a way similar to the cationic polymer.

The short paper presented here is part of a larger study including results from many different endpoints (20). Despite the large number of studies published using different types of nanoparticles, there is still a lack of studies in which an in-depth analysis of the overall toxicological profile of nanoparticles is performed. Most studies are limited to the determination of the cytotoxicity without addressing the eco-, geno- and human toxicology of the developed nanoparticles. Nanomaterial researchers

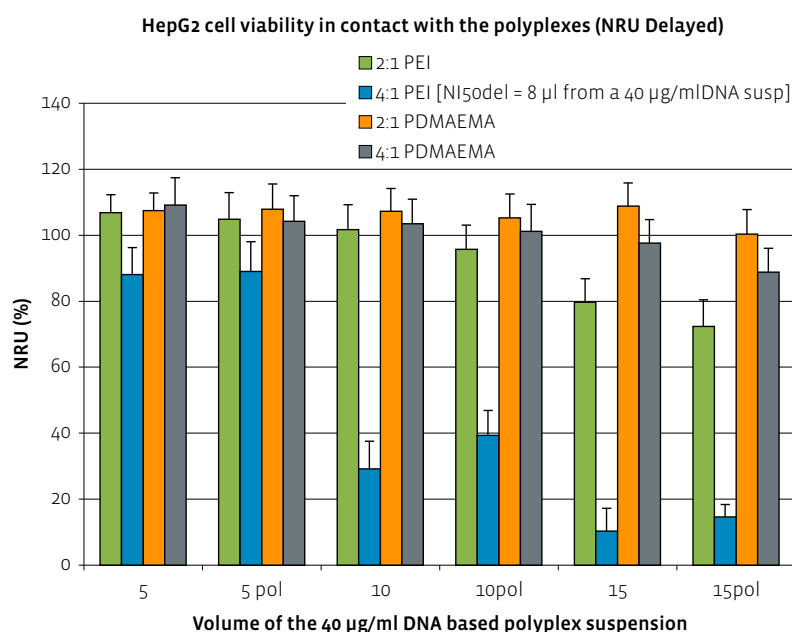


Figure 2: Effect of the 2:1 and 4:1 polyplexes on the HepG2 cell viability, 5 days after a 24 hour exposition. The cells were incubated for 24 hours with three different amounts of polyplexes (5 µl, 10 µl, 15 µl from a complex suspension with a base of 40 µg/ml of DNA), then the medium was renewed and the cells kept in culture for five more days. On the fifth day, the ability of the cells to uptake the neutral red was assessed. Each amount of polyplexes was tested 7 times per experiment; data are representative of three experiments. Bars significantly different from control are marked with a * ($p < 0.05$). 5 is for 5 µl, 5 pol is for 5 µl from a solution with the same amount of polymer that was used for the complexation and without DNA, idem for 10/10pol, 15/15pol.

also face increasing concern from the governmental, environmental and industrial groups regarding the potential health hazards of nanoparticles. Despite these concerns and the will of the EU to identify possible risks related to nanoparticles [COM (2004) 338], there are as yet no regulations for the safe use of nanoparticles at the European level.



IMPACT ON PUBLIC HEALTH

The important message is that the risk assessment of nanoparticles is a complex task and several test systems have to be used in order to draw a thorough conclusion. Conclusions based on a single test need to be approached with caution. This work gives grounds for performing a whole battery of tests for a comprehensive characterisation of risks.

ACKNOWLEDGMENTS

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SOURCE

Robbens J, Vanparys C, Nobels I, Blust R, Van HK, Janssen C, *et al.* Eco-, geno- and human toxicology of bio-active nanoparticles for biomedical applications. *Toxicol* 2010;269(2-3):170-81.

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HIV infections and STI co-infections in men who have sex with men in Belgium: sustained increase in HIV diagnoses

A. Sasse

A. Defraye

INTRODUCTION

Re-emergence of HIV epidemic and continuous high notification rates of newly diagnosed HIV cases in men who have sex with men (MSM) have been observed in many Western European countries (1-6). Diagnoses of concurrent other sexually transmitted infections (STI) have also increased substantially. In this paper, based on surveillance data collected by the Epidemiology division at the Scientific Institute of Public Health in Brussels, we describe the trends and epidemiological features of HIV and STI among MSM in Belgium.

MATERIAL AND METHODS

HIV infection

The total number of screening tests was provided by the National Institute for Sickness and Invalidity Insurance (INAMI-RIZIV) based on reimbursement figures. HIV testing is widely available and used in Belgium. People may seek HIV testing from their general practitioner, a hospital or a family planning centre.

All serums with positive screening test results are submitted for confirmation to one of the seven AIDS Reference Laboratories. For each confirmed test, a form is sent to the patient's clinician. Based on biology results and information collected at the consultation, the clinician provides data on age, sex, nationality, residence, sexual orientation, probable mode of HIV transmission and CD4 count at the time of HIV diagnosis. Data are validated for duplicate recording and included in a HIV database maintained at the Scientific Institute of Public Health in Brussels since 1985. In 1990, HIV and AIDS databases were integrated.

Sexually transmitted infections

Data on co-infections of HIV and other STI in MSM were collected from the Belgian AIDS Reference Centres. These centres are specialised in STI/HIV counselling and treatment. Seven of the nine medical Centres have participated in the ongoing surveillance of STI since the beginning of 2007. The reported STI included chlamydia, gonorrhoea, syphilis, *lymphogranuloma venereum* (LGV), hepatitis B virus infection (HBV) and sexually acquired hepatitis C virus infection (HCV). In the surveillance system, only recent, active syphilis infections have to be reported; no information on the stage of disease is collected. For hepatitis B and C, only acute infections have to be reported. In order to minimise the workload for the voluntarily participating physicians, no information on the laboratory testing results is collected.

Statistical methods

Stata 10 (Statacorp, College Station, Texas, US) was used for analysis and processing. Logistic regression was used to analyse factors for late HIV diagnosis.

RESULTS

HIV infection

In 2008, there were 1,079 persons newly diagnosed with HIV in Belgium. This is the highest number ever reported. During the period 2003-2008, a plateau was observed; the rate of newly diagnosed cases of HIV infection fluctuated between 96 and 102 per million population. Before this plateau phase, the rate of newly diagnosed cases had increased by 47%, from 69 per million

in 1997 to 101 per million in 2003.

In the past decade, the yearly number of new HIV diagnoses in MSM increased by 228%, from 101 cases diagnosed in 1999 to 332 cases in 2008 (*Figure 1*). The proportion of MSM among all newly diagnosed HIV cases, for whom the probable transmission mode was known, increased from 23% in 1999 to 46% in 2008. During the same period, the trend in new HIV diagnoses in heterosexuals was reversed: in a first phase the yearly number of new diagnoses increased by 61%, from 298 cases diagnosed in 1999 to 480 cases in 2003; in a second phase, this number decreased by 27% to 350 cases diagnosed in 2008 (*Figure 1*).

In 2008, almost half of HIV diagnoses were in MSM (46%), even if heterosexual transmission remained the predominant reported mode of infection (48%). Only 2% of patients were infected by intravenous drug use. Transmission mode data were available for approximately 70% of new diagnoses.

During the period 1999-2008, increasing rates of newly diagnosed HIV cases in MSM per 100,000 male population were observed among all age groups: the rate in age group 20-29 years increased by a factor of 4.5, and the rates in age groups 30-39 years and 40-49 years by factors of 2.8 and 3.5 respectively. The median age of MSM at time of diagnosis remained constant. For period 1999-2008, the median age was 37 years.

The majority of new HIV infections in MSM were diagnosed among Belgian citizens (72%), followed by other European nationalities (13%). Among new diagnoses in heterosexuals, 59% were patients from sub-Saharan Africa, and 27% were of Belgian nationality.

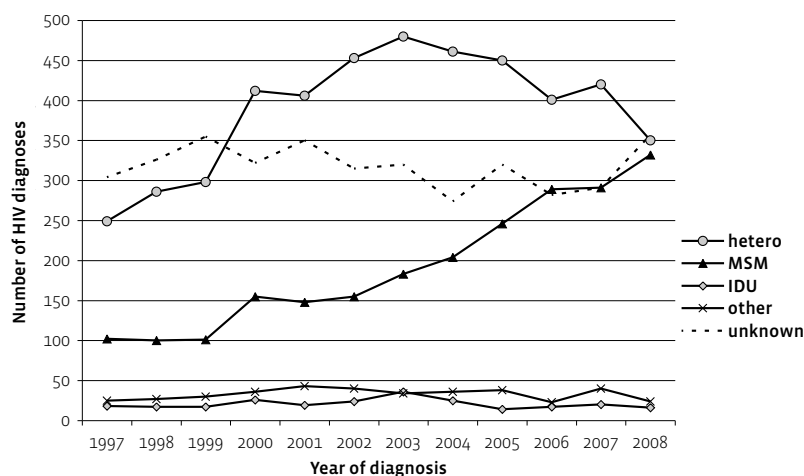


Figure 1: New HIV diagnoses per mode of transmission in Belgium, 1997-2008.

Surveillance data suggest that HIV testing behaviour has evolved in MSM and that a significant improvement was made regarding early HIV diagnosis during the last few years. A steady and significant increase of CD4 count at HIV diagnosis was observed between 2001 and 2008, suggesting that HIV infections were diagnosed earlier in recent years. The mean CD4 count at HIV diagnosis among MSM was 526 in 2008 versus 395 in 2001 ($p < 0.001$).

Furthermore, the proportion of late HIV diagnoses (defined as CD4 cell count < 200 per mm^3 at diagnosis or AIDS diagnosed within three months) among MSM significantly decreased over time (Figure 2). In a multivariate analysis, controlling for time of diagnosis and nationality, older age was independently associated with late HIV diagnosis ($p < 0.001$).

HIV testing

An average of 56 HIV tests per 1,000 population are performed yearly nationwide, excluding tests related to blood donations. The number of tests performed remained remarkably stable over time, varying between 51 and 57 HIV screening tests per 1,000 individuals per year during the period 1997-2008.

Sexually transmitted infections

For 2008, seven of the nine AIDS Reference Centres reported 267 episodes of STI diagnosed in 241 MSM living with HIV. Numbers of episodes per STI diagnosis were as follows: syphilis: 162; chlamydia: 29; gonorrhoea: 29; LGV: 31; hepatitis C: 14. MSM represented 95.6% of all HIV-positive patients reported with a new STI episode in these centres for 2008. In 215 cases, patients were aware of their HIV status at the time of the STI consultation. In 26 cases (10.8%), HIV infection was diagnosed at the STI consultation.

Among 162 syphilis diagnoses, 67 (41.4%) were reported as re-infections. In 26 patients (10.8%), two STI other than HIV were diagnosed at the same time. Syphilis was associated with chlamydia in eight cases, with LGV in six cases, with HCV in three cases and with gonorrhoea in one case. Gonorrhoea was associated with chlamydia in five cases and with LGV in three cases.

DISCUSSION

After years of steady decrease, from 1997, a reverse has been observed in the number of newly diagnosed HIV infections in Belgium (6).

In 2008, the number of reported new HIV diagnoses among MSM was higher than ever since the beginning of the epidemic, including among young MSM. The increasing trend in Belgian MSM was continuous from 1999 until 2008, while the numbers reported in heterosexuals of sub-Saharan origin seem to decrease in recent years.

Changes in HIV testing may influence trends in HIV diagnoses (7). In Belgium however, the increase in HIV diagnoses does not reflect an increase in HIV testing since the number of tests performed nationwide has remained remarkably stable over time.

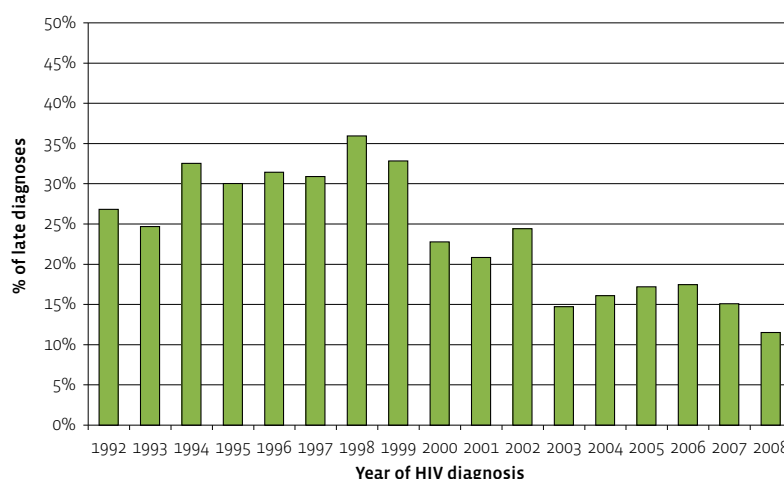


Figure 2: Proportion of late* HIV diagnoses among MSM, 1992-2008.
* $< 200\text{CD4}$ at HIV diagnosis or HIV & AIDS diagnoses within 3 months

On the other hand, increasing trends may partially reflect changes in the targeting of most at-risk groups during the period 1997-2008, as suggested by the fact that MSM are being diagnosed earlier in recent years. Testing promotion campaigns focused on MSM have been launched in recent years.

The steadily increasing trend in newly diagnosed HIV infections among MSM, and the high rate of co-infections with other STI are worrying (8, 9). In 2008, in 11% of HIV-positive MSM co-infected with STI reported by the participating AIDS Reference Centres, HIV seropositivity was discovered following the STI consultation. This finding underlines the importance of offering an HIV test to patients presenting with a STI.

These observations corroborate recent studies indicating increasing prevalence of sexual risk behaviour among MSM, including those who are aware of their HIV-positive status. A survey in the French Community of Belgium carried out in 2004 and 2005 among 942 MSM pointed out that, although the majority of the respondents mentioned a high level of protection during anal intercourse, a quarter of respondents had at least one unprotected anal

intercourse in the last year with a partner whose status was unknown or different from their own (10). Moreover, HIV-positive men and men who were not sure about their HIV status were found to be more likely to admit unprotected anal intercourse (10). In the Flemish Community of Belgium, 1,793 MSM completed an internet questionnaire in 2007; this study found a higher rate of STI infections among HIV-positive MSM compared to HIV-negative MSM. The characteristics associated with sexual risk behaviour in this study were drug use, lower educational level, lower score on mental health, less social support, more sensation seeking and more sexual partners (8).

It is essential to adapt and to reinforce prevention interventions aimed at high risk groups. The survey conducted in the French Community showed that knowledge of ways of transmission and treatment of HIV among respondents was good, nevertheless many engaged in high-risk sexual practices. Hence, while providing information on HIV and STI remains necessary, it is not sufficient and has to be combined with other preventive interventions. Development of behavioural surveillance and more qualitative research on reasons why people practice unsafe sex are needed in order to develop more effective prevention strategies.



IMPACT ON PUBLIC HEALTH

The analysis of HIV/AIDS trends in Belgium, determining factors of the epidemic and risk factors, as well as the communication of this information, contributes to the setting up of prevention programs and forecasting in terms of caring for and monitoring patients carrying the infection.

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Human biomonitoring of heavy metals in the vicinity of non-ferrous metal industries in Ath, Wallonia

S. Fierens

INTRODUCTION

The quantification of pollutants or their metabolites in biological samples, known as human biomonitoring, is a reliable and sensitive approach to assess the degree of contamination of exposed populations and to estimate their possible health consequences. Originally limited to occupational medicine, biomonitoring is now largely used for assessing environmental exposure. Moreover, the usefulness of a systematic implementation of this approach on a large scale is becoming increasingly recognised. This is the case in Flanders with the Flemish biomonitoring program (Flemish Centre of Expertise on Environment and Health), in Germany with the German Environmental Survey on Children (GerES), and in the U.S. with the National Health and Nutrition Examination Surveys (NHANES).

In industrialised countries, environmental pollution by heavy metals is mainly the consequence of past emissions from non-ferrous metal industries, emitting large amounts of pollutants during the 19th century. Although emissions have declined overall, soils can remain contaminated for decades, which is still a concern for populations living in these polluted areas. Furthermore, in the case of contaminated soils, pollutants can be captured by plants and enter the food chain, where bioconcentration of pollutants can occur, leading to increased exposure of people eating contaminated foods.

The presence of two non-ferrous metal industries in a residential area led the authorities of the municipality of Ath to undertake an assessment of the potential environmental pollution. Relatively high levels of heavy metals had been detected in surface soil samples collected near houses

located in the vicinity of these industries (1). The present study was conducted in order to evaluate the potential transfer of this contamination into the human body and, consequently, the health impact on the population.

METHODS

We performed a population based cross-sectional study. Human biomonitoring was used to assess environmental exposure to five metals. This study was submitted to the Belgian Commission for the Protection of Privacy and received the agreement of the Ethical Commission of the Medicine Faculty of the Université catholique de Louvain (UCL). Each participant has given his signed informed consent. For children, the informed consent was given by their parents or tutors.

Study area

The study was carried out in the municipality of Ath, in the Walloon Region of Belgium. The region of Ath is mainly agricultural. The city is mostly residential but has two non-ferrous metal industries, located in the heart of the city. We defined two areas of investigation. The first is the 'central area', formed by a circle of 1 km radius around each of the two industries that are ± 500 m away from each other. The central area is where we expected to find the population most exposed to metals. The second area is the 'peripheral area', with a population expected to be little exposed or not exposed to the metals emitted by the industries of interest. The peripheral area is composed of six villages within the municipality of Ath, located more than three kilometres away from the industries.

Study population

The study population was restricted to environmentally exposed persons. Potential study subjects were screened for occupational exposure and, if present, were therefore excluded. Three target groups were investigated: young children aged 2.5 to 6 years, children aged 7 to 11 years, and adults aged 40 to 60 years. The time of residence in the study area had to be at least 10 years for adults, 1 year for children aged 7 to 11 years, and 6 months for children aged 2.5 to 6 years. Participants were recruited at random, stratified by the 3 target groups and with an equal sample size between the two studied areas.

Questionnaire

Participants were asked to fill in a questionnaire, adapted differently for adults or children. The questionnaire consisted of five parts: i) general and socio-demographical questions, ii) questions on housing and surroundings, iii) lifestyle and behaviour, iv) health status, and v) food consumption habits.

Biological samples

All samples were taken in the same place in Ath, between 26 February 2009 and 20 March 2009. Adults and children aged 7 to 11 years provided a urine sample in a metal-free container by the midstream technique. They also gave two tubes of venous blood taken by a physician. The tubes were trace element free. The first, containing EDTA, was used for the measurement of lead and cadmium. The second, containing lithium and heparin, was used for the measurement of serum ferritin. This protein, correlated with iron levels in the body, is used as

a determinant of anaemia, which is known to increase gastro-intestinal absorption of cadmium and lead. A distinct, less invasive procedure was adopted for young children aged 2.5 to 6 years. Approximately 10 drop-lets of capillary blood were taken by a little prick at the end of the digit. This sample was intended to measure lead, the only biomarker quantified for this age group.

All the biological analyses were done by the Laboratory of Industrial and Environmental Toxicology, Department of Clinical Biology, University Hospital Saint-Luc, Brussels. Blood cadmium and blood lead were quantified by Graphite Furnace Atomic Absorption Spectrometry (GFAAS). Urinary cadmium, chromium, cobalt, and nickel were quantified in urine by Inductively

Coupled Plasma – Mass Spectrometry (ICP-MS). Urinary parameters were expressed in µg/g creatinine. Urinary samples with creatinine concentrations < 0.3 g/l or > 3 g/l (10 children and 14 adults) were excluded from statistical analyses.

Table 1: Baseline characteristics of biomonitoring study participants, Ath, 2009.

	Central area	Peripheral area	Total
Children aged 2.5 to 6 years	n = 49	n = 49	n = 98
Age, years *	4.6 (1.4)	4.5 (1.5)	4.6 (1.5)
Gender, n			
Male	27 (55%)	24 (49%)	51 (52%)
Female	22 (45%)	25 (51%)	47 (48%)
BMI, kg/m ² *	16.0 (2.1)	16.1 (1.2)	16.0 (1.7)
Higher education of parents, n	43 (88%)	42 (85%)	85 (88%)
School in the city of Ath, n	36 (78%)	6 (12%)	42 (44%)
Locally grown vegetables consumption, n	17 (35%)	33 (67%)	50 (51%)
Children aged 7 to 11 years	n = 38	n = 36	n = 74
Age, years *	9.4 (1.5)	9.6 (1.5)	9.5 (1.5)
Gender, n			
Male	22 (58%)	20 (56%)	42 (57%)
Female	16 (42%)	16 (44%)	32 (43%)
BMI, kg/m ² *	17.8 (3.5)	17.4 (2.4)	17.6 (3.0)
Higher education of parents, n	28 (78%)	28 (80%)	56 (79%)
School in the city of Ath, n	27 (77%)	6 (18%)	33 (48%)
Locally grown vegetables consumption, n	19 (50%)	25 (69%)	44 (60%)
Men	n = 27	n = 25	n = 52
Age, years *	52.8 (5.7)	50.3 (6.2)	51.6 (6.0)
BMI, kg/m ² *	30.9 (3.0)	28.0 (3.4)	31.1 (2.2)
Higher education, n	14 (52%)	13 (54%)	27 (53%)
Work in Ath, n	10 (37%)	6 (25%)	16 (31%)
Locally grown vegetables consumption, n	14 (52%)	11 (44%)	25 (48%)
Daily consumption of alcohol, n	9 (35%)	11 (44%)	20 (39%)
Current smokers, n	5 (19%)	5 (20%)	10 (20%)
Women	n = 28	n = 26	n = 54
Age, years *	50.3 (5.7)	51.3 (6.3)	50.8 (6.0)
BMI, kg/m ² *	28.1 (5.7)	27.4 (7.1)	27.8 (6.3)
Higher education, n	21 (75%)	16 (62%)	37 (69%)
Work in Ath, n	16 (59%)	9 (39%)	25 (50%)
Locally grown vegetables consumption, n	11 (39%)	20 (77%)	31 (57%)
Daily consumption of alcohol, n	7 (25%)	9 (35%)	16 (30%)
Current smokers, n	6 (21%)	4 (15%)	10 (19%)

* Arithmetic means (standard deviation). BMI: Body Mass Index

Statistical analyses

Data were encoded with the software EPI-DATA 3.0. Statistical analyses were done with the software SPSS 16.0, SPSS 18.0, and STATA 10.1. A p -value ≤ 0.05 was taken as the level of significance. When needed, log normal transformation was performed. Outliers were excluded from analyses. The Student's t -test and analysis of variance (ANOVA) were used for mean comparisons. The Pearson's or Spearman's correlation coefficient was used to test association between quantitative variables. Proportions between groups were compared by the Chi-square test or the exact Fisher's test. Stepwise multiple linear regression analyses with biological parameter concentrations as dependent exposure variables were applied on the combined groups of central and peripheral areas, for adults, children aged 7 to 11 years, and children aged 2.5 to 6 years separately. For urinary parameters, the values under the limit of quantification (LOQ) were estimated by giving them the value of $1/2$ LOQ.

RESULTS

The response rate was 24%. This rate is comparable to that found in other human biomonitoring studies in the general population, such as the Flemish biomonitoring (response rate of 22.3%). The final number of participants who have taken part in our study is 278, composed of 98 young children aged 2.5 to 6 years, 74 children aged 7 to 11 years and 106 adults (52 men and 54 women). The most relevant characteristics of the study population are given in table 1.

Table 2 shows the values of the biological parameters in the central and peripheral areas, by age group and by gender in adults. The metal concentrations observed in this study are within the same range as those found in the general population in Belgium or other industrialised countries, as reported by other studies. Furthermore, if we compare the blood lead and urinary cadmium levels from this study to those

found by previous studies several years or decades ago, we note that concentrations of these metals have decreased with time in the general population (2). This reflects a global decrease in exposure that has already occurred over several decades.

The situation for small children is however particular. The mean blood lead level of children aged 2.5 to 6 years is significantly higher in the central area than in the peripheral area. Even if the difference of means is very weak and if the range of values is comparable between the two areas, this difference could be the consequence of the pollution from the industries. Our results, showing an effect of the location of residence in children aged 2.5 to 6 years but not in older children or adults, confirms the importance of studying young children, as a risk group of the population. While for cumulative metals such as cadmium and lead, concentrations observed in children are, as expected, lower than those observed in adults, the observed concentrations of cobalt, chromium and nickel, which reflect recent exposure, are similar or even higher in children than in adults. For nickel, 12% of the children values are over the adult reference value of $3 \mu\text{g/g}$ creatinine, while only one adult reaches this threshold. This could reflect differences in exposure, but also in physi-

ology between children and adults. As less is known about the safety factors concerning health effects in children, this underlines the need for specific reference values for children. More broadly, a systematic evaluation of baseline values for a panel of pollutants in the general population is lacking in Wallonia. Our study stresses the usefulness of a human biomonitoring programme at a regional or greater level.

We performed multiple regression analyses, stratified by age group, in order to identify determinants from the biological parameter concentrations. Table 3 shows the significant determinants identified by the models. The only parameter for which the geographical area is a significant determinant is, as expected, blood lead in children aged 2.5 to 6 years. For this age group, no other significant determinant was found by this analysis. No significant determinant was found for cobalt, chromium or nickel in this age group. In children aged 7 to 11 years, blood lead concentrations were significantly influenced by the housing age, children living in houses built before 1950 having higher concentrations. Blood lead concentrations in adults were not only influenced by gender (men having higher values) but also positively related with alcohol consumption and age, and negatively related with BMI. In adults, while



Table 2: Biological parameters of biomonitoring study participants, Ath, 2009.

	Central area	Peripheral area	Total
Children aged 2.5 to 6 years	n = 49	n = 49	n = 98
Blood lead, µg/l	18.2 (15.9-20.9) [6-66]	14.8 (12.6-17.4) [3-68]*	16.6 (14.8-18.2) [3-68]
Children aged 7 to 11 years	n = 38	n = 36	n = 74
Blood lead, µg/l	15.5 (13.2-18.2) [4-47]	14.1 (11.8-17.4) [4-50]	14.8 (13.2-16.6) [4-50]
Blood cadmium, µg/l	0.13 (0.11-0.15) [0-0.3]	0.14 (0.12-0.16) [0-0.2]	0.13 (0.12-0.15) [0-0.3]
Urinary cadmium, µg/g cr	0.07 (0.05-0.09) [0.01-0.22]	0.06 (0.04-0.07) [0.02-0.20]	0.06 (0.05-0.07) [0.01-0.22]
Urinary cobalt, µg/g cr	0.38 (0.29-0.50) [0.08-1.27]	0.29 (0.23-0.36) [0.09-0.68]	0.33 (0.28-0.39) [0.08-1.27]
Urinary chromium, µg/g cr	0.18 (0.15-0.21) [0.06-0.47]	0.18 (0.15-0.23) [0.07-0.48]	0.18 (0.16-0.21) [0.06-0.48]
Urinary nickel, µg/g cr	1.75 (1.46-2.09) [0.37-3.92]	1.75 (1.37-2.26) [0.30-5.74]	1.75 (1.51-2.03) [0.30-5.74]
Men	n = 27	n = 25	n = 52
Blood lead, µg/l	31.2 (26.4-36.9) [14-85]	32.3 (26.1-40.0) [12-118]	31.7 (27.9-36.1) [12-118]
Blood cadmium, µg/l	0.25 (0.19-0.33) [0.1-1.2]	0.38 (0.29-0.50) [0.1-1.7]	0.30 (0.25-0.37) [0.1-1.7]
Urinary cadmium, µg/g cr	0.22 (0.16-0.30) [0.05-0.87]	0.20 (0.14-0.30) [0.04-0.78]	0.21 (0.17-0.27) [0.04-0.87]
Urinary cobalt, µg/g cr	0.15 (0.11-0.20) [0.06-0.58]	0.18 (0.13-0.25) [0.06-1.84]	0.16 (0.13-0.20) [0.06-1.84]
Urinary chromium, µg/g cr	0.13 (0.10-0.16) [0.05-0.33]	0.16 (0.13-0.21) [0.05-0.35]	0.14 (0.12-0.17) [0.05-0.35]
Urinary nickel, µg/g cr	0.65 (0.51-0.84) [0.21-2.58]	0.69 (0.51-0.92) [0.20-2.16]	0.67 (0.56-0.80) [0.20-2.58]
Women	n = 28	n = 26	n = 54
Blood lead, µg/l	22.5 (18.0-28.2) [4-57]	20.3 (15.6-26.5) [6-93]	21.4 (18.1-25.3) [4-93]
Blood cadmium, µg/l	0.37 (0.29-0.47) [0.2-1.9]	0.39 (0.32-0.47) [0.2-1.6]	0.38 (0.33-0.44) [0.2-1.9]
Urinary cadmium, µg/g cr	0.23 (0.17-0.33) [0.04-0.78]	0.25 (0.18-0.34) [0.05-1.0]	0.25 (0.20-0.30) [0.04-1.0]
Urinary cobalt, µg/g cr	0.20 (0.16-0.26) [0.07-0.40]	0.22 (0.17-0.29) [0.09-1.12]	0.21 (0.18-0.25) [0.07-1.12]
Urinary chromium, µg/g cr	0.17 (0.14-0.20) [0.06-0.32]	0.18 (0.15-0.21) [0.07-0.29]	0.17 (0.15-0.20) [0.06-0.32]
Urinary nickel, µg/g cr	1.30 (1.05-1.61) [0.66-3.25]	0.96 (0.72-1.29) [0.17-2.02]	1.10 (0.91-1.33) [0.17-3.25]

Data are geometric means (95% Confidence Interval) [minimum-maximum].

* Statistically significant difference ($p < 0.05$) between central and peripheral area using the Student's t-test.

cr: creatinine. RBP: retinol-binding protein.

Table 3: Multiple linear regression analyses of the biological parameter determinants of biomonitoring study participants, Ath, 2009.

Dependent variable	Independent variable	Partial r^2	β estimate	p-value
Children aged 2.5 to 6 years				
Blood lead, µg/l	Geographical area	0.400	-0.092	0.050
Children aged 7 to 11 years				
Blood lead, µg/l	Housing age	0.129	-0.141	0.005
	BMI	0.081	-0.020	0.016
Urinary cadmium, µg/g cr	BMI	0.159	-0.038	0.002
Adults				
Blood lead, µg/l	Gender	0.115	-0.158	0.001
	Alcohol consumption	0.072	0.010	0.027
	Age	0.057	0.010	0.020
	BMI	0.038	-0.010	0.033
Blood cadmium, µg/l	Smoking status	0.304	0.388	0.001
	Ferritine	0.046	-0.120	0.008
	Age	0.030	0.011	0.005
Urinary cadmium, µg/g cr	Age	0.211	0.022	0.001
	Smoking status	0.056	0.219	0.016

cr: creatinine. BMI: Body Mass Index.

smoking status is the main determinant of blood cadmium (explaining 30% of the variation of concentrations), urinary cadmium, more closely related to the body burden, is mainly influenced by age (explaining 20% of the variation of concentrations).

The effect of the consumption of locally grown vegetables was further assessed by testing mean differences of adjusted metal values between categories of locally grown vegetables consumption (no consumption, < 50% of the total consumption, and ≥ 50% of the total consumption) separately in central and peripheral areas. We did not observe any significant difference between consumption categories, whatever the metal, category of age, or geographical area.

Lifestyle determinants of blood lead levels in children aged 2.5 to 6 years were further assessed by area. No effect of sucking one's thumb, biting one's nails, or of the frequency of hand washing was observed. On the other hand, time spent outdoors is a significant determinant of blood lead levels in the central area, while not in the peripheral area. In the central area, children spending more than 5 hours/week outdoors have significantly higher blood lead levels than those spending less or

equal than 5 hours/week outdoors (24.8 µg/l vs. 16.5 µg/l respectively, $p = 0.009$). Moreover, the same effect of time spent outdoors is seen in children aged 7 to 11 years in the central area (19.4 µg/l when > 5 hours/week vs. 13.18 µg/l when ≤ 5 hours/week, $p = 0.015$) but not in the peripheral area. The link between blood lead levels in children and the time they spent outdoors, existing in the central area but not in the peripheral area, reinforces the hypothesis of exposure from environmental contamination that could come from the industries. However, even in the central area, and even in children who spent more time outdoors, the blood lead levels observed are comparable to levels observed in children of the same age in the general population, not exposed to a known specific environmental exposure (3, 4).

CONCLUSION

The metal concentrations found in the population of Ath are comparable to those found in other similar biomonitoring studies in the general population.

With the exception of the small difference in blood lead levels in children aged 2.5 to 6 years, human exposure to heavy metals does not differ significantly between the central area, where two non-ferrous metal industries are located, and the peripheral area. The consumption of locally grown vegetables in the surroundings of the industries does not appear to influence heavy metal concentrations of inhabitants. However, spending ≥ 5 hours/week outdoors is associated with an increased lead exposure of children living in the vicinity of these industries.



IMPACT ON PUBLIC HEALTH

This study allowed us to assess heavy metal exposure of the population of Ath, in a contaminated environment. Human biomonitoring is a useful tool providing authorities and affected populations with information on human load and consequential health effects of pollutants from the environment.

ACKNOWLEDGEMENTS

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Monitoring illicit drug use among female sex workers in Europe, 2000-2008

M. Roelands

INTRODUCTION

Popular beliefs as well as opinions from medical staff have suggested a relationship between illicit drug use (hereafter referred to as “drug use”) and sex workers (SW). From a public health perspective, it is important to investigate its validity, as decision makers in the field of prevention and treatment need to have a clear view on the needs of this vulnerable group, in order to provide the necessary services. Drug use among SW can endanger their health as it interferes with safe sex practices and can facilitate physical and mental abuse, and violence. Moreover, unsafe injecting drug use (IDU) is a risk factor for the spread of infectious diseases to the general population. The aims of this study were to describe the current status regarding the monitoring of illegal drug use among SW in European countries and to present an overview of the prevalence estimates.

METHODS

Sources of references

Literature was reviewed with PubMed to find peer-reviewed articles. Additionally, all yearly National Reports that the National Focal Points of all European countries provide to the European Monitoring Centre on Drugs and Drug Addiction (EMCDDA), in the years 2000 to 2008, were searched in order that unpublished reports from health care services were included. The National Reports ought to describe findings of all studies on the epidemiology of drug use in sex workers that are reported in the scientific and grey literature.

Inclusion criteria

A publication was included if it met all of the following criteria: it included information on female SW; an estimate of the prevalence of drug use in SW was provided; it was a scientific article or an (un)published report; the original document or the referring document was published between 2000 and 2008; and the geographical area was restricted to Europe.

RESULTS

Using PubMed over the period 2000- 2008, the 165 hits provided three studies that met the inclusion criteria. Moreover, information about the prevalence of drug use among SW was presented in 29 of the 222 National Reports on Drugs that were published between 2000 and 2008, considering 14 of 32 countries.

Prevalence of drug use in the total group

Only indirect estimates were found for some countries, such as personal estimates provided by outreach workers (Slovakia) (1), or estimates based on the assumptions that all SW on the street were known to a health service and all were drug users, and on an estimate of all SW in the country (Denmark) (2) (Table 1). In other countries, continuous recording of drug use in this population exists but it is very limited. It is only recorded whether the use of alcohol or illegal drugs was discussed when SW ask for information or help (Belgium) (3). Unfortunately, information on the prevalence of drug use and on the substances discussed was not available, despite the large samples of SW that were contacted every year.

In countries where some information on the prevalence of drug use in SW is available, the subject is studied through single surveys or through surveillance systems in the context of interventions focussing on the prevention of infectious diseases. Surveillance by health care and prevention services can be successful, suggesting increased use compared to the general population. However, apart from a general measure of the prevalence of drug use in SW, some sources provide no further information on the methods used or more detailed results (Italy) (4). Another source studied a vaccination campaign against hepatitis B in SW in three cities (Belgium) (5). Drug use and IDU were investigated through face-to-face interviews on an ongoing basis. However, only pooled data covering a 10-year period were presented. The working environment and the prevalence of IDU and non-IDU were mentioned, but no information was provided regarding specific substances.

More frequently, the topic has been studied through single surveys, which also suggest a high level of drug use. However, the scopes of the studies differ and often little information is available on the substances used and on the study methods. In one of the earlier studies, earning money to buy drugs or alcohol is mentioned as a reason for sex work, which is the only estimate of drug use prevalence (Ireland) (6, 7). A lack of information on methods and results was also the case in a study performed by health services in 3 cities (The Netherlands) (8). Prevalence of unspecified drug use was reported, but not the prevalence of specific substances, the number of IDUs and the time frame of the prevalence measure.

Some single surveys of SW presented more detailed information on the substances used. However, most often the study investigated the use of a limited number of substances, most often hard drugs. Moreover, the prevalence of using at least one substance was not stated and sometimes the prevalence of IDU was absent (Belgium, United Kingdom and France) (3, 9-12). On the contrary, some studies are, by their scope, e.g. HIV infection, limited to IDU (Czech Republic) (13). This study distinguished between injecting methamphetamine, heroin and cocaine.

Two studies described drug use in SW more fully. The *Lithuanian* AIDS Centre reported on the prevalence of drug use in the last 12 months and the use of specific drugs (cannabis, tranquillisers and heroin) among SW, including also the prevalence of IDU (14). And the *Latvian* Aids Prevention Centre performed a survey that investigated lifetime prevalence and last month prevalence regarding heroin, amphetamines, tranquillisers, cannabis, cocaine, ephedrine, heroin plus cocaine, and barbiturates (15). Last month prevalence of IDU was also included.

Prevalence of drug use by age group

Four studies took SWs' age into consideration and found a higher prevalence in younger age groups (Latvia, Estonia, Belgium and Estonia) (15-20).

Prevalence of drug use by working environment

A high level of hard drug use in street SW has been found repeatedly (The Netherlands) (8, 21-23). SWs' working environment proved to be an important factor in those studies that included it as a variable, allowing comparisons among subgroups within the same study to be made. Prevalence of IDU has been found to differ in persons depending on whether they work in private places, in bars or on the street (Belgium) (24). The importance of careful sample selection and reporting was also demon-

strated in another study. Prevalence of the use of any hard drug in the last 6 months was found to differ by working environment (street prostitution districts versus clubs) (The Netherlands) (8). In Estonia (19, 20) prevalence of lifetime use was found to be higher in SW who were offering their services in public places compared to SW who were selling their services via phone/advertisement and SW who were working for a company.

Few surveys included different substances, but regarding all substances, all of these studies found the highest prevalence of use among street prostitutes. All were conducted in the United Kingdom. Outdoor workers were found to use more crack and cocaine in the past six months compared to indoor workers (25, 26). Information on the sample, estimates of the prevalence of specific substances and information on the number of IDUs were absent. Prevalence of daily use of crack and heroin in the last 30 days was found to be higher in street SW compared to sex parlour workers (27, 28). Similar differences were found in a small study among street-based and indoor SW (29).

Prevalence of drug use by geographical area

A *Romanian* study in 12 cities took geographical area into account (30). Injecting heroin was clustered in one city. Although information on the composition of the sample regarding the working environment and on the use of other substances was not available, the results suggest differences in drug use prevalence and patterns of use between cities and regions in one country.

Trends in drug use

Although information on trends in drug use in this population is scarce, available data show a considerable increase in drug use. A surveillance system of drug use in SW in three cities found an increased prevalence of drug use between 1998 and 2006 (Bel-

gium) (18). A survey of street SW from most cities in Lithuania compared the prevalence of illegal drug use in 1998 with that of 2001 (Lithuania) (14). However, it was not reported whether this increase was found for all substances and was equally found for IDUs. The level of crack use among SW has been reported for two cities in the United Kingdom (10, 31). An opposite trend was noticed in Norway (32), but little information about the study is available.

CONCLUSION

A limitation of this literature review is the inclusion of mainly unpublished reports. The use of grey literature has some inherent limitations: information on the study methods (sample, measures, data collection) is often not available as it is often not included in these reports, a copy of the original report is hard to find and the report is often unavailable in English. This hampers critical reflection on the methods used. However, the lack of research published in peer-reviewed journals necessitated this approach. The National Reports to the EMCDDA proved to be a useful additional source of additional references and information in this context, including information from the grey literature.

In spite of this intensive search, this review showed that information on the situation is completely absent in many countries. Moreover, in most countries where data on the subject was available, information was limited. Single studies were few in almost all countries. Recording drug use in the context of interventions focussing on the prevention of infectious diseases was only exceptionally a source of information on this subject.

It was also a significant finding that, in most cases, the information presented in the National Reports was incomplete. Regarding the content of the studies, there were various shortfalls including: the prevalence of using any drug was often missing; only a limited number of substances

Table 1: Prevalence of illicit drug use in sex workers by study, presented in National Reports 2000 to 2008.

Country	Name of study	Year of data collection	Sample	Measure	Prevalence
Belgium	EUROPAP	2001	City: unknown N = 83;	Current use	Marijuana: 18% (15/83) Cocaine: 6% (5/83) Speed: 5% (4/83)
	Espace P	1998-2002	3 cities (Liège, Namur, Charleroi) N = 934; Mixed environments Vaccination campaign of outreach service	Unknown	IDU Total sample: 7.4% (49/664) In private places: 4% In bars: 7% In street walkers: 32%
		1998-2007	3 cities N = 1649; 97% females Mean age = 31.4 y 25% missing information on drug use Mixed environments (details available: a.o. 4% street walkers) Vaccination campaign of outreach service	Unknown	Non-injecting drugs: 11.5% IDU: 5.6% Non-IDU in period 1998-2006 < 25 y: 9.8% 25-34 y: 8.4% ≥ 35 y: 1.7%
	PASOP	2005 and 2006	In 2005: N contacts = 2,674 N SW = 745 In 2006: N = 2,371 N SW = 703 Registration with administrative aim	“Use of drugs or alcohol was discussed in contacts when SW asked for information or help”	In 2005: 2.1% In 2006: 1.8%
Czech Republic	Bruckova <i>et al</i> , 2006		Multi-cities survey N = 585	Lifetime prevalence	IDU: 10.1% (59/585)
Denmark		1999 and 2001	Estimates by drop-in centre for drug-using SW	Number of drug-using SW on street divided by number of SW in country	Drug using street SW: 10% (620/5,450 to 7,800)
Estonia	Trummal	2005	City: unknown N first time = 106; N repetitive clients = 72 SW that are clients of health care centre Survey	Last 6 months prevalence	In first time clients: 30% In repetitive clients: 28% “Half” of the 18-24 year olds Above 24 years old: 6% Amphetamines and cannabis; only few cocaine.
	Trummal	2006	N = 227 RDS and interviewers' contacts	Lifetime prevalence	Drugs: 66% ≤ 24 y: 75% 25-34 y: 70% Offering in public places: 84% Selling via phone/advertisement 53% Working for company: 49%
				Last 4 weeks prevalence	Drugs: 26% (60/227)
France	Cagliero & Lagrange	2002	Paris, Marseille, Lille N = 165 Survey	Lifetime prevalence (“experimented”)	Cocaine or crack: 20% (30/165)
				Recent use	Heroin: 5%
Ireland	O'Connor	1995	Region: unknown N = 84 Survey	Current use? (not explicit)	Drugs or alcohol: 11% (9/84)

Country	Name of study	Year of data collection	Sample	Measure	Prevalence
Italy		1996	Region: unknown N = 334 Outreach service	Drug dependence	Drugs: 20% (67/334)
Latvia		2002	N = 92 Mean age = 25.7 y (min 15, max 47) Survey	Lifetime prevalence	Drug use: 75% 15-19: 100% (14/14) 20-24: 89% (31/35) 25-29: 75% (12/16) 30-34 y: 41% (7/17) ≥ 35 y: 50% (5/10)
				Last month prevalence	Drug use: 63% IDU: 62% Heroin: 90% Amphetamines: 26% Tranquilisers: 16% Cannabis: 14% Cocaine: 4% Ephedrine: 4% Barbiturates: 2% Heroin + cocaine: 4%
Lithuania		1998	Region: unknown N = 73 Street sex workers Survey	Unknown	Use of drugs or psychotropic substances: 23%
		2001	Region: unknown N = 220 Street sex workers Survey	Unknown	Use of drugs or psychotropic substances: 65%
		2000	Vilnius N = 96 Survey???	Last 12 months prevalence	Drugs: 80% Cannabis: 65% Tranquillisers: 45% Heroin: 37% IDU: 34%
Slovakia		Probably 2003	Estimates provided by out-reach workers	Unknown (current use?)	IDU: between 5% and 53% depending on working environment
The Netherlands	EUROPAP	2002-2003	Amsterdam, Heerlen, Twente N = 80+ 80 + 40	Current use? (not explicit)	Drugs: 34%
		2002	Rotterdam N = 109	Last 6 months prevalence	Hard drugs: 32% Cocaine: 26% Crack: 24% Heroin: 20% Street sex workers: 54% hard drugs Sex workers in clubs: 10% hard drugs
	Korf <i>et al.</i>	2004	Amsterdam N = 92 Median age = 40 y (min 13, max 58) Street sex workers "Field study"	Last month prevalence	Cannabis: 51% Cocaine: 9% Crack: 89% Heroin: 68%
				Lifetime prevalence	Cannabis: 87% Cocaine: 61% Crack: 91% Heroin: 81%
Norway		2003-2004 2-3 years earlier	Oslo SW in contact with prostitution competence centre	"Drug addiction"	Drugs: about 30% Drugs: nearly 80%

Country	Name of study	Year of data collection	Sample	Measure	Prevalence
Romania	ARAS	2005	12 cities N = 395 Survey Snowball sampling	Previous year prevalence (in 2004)	IDU heroin (total sample): 11% IDU heroin in Bucharest: 33%
United Kingdom	Chrurch <i>et al.</i>	unknown	Leeds, Glasgow and Edinburgh N = unknown Outdoor and indoor workers Survey	Last 6 months prevalence	Illegal drugs Outdoor workers: 93% Indoor workers: 69% Crack Outdoor workers: 32% Indoor workers: 4% Cocaine Outdoor workers: 17% Indoor workers: 15%
	Jeal & Salisbury, 2004		N = 71 sex parlour workers N = street workers	Daily use in last 30 days	Parlour workers Drugs: 23% Crack: 7% Heroin: 6%
					Street workers Drugs: 96% Crack: 87% Heroin: 85%
	Jeal & Salisbury, 2004		N = 71 street ers	Current dependency problems	Alcohol or drugs: 96% (68/71) Heroin: 83% (59/71) Heroin and crack: 78% (55/71) IDU: 60% (41/71)
	Ward <i>et al.</i>	1989 – 91	London	Lifetime prevalence	Crack cocaine: 11%
		1995 – 96	London N = 143	Last 3 months prevalence Lifetime prevalence	Crack cocaine: 10% (14/143) Crack cocaine: 34% (48/143)
	McCullagh <i>et al.</i>	1996 2004	Liverpool N = unknown Street sex workers	unknown	Crack cocaine: 17% Crack cocaine: 75%

were reported; when using a general measure of drug use, the substances included were not specified; the focus of the study was limited to IDU or, in other cases, IDU was not studied/reported; only a few studies presented prevalence estimates by age group, although age is an important background variable.

Moreover, basic aspects of the methods used were often not described, such as a definition of the study population, the sample selection method, the sample size and the level of non-response. The time frame of the prevalence measure was not made explicit or loosely defined, such as current use or recent use. Therefore, it was not possible to reflect on the validity and reliability of the estimate presented. Often infor-

mation on the unpublished source could not be retrieved. References were difficult to trace and to control by researchers. A multitude of factors are responsible for the existing lack of information on the prevalence of drug use in SW. An important factor is situated at the cultural level: currently in Europe, SW, mostly called prostitution, as well as illegal drugs are sensitive subjects. Researchers and politicians

IMPACT ON PUBLIC HEALTH

The project revealed:

- Increased prevalence of drug use in sex workers compared to the general population;
- That most countries lack surveys and surveillance systems providing estimates of sex workers' drug use;
- The need to include more extensive presentation of study methods in National Reports to facilitate interpretation of results.

deciding on research financing are affected by this factor. Moreover, in many countries, SW who use drugs, attempt to remain undercover because of the double illegality. Apart from being liable to prosecution because of drug use, in many countries they face legal prosecution because of their job, i.e. sex work. A third factor is the setting of

the interview, which involves time, as SW are contacted at their place of work. Fourth, the main focus of organisations in the field is on performing preventive interventions, such as vaccination and providing harm reduction information, not on research. In this multi-cultural group, language barriers can also hamper an interview.

As valid as these barriers may be, up-to-date information on the situation is needed to develop preventive and curative health interventions for SW. Surveillance or repeated studies of drug use in SW should be considered in order to provide this information in the future.

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The results of successive health interview surveys show that socio-economic inequalities in health-related lifestyles have persisted over the last decade

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INTRODUCTION

Numerous studies show, almost without exception, that people with a low social status have a poorer state of health, poorer living habits and lower life expectancy than people with a high social status. These social inequalities can be found in most European countries (1). Belgium is, in this context, not an exception. Recent research in Belgium has shown inequalities in morbidity and disability free life expectancies (2), mental health (3), access to health care and mortality (4-6) and several health-related lifestyles. An essential question is whether social inequalities in health are a steady phenomenon or whether indications can be found showing that these inequalities tend to increase or decrease. Recent European research indicates that social inequalities are quite stable or do tend to increase (7, 8). In this contribution, results of the successive Belgian Health Interview Surveys (HIS) will be analysed to assess if, and to what extent, social inequalities in health-related lifestyles have changed during the last decade.

DATA AND METHODS

The HIS is a large scale, recurrent and cross-sectional survey performed in 1997, 2001, 2004 and 2008. Since the results of the last survey have not yet been completely analysed, only the three first surveys will be addressed. Methodological details of the HIS can be found on the website (<http://www.wiv-isp.be/epidemio/epinl/index4.htm>).

Alongside extensive data on health related topics, data on the socio-economic profile of the participants are also gathered in the survey (education, income, profes-

sional status, etc.). In this contribution, we have opted to assess the social position by means of the equivalent household income. In order to make the income of households of different size and composition comparable, the adjusted OECD equivalence scale was used in which the monthly available income is divided by the sum of 1.0 for the first adult in the household, 0.5 times every additional adult and 0.3 times every child (9). For each survey year, income quintiles were calculated, resulting in 5 groups, each covering 20% of households: the first quintile refers to the 20% of households with the lowest equivalent income, the fifth to the 20% of households with the highest equivalent income. By using year-specific income-quintiles, changes in the global level of incomes and in the distribution of incomes are taken into consideration. When referring to the income quintiles it should be understood as 'members of the household that belong to a specific quintile'.

When analysing the relationship between social position and health-related lifestyles throughout time, one has to account for changes in age- and gender composition of the population. To account for this, it was decided to present the results stratified for gender and standardised for age, using the 2004 population as the standard population.

To assess differences in social inequalities in health, three parameters will be used:

- A first parameter is the difference in prevalence between those in the lowest income quintile and those in the highest quintile. This parameter is quite straightforward, yet it does not take into account the prevalence of the remaining income quintiles.
- A second parameter is the population attributable risk, calculated as the difference between the overall prevalence and the prevalence in the highest income quintile, expressed as a percentage of the overall prevalence. It can be understood as the proportional reduction of the overall prevalence that could occur in the hypothetical case that the prevalence in all income quintiles would be the same as the one of the highest quintile.
- The third parameter is the relative risk (RR) between the lowest and the highest income group and can be understood as the proportion of persons with a worse health related life style among those in the lowest income quintile divided by the same proportion among those in the highest income quintile. An RR of 1 means no differences in lifestyle between the lowest and the highest income group. An RR higher than 1 implies that the risk of a worse lifestyle is higher among those belonging to the lowest quintile. RRs will be presented with their 95% confidence intervals (CI). An easy-to-grasp interpretation of a 95% CI is that in 95% of all possible surveys using the same method in the same population, the value of the estimated RR will belong to the interval.

The HIS provides many indicators of health status, lifestyle and medical consumption. In this contribution, only social inequalities in four lifestyle indicators in the Belgian population are presented:

- The prevalence of *obesity* in adults (18+ years), based on the (self reported) height and weight. The Body Mass Index is the individual's body weight divided by the square of his/her height. In accordance with the criteria defined by the World Health Organisation, a BMI of 30 or higher, suggests that the person is obese.
- Prevalence of smoking is assessed by comparing daily or occasional smokers with current non smokers (15+ years)
- The prevalence of binge drinking is measured by asking respondents whether, in the last six months, they have ever drunk six alcoholic beverages or more on one day. This group is compared to abstainers and those not indicating binge drinking in this reference period.
- Information on leisure time physical inactivity is collected by asking respondents to select what best describes their leisure time physical activity, ranging from heavy sports activities to merely sedentary activities. Comparable data on the level of physical activity throughout the different survey years is only available for leisure time physical activity and is based on how respondents describe their leisure time. For the 2004 survey, it was decided change the mode of data collection concerning these questions. Comparing the results from 2004 with those from 1997 and 2001 is consequently affected.

RESULTS

Concerning the prevalence of obesity, an unusual development was found in the male population: in 1997, the prevalence of obesity seemed not to be related to the income position (*Table 1*). This is reflected in all parameters: a prevalence difference of only 1%, a limited (and negative) population attributable risk and a non significant relative risk. In 2004 the situation changed; compared to the highest income quintile, the prevalence of obesity is 4.3% higher in the lowest quintile. From a negative value in 1997, the population attributable risk is 43.1% in 2004. This implies that, in the case where the prevalence of obesity for quintiles being the same as the one for the highest quintile, the overall prevalence would drop by 43.1%. The relative risk, non significant in 1997, is 2.4 (95% CI: 1.7 – 3.4) in 2004.

This change of prevalence in the male population is not reflected in the female population: social inequalities between the lowest and highest quintiles were already observed in 1997 and appear to be quite stable over time. In 2004 the relative risk of being obese in the female population in the lowest income quintile is twice as high (RR: 2.95% CI: 1.5 – 2.6) compared to this risk in the female population belonging to the highest quintile.

During the last decade, the percentage of males (aged 15+) that describe themselves as daily or occasional smokers has decreased from 29.3% in 1997 to 26.7% in 2004 (non significant decrease). For all years of the survey, the picture remains similar with a higher prevalence of smokers in the lowest income quintiles. For example, in 2004, 35.4% of males in the first income quintile describe themselves as being a smoker, whereas for the same year, this value is 23.9% for the highest quintile, providing a prevalence difference of 11.5% and a relative risk of 1.4 (95% CI: 1.3 – 1.7).

The prevalence of (daily or occasional) smoking in the female population is lower than in the male population. In 2004, 18.6% of all females indicated being a smoker, a percentage that hardly changed between 1997 and 2001. Like in the male population, clear social inequalities in smoking behaviour can be observed in females, with higher prevalences in lower income quintiles. For example, in the hypothetical case that the prevalence of smoking in all quintiles would be the same as the one observed for the highest quintile, the overall prevalence of smoking would drop to not less than 36.5% in 2004. The inequalities in smoking behaviour by income quintiles have not changed over time.

The indicator on excessive alcohol consumption is interesting since it gives an example for which social inequalities are inverted; in the male population the prevalence is higher in the highest income quintiles. This phenomenon is observed in all 3 surveys. In males, the overall prevalence of the excessive use of alcohol is quite stable over the years (around 35%). The population attributable risk shows that, in the case where all males would follow the “example” of males belonging to the highest income quintile, the overall prevalence of excessive alcohol consumption would increase by 10% (figures for the survey year 2004). In females, the rise in the prevalence of people reporting binge drinking has increased over the years; from 9.4% in 1997 to 13.9% in 2004. This increase can be found across all income quintiles but is more pronounced in the highest quintiles. This is reflected both in the prevalence differences (-3.4% in 1997, -8.2% in 2004) and especially in the evolution of the population attributable risk (-9.6% in 1997, -32.4% in 2004).

Table 1: Inequalities in health damaging lifestyle (summary) by income quintile, year and gender, Health Interview Survey, Belgium, 1997, 2001 and 2004.

	Males			Females		
	1997	2001	2004	1997	2001	2004
Obese						
Overall prevalence	9.5	10.5	10.2	9	11.3	11.5
Prevalence difference	-1	1.1	4.3	6.8	8.6	7.8
PAR	-7.4	21.9	43.1	53.3	47.8	37.4
Relative Risk (95% CI)	0.9 (0.7 – 1.3)	1.1 (0.8 – 1.5)	2.4 (1.7 – 3.4)	2.6 (1.8 – 3.7)	2.4 (1.7 – 3.4)	2 (1.5 – 2.6)
Smoker						
Overall prevalence	29.3	26.8	26.7	18	18.4	18.6
Prevalence difference	14.7	6.8	11.5	7.1	7.9	11.2
PAR	18.4	7.8	10.5	48.9	34.8	36.5
Relative Risk (95% CI)	1.5 (1.3 – 1.7)	1.2 (1.1 – 1.4)	1.4 (1.3 – 1.7)	1.6 (1.3 – 1.9)	1.5 (1.3 – 1.8)	1.8 (1.6 – 2.2)
Excessive use of alcohol						
Overall prevalence	35.1	39.3	37	9.4	13.8	13.9
Prevalence difference	-9.3	-16.3	-13.2	-3.4	-3.5	-8.2
PAR	-19.1	-19.6	-10.5	9.6	7.2	32.4
Relative Risk (95% CI)	0.8 (0.7 – 0.9)	0.7 (0.6 – 0.8)	0.7 (0.6 – 0.8)	0.6 (0.5 – 0.8)	0.8 (0.6 – 1.0)	0.7 (0.6 – 0.9)
Physical inactivity						
Overall prevalence	34.7	32.5	23.1	43.4	42.7	34.1
Prevalence difference	11.5	15	10.9	15.7	15	19.6
PAR	27.1	22.7	22.1	19.8	19.7	21.4
Relative Risk (95% CI)	1.5 (1.2 – 1.8)	1.7 (1.4 – 2.0)	1.7 (1.3 – 2.1)	1.5 (1.3 – 1.7)	1.5 (1.3 – 1.7)	1.7 (1.5 – 2.0)

Considerable social inequalities can be observed in both males and females for all survey years in regard to leisure time physical activity. Males and females in the lower income quintiles more frequently report that they hardly do any leisure time physical activity. The prevalence difference is more pronounced in females than in males. Also, the relative risk remains quite stable; the risk of physical inactivity during leisure time is 70% higher in the lowest quintile compared to the highest quintile.

CONCLUSION

Despite the limited number of health-related lifestyle indicators addressed in this contribution and the restricted character of the applied statistical analysis, the results show that, in general terms, social inequalities in lifestyle do exist and persist over the years. It should be mentioned that the reference period of 7 years (1997 – 2004) is perhaps too short to adequately describe trends in social inequalities.

As mentioned in the introduction, persisting social inequalities in health-related lifestyles are not a specific Belgian phenomenon, but can be found in all other European countries and regions. The complex mechanisms responsible for this remain under debate. Some authors stress the importance of 'cultural capital' that interacts

with the economic and social capital in the social structuring of people's health chances and choices while others underline the phenomenon of 'reproduction' of health-damaging behaviour in the socialisation process (with parents as the peer socialisation agents) (10). An interesting perspective is the one of the (partly social determined) future time perspective, a disposition to ascribe high value to goals in the future and to anticipate, in the present, the long-term consequences of a potential action (11). Perhaps in lower income groups, this perspective is less prevalent. It could explain why a policy aimed at preventing unhealthy behaviour – by pointing out its long-term health consequences – risks becoming more successful in advantaged groups, with growing inequalities as a latent result.



IMPACT ON PUBLIC HEALTH

Even in a modern welfare state such as Belgium, social inequalities in health-related lifestyles exist and seem to persist over time. Preventive action should take account of the economical, social and cultural specificities of their target groups.

SOURCE

Vranken J, Campaert G, Dierckx D, Van Haarlem A, (red). Armoede en sociale uitsluiting – Jaarboek 2009. Leuven: ACCO, 2009, 382 pages.

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Tackling health inequalities in Belgium: some results and policy recommendations

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INTRODUCTION

It has been established in the scientific literature that, in general, mortality and morbidity rates follow a social gradient whereby those at the top of the socio-economic hierarchy (high educational level, high revenues or high professional grade) have lower mortality and morbidity rates compared to those at the bottom of this hierarchy. Such health inequalities have been detected in Belgium where studies have found socio-economic disparities using a series of health indicators including life expectancy, healthy life expectancy, self reported health, access to health care, and health behaviours such as tobacco use, eating habits and physical activity (1, 2). These health inequalities are a major challenge for policy makers as they are the causes of inequitable and preventable health problems in the population. Therefore, there is a critical need to develop effective policies and interventions to reduce these inequalities in health.

It is within this context that the TAHIB project (Tackling Health Inequalities in Belgium) has been initiated in 2006. This project aims at generating a better understanding of the determinants of health inequalities in Belgium and at proposing effective policy recommendations. Researchers from three institutions have collaborated on this project: the Scientific Institute of Public Health (WIV-ISP) that is also the coordinator, the Vrije Universiteit Brussel (VUB), and the Université

Catholique de Louvain (UCL). One of the objectives* of this project is to describe the time trends in social inequalities in mortality and morbidity. This is an important endeavour for several reasons. Firstly, exploring the trends in health inequalities provide policy makers with up-to-date information that can guide the development of effective intervention programmes and policies. Secondly, such research can help predict future trends. Thirdly, findings from such work enable researchers and policy makers to assess the impact of different health promotion programmes on different population subgroups and to identify which interventions are more effective in reducing social inequalities.

As part of this project, Deboosere and colleagues (3) have examined the evolution of life expectancy by educational level at the turn of the century in Belgium. They found that life expectancy increased for all educational groups, but this increase was more marked for those with higher educational levels. As a result, inequalities in life expectancy by educational level increased between 1991 and 2001. Yet, studying the trends in mortality is not sufficient to examine the trends in the health of the population. To add a quality dimension to the quantity of years lived, Van Oyen and colleagues (4, 5) examined the evolution in socioeconomic inequalities in healthy life expectancy between 1997 and 2004. For this, they used the disability free life expectancy (DFLE) indicator, which divides the years of remaining life in disability free

years and years spent in disability. In this paper, we will present the research described above conducted by Van Oyen and colleagues^o.

METHODS

To assess DFLE, two types of data are needed. Data on mortality enable the estimation of the total life expectancy. Data on the prevalence of different health states are applied to the life table to estimate the person-time lived with and without disability.

Mortality data by educational level were generated based on two sources of data: the national censuses of 1991 and 2001 that provide information on the distribution of the population by educational level, and the national register that provides information on mortality and emigration for the periods 1991-1994 and 2001-2004. These two datasets were linked based on a unique identifier present in both the census and the register files. As a consequence, the socio-demographic characteristics in the census were unambiguously matched to mortality and emigration data in the population register with a follow-up period of three years after each census.

The prevalence of the disability was obtained from the 1997 and 2004 Health Interview Surveys. The methodological approach did not change between the two surveys[•]. The National Register was used

* The three other objectives are: 1. Analyze social inequalities in health in relation to the longitudinal changes in social position and study the effect of changing social position on health, 2. Study the effect of macro-social factors on social inequalities in mortality and morbidity, 3) Evaluate the time trends in the association between social inequalities in health and social inequalities in health behaviors.

^o Seven other studies were conducted in the context of the TAHIB project. Details of these studies are found in the final report (5).

[•] <http://www.wiv-isp.be/epidemiologie/index4.htm> (see protocol) (accessed May 5, 2009)

as a sampling frame to select a sample using a multistage sampling method (6). The number of subjects aged 25 years and over in the 2 surveys was respectively 6,763 and 9,271. Life expectancies without limitations were estimated by gender and educational level starting by age 25 up to age 100 using the Sullivan method (7).

Socio-economic position

The socio-economic position was defined by the highest level of educational attainment. The census and Health Interview Survey used the same question and response categories. Based on the International Standard Classification of Education (ISCED) (8), the educational attainment was recoded as one of five categories: no diploma, primary education, lower secondary, higher secondary and tertiary education.

Health domain

Activity restriction was used to define disability and was classified based on difficulties in doing one of 7 ADL-functions (transfer in and out bed, transfer in and out chair, dressing, washing of hands and face, feeding, going to the toilet, continence), or having mobility problems (walking a distance of maximum 200 metres) or having problems in seeing (ability to recognise a person at a distance of 4 metres even wearing glasses) or in hearing (ability to follow a TV program with the volume turned higher than acceptable to others even with a hearing aid) (9).

Summary measures of socio-economic inequalities

Guidance for measuring inequalities in health suggests using both absolute and relative measures (10). We firstly used a population attributable life loss index (PALL). This index represents the increase in overall DFLE that would be achieved if all groups had the DFLE of the best group. As the distribution of educational level in the population has changed between 1990 and 2000, we need to account for this change

while studying the evolution in inequalities. For this, we used the Composite Index of Inequality, which accounts for the population size of the educational classes. The absolute Composite Index of Inequality (CII_{abs}) is the sum of the weighted (w_i) difference in the DFLE between the educational groups and the highest educated category, with w_i proportional to the population size of the educational categories. The relative Composite Index of Inequality CII_{rel} is the CII_{abs} divided by the overall population's values and is interpreted as the percentage change in the overall health expectancy that would occur if all socio-economic groups had the health expectancy of the population with highest educational level. For example, for the DFLE, the CII_{abs} and CII_{rel} are estimated as:

$$CII_{abs} = \sum_i (DFLE_{Highest} - DFLE_i) * w_i$$

$$CII_{rel} = \frac{CII_{abs}}{DFLE_{Belgium}}$$

RESULTS

The distribution of the educational attainment in the census of 1991 and the census of 2001 is presented in Table 1. Between the two censuses there was a substantial increase in the educational attainment. In 2001, about one quarter of the population has a tertiary education degree compared to less than 20% ten years earlier. There are fewer people without a diploma in 2001 (males 10%, females 12%) compared to 1991 (males 26%, females 32%).

The results show that in 1997, there is a gradient in DFLE where those at the top of the educational hierarchy live longer without disability than those at the bottom of this hierarchy. In 2004, men and women with the highest educational level continue to live even longer without disability than in 1997 (Table 2). For instance, men with a tertiary education have an increase of 2.86 years in their DFLE while those without a diploma had an increase of 1.28 years. When we look at the absolute inequality at the population level, we note an increase in educational inequalities in DFLE by 0.44 for males and 0.86 for females.

A different picture emerges when we take account of the general improvement in the educational distribution of the population. When we account for these changes, we see for men decreasing inequalities in absolute (-1.15 years) and relative terms (-4.01%). For women, inequalities persist even after accounting for the changes in the educational distribution (absolute difference is 0.44 years and the relative difference is 0.50%). These results were not statistically significant. Therefore, we can conclude that at best there is no evidence to suggest that social inequalities in DFLE have been reduced in the last decade.

As a conclusion, findings from the TAHIB project suggest that inequalities in mortality and morbidity between educational groups have increased during the last decade in Belgium. However, when we account for the general improvement in the educa-

Table 1: The distribution by educational level of the total Belgian population aged 25+, 1991 and 2001.

Education	Men	Men	Women	Women
	1991	2001	1991	2001
	%	%	%	%
No diploma	26.1	10.3	32.0	11.8
Primary education	14.9	15.5	17.2	19.2
Lower secondary	18.3	23.7	16.0	23.0
Higher secondary	23.2	25.0	19.7	22.3
Tertiary	17.6	25.4	15.1	23.8

Table 2: Disability Free Life expectancy (DFLE) and summary indices of inequality at age 25 by educational level and sex, 1991 and 2001.

Education	Men	Men	Change	Women	Women	Change
	1991	2001	91-01	1991	2001	91-01
	%	%	%	%	%	%
No diploma	26.5	27.8	1.3	33.3	28.9	-4.4
Primary education	32.6	36.7	4.1	34.7	36.3	1.6
Lower secondary	37.4	39.7	2.3	40.9	42.0	1.1
Higher secondary	42.6	41.5	-1.1	43.4	41.3	-2.1
Tertiary	43.5	46.3	2.8	44.7	47.1	2.4
Total	38.1	40.5	2.4	38.9	40.4	1.5
PALL	5.4	5.9	0.5	5.8	6.7	0.9
CI _{abs}	7.3	6.2	-1.1	6.3	6.7	0.4
CI _{rel} (%)	19.3	15.3	-4.0	16.1	16.6	0.5

tional level of the population, we find that, in some cases, inequalities did not change overtime or they even decreased. This finding points to the importance of tackling upstream factors, such as education for reducing health inequalities in the population.

POLICY RECOMMENDATIONS

The findings reported above examine the trends in socio-economic inequalities in mortality and morbidity. In the TAHIB project, other issues have also been examined resulting in four policy recommendations. These recommendations are not only based on the findings of TAHIB, but also on the European experience in setting policies to reduce health inequalities and on the discussions with the steering committee that followed up the project for four years.

Raise the awareness on the issue of the social gradient in health in contrast to the binary approach focusing on poverty, which is often the most common political approach

The presence of the social gradient in health is not new. It has been repeatedly shown in the scientific literature that health inequalities do not only exist be-

tween the extreme categories of the society (rich versus poor or educated versus non-educated); rather social inequalities in health follow a distribution that is socially stratified in the population where each social category shows mortality and morbidity rates that are higher than the social category that precedes it on the hierarchy.

It is important to highlight this issue in order to avoid the focus being on the issue of poverty but rather discuss poverty in the context of health inequalities.

Appoint a governance body to tackle health inequalities

In Belgium there is no institutional stakeholder responsible for tackling health inequalities. This is an important issue because tackling inequalities is not the responsibility of one sector but rather a number of sectors such as health, social work, and employment. Therefore we need an institutional body that coordinates and supports policy actions, interventions, and research in this area.

The need for such a body is based on the experience of other European countries in reducing health inequalities. A main component of successful policies is political commitment (11) and political commitment cannot exist without responsibility.

Develop a national action plan to reduce health inequalities

In Belgium, there are a number of initiatives to reduce inequalities, but these initiatives are scattered. Therefore, there is a need to develop a national action plan to coordinate and develop actions to reduce inequalities. The basis of this plan may be the conceptual framework used by Michal Marmot and his colleagues in the WHO Commission for Social Determinants on Health (12). This conceptual framework recommends tackling inequalities at the root of the problem by tackling upstream factors such as education.

Develop a research strategy to better understand, monitor and reduce health inequalities and to provide the framework for evidence-based policy and policy evaluation

The knowledge about health inequalities in Belgium is already sufficient to be able to start tackling health inequalities. However, a number of questions need further exploration to effectively reduce health inequalities. One of the issues that requires greater insight is how to intervene to reduce health inequalities. In other words, we already know a lot about the problem but not enough about the solution. This recommendation is echoed by major

European and international researchers in health inequalities such as Michael Marmot (12) and Johan Mackenbach (11).

entry points that are evidenced based. This takes political commitment, strategies that are effective, sustainable and integrated, and the readiness to engage in the long term.

CONCLUSION

Finally, it is important to emphasise that the elimination of these inequalities cannot be achieved in the near future, but it is possible to reduce them to levels that are more acceptable. The appropriate solution is not solely to invest even more in the health care system and in new technologies, rather it is important to tackle the roots of these inequalities and implement focused actions based on a range of policy

IMPACT ON PUBLIC HEALTH

The results and policy recommendations of this project will be used by the King Baudouin Foundation to raise awareness about social inequalities in health among the general public and among politicians, and to propose solutions to reduce these inequalities.

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SOURCE

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COMMUNICABLE AND INFECTIOUS DISEASES DIRECTORATE

Introduction by Dr Michaël Kalai

The Communicable and Infectious Diseases Directorate (OD) was officially established by a Ministerial Decree on the 26th of August 2008. It represents the final stage of a major reform project carried out by the WIV-ISP leading to the merger between the former Pasteur Institute Department and the Bacteriology, Virology and Mycology divisions of the Microbiology Department. This project, carried out in the frame of the business plan re-engineering-Project Jenner, started in 2007 and its recommendations were validated in 2008. The primary phase defined the medium-term research strategy of the new scientific direction and aimed to answer two questions:

1. What are the scientific themes that should be developed, maintained, reduced, transferred or abandoned?
2. What means - human and financial - specific (per theme) and general (the entire scientific direction) are deemed necessary to achieve the strategic orientations proposed?

Answers to these questions led to the second phase concerning the ongoing reorganization of the OD, which was one of the main challenges facing the WIV-ISP in 2009.

The primary mission of the new Communicable and Infectious Diseases Directorate is the early and rapid detection of existing or (re)-emerging infectious and communicable diseases in Belgium, their prevention and treatment. The Directorate is a recognized centre of excellence for the research of infectious diseases. It plays an important role in the Belgian, European and international network of surveillance, reference laboratories and centres of expertise for infectious diseases and food safety. Through its work and expertise, or in collaboration (coordination of surveillance and crises management) with other Directorates (notably the OD Public Health and Surveillance), the Communicable and Infectious Diseases Directorate reliably informs policymakers and the public on new developments in public health, security of the food chain, food and the environment.

The OD assures national and international surveillance of multiple pathogens via its National Reference Laboratories (NRL) including: NRL-TB and mycobacteria, NRL-Salmonella and Shigella, NRL-Listeria, NRL-Neisseria, NRL-Meningococcus, NRL-aminoglycosides resistance (resistance to antibiotics: *Streptococcus pneumoniae* and *Staphylococcus aureus*), NRL-Phage Typing [*Salmonella* and *Staphylococcus aureus* (MRSA)], NRL-food microbiology, NRL-botulism & tetanus, NRL-Human or animal toxoplasmosis, NRL-Influenza, NRL-Measles & Rubella, NRL-viral hepatitis, NRL-Rabies, NRL-Medical Mycology.

The multiple services provided by the OD include: identification, genotyping, serological & molecular diagnosis, molecular epidemiology and surveillance of bacterial, viral, fungal or parasitic pathogens, in human and animal clinical samples and in food. The OD conducts investigations and identifies pathogens in indoor environments: hospitals, workplaces,

schools and housing. Through a network of four sampling stations, it monitors air quality by identifying and measuring the quantity of allergenic pollen and fungal spores in the open air in Belgium.

The research projects carried out by the OD focus on: research and development of extraction and identification methods of pathogens, biotoxins, rapid identification of markers of resistance to treatment, development of animal and cellular models of infection for the study of virulence mechanisms and development of new prevention (vaccine) and treatment methods. The OD participates in epidemiological surveys on the prevalence and immunization coverage of viral or bacterial diseases. It also studies the immunological mechanisms responsible for allergy and pulmonary hyperreactivity associated with mould spores.

These scientific activities, realised in the two WIV-ISP sites (Ixelles/Elsene and Uccle/Ukkel), were organised within 5 functional programmes (Bacteriology, Virology, Mycology & Aerobiology, Immunology & Vaccinology and Tuberculosis & Mycobacteriology) which are supported by transversal platforms and themes including an animal facility. Access to high containment biosecurity level L3 laboratories is provided for handling (re)emerging pathogens representing an important risk to public health.

Bacteriology: The programme regroups the reference and research laboratories assuring the surveillance, diagnosis, typing and study of bacterial pathogens and toxins (excluding mycobacteria), foodborne pathogens and human and veterinary parasites. The programme monitors and identifies markers of antibiotic resistance and studies mechanisms of virulence and prevention approaches for toxoplasmosis.

Virology: The programme regroups all the reference and research laboratories assuring the surveillance, diagnosis and study of human and zoonotic viral pathogens including: Influenza virus, Hepatitis A, B, C, D and E viruses, Measles, Rubella and West Nile virus. It develops new diagnostics and studies new prevention and treatment techniques. It provides diagnosis and treatment of rabies in humans and serological monitoring of vaccination in humans and animals.

Mycology & Aerobiology: The programme hosts the medical mycology reference centre and the national collection (BCCM/IHEM) of filamentous fungi and yeasts presenting an interest for public health (humans and animals fungal pathogens, fungi isolated from the environment of atopic patients and mycotoxin-producing fungi). It investigates the microbial quality of indoor and outdoor environments and assures the identification of filamentous fungi and yeasts in human, animal, food and environmental samples.

Immunology & Vaccinology: The programme includes three research laboratories: an Allergy Laboratory studying the immunological mechanisms involved in pulmonary hyperresponsiveness associated with mould spores; a Laboratory of Mycobacterial Immunology centred on the development of immuno-diagnostic and more effective vaccines against tuberculosis, paratuberculosis cattle, and Buruli ulcer, and a Laboratory of Mycobacterial Biochemistry, which studies the importance of the biosynthesis of essential elements (vitamins, amino acids...) for the virulence and survival of *Mycobacterium tuberculosis*.

Tuberculosis & Mycobacteriology: The programme hosts the National Reference Centre assuring the microbiological diagnosis of tuberculosis and mycobacterioses, their molecular identification and typing, and the study of their susceptibility to antibiotics. It includes several research laboratories mainly interested in the study of the mechanisms of virulence and drug resistance of *Mycobacterium tuberculosis*. The programme is developing and studying both *ex vivo* and *in vivo* models of infection to evaluate the effectiveness of new antituberculosis therapies.

Current status of botulism intoxication in Belgium: the need for monitoring

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INTRODUCTION

Botulism is a severe intoxication caused by seven different heat-labile neurotoxins (A to G) produced by different strains of *Clostridium botulinum*, a spore-forming obligate anaerobe. Botulism is not spread from one individual to another. The three main forms of “natural” human botulism are: **foodborne botulism** caused by the ingestion of botulinum neurotoxins produced during the growth of *C. botulinum* spoiling preserved or stored food, **infant botulism** resulting from the colonisation of the intestine by *C. botulinum* and the *in situ* production of toxins, and **wound botulism**, the rarest, due to toxins produced by bacteria growing in a contaminated wound. The botulinum neurotoxins are considered to be the most lethal substances known, since as little as about 1 ng/kg can be lethal to an individual. All types of neurotoxins produce the same illness. They bind to and enter inside peripheral cholinergic nerve terminals, from which they inhibit the release of acetylcholine, with ensuing flaccid paralysis. If the paralysis extends to respiratory muscles, the individual dies of respiratory failure. Botulism can occur in humans as well as in animals with one difference of susceptibility to neurotoxins. Types A, B, E, and rarely F cause disease in humans. Type C is the most common cause of botulism in animals. Mainly, this type has been associated with botulism of birds. Type D is often seen in cattle, and type B can occur in horses. Type G rarely causes disease, although a few cases have been seen in humans. The current report analyses the results obtained by the National Reference Laboratory for Botulism during the years 1988-2009.

The objectives for the National Reference Laboratory for Botulism

The National Reference Laboratory (NRL) for Botulism, of the Scientific Institute of Public Health (ISP-WIV), is the only laboratory in Belgium to perform all laboratory tests for biological confirmation of botulism in human, animal and food specimens. The NRL has the following objectives:

- To assist physicians and veterinary surgeons when botulism is suspected in an individual by the examination of suspect foods and clinical specimens submitted for analysis;
- To rapidly alert the Federal Agency for the Safety of the Food Chain (FASFC) when food contains botulism;

- To take part in the monitoring of an outbreak of animal botulism in the national programs “cattle sounder” of DGZ (Dierengezondheidszorg Vlaanderen) and ARSIA (Association Régionale de Santé et d'Identification Animales) including control of the milk in affected farms;
- To take part in the annual monitoring programs of FASFC for *C. botulinum* and its toxins in food;
- To maintain reference strains of *Clostridium botulinum*;
- To publish an annual epidemiologic report of the botulism in Belgium;
- To develop new *in vitro* laboratory tests for the identification of all forms of botulism.



METHODS

Laboratory tests for the confirmation of botulism

A case of botulism requires definitive laboratory confirmation:

- Human foodborne botulism requires confirmation of intoxication in a person by the detection of the botulinum toxin in serum, stool, gastric aspirate or food or by the isolation of *C. botulinum* from stool or gastric aspirate, in addition to biological confirmation that the patient ate food containing the toxin or the bacteria;
- Infant botulism requires confirmation of intoxication in a baby less than one year of age by the detection of botulinum toxin in stool or serum or the isolation of *C. botulinum* from stool;
- Wound botulism requires confirmation of intoxication in a person by the detection of botulinum toxin in serum or the isolation of *C. botulinum* from a wound;
- Animal botulism requires confirmation of intoxication in the individual by the detection of botulinum toxin in serum, stool, gastric aspirate or organs after the autopsy or the isolation of *C. botulinum* from stool, gastric aspirate or organs after the autopsy. In addition, the origin of the intoxication may be detected in feed, water or environment. Absence of toxin in milk of positive farms has to be confirmed.

The NRL performs all the tests to analyse human, animal and food specimens for biological confirmation of botulism by the very sensitive reference method, i.e. the mouse bioassay. After the extraction from the samples, the neurotoxins are detected by the lethal test in mice and the identification of the toxin type by neutralisation with specific antitoxins. After enrichment in anaerobic conditions, the presence of the causative organisms in specimens is demonstrated by specific toxicity of the culture supernatants and by further characterisation of the selected *C. botulinum* bacteria. These laboratory tests allow the identification and the typing of the neuro-

Table 1: Cases of Human Botulism in Belgium (1988-2009).

Database for National Reference Laboratory for Botulism of the Scientific Institute of Public Health			
Year	Number of cases	Toxin type	Food source
1988	0		
1989	2	B	ham
1990	1	B	ham
1991	0		
1992	1	B	ham
1993	1	?	unknown
1994 a	1	?	unknown
1995	0		
1996	1	A	potatoes with onions and bacon
1997	3	B	ham
1998	1	B	olives
1999	0		
2000	0		
2001	0		
2002	0		
2003	0		
2004	1	B	ham
2005	0		
2006	0		
2007	0		
2008	1	B	
2009	0		
a: 1 case declared by the French community			

toxins A, B, C, D, E and F, and the identification and the characterisation of *C. botulinum* strains. *Clostridium tetani* can also be identified in this way.

National botulism database and surveillance

The NRL has had a National Botulism Database since 1987, collected from all cases of botulism suspected and confirmed in Belgium. Each year, the cases of human, animal botulism and food positive for botulinum toxin, the botulinum toxin types and the *C. botulinum* strains identified are recorded and used for further analysis and surveillance of botulism in Belgium. This database is used to follow the evolution of the outbreaks of human and animal botulism and the identification of foodborne botulism.

RESULTS AND DISCUSSION

Surveillance and notification of human botulism (NRL database: 1988-2009)

The majority of European countries have their own NRL for botulism. All of which have developed their scientific expertise, in parallel to their surveillance programs. Since 1971, human botulism is a legally declarable disease and its notification in Belgium is obligatory. The NRL is in charge of confirming diagnosis of all cases of botulism, by identification of toxin and *Clostridium botulinum*, and of further scientific investigation. The NRL database shows that human botulism is rare in Belgium (Table 1); only 13 cases of foodborne botulism have been confirmed during the last 22 years. 10 cases of botulism type B were associated with the consumption of

ham (8 cases), olives (1 case) and 1 case of which the origin was not identified, 1 case of botulism type A is associated with the consumption of potatoes with onions and bacon. For one case, the origin and toxin type were not identified and another was declared by the French speaking community without notification of origin and type. The evolution towards a reduction of cases of human foodborne botulism over the last decade suggests that the awareness of the public about the risk of botulism in food, improvements in food conservation methods and the treatment of industrial food products have succeeded in making human botulism rare in Belgium.

Detection of botulism in foodstuffs (NRL database: 2000-2009)

To maintain the safety of the industrial food chain, the NRL received specimens for the detection and the identification of botulinum neurotoxins and *C. botulinum* bacterial strains. These analyses were requested by industrial food companies or by the FASFC. During the last decade, no massive contamination of foodstuff by botulism was detected (NRL database). However, from 18 honey samples analysed for the FASFC (in 2005 and 2006), 2 samples appeared positive for *C. botulinum* type D and type B. In 2009, another honey sample positive for *C. botulinum* type C was identi-



fied. Consequently the Scientific committee of the FASFC recommended that all honey containers on sale in retail markets carry the label **“do not administer to children of less than one year old”**. Several studies showed that honey can sometimes contain dormant spores of *C. botulinum* (1). This bacterium can be dangerous to infants as this vegetative form can be rapidly transformed into toxin-producing

bacteria in the infant's immature intestinal tract, leading to illness and even death. Until now, no infant botulism has been reported in Belgium. Thus, the monitoring of botulism in foodstuffs by industrial food companies and by control of the food by the FASFC is important to avoid the appearance of human foodborne botulism in Belgium.

Table 2: Cases of botulism in Belgium (NRL database: 2000-2009).

Bird botulism	2000	2001	2002	2003	2004	2005	2006	2007	2008	2009
Number of samples	60	36	48	44	49	52	29	22	16	16
Toxin type C	18/60 30%	12/36 33%	11/48 23%	12/44 27%	18/49 36.7%	11/52 21%	16/29 55%	6/22 27.2%	3/16 18.7%	7/16 43.7%
Toxin type D	0/60 0%	0/36 0%	0/48 0%	4/44 9%	2/49 4%	0/52 0%	0/29 0%	1/22 4.5%	0/16 0%	0/16 0%

Bovine botulism	2000	2001	2002	2003	2004	2005	2006	2007	2008	2009
Number of samples	9	11	41	65	26	43	81	157	195	283
Toxin type C	0/9 0%	0/11 0%	0/41 0%	0/65 0%	0/26 0%	0/43 0%	4/81 4.9%	3/157 1.9%	2/195 1.02%	2/283 0.7%
Toxin type D	0/9 0%	0/11 0%	6/41 14.6%	8/65 12.3%	0/26 0%	5/43 11.6%	6/81 7.4%	17/157 10.8%	16/195 8.2%	56/283 19.8%

Evolution of outbreaks of animal botulism (NRL database: 2000-2009)

Each year, many cases of animal botulism are identified in Belgium by the NRL. Bird botulism is often associated with toxin and *C. botulinum* Type C (Table 2). In spite of the low number of samples analysed, our results suggest that this type of botulism is on the rise in birds in Belgium. The first cases (4 cases) of bird botulism type D were detected in 2003, but the outbreak of this type of botulism remains sporadic (Table 2).

Bovine botulism is often associated with toxin and *C. botulinum* Type D (Table 2). The first cases (6 cases) of bovine botulism type D were recorded in 2002. Since then, the number of analysed samples and positive cases has increased. In 2006 the first cases (4 cases) of bovine botulism type C were detected and, from then on, sporadic cases of this botulism type have been detected in cattle (Table 2). The origin of this outbreak has not been found. Many published reports suggest that the source of toxins or *C. botulinum* strains type C, and type D are associated with carcasses of birds, particularly of poultry, or small animals that may have contaminated the animal feed, water, pasture or litter (2, 3). This is also described in Belgium (4).

Animal botulism type B is rare in Belgium. This botulism is sporadic in horses and only 2 cases were identified in bovines; one in 1998 (5) and another in 2009 (NRL database). Botulism Type B is often associated with the ingestion of putrefied vegetables, containing the toxin or the *C. botulinum* type B, present in the ensilages (5, 6). *C. botulinum* type B contaminated bovines may eliminate the bacteria in their faeces (6). Consequently, their milk may then be contaminated by the faeces through the cow's udder. The presence of neurotoxin type B in milk from a cow presenting mastitis has been described (7), which clearly shows a possibility of toxin passage in milk. This neurotoxin type B may cause human botulism.

Research and development

Currently the mouse bioassay method is the only reference method for detecting botulinum toxin during botulism investigations. Although highly sensitive (5–20 pg/ml), this method is time-consuming and requires specific authorisation and facilities such as mouse breeding in order to perform the test. The test takes a minimum of 4 days to detect a neurotoxin in clinical or food specimens. The identification of *C. botulinum* neurotoxin after culture of clinical and food samples can take a minimum of 9 days. The routine availability of alternative, rapid, sensitive and specific methods for detection of both botulin toxins and botulinogenic bacteria is an urgent matter. Therefore, we are developing such *in vitro* methods.

IMPACT ON PUBLIC HEALTH

Although the incidence of human botulism remains relatively low, the incidence of animal botulism is on the rise. This may provoke foodborne botulism by human consumption of products derived from contaminated animals. Thus, surveillance of botulism in humans, animals, food and environment remains important for public health in Belgium.

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Development of rat monoclonal antibodies against moulds and their toxins

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INTRODUCTION

The causal link between fungal exposure and allergic problems has been clearly demonstrated by numerous international studies as the association between asthma severity and mould (but not pollen or animal) allergen sensitisation (1). Strong evidence for an association between sensitisation/exposure to moulds and life threatening exacerbations of asthma has also been obtained, further highlighting the detrimental properties of these allergens (2). The reported worldwide prevalence of sensitisation to mould allergens is quite variable and evidently depends on differences in exposure. Nevertheless, an overall prevalence of fungal immunologic sensitisation determined as the presence of specific IgE as opposed to atopic disease, ranging from 3% to 9% in the general population and from 7% to 50% in asthmatic children is often acknowledged (3). In the general population of the United States the prevalence of sensitisation to *Alternaria*, one of the main allergenic moulds, was reported to be 12.9% (4). This prevalence is only an approximation since the diagnosis of mould sensitisation is hampered by the variability, complexity and lack of standardisation of available fungal extracts and therefore, for practical reasons, the measurement of mould sensitisation is very often restricted to some species only.

Moulds are ubiquitous in nature and are present not only outdoors but very often also in the indoor environment, due to the colonisation of damp dwellings. If present in large numbers, especially in the case of dwelling colonisation, some mould species can represent a health hazard potentially causing or triggering allergic reactions and respiratory problems due to inhalation of allergens. Moulds also produce

secondary metabolites such as macrocyclic trichothecene mycotoxins which are known protein synthesis inhibitors. Some case studies and laboratory evidence suggest that these toxins may contribute to reported complaints such as headache, eye and throat irritation, nausea, dizziness, nose bleeds, and both physical and mental fatigue in subjects occupying a mouldy environment (5). Because of the increasing frequency of allergic diseases and the recognition of moulds and their metabolites as inducers or triggers of allergic and respiratory problems, the presence of moulds in houses, schools and other dwellings has become a major issue of public concern. In Northern Europe and Canada it has been estimated that 20 to 40% of house dwellings are contaminated with moulds (6). Therefore it has become increasingly important to control the indoor environment, i.e. to identify mould contamination and quantify the levels in indoor environments using validated methods.

Current assessment methods of indoor mould contamination are based on sample cultivation or microscopic spore counts. Although these techniques may be informative on a case by case basis they have major drawbacks. The direct microscopic spore count method is time consuming, subjective, shows a low sensitivity and high data variability. Methods based on culture analysis can overlook fungal species that are not easily cultured, may give an underestimate of those fungal types that grow slowly because they are overtaken by faster growing colonies and ignore the presence of nonculturable and non-viable spores or mycelia fragments (7). Recent developments in molecular techniques have provided significant advances in rapid detection and characterisation of micro-organisms irrespective of their viability

or cultivability. However, these molecular assays require skilled laboratory personnel, are not easily implemented for routine analysis and more importantly are expensive. Surrogate markers of mould contamination that measure quantitative loads of indoor fungal biomass such as β -glucan or ergosterol are useful for providing information about the global amount of fungi. However their measurements are also very expensive and they require highly trained personnel and sophisticated analysers (HPLC and mass spectrometer) which hamper the routine use of these markers (8).

Therefore there is a need for the development of better techniques able to identify environmental exposure to moulds and it is clear that the development of new assays will allow the development of new preventive measures for public health purposes. Indeed, in its report "Damp Indoor Spaces and Health", the Institute of Medicine of the US National Academy of Sciences identified the development of valid and standardised quantitative exposure assessment methods (particularly methods based on non-culture techniques and measuring constituents of micro-organisms such as allergens, β -glucans, fungal spores, etc.) as a high research priority (9).

Monoclonal antibodies (MoAbs) are powerful tools for the detection, quantification, and targeting of specific molecules. Therefore, immunoassays have become common techniques in diagnostic laboratories. Nowadays MoAbs are frequently used for the detection of numerous compounds and for the assessment of exposure to numerous agents. Immunodetection techniques are very flexible, inexpensive, easily implementable and standardisable and could be interesting tools for the development of new assessment methods of indoor fungal contamination. Therefore

we developed a panel of rat MoAbs specific for mould antigens and for the Verrucarin-A, an important trichothecene mycotoxin. We also established immunoassays for the detection of these compounds. We will describe here two immunoassays that we established and which would be helpful for the rapid assessment of mould contamination in dwellings.

METHODS

LOU/c rats were immunised weekly in both footpads with 10^6 spores of *Alternaria alternata* (IHEM 18586) or with $20 \mu\text{g}$ of Verrucarin-A coupled to bovine serum albumin (BSA). After the third immunisation, lymphocytes were obtained from the popliteal lymph nodes and fused using the polyethylene glycol method, with HGPRT mutated IR-983F myeloma cells. The cell mixture was then cultured in microplates in HAT medium containing the selective drug. As immune cells, although not sensitive to the HAT medium, survive for only about one week in culture and the IR-983F myeloma cells are drug sensitive they will all die within a week or so. The only cells that can survive during a culture in the HAT medium are those hybrid myeloma cells that obtained a normal HGPRT gene from the immune cells.

The supernatants of growing hybridoma were collected and analysed for the presence of mould specific or verrucarin-A specific MoAbs. The presence of mould specific MoAbs in the supernatants was analysed by fluorocytometry on spores from relevant mould species contaminating indoor environments (5 different *A. alternata* strains; *C. herbarum*, *cladosporioides*, *sphaerospermum*; *P. chrysogenum*, *brevicompectum*; *A. niger*; *S.chartratum*; *U.botrytis*). Two yeasts were used as negative controls (*C. albicans* and *S. cerevisiae*). Spores were obtained from 15-day cultures on PDA medium. Moulds antigens were extracted from the spores by overnight agitation in PBS. The presence of Verrucarin-A specific MoAbs in the supernatants was

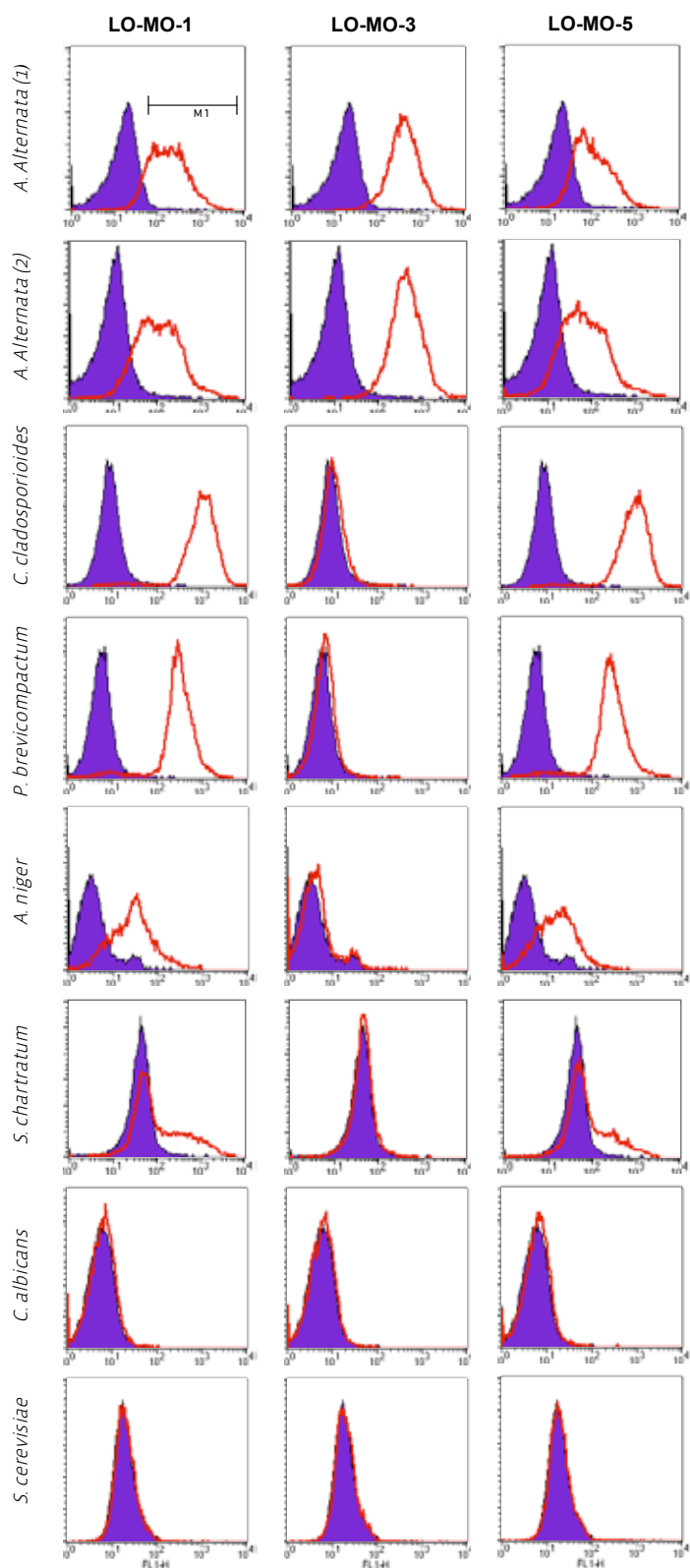


Figure 1: Flow cytometric analysis of the specificities of LO-MO-1, -3 and -5. Mould spores were incubated with the indicated antibodies. Bound antibodies were detected using an FITC labelled anti-rat antibody. The fluorescence was analysed on a FACSCalibur flow cytometer. The blue histogram gives the non-specific binding. The red-line histogram indicates the specific binding of the LO-MO-1, -3 and -5 respectively.

detected using an indirect immunoassay. ELISA plates were coated with Verrucar-A coupled ovalbumin (OVA). Then supernatants were added and the binding of MoAbs was detected using a peroxidase labelled mouse antibody directed against the kappa light chain of rat immunoglobulins. Positive clones were subcloned and then expanded in small bioreactors. The medi-

um containing the MoAbs were repeatedly collected and the antibodies were purified by immunoaffinity. Mould antigens were detected using a sandwich ELISA. "In-house" developed mould specific antibodies were used as capture antibodies and biotinylated antibodies and streptavidin labelled peroxidase were used for the detection. Verrucar-A was

detected using a competitive ELISA. Plates were coated with OVA labelled Verrucar-A. The binding of Verrucar-A specific MoAbs to the coated antigens was inhibited by the free Verrucar-A added in solution. The bound MoAbs were detected using a mouse antibody directed against the kappa light chain of rat immunoglobulins.

RESULTS

Popliteal lymph node cells from LOU/c rats immunised in the footpads with spores of *A. alternata* were fused with IR-983F cells. Growing hybridomas were selected in HAT medium and positive clones were selected by flow cytometry. Five different MoAb were obtained. LO-MO-1, -3 and -5 were able to bind *Alternaria* spores in flow cytometry and also recognised *Alternaria* mould extract using an indirect ELISA. These MoAb are all IgM. LO-MO-2 and -4 were of IgG2c and IgG1 isotype respectively and did not recognise mould extract by ELISA.

The specificities of LO-MO-1, -3 and -5 were further analysed using a FACSCalibur flow cytometer (Figure 1). LO-MO-3 binds to *Alternaria* spores IHEM 18586 and also to four other *Alternaria* strains (not shown). However this antibody does not bind *Cladosporium*, *Penicillium*, *Aspergillus*, *Stachybotrys* nor *Candida* or *Saccharomyces* strains demonstrating that it is species specific. Nevertheless, it also recognises *Ulocladium botrytis*, a species phylogenically very close to *Alternaria* ssp. The two other MoAbs (LO-MO-1 and -5) recognise all mould strains tested so far but not the two yeast strains (*C. albicans* and *S. cerevisiae*) indicating that these two antibodies bind an antigenic determinant common to moulds but not to yeasts. These two antibodies may therefore be used to determine the total mould biomass in dwellings while the LO-MO-3 could be used for quantifying specifically *Alternaria* antigens.

A sandwich ELISA using the LO-MO-5 was established and used to detect the presence of the recognised antigen in various mould extracts prepared in the laboratory. As shown in Figure 2A, this assay was

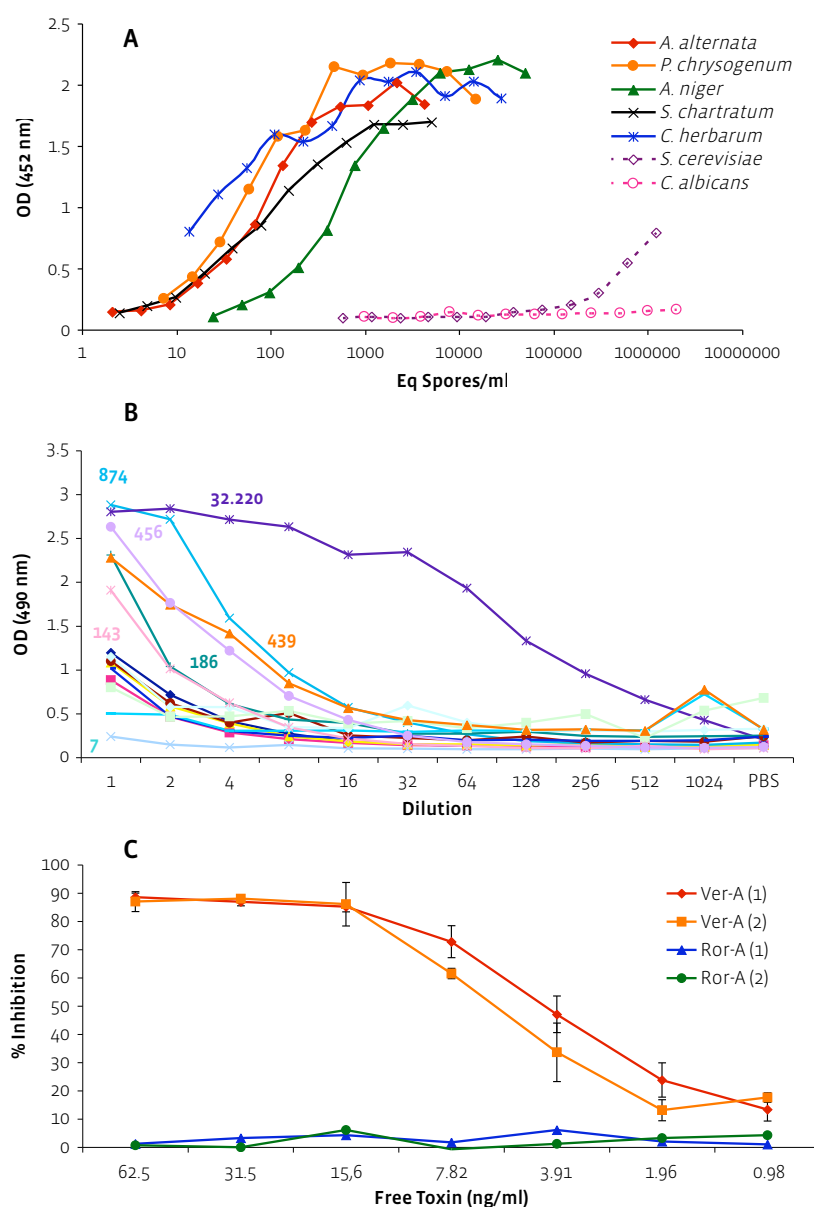


Figure 2: Mould specific ELISA. A. Detection of mould but not yeast antigens in various extracts prepared in the laboratory using a LO-MO-5 based sandwich ELISA. B. Detection of mould antigens in dust vacuum-cleaned from dwellings. The numbers indicate the equivalent *C. herbarum* spores ($\times 10^3$)/ml in these field samples. C. Detection of Verrucar-A in solution using a competitive F24-1G2-based ELISA. Two samples containing the free Verrucar-A or the free Roridin-A were analysed. Only the free Verrucar-A was able to inhibit the binding of the F24-1G2 MoAb to the Verrucar-A labelled OVA.

able to efficiently detect mould antigen in extracts from *A. alternata*, *C. herbarum*, *S. chartratum*, *P. chrysogenum* and *A. niger* while extracts from *C. albicans* and *S. cerevisiae* were not recognised, confirming the flow cytometric results.

Using this LO-MO-5 based ELISA we are now determining the quantity of mould antigens in air and dust samples from dwellings (Figure 2B). An extract of *C. herbarum* spores is used as an internal standard for the quantification of field samples and a "logit" regression is applied to calculate the standard curve. The intra-assay coefficient of variation (CV) for this assay is below 10% and the inter-assay CV is 20.58%. The sensitivity of this assay was estimated to be between 4000 and 2000 equivalent spores per ml. We are currently comparing the results obtained with this rapid assay with those obtained with the classical microbiological techniques currently used for the determination of mould biomass in the air.

Another interesting antibody has been obtained from rats immunised with bovine serum albumin coupled to the trichothecene mycotoxins Verrucar-A. Of the 553 tested clones, 70 clones (13%) produced antibodies recognising the Verrucar-A bound to OVA. Only one of these clones (F24-1G2) produced a MoAb which

was inhibited by free Verrucar-A in solution. This antibody is able to bind to OVA or BSA labelled Verrucar-A but not to these same proteins labelled with Roridin-A (another trichothecene mycotoxin with a very similar structure) or to the unlabelled proteins, demonstrating the specificity of this MoAb towards the Verrucar-A only. In a competitive ELISA, free Roridin-A was unable to inhibit the binding of the F24-1G2 antibody to the OVA-Verrucar-A while this binding was efficiently inhibited by free Verrucar-A (Figure 2C). Once optimised, this assay showed an intra-assay CV below 15% and an inter-assay CV of 29.9%. The overall sensitivity of this competitive ELISA ranged between 1 and 2 ng/ml of free Verrucar-A.

CONCLUSION

We have developed rat MoAbs recognising ubiquitous mould antigens (LO-MO-1 and -5), an *Alternaria* specific antigen (LO-MO-3) or Verrucar-A, an important trichothecene mycotoxin. Using these MoAbs we have developed several sensitive and reproducible ELISAs able to detect these components. We are currently comparing the efficacy of this kind of detection with the classical microbiological techniques for evaluation of mould biomass in dwellings. We hope that these MoAbs will allow the development of new rapid methods for the efficient evaluation of indoor mould contamination, permitting a better estimation and control of microbiological pollution in dwellings.



IMPACT ON PUBLIC HEALTH

The association between fungal exposure and allergic-respiratory problems has been clearly established. Moulds are ubiquitous and can be present indoors due to their colonisation of damp dwellings. The development of new methods allowing an easy and rapid testing of mould contamination will contribute to the safety of our environment.

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Discovery of a fascinating thiamine- and siderophore-auxotrophic fungus

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INTRODUCTION

Exposure to fungi may induce respiratory or allergic diseases. During an investigation of indoor fungi as potential allergens, we isolated a hyphomycetous species parasitising a *Penicillium* sp., apparently causing no harm or inhibition to the host (Figure 1). This mycoparasite was deposited in the BCCM/IHEM Culture Collection hosted by the Scientific Institute of Public Health, Brussels, Belgium.

The first attempts to obtain this mycoparasite in axenic culture failed. This fungus thus had an absolute requirement for one or more nutrients produced by the *Penicillium* host, just like biotrophs (obligate parasites with balanced relationships) depend on nutrients from their hosts. Biotrophic mycoparasites are divided into two main groups: filamentous haustorial biotrophs belonging to families formerly classified in Mucorales, and contact biotrophs, all hyphomycetous species producing specialised branches to contact the host hyphae to absorb nutrients. The most frequent growth factor of contact biotrophs, referred to in the literature as “mycotrophein”, is present in hot water extracts of their hosts, in particular in *Alternaria alternata*. Other fungi release this growth factor into the substrate, thus supporting saprobic growth of these biotrophs. Until now, the chemical nature of this growth factor had remained unknown.

Among the substrates tested, the isolated mycoparasite was able to grow on rabbit droppings. In this regard, Hesselstine and co-workers (1) have shown that dung or dung extracts, required for the growth of some strictly dung inhabiting fungi, could be replaced by an iron containing com-

pound from the fermentation liquors of a number of bacterial and fungal species. They named this growth factor ‘coprogen’. Another study reported that ferrichrome could replace coprogen or dung decoction in culture media for culturing these species (2). Later, it was shown that coprogen and ferrichrome functioned as iron-transporting agents in microbial metabolism. Obviously, the *Penicillium* culture filtrate, the growth factor mycotrophein, and the commercially available compound ferrichrome had to be tested on our newly isolated fungal strain.

The purpose of this study was to determine the requirements for the axenic growth of this mycoparasite, and to try to clarify its nutritional relationship with its host.

MATERIALS AND METHODS

The host *Penicillium* strain was cultivated in a 1,000 mL Erlenmeyer flask containing 250 mL of Sabouraud Glucose Broth, and stationary incubated in continuous darkness at 25°C. The liquid phase was recovered 15 days later and sterilised by filtration (single use filter unit of 0.2 µm from Sartorius). To assess the effects of this culture filtrate on the mycoparasite growth, decreasing quantities of the filtrate were mixed in 9 cm Petri plates to approximately 30 mL of melted malt extract agar (MEA), or the glucose-casein hydrolysate agar (GCH), a vitamin-free medium supplemented or not with biotin and thiamine at concentrations of 20 and 100 µg L⁻¹ respectively. After cooling, the mycoparasite conidia were inoculated alone or with those of *Penicillium* sp. (at the same time and the same place), and Petri plates were incubated at 25°C in continuous darkness for 21 days.

Hot water extract of *A. alternata* was obtained as described by Hwang *et al.* (3). This extract and ferrichrome were blended into Petri plates to the basal medium MEA, singly or together, in various concentrations. Mycoparasite conidia were then inoculated, and Petri plates incubated at 25°C, for 21 days.

The ferrichrome stock solution (ferrichrome iron-free from *Ustilago sphaerogena*, Sigma), was prepared by dissolving 1 mg in 50 mL distilled water (2 µg mL⁻¹). Successive concentrations from 0.001 mg L⁻¹ to 1 mg L⁻¹ were tested.

All experiments were repeated three times or more.

RESULTS

Peculiar properties of the *Penicillium* culture filtrate

Mycoparasite conidia deposited on semi-synthetic culture media (GCH, GCH + thiamine & biotin, and MEA) germinated but the germination process stopped immediately; only relatively long germ tubes were formed. The fungus grew on media supplemented with 1 mL of a culture filtrate from the 15-day-old *Penicillium* liquid culture. However, sporulation observed on the vitamin-free medium GCH to which the filtrate was added was abnormally low. Unlike biotin, the injection of thiamine alone to the mixture mentioned above supported the normal growth of the fungus. When the *Penicillium* sp. and the mycoparasite conidia were inoculated jointly, the latter appeared only after the fourth day on the top of the *Penicillium* colonies, without showing a significant difference accord-

ing to the culture medium. The mycoparasite then received the nutrient(s), possibly mycotrophein, as well as a vitamin essential to its growth from the host mycelium via the culture medium or via absorptive structures.

Presence of particular morphological structures

Microscopic observation of cultures showed no tropism between germ tubes of the mycoparasite conidia and hyphae of the host. The mycoparasite did not form structures resembling haustoria or absorptive branches which would indicate it was a haustorial or contact biotroph.

Growth factors activity

Hot water extracts from *A. alternata* acted at infinitesimal concentrations since a 1,000-fold dilution of the extract (less than 0.1 mL) in the basal medium MEA already induced an axenic growth of the mycoparasite. However, sporulation of colonies formed by the fungus with this extract was weak (even when more extract was incorporated into the medium). Moreover, the conidia from these colonies were unable to germinate on a culture medium, meaning that they were lacking something crucial for germination. Successive hot water extracts of *Alternaria* produced the same result and affected identically the growth of the parasite. Since they came from a fungus known to produce mycotrophein, these extracts theoretically contained this growth factor. Similarly to mycotrophein, these extracts acted at infinitesimal concentrations, were heat stable, and soluble in water and acetone. This suggested that mycotrophein was not the exact requirement of the fungus, or that it needed an additional substance (a third after thiamine and mycotrophein). However, when ferrichrome was added alone in the MEA medium, the mycoparasite grew normally. A concentration of 0.1 mg L⁻¹ ($\approx 10 \mu\text{M}$) gave the same sporulation as that observed with the *Penicillium* culture filtrate. Moreover, conidia produced with the *A.*



Figure 1:
A five-week-old culture of the mycoparasite and its host *Penicillium* sp. on MEA. The mycoparasite (coloured ochreous) appeared a few days after inoculation in the middle of the *Penicillium* colony.

alternata extract germinated on MEA supplemented with ferrichrome, or with *Penicillium* culture filtrate. However, addition of ferrichrome to the vitamin-free medium GCH failed to support axenic growth of the mycoparasite. Unlike biotin, the injection of thiamine alone to the same mixture supported the normal growth of this curious fungus.

These experiments showed that our mycoparasite required thiamine and a growth-promoting iron chelate (ferrichrome or a related compound).

DISCUSSION AND CONCLUSIONS

Thiamine, a water-soluble vitamin (vitamin B₁) plays a pivotal role as coenzyme in intermediary carbon metabolism. Although, most fungi are able to synthesise this vitamin, our parasite cannot! Knowledge on thiamine production pathways in fungi mainly comes from the yeast *Saccharomyces cerevisiae*, which either utilises external thiamine or can synthesise the

cofactor itself (4). Thiamine is complicated to synthesise, its biosynthesis is an energy-costing process (4, 5), and exogenous thiamine represses genes encoding the enzymes involved in the metabolism of thiamine (6). Therefore, our mycoparasite probably became thiamine auxotroph following a continuous external supply as in a close and long term relationship between a mycosymbiont and its host. This hypothesis is all the more plausible since several studies have shown that concentrations of thiamine in the natural environment are much lower than those normally required by thiamine dependent organisms (7).

Most fungi produce distinct cellular and extracellular siderophores in order to solubilise environmental iron, particularly in low-iron environments. These low-molecular-mass complexing agents, covering a wide range of physico-chemical properties, have an extremely high affinity for extracellular ferric iron and bind this metal avidly (8, 9). Approximately 100 to 150 different fungal siderophore structures have already been described (9). The ferrichromes,

coprogens, fusarinines and rhodotorulic acid represent the four structural families of the hydroxamate-type identified in the asco- and basidiomycota (10). Our parasite is unable to acquire iron in an independent manner since its growth is dependent on the presence of the siderophore ferrichrome in the culture medium.

If this mycoparasite was able to grow with the hot water extract of *Alternaria*, it was because an iron-chelating agent present in this extract had provided it with iron. *Alternaria* is known to make only ferricrocin and coprogen-type siderophores (8). Assuming that siderophores are interchangeable as growth factors for the mycoparasite, our results suggest that ferrichrome is a far more efficient iron donor. In addition to capturing and transporting iron, siderophores store solubilised iron in the fungal cells. Since conidia are dormant cells that require a large input of iron for germination, intracellular siderophores are essential for an iron storage function, and have been recognised as conidial germination factors of some fungi (11, 12). Most conidia still contain a certain amount of siderophores that are released during germination (13). Moreover, intracellular siderophores have a crucial role in conidiogenesis, and a deficiency in these cellular compounds leads to reduced conidia production in *Aspergillus nidulans* and *Aspergillus fumigatus* (14, 15). Such phenomena were observed during this study. When the mycoparasite grew

with the siderophore ferrichrome, sporulation and conidial germination were normal, whereas with the *Alternaria* extract the sporulation was reduced, and conidia were unable to germinate. These conidia had become siderophore-dependent. Just like conidia without intracellular siderophores, lacking an active iron transport mechanism. The most plausible explanation would be that, whereas the ferrichrome was transported inside the cells, which implied that conidia contained some, the unspecified siderophore present in hot water extract of *Alternaria* yielded iron to the cells without concomitant uptake of the ligand.

Symbiosis is, in its broad sense, an association between different organisms living together that range from parasitism to mutualism. This study showed that the major (or the only) route for thiamine acquisition by the mycoparasite came from an exogenous source via a symbiotic re-

lationship. Since energy is required for the siderophore biosynthesis and for the essential mechanisms intended to avoid toxic accumulation of intracellular iron, and because its host excreted enough hydroxamate ligands, the mycoparasite probably also became siderophore auxotroph. In mycosymbionts of fungi, an association in which one symbiont benefits and the other suffers no adverse effects is known as a biotrophic fungal symbiosis (16). Such is the case for the relationship between this mycoparasite and the *Penicillium* sp. However, an interfungal association based on utilisation of siderophores has not been previously reported. Moreover, this thiamine- and siderophore-deficient mutant does not live in intimate association with the cytoplasm of its host. Indeed, it is neither a contact biotroph nor a haustorial biotroph, the two groups in which the mycosymbionts of fungi are classified. Therefore, our fungus represents a new type of biotrophic mycosymbiont.

IMPACT ON PUBLIC HEALTH

Iron plays a central role in fungal-host interactions. Essential mechanisms intended to ensure the iron homeostasis in pathogenic and symbiotic fungi are still little known and their understanding requires the study of these associations. Siderophore biosynthesis pathways are currently the subject of several studies, in order to find new antifungal agents.

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Effect of the neutralisation of IL-17A on the immune response to *Mycobacterium tuberculosis* infection

INTRODUCTION

With about 9 million new cases and 2 million deaths each year, tuberculosis (TB), a disease caused by infection with *Mycobacterium tuberculosis* (*Mtb*), remains a major health problem. It is estimated that a third of the world population is latently infected with *Mtb* (1). Among infected individuals, 10% are susceptible to develop symptomatic TB during their lives and thus may become contagious (2). A major risk factor for the development of TB is HIV co-infection (3). The attenuated *M. bovis* BCG (Bacille Calmette-Guérin) strain is still the only available TB vaccine. BCG is however poorly efficient against pulmonary TB, the most common form of TB in adults (4). Development of more effective TB vaccines is essential and requires a better understanding of the immune response against *Mtb*. *Mtb* is an intracellular pathogen able to replicate and to enter into a dormant state in pulmonary macrophages. Protective immunity against *Mtb* depends on interactions between T cells (CD4⁺ Th1, CD8⁺, CD1-restricted and $\gamma\delta$) and macrophages. Interleukin-12 (IL-12) produced by infected macrophages and dendritic cells (DCs) is required for the induction of IFN- γ and TNF- α , which are both essential in controlling *Mtb* growth and granuloma formation (5, 6). The role played by other cytokines such as IL-17A in the response against *Mtb* is still not fully understood.

IL-17A is the founding member of a newly described family of cytokines (IL-17A to IL-17F). IL-17A is a proinflammatory cytokine primarily produced by CD4⁺ T cells (Th17) that promotes the granulopoiesis and the recruitment of neutrophils to the site of infection through the induction of CXC chemokines LIX (CXCL5), KC (CXCL1), IL-6, IL-8, G-CSF and TNF- α . In the context of auto-

immune diseases (such as experimental auto-immune encephalitis (EAE) and rheumatoid arthritis), IL-17A has detrimental effects by promoting inflammation. IL-17A plays an important role in the protection against different pathogens like *C. albicans* or *K. pneumoniae* and is produced during *Mtb* infection (7). However, little is known about the role of IL-17A in *Mtb* control and published data is controversial. Studies by Khader *et al.* have shown that although IL-17A response is dispensable for protection against *Mtb* during primary infection, IL-17A response is nevertheless implicated in an optimal IFN- γ recall response in the lung (8, 9). Moreover, lung-infiltrating T cells of IL-17A-deficient mice challenged with BCG produce less IFN- γ in comparison to wild type mice and a defective granuloma formation is observed (10). Our laboratory has shown that boosting of BCG vaccinated mice by subsequent Modified Vaccinia Ankara virus expressing antigen 85A (MVA-Ag85A) resulted in increased IFN- γ responses and induced an IL-17A response. This BCG-MVA-Ag85A vaccination protocol did not result in increased protective efficacy of BCG and we hypothesised that amplified inflammation possibly linked to the observed IL-17A responses had occurred with this protocol (11). Hence our interest in analysing in more detail, the role of IL-17A in the context of *Mtb* infection and *Mtb* vaccine development.

Here, we have analysed the effect of the neutralisation of IL-17A on the course of *Mtb* infection in mice. IL-17A was neutralised using an anti-IL-17A auto-vaccine developed by Drs Uyttenhove and Van Snick (Institut De Duve, UCL, Bruxelles) (12). The aim of this preliminary study was to understand the role of IL-17A in *Mtb* infection and to evaluate the potential effect of a treatment neutralising IL-17A on the

susceptibility to *Mtb* infection. Indeed, development of therapeutic agents able to block IL-17A bioactivity is in progress to treat auto-immune diseases.

MATERIALS AND METHODS

Mice

7 weeks old DBA/2 (H-2d) female mice were used (obtained from Janvier).

IL-17A neutralisation

To neutralise IL-17A bioactivity, mice were vaccinated with anti-IL-17A auto-vaccine. This vaccine is composed of recombinant murine IL-17A dimer cross-linked to ovalbumin with glutaraldehyde and is administered subcutaneously with Gerbu100 adjuvant (Gerbu Biotechnik). Mice were injected seven times at two-week intervals with 2 μ g IL-17-OVA complexes suspended in 50 μ l PBS and 50 μ l Gerbu100 adjuvant. The neutralisation of IL-17A was checked by Drs Uyttenhove and Van Snick for each mouse using an IL-6 bioassay (12). Uninjected mice were used as control.

M. tuberculosis infection

Mice were inoculated intratracheally three weeks after the last anti-IL17A vaccination with 104 CFU of luminescent *Mtb* H37Rv. This strain is transformed with the pSMT1 plasmid, which encodes a bacterial luciferase under the control of the constitutive mycobacterial hsp60 promoter. Bacterial load in lung and spleen homogenates was quantified by enumerating the number of bioluminescent organisms (determined as relative light units [RLU]). 1% n-decanol in ethanol was used as substrate. We have

previously shown that RLU counting correlated with CFU enumeration on agar plates (13). Animals were sacrificed by cervical dislocation 2, 4 and 13 weeks after challenge.

Isolation of cell populations from lung tissue

CD11c⁺ cell fraction was isolated from homogenised lung tissue samples using magnetic beads coupled with antibody (Becton-Dickinson). Single-cell suspensions of lung tissue were prepared by digestion with RPMI 1640 medium supplemented with 2.4 mg/ml collagenase (Sigma-Aldrich) and 1 mg/ml DNaseI (Roche) for 30 min at 37°C. The digest was passed through a 70-µm cell strainer, and erythrocytes were lysed with NH₄Cl-Tris buffer.

RT-qPCR

RNA was extracted using a commercially available RNeasy kit according to the manufacturer's instructions (BIO RAD). cDNA was synthesised using a TaqMan Reverse Transcription Reagent kit (Roche). RT-qPCR was performed on an ABI Prism 7700 Sequence Detector (Applied Biosystems) using a qPCR Core Kit for Sybr Green I (Eurogentec). Each RT-qPCR amplification was performed under the following conditions: 50°C for 2

minutes and 95°C for 10 minutes, followed by 40 cycles at 95°C for 15 seconds and 60°C for 1 minute. Hydroxymethylbilane synthase (HMBS) mRNA was used as reference housekeeping gene for normalisation. The level of target mRNA, relative to the mean of the reference housekeeping gene, was calculated as previously described (14).

Assessment of leukocyte distribution in BAL fluid

Mice were killed and broncho-alveolar lavage (BAL) was performed. The total volume and cell number recovered from the BAL were recorded. Number and composition of cells infiltrating the lungs were determined using cytopsin for individual mice. Cells were classified as mononuclear cells, neutrophils and lymphocytes using standard morphological criteria after staining with Kwikdiff coloration (Thermo). At least 200 cells were counted per cytopsin preparation, and the absolute number of cells for each cell type was calculated.

RESULTS

Vaccination with the anti-IL-17A auto-vaccine induces the production of IL-17A antibodies

To block biological activity of IL-17A, DBA/2 mice were injected seven times with an anti-IL-17A auto-vaccine formulated with Gerbu100 adjuvant before *Mtb* infection. This immunisation induced the production of antibodies able to neutralise the biological activity of IL-17A (data not shown). Anti-IL17A antibodies levels in the sera remained stable throughout the infection and were comparable to the levels obtained in SJL mice, which were vaccinated with the same auto-vaccine and could be protected against EAE (12).

IL-17A neutralisation has a moderate effect on the susceptibility to *Mtb* infection

Control mice and mice vaccinated with the anti-IL17A auto-vaccine were infected by the intratracheal route with a low dose of 104 CFU of luminescent *Mtb* H37Rv. 2, 4 and 13 weeks after infection, mice were sacrificed and the number of bioluminescent bacilli was evaluated in spleen and lungs. The bacterial burden in the spleens of the IL-17A neutralised mice and control ani-

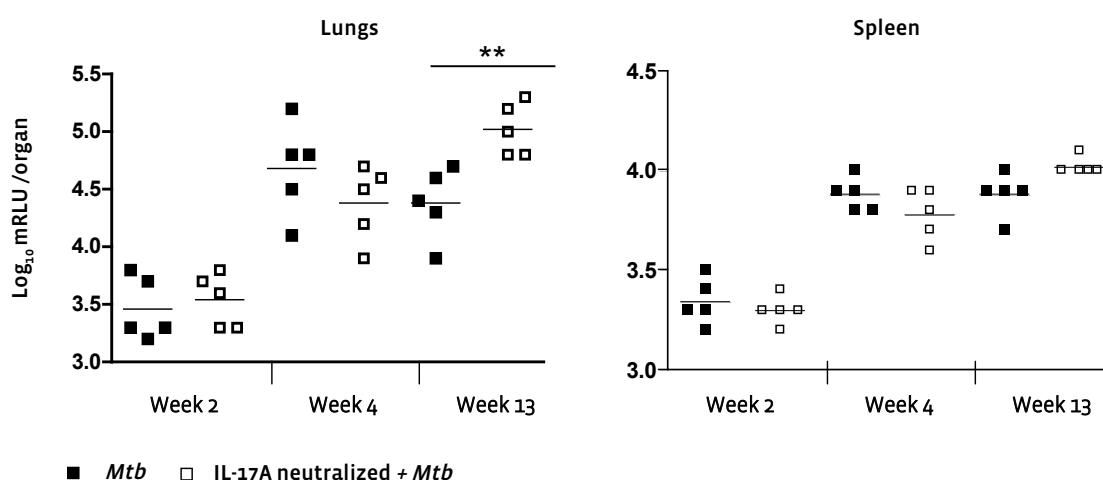


Figure 1: Bacterial counts in the lungs and spleen of DBA/2 mice with IL-17A neutralised or not at different time points after intratracheal instillation of luminescent *Mtb* (104 CFU). Dots represent the mean log₁₀(mRLU) values obtained for individually tested animals and the bar represents the mean value for each group. Statistical significance between groups is represented in the figure by ** (0.005 < p < 0.010) and was evaluated by Student's *t*-test.

mals were comparable at 2, 4 and 13 weeks post-infection. Regarding bacterial burden in lungs, no effect was observed at weeks 2 and 4 post-infection but a moderate increase in the anti-IL-17A vaccinated mice ($0.5 \log_{10} \text{mRLU}$) was observed at week 13 (Figure 1). Moreover, IL-17A neutralisation had no effect in terms of weight loss and lung histological analysis did not reveal any difference between the two experimental groups (data not shown).

IL-17A neutralisation induces a loss of neutrophils recruitment in the lungs during the first 4 weeks after *Mtb* infection

IL-17A is implicated in the recruitment of neutrophils to the site of infection via its induction of the production of neutrophil recruiting chemokines such as CXCL5/LIX (7). We have therefore performed an analysis of the composition and the number of cells infiltrating the lungs, by performing a cytopspin analysis of the BAL cells. Our results demonstrate that neutrophils are absent at least during the first 4 weeks after infection in the lungs of IL-17A neutralised mice as compared to control mice, nevertheless neutrophils infiltrating the lungs were observed 13 weeks after infection in

these mice (Figure 2). No differences were observed for lymphocyte or macrophage recruitment between the two groups (data not shown). Moreover, 4 weeks after infection, a lower CXCL5/LIX mRNA expression was observed in CD11c+ lung cells of the IL-17A neutralised group versus control. These results confirmed the specific role of IL-17A in neutrophil recruitment (Figure 2).

CONCLUSION

A clear understanding of the role of IL-17A during *Mtb* infection is still not available. In this study, we have investigated the effect of the neutralisation of IL-17A during *Mtb* infection in mice. For that purpose, we have used an anti-IL-17A auto-vaccine inducing long-lasting IL-17A-neutralising antibodies that block the biological activity of IL-17A (12). To perform this study DBA/2 mice were selected. Indeed, among the mouse strains generally used to study *Mtb* infection, DBA/2 mice are more susceptible to TB than C57BL/6 or BALB/c (15) and this has been attributed to an increased neutrophil recruitment in the lungs of DBA/2 mice after infection (16). Our results show that the number of neutrophils infiltrating

the lungs during the first 4 weeks of infection was decreased in the group of mice that had received the IL-17A auto-vaccine compared to infected control mice. This can be attributed to the fact that IL-17A is implicated in neutrophils recruitment. Indeed, CXCL5/LIX expression in the IL-17A neutralised mice was lower compared to infected control mice. LIX has chemoattractant activity for neutrophils and is induced by IL-17A. Therefore these results both confirm that vaccination with IL-17A-OVA + Gerbu100, indeed induced the neutralisation of IL-17A. However, at 13 weeks post infection, neutrophils were observed in these anti-IL-17A vaccinated mice despite similar IL-17A antibody levels at week 13 post-infection and before infection. It is likely that neutrophils are recruited by other factors at this late time point. Absence of neutrophils at the early time points after infection did not correlate with differences in the bacterial load in the lungs. However, the bacterial load 13 weeks after infection was higher in IL-17A neutralised mice compared to control infected mice. The role of neutrophils in mycobacterial infections remains a subject of controversy. One study showed an increased bacterial load when neutrophils

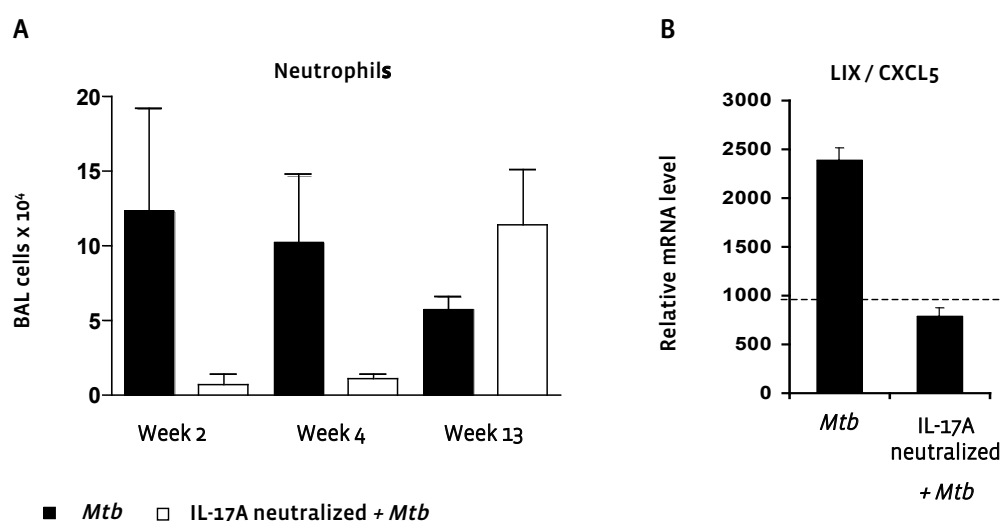


Figure 2:
A. Neutrophil infiltration in the lungs of DBA/2 mice with IL-17A neutralized (□) or not (■) at different time points after intra-tracheal instillation of luminescent *Mtb* (10^4 CFU). Shown are absolute numbers of neutrophils. Data are mean \pm SEM ($n = 4-5$).
B. mRNA expression of LIX/CXCL5 by CD11c+ pulmonary cells of DBA/2 mice with IL-17A neutralized or not, 4 weeks after intra-tracheal instillation of luminescent *Mtb* (10^4 CFU). Data are expressed as relative mRNA levels, normalized against reference housekeeping gene. Dotted lines denote mRNA levels obtained from non infected mice.

are depleted at early time points after infection (17) but another study failed to detect any difference in bacterial counts in neutrophils-depleted mice (18). A recent study demonstrated that neutrophils have the capacity to activate DCs. BCG-infected neutrophils facilitate full maturation, cytokine production and antigen presentation by DCs. Therefore, it was suggested that neutrophil deficiencies lead to non optimal protective antimycobacterial immune responses (19). The decreased neutrophil infiltration observed in our study in the first 4 weeks post-infection may thus explain the differences in bacterial loads observed at week 13. Further experiments are needed to confirm this hypothesis. The aim of this preliminary work was not only to acquire knowledge about the role of IL-17A during *Mtb* infection, but also to investigate potential effects of the IL-17A auto-vaccine on *Mtb* infection. Indeed,

this auto-vaccine is protective against EAE in SJL mice and has a potential as a future treatment against auto-immune disease (12). In this context, it was interesting to analyse the impact of this IL-17A neutralisation on *Mtb* infection susceptibility. In future experiments, it will be interesting to use the auto-vaccine after infection to analyse if this treatment has an effect on the reactivation of a latent infection.

In summary, our data show that the neutralisation of IL-17A induces depletion of neutrophils in the lungs during at least 4 weeks after *Mtb* infection and leads to a moderate increase in lung bacterial loads at the latest time point tested. However, more experiments are needed to conclude on the impact of IL-17A neutralisation on *Mtb* infection.

IMPACT ON PUBLIC HEALTH

Tuberculosis is a worldwide health problem. Investigating immune factors involved in host response to *Mycobacterium tuberculosis* is important for the development of new, more efficient tuberculosis prevention strategies. It is also essential for determining the impact of new immunotherapy, under development to treat other diseases, on the susceptibility to tuberculosis.

ACKNOWLEDGEMENTS

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SOURCE

(Conference presentation)

- Meeting GREMI Th17-derived cytokines: “New kids on the block of inflammation” (December 2008; Paris)
- Keystone “Th17 cells in health and disease” (February 2009; Vancouver)
- European Congress of Immunology (September 2009; Berlin)

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Environmental *Staphylococcus* contamination in a production plant at the origin of a foodborne outbreak

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INTRODUCTION

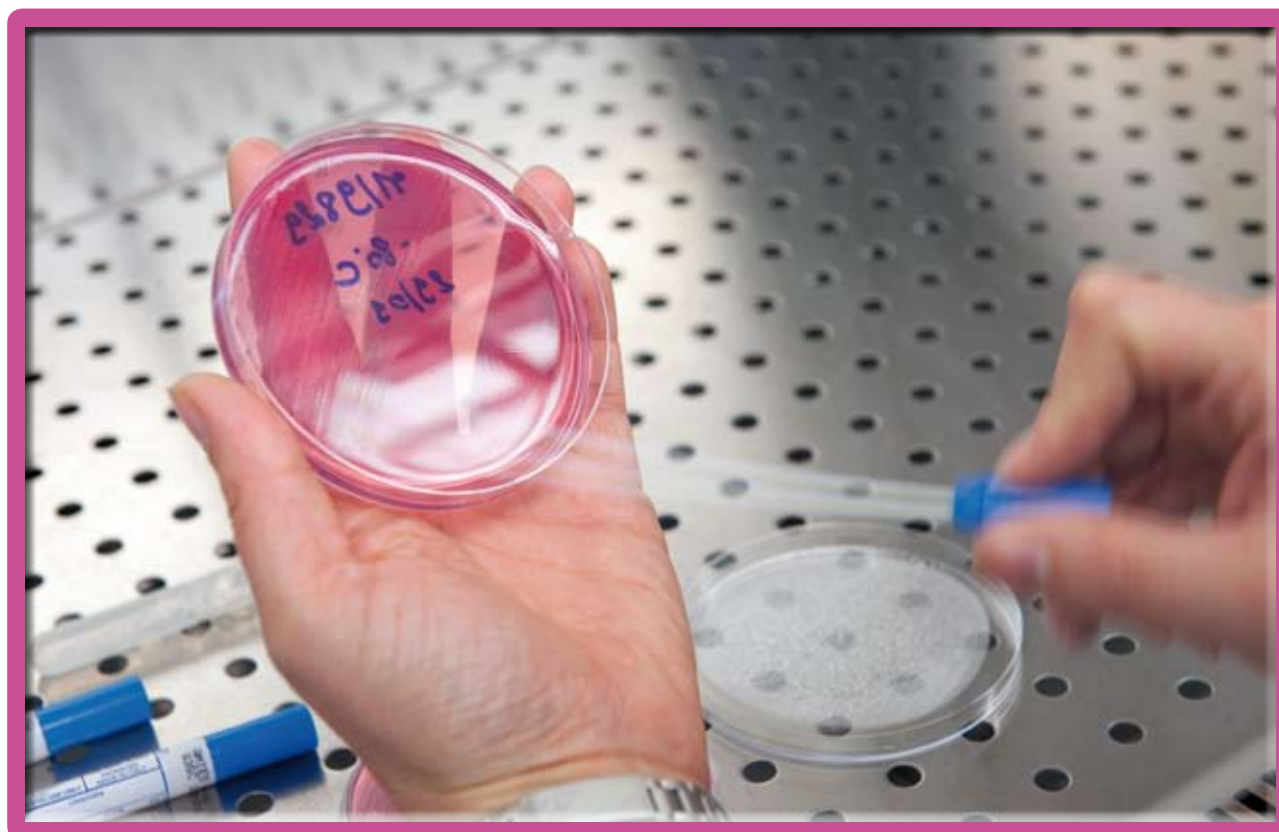
Staphylococcal food poisoning outbreaks are one of the most significant among foodborne outbreaks in Europe and are characterised by nausea, vomiting, abdominal pain and diarrhoea. The incubation period is short (2-8 hours) after the ingestion of contaminated food. The symptoms are caused by the staphylococcal enterotoxins (SETs) produced during the massive growth of *Staphylococcus aureus* in the foodstuff. The most susceptible food items are dairy products such as raw milk cheese, sliced soft cheese and meat products. The main factors contributing to outbreaks are poor temperature control during preparation, cooking and storage of the food. Epide-

miological studies report that an ingested dose ranging from 20 to 100 ng of toxin types SEA and SEH is sufficient to cause initial symptoms in the most sensitive people. During the investigation of the current outbreak, different *Staphylococcus* isolates were characterised by toxin production, presence of the toxin genes (*sea* to *seo*), drug susceptibility testing, phage typing and MLST.

Case study

In the summer of 2007, a foodborne outbreak occurred at a Scout Camp at Latinne, in the south of Belgium. About 4 hours after a lunch consisting of hamburgers with cheese, the children, aged between 5 and 8,

developed symptoms of vomiting, abdominal pain and diarrhoea. In total, 15 children out of 34 and one of the adults became ill. The Belgian Health Inspectorate was alerted and decided to hospitalise the children. The children recovered very quickly and left the hospital the same day, except for one who was hospitalised for one night. Because the symptoms started almost simultaneously in all children, it was obvious that food was probably at the origin of the infection. The inspectors of the Belgian Federal Agency for the Safety of the Food Chain (FASFC) were contacted immediately and started an investigation. The hamburgers had been bought frozen in a supermarket. They were grilled for 12 minutes and then stored in a casserole at ambient tem-



perature until all hamburgers were cooked. Although it was a warm summer period with temperatures at noon reaching 30°C, the camp site was equipped with minimal infrastructure; only one refrigerator was present for the storage of leftover food. All of the leftovers from the lunch in the refrigerator at the camp site were sampled. These included hamburgers, cheese and ketchup. Leftover boiled pasta from the previous evening, water and UHT milk, which were present in the refrigerator were also collected. All samples were taken to the National Reference Laboratory (NRL) of foodborne outbreaks for analysis at the WIV-ISP. The next day, hamburgers from the same production batch at the supermarket where the original hamburgers had been bought were sampled.

A high level of contamination with coagulase positive *Staphylococci* (CPS) ($> 10^8$ cfu/g), was detected in the leftover hamburgers together with the presence of enterotoxins. The hamburgers sampled at the supermarket were also contaminated by coagulase positive *Staphylococci*. However, the contamination level was very heterogeneous, lower than 10^5 cfu/g, and no detectable toxins were present in the samples. According to the European legislation, no recall of the hamburgers was necessary. Nevertheless, the supermarket did recall all the hamburgers from the same production batch, which were imported from the

Netherlands. After the European Rapid Alert System for Food and Feed (RASFF), international recalls soon followed. In the Netherlands the officers of the “Voedsel en Waren Autoriteit” (VWA) performed a thorough investigation at the production plant. Other food products (fricandel, chicken nuggets) prepared at the same production site were found to be contaminated with *Staphylococcus aureus*. In the manufacturing environment, *Staphylococcus aureus* was found in 2 of the 5 production lines at the rapid spray cooling stage of the food products following pasteurisation. This led to heterogeneous surface contamination of the products. Molecular and phenotypical characterisations were performed on a selection of *Staphylococcus* strains to determine the relation between the strains and to confirm the origin of the outbreak.

MATERIALS AND METHODS

Microbiological examination of food and environment

The suspected food and environmental samples were analysed according to the EN ISO methods 6888-1 (Belgium) or 6888-2 (The Netherlands) for the enumeration of coagulase positive staphylococci. Typical colonies were further used for molecular characterisation.

For the detection of the enterotoxins, the method described in the European screening method version 2 (EURL-Staphylococcus) was used. After extraction and concentration of the toxins, detection of the enterotoxins was done using the Vidas SET2 (BioMérieux, Marcy-l’Etoile, France) automated kit.

Further differentiation of the staphylococcal enterotoxins types A to D was performed on samples detected positive by the Vidas SET2, using the SET-RPLA (Oxoid, Basingstoke Hampshire, UK) detection kit which is based on an agglutination reaction. Positive samples were also sent to the Community Reference Laboratory (Maisons-Alfort, France) for confirmation of the results and for a quantitative detection of the toxins by their in house ELISA method (AFSSA LERQAP/CAT BAC/nr 3).

Other bacterial parameters measured in the food were: total colony plate count at 30°C, enumeration of *Bacillus cereus*, enumeration of *E. coli*, detection of *Salmonella* and *Campylobacter*. These were performed according to their respective ISO methods 4833:2003, 7932:2004, 16649-2:2001, 6579 and 10272-1.

Molecular and phenotypical typing of the strains

For further differentiation the strains isolated in the outbreak, several approaches were followed. Besides molecular typing

Table 1: Bacteriological and toxin results of the hamburgers.

Parameters analysed*	Frozen hamburgers from the same production batch (highest contamination level)	Contamination level of leftover hamburgers at the camp
TCC	$7.1 \cdot 10^4$ cfu/g	$3.9 \cdot 10^8$ cfu/g
<i>E. coli</i>	< 10 cfu/g	$3.8 \cdot 10^8$ cfu/g
CPS	$6.5 \cdot 10^4$ cfu/g	$8.1 \cdot 10^7$ cfu/g
SET	Absent in 25g	present in 25g (A: +++, D: +)
SET –RPLA production by isolated strain	enterotoxin A +	enterotoxin A +
BC	80 cfu/g	< 100 cfu/g
<i>Campylobacter</i>	Absent in 25g	Absent in 25g
<i>Salmonella</i>	Absent in 25g	Absent in 25g

*TCC: total colony count at 30°C (ISO 4833:2003); CPS: Coagulase positive *staphylococci* ISO 6888 part1); SET: *staphylococcus* enterotoxin (European screening method version 2); BC: *Bacillus cereus* (ISO 7932); *E. coli* 16649-2: 2001; SET-RPLA: differentiation of the enterotoxins of *Staphylococcus* by the reversed latex agglutination test kit; *Salmonella* detection (ISO 6579); *Campylobacter* detection (ISO 10272-1)

Table 2: In-depth characterisation of the *Staphylococcus* isolates from hamburgers and the production plant.

Isolate number	Origin	Country isolated	Atypical growth in BHI	Sequence type (MLST)	Phage type	Toxin genes present
162	Hamburger camp	BE	No	1	53	<i>sea+seh</i>
164	Hamburger same batch	BE	No	1	53	<i>sea+seh</i>
223	Production line	NI	Yes	1	53	<i>sea+seh</i>
224	Production line	NI	Yes	Other ST	53	<i>sea+seh</i>
227	Fricandel	NI	Yes	7	NT	-
228	Hamburger	NI	Yes	1	53	<i>sea+seh</i>
225 and 226 *	Production line	NI	Yes	ND	81	-

*cns: coagulase negative *Staphylococcus aureus*

of the strains with Multi Locus Sequence Typing (MLST), phenotypical methods such as phage typing and typical characteristic growth in BHI at 37°C, were also used.

The sequence type (ST) of five *Staphylococcus* isolates was determined by the MLST method on seven housekeeping genes using the primers described by Enright *et al.* (1). For each locus, sequences were compared and distinct sequences were assigned allele numbers (<http://saureus.mlst.net>). For each isolate the alleles at each of the seven loci defined the allelic profile corresponding to its ST.

Phage typing was performed as described by Blair and Williams (2) using the International Typing set of bacteriophages at the routine test dilution (RTD) and the 100-fold concentration (RTDX100).

The presence of different enterotoxin genes was tested by PCR. Primers used were described previously in PCR methods for the detection of the *seo* and *sen* genes (3) *sea*, *seb*, *sec*, *sed*, *seg*, *seh*, *sei*, *sej*, *sek*, *sel* and *sem* genes (4).

All strains were tested by PCR for the presence of the *mecA* resistance gene which codes for methicillin resistance using the primers published by Perazzi *et al.* (5)

RESULTS

Table 1 gives an overview of results from the hamburgers analysed in the scope of this outbreak. All other food items were found to be negative. The leftovers of the hamburgers were highly contaminated with CPS and toxins were also present in the analysed food extract. Hamburgers from the same production batch were contaminated with CPS at a level that almost corresponded with the total aerobic colony count at 30°C. Therefore, we can assume that the contamination was almost exclusively due to CPS. The *Staphylococcus* contamination of the batch was very heterogeneous as determined on 10 different samples and ranged from 100 to 65,000 cfu/g. In these hamburgers, no enterotoxins were detected. However, enterotoxin A production by the isolates was observed. Using PCR, the *seh* gene was detected besides the *sea* gene in all *Staphylococcus* isolates from this outbreak.

The concentration of SEA found in the leftovers of the hamburgers was 0.93 ng/g. Assuming that 100 g of hamburger is consumed per person, the dose causing the infection was approximately 100 ng. This is almost twice the toxic dose described by Ikeda *et al.* (6), where only 40 ng of SEA and SEH was enough to induce symptoms such as vomiting and diarrhoea.

The CPS isolated from the environment of the production plant in the Netherlands showed an aspecific morphology on solid RPF agar and in Brain Heart Infusion broth.

Growth resulted in agglutination of the isolates from the production line whereas this phenotype was not observed for any of the hamburger isolates. To provide evidence of the origin of the outbreak, 7 isolates were further characterised by phage typing and genotypic typing. All strains isolated from the food and production plant belonged to the same MLST type (ST1) and also to the same phage type 53, indicating a common source of contamination. Some isolates revealed to be coagulase negative staphylococci, were classified by phage typing as phage type 81. All tested isolates were *mecA* negative.

The results indicate that different *staphylococcus* strains circulated in the production plant and that the isolates from the leftovers at the camp site were identical to those isolated at the production site.

Looking back at results of analyses performed at the production plant on behalf of quality control, it was obvious that the staphylococcal contamination had existed for a long time prior to this outbreak. A thorough investigation by the Dutch food safety authorities revealed that the spray cooling system, which was used to rapidly cool the pasteurised hamburgers, was contaminated with high levels of *Staphylococcus aureus*. *Staphylococcus aureus* embedded in biofilms is difficult to eradicate and can cause long lasting problems in a manufacturing plant. Further phenotypic and molecular characterisations of the *Staphylococcus aureus* isolates collected during this outbreak, demonstrated that the

strains isolated in the production line in The Netherlands showed atypical growth in brain heart infusion broth (clumping) which might be correlated with a stressed phenotype or a phenotype which is expressed in a biofilm. In clinical microbiology, the formation of biofilm represents an important virulence factor of certain strains of *Staphylococcus aureus*. The ability of bacteria to aggregate, forming biofilms, is strictly related to the capacity of producing an extracellular polysaccharide (7). Little information is available regarding biofilm formation by *Staphylococcus aureus* strains isolated from food or food production environments. Recently Rode *et al.* (8) indicated that food processing conditions such as temperature, salt and glucose concentrations relevant for food processing could promote biofilm formation. Nevertheless, biofilm formation was not further investigated in this study. In the production plant coagulase negative *Staphylococcus aureus* strains, which may also be the origin of contamination of meat products, also circulated. However, these strains were negative for the tested enterotoxin genes. Both phenotypical and genotypical typing techniques did further discriminate the *Staphylococcus aureus* strains.

CONCLUSION

We demonstrated that in depth characterisation using different techniques is useful to confirm the common origin of a contamination source. From the investigation of this outbreak, we can conclude that an initial staphylococcal contamination level of hamburgers below the legal limit was at the origin of a foodborne outbreak, probably due to growth during thawing of the hamburgers and storage at ambient temperatures before and after cooking. The contamination was caused by spray cooling of the hamburgers after the pasteurisation

step in the production line, and the presence of a biofilm containing staphylococci in the cooling system. Because the contamination of the product was never higher than the 10^5 cfu/g legal limit, no actions were taken by the manufacturer after detection of positive samples in batch controls. Nevertheless, the manufacturer should have taken measures to reduce the *Staphylococcus* contamination in the environment by applying strict hygiene rules. However, this was not evident and the real source of contamination was not found until the current accidental foodborne outbreak.

IMPACT ON PUBLIC HEALTH

This accidental foodborne outbreak investigation demonstrated that using different in depth characterisation techniques is useful in identifying the common origin of a contamination and allowed the detection of the source in a food production plant. This resulted in a recall of contaminated meat products and thus prevented further staphylococcal intoxication.

ACKNOWLEDGEMENTS

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Immunogenicity and protective efficacy of DNA vaccines encoding MAP0586c and MAP4308c of *Mycobacterium avium* subsp. *paratuberculosis* secretome

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INTRODUCTION

Johne's disease, or paratuberculosis, caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP) was first described as an atypical case of intestinal bovine tuberculosis by H.A. Johne and L. Frothingham in 1895. MAP is the etiological agent of severe chronic enteritis of the small intestine in domesticated and wild ruminants. Disease is prevalent worldwide though is mainly found in temperate and humid areas (1, 2). It causes substantial financial losses in the livestock industry, particularly the dairy sector, due to weight loss, reduced milk production, premature culling and reduced fertility of affected animals (3, 4).

MAP specific vaccines are composed of whole bacteria (killed or attenuated) formulated in mineral oil adjuvants. These vaccines confer partial protection against MAP, thus containing the excretion of bacteria in faeces and reducing the number of diseased animals. However, these vaccines do not protect against infection, they interfere with the tuberculin skin test used for the diagnosis of bovine tuberculosis and with indirect tests used for the diagnosis of paratuberculosis (antibody and IFN- γ ELISA). Therefore, the development of sub-unit based vaccines using MAP specific antigens, which would not interfere with existing diagnostic tests for bovine tuberculosis or paratuberculosis, may contribute actively to the control of Johne's disease.

Recently, we carried out a large-scale post-genomic analysis of MAP proteins, to identify MAP specific antigens that could potentially improve the serodiagnosis of bovine paratuberculosis. Performing systematic

proteomic identification and immuno-proteomic analysis of MAP protein extracts and MAP culture filtrates with sera from MAP and *M. bovis* infected cattle, we have identified 25 candidate antigens. Five of these, i.e. MAP0586c, MAP1693c, MAP2677c, MAP3199 and MAP4308c were tested in an antibody ELISA assay for their diagnostic potential on a limited panel of field sera and the combination of three of them competed in performance with available commercial assays, reaching a test sensitivity of 94.7% and a specificity of 97.9% (5). These five antigens were also evaluated for their vaccine potential as plasmid DNA vaccines in an experimental mouse model. We present here the results obtained for two of them, namely MAP4308c and MAP0586c. Sequence analysis identified a possible lytic transglycosylase SLT domain in MAP0586c protein and a class I fructose-bisphosphate aldolase activity for the MAP4308c protein. BALB/c mice were immunised with either plasmid DNA alone or immunised with a DNA prime-recombinant protein boost protocol. Vaccinated mice were challenged by the intravenous route with MAP and bacterial replication in the spleen was monitored by luminometry and CFU plating on solid Middlebrook 7H11 agar as previously reported (6).

MATERIALS AND METHODS

Mice

Female BALB/c mice were bred in the Animal Facilities of our Institute, from breeding couples originally obtained from Bantin & Kingman (United Kingdom). All animals were 6 to 8 weeks old at the start of the experiments.

Preparation of genomic DNA from *M. avium* subsp. *paratuberculosis* ATCC 19698

Genomic DNA of *M. avium* subsp. *paratuberculosis* ATCC 19698 was prepared as described by Tanghe *et al.* for *M. ulcerans* (7).

Construction and preparation of DNA vaccines encoding MAP0586c and MAP4308c

MAP0586c and MAP4308c genes were cloned as *Bgl*II/*Eco*RI fragments in pV1Jns-tPA-his containing a hexa-histidine tag coding sequence in 3' of the *Bgl*II restriction site of the pV1Jns-tPA vector (Merck Research Laboratories, PA, USA) (8). Briefly, MAP0586c and MAP4308c genes were amplified by PCR (Expand Polymerase; Roche) using *M. avium* subsp. *paratuberculosis* ATCC19698 genomic DNA as a template and using primers:

5'-GGAAGATCTTGTGCGGTGTGCCGTGAGGG-3' (forward MAP4308c),
5'-ATAGAATTCGCCGCGACCGAGGCGTCGTA-3' (reverse MAP4308c),
5'-GGAAGATCTTGGTGAGCAATCGGCGCACC-3' (forward MAP0586c),
5'-TATAGAATTCCTGCGGGTGCGCCGCCACGTAGTCGG-3' (reverse MAP0586c).

PCR fragments were purified on column, digested with *Bgl*II/*Eco*RI and ligated into *Bgl*II/*Eco*RI digested pV1Jns-tPA-his. After ligation (T4 DNA ligase, Fermentas) and transformation into DH5- α chemical competent *E. coli* cells (Invitrogen), positive clones were screened on LB-kanamycin medium (50 μ g/mL) and plasmid was checked by restriction digestion and sequencing. Finally, sufficient amounts of these DNA plasmids for vaccination experiments were purified using the PureLinkTM HiPure Plasmid DNA Gigaprep kit (Invitrogen).

Cloning, expression and purification of recombinant MAP0586c and MAP4308c proteins

The genes coding for histidine-tagged MAP0586c and MAP4308c were cloned in *E. coli* expression plasmid pQE-80L (Qiagen) from pV1.Jns-tPA-MAP0586c and V1.Jns-tPA-MAP4308c respectively using primers: 5'-GGAAGATCTGTGAGCAATCGGCGCACCGCA-3' (forward MAP0586c), 5'-TATAAGCTTCACTGCGGGTGC GCCGCCACG-TAGTCGG-3' (reverse MAP0586c), 5'-TATAGGATCTGCGGTGTGCCCGTGAGG-3' (forward MAP4308c), 5'-TATAAGCTTCAGCCGGCGACCGAGGCGTCGTA-3' (reverse MAP4308c).

Plasmids were checked by restriction digestion and sequencing. Recombinant MAP0586c and MAP4308c were expressed as his-tagged protein in Top-10F' *E. coli* after IPTG induction and purified by affinity chromatography on immobilised nickel-chelate (Ni-NTA) column, as described previously (9).

Vaccination

Mice were vaccinated four times (MAP0586c) at 3 week intervals. For DNA immunisations, mice were sedated with ketamine/xylazine and injected intramuscularly in both quadriceps muscles with $2 \times 50 \mu\text{g}$ of pDNA. For protein boosting (last immunisation only), mice were injected subcutaneously (s.c.) in the back with $20 \mu\text{g}$ of purified protein emulsified in incomplete Freund adjuvant (IFA).

Cytokine production

DNA vaccinated mice were sacrificed 3 weeks after the last immunisation, Spleens from three to four individual mice per group were removed aseptically and homogenised by gentle disruption in a Dounce homogeniser, and cells were adjusted to 4×10^6 white blood cells/ml in RPMI 1640 medium (Gibco, Grand Island, NY) supplemented with 10% foetal calf

serum (FCS), 5×10^{-5} M 2-mercaptoethanol, penicillin, streptomycin and fungizone.

Cells were stimulated with purified recombinant antigens ($5 \mu\text{g}/\text{ml}$) and incubated at 37°C in round-bottom, 96-well microtiter plates in a humidified CO_2 incubator. Cytokine responses against whole protein were tested on spleens from individual mice. Culture supernatants were harvested after 24 hours for interleukin-2 (IL-2) assays and after 72 hours for IFN- γ assays, when peak values of the respective cytokines can be measured. Supernatants were stored frozen at -20°C until testing.

IL-2 bio-assay

IL-2 activity was measured using a bio-assay on IL-2 dependent CTLL-2 cells, as described before (10). Each sample was tested in duplicate. IL-2 levels are expressed as mean counts per minute (cpm) of incorporated (^3H) thymidine.

IFN- γ ELISA

IFN- γ activity was quantified by sandwich ELISA using coating antibody R4-6A2 and biotinylated detection antibody XMGI.2 (both BD Pharmingen). Antigen-specific cytokine levels were considered positive when values were at least fivefold higher than those of unstimulated cells.

Mycobacterium avium subsp. *paratuberculosis* challenge

Immunised BALB/c mice were challenged 6 weeks after the last immunisation. Luminescent MAP ATCC 19698 (11) were grown in Middlebrook 7H9 medium supplemented with OADC, mycobactin J (Allied Laboratories Inc, Synbiotics Europe, $2 \mu\text{g}/\text{ml}$) and hygromycin ($100 \mu\text{g}/\text{ml}$), to an optical density ranging between 0.6 and 0.8. Bacteria were centrifuged for 30 minutes at 2000

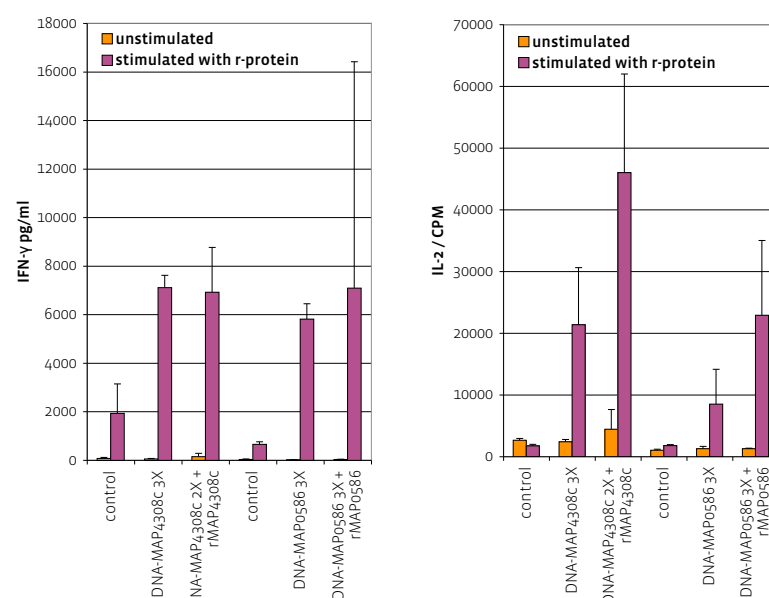


Figure 1: MAP 4308c- and MAP0586c- specific IFN- γ (A) and IL-2 (B) production in spleen cell cultures from mice vaccinated only with V1.Jns-tPA-his-MAP4308c or V1.Jns-tPA-his-MAP0586c DNA or boosted with MAP4308c or MAP0586c protein
A. IFN- γ levels (picograms per millilitre (mean \pm S.D.) for three to four mice tested individually) in spleen cell cultures stimulated or not for 72 hours with purified MAP4308c or MAP0586c protein ($5 \mu\text{g}/\text{ml}$). Mice were sacrificed 3 weeks after the last immunisation.
B. IL-2 levels (counts per minute (mean \pm S.D.) for three to four mice tested individually) in spleen cell cultures stimulated or not for 24 hours with purified MAP 4308c or MAP0586c protein ($5 \mu\text{g}/\text{ml}$). Mice were sacrificed 3 weeks after the last immunisation.

rpm, suspended in PBS to a concentration of 107 CFU/ml (8.5×10^6 RLU/ml). Luminescence was measured in a Lumat LB 9507 Luminometer (Berthold Technologies) as flash emission (15 sec. integration time) using 1% n-decanal (Sigma) in ethanol as substrate. Mice were infected intravenously in a lateral tail vein with 0.2 ml of bacteria and eight weeks after challenge, mice were sacrificed and the number of bioluminescent bacteria was determined in spleen homogenates. For statistical analysis (one way ANOVA, Tukey's Multiple Comparison Test), results obtained in Relative Light Units (RLU) by spleen were converted to \log_{10} values (6).

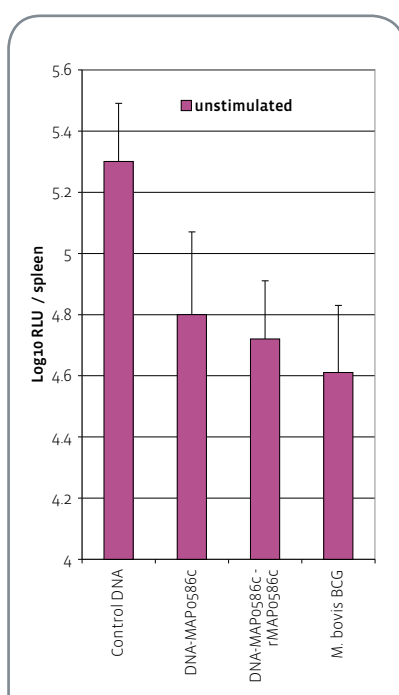


Figure 2: Protective efficacy of MAP0586c vaccine in BALB/c mice. Bacterial replication in spleen from BALB/c mice vaccinated with control DNA, MAP0586c-DNA, MAP0586c-DNA boosted with recombinant MAP0586c protein in IFA adjuvant, and vaccinated with *M. bovis* BCG and infected with bioluminescent *M. avium* subsp. *paratuberculosis* ATCC 19698 8 weeks before. Spleens from individual infected mice were homogenised, and the number of bacteria/organ was enumerated by luminometry (results in RLU converted to \log_{10}). Data represent mean \pm S.D. values of 3–5 mice (numbers given in parentheses). Statistical analysis was performed using GraphPad Prism 4: One-way-ANOVA Tukey: *** ($p < 0.001$), ** ($0.001 < p < 0.01$), * ($p < 0.05$) as compared to \log_{10} RLU values in control DNA vaccinated mice.

RESULTS

Th1 type cytokine secretion in response to MAP0586c and MAP4308c in plasmid DNA-vaccinated BALB/c mice

Vaccination of BALB/c mice with the MAP0586c DNA vaccine (including a vaccination regimen in which DNA vaccination was combined with a recombinant protein boost for the last immunisation) induced elevated levels of antigen-specific IL-2 and IFN- γ (Figure 1). IFN- γ ELISPOT levels were two to threefold higher in mice that had received the combined DNA/protein immunisation. IL-2 levels also tended to be higher in BALB/c mice that had been vaccinated with the DNA/protein combination, but this difference was not statistically significant.

MAP4308c-specific immune responses after vaccination of BALB/c mice are summarised in Figure 1. As for MAP0586c, elevated IL-2 and IFN- γ levels could be detected in mice and again IL-2 levels were increased by the DNA/protein combination.

Protective efficacy of DNA vaccines encoding MAP0586c and MAP4308c

Immunisation with MAP0586c conferred partial protection to BALB/c mice from an experimental challenge infection with luminescent MAP (administered 6 weeks after the last immunisation), as analysed at 8 weeks post-infection and compared to mice vaccinated with empty vector (Figure 2). Both the DNA and the DNA/protein combination effectively reduced the number of bacteria in the spleen, as determined by luminometry and CFU plating.

DNA vaccine encoding MAP4308c did not confer any protection against an experimental challenge infection with luminescent MAP at 8 weeks post-infection (data not shown).

DISCUSSION

Several countries have initiated control programs to stop the spreading of Johne's disease. However, increasing national and international trade, the extremely long incubation period, the long survival time of the bacteria in the environment and the large proportion of subclinically infected animals (difficult to identify with present diagnostic tools) seriously hamper the control of this disease (3).

The development of a marked subunit vaccine composed of one or more MAP specific and protective antigens, which would not interfere with current immunodiagnosis of bovine tuberculosis and paratuberculosis, would be an invaluable tool for the control of Johne's disease. Several research groups have identified MAP antigens for use in serodiagnosis (5, 12–15) and have identified antigens for T cell based diagnosis with a strong IFN- γ inducing activity (9, 16–21). However, only few of these antigens have been analysed for their actual vaccine potential, particularly in target species.

In order to analyse the vaccine potential of MAP0586c and MAP4308c, we used the technique of DNA vaccination. It is well established that intramuscular DNA vaccination of mice is an easy and effective method for generating strong humoral and Th1 biased cellular immune responses, enabling high throughput screening of possible vaccine candidates (22). Here we have shown that DNA vaccination and homologous DNA-prime/recombinant protein-boost vaccination induced substantial production of antigen-specific IFN- γ and IL-2 in BALB/c mice and MAP0586c DNA vaccine conferred significant protection against MAP challenge in BALB/c mice.

In conclusion, our results show that plasmid vaccination with MAP4308c and MAP0586c DNA induced strong Th1 type immune responses and a partial protection from infection with MAP in mice. Preliminary testing of these two antigens in separate herds of naturally MAP and *M.*

bovis infected cattle also showed a very limited cross-reactivity in the *ex vivo* 20h-IFN- γ assay and a clear potential as specific diagnostic reagents (M. Govaerts *et al*, data not shown). It is obvious that more work is needed to confirm these findings in target species of Johne's disease, such as sheep, goats and cattle.

IMPACT ON PUBLIC HEALTH

Crohn's disease is a human illness with similar histopathological findings and symptoms to those observed in Johne's disease in cattle. Discovery of new specific antigens may enable a possible link to be found between these diseases and eventually confirm the implication of *Mycobacterium avium* subsp. *paratuberculosis* in Crohn's disease.

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SOURCE

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Molecular genetics of *para*-aminosalicylic acid resistance in clinical isolates and spontaneous mutants of *Mycobacterium tuberculosis*

INTRODUCTION

Tuberculosis is a highly contagious pulmonary infection caused by *Mycobacterium tuberculosis*. With one third of the world's population infected and around 1.7 million deaths annually, this disease represents an important epidemiological problem (1). The recrudescence of HIV co-infection and the emergence of drug-resistant strains are also worrying. A growing source of public health concern has been the emergence of resistance to multiple drugs (MDR-TB), defined as an isolate resistant to at least isoniazide and rifampin, the two most potent anti-tuberculosis drugs (2). MDR-TB is associated with very high morbidity and mortality, prolonged treatment to cure, and an increased risk of spreading infection with drug resistant isolates in the community. Today, the emergence of *M. tuberculosis* strains resistant to first-line antibiotics has renewed interest in second-line anti-tuberculosis agents, such as *para*-aminosalicylic acid (PAS).

In 1943, Lehmann discovered that an aspirin-like drug called *para*-aminosalicylic acid (Figure 1A) presents anti-tuberculosis activities (3). PAS was successfully introduced to clinical use as far back as 1948 (4). It was the second antibiotic found to be effective in the treatment of tuberculosis, after streptomycin. Combination therapy of tuberculosis with streptomycin and PAS brought much hope in the fight against tuberculosis (5). With the advent of antimycobacterial agent isoniazid in 1951, triple or combination therapy, including PAS, was maintained until the mid-60s. Although PAS-combination therapy proved highly successful, side-effects attributed to PAS were documented as early as 1951. In addition to PAS-associated gastrointestinal toxicity, elevated and repetitive dosing com-

plicated therapeutic regimens. PAS therapy was discontinued after the introduction of rifampin (rifampicin) and pyrazinamide to the treatment. PAS tuberculosis treatment was reintroduced in the United States in 1992, following several outbreaks of MDR-TB isolates (6). Since then, the need for new antibiotics for the treatment of MDR-TB has led to the development of novel formulations of PAS, which were proven to be less toxic (7). Today, PAS is used primarily as a second line drug to treat MDR tuberculosis but despite its introduction to clinical use in 1948, the exact mode of action of PAS is not yet known (8).

Using transposon mutagenesis, Rengarajan and colleagues have shown that PAS resistance is associated with mutations of thymidylate synthase A, encoded by the *thyA* gene and required for thymine biosynthesis in the folate pathway (Figure 1B) (9). *ThyA* catalyses the reductive methylation of dUMP (deoxyuridine monophosphate) to yield dTMP (deoxythymidine monophosphate), required for *de novo* thymidine synthesis. It is hypothesised that the bacteriostatic activity of PAS results from perturbation of the folate pathway, although the underlying mechanism has yet to be elucidated. In our laboratory, we aimed to extend the understanding of mechanisms underlying PAS resistance by analysis of six genes of the folate metabolic pathway and biosynthesis of thymine nucleotides (*thyA*, *dfrA*, *folC*, *folP1*, *folP2*, and *thyX*) and three N-acetyltransferase genes (*nhoA*, *aac1* and *aac2*) within the genome of 118 well-characterised PAS-resistant clinical isolates and spontaneous mutants. To better understand PAS resistance, the identified mutations were correlated with the Minimal Inhibitory Concentrations (MIC) and the protein three-dimensional (3D) *ThyA* structure (10).

MATERIALS AND METHODS

Samples

Fifty-one PAS-resistant isolates were selected from the *M. tuberculosis* collection maintained in the Public Health Research Institute (PHRI), Newark, NJ. All of the isolates were typed by multiple genetic techniques in order to establish their genetic diversity or relatedness. In addition, 55 spontaneous mutants resistant to PAS were selected on agar plates containing PAS (11, 12). To avoid strain bias, spontaneous mutants of eight well-characterised clinical strains were selected. Before DNA extraction and stocking, colonies were subcultured in the presence of various concentrations of PAS for determination of the MIC. Twelve PAS-resistant and PAS-susceptible reference strains were also used as positive and negative controls, respectively. In total, 118 samples were analysed.

Sequencing drug target regions

Mutations in the *thyA* gene (Rv2764c) were identified by sequencing. The loci *folP1* (Rv3608c), *folP2* (Rv1207), *thyX* (Rv2754c) and *dfrA* (Rv2763c) were also analysed. The *folC* (Rv2447c) gene was sequenced in 12 PAS-resistant isolates that did not include any other mutations in genes of the folate biosynthetic pathway. Three additional genes were further sequenced in 5 of these PAS-resistant isolates (without mutation in the other studied genes), including the *nhoA* (Rv3566c), *aac1* (Rv1347c) and *aac2* (Rv0262) recently showed to be implicated in some mechanism of resistance to anti-tuberculosis drugs.

MIC

The Minimal Inhibitory Concentration (MIC) of all the clinical strains and spontaneous mutants were determined by the agar dilution method and Bactec/Alert 3D system (bioMérieux, France). Growth curves were determined for 6 clinical isolates, 2 spontaneous mutants with an early stop codon in the *thyA* gene, 6 spontaneous mutants with other mutations within the *thyA* gene, and 6 PAS-resistant isolates including wild-type genes in the folate and pyrimidine biosynthesis pathway. Growth curves were done on 7H9 broth containing 0, 16, 32, 64 and 128 µg/ml of PAS.

Structural analysis

A 3D model of the *M. tuberculosis* thymidylate synthase *ThyA* homodimer was built with the Modeler program using the X-ray structure of *Escherichia coli* thymidylate synthase as a homologous protein template (13). Thermodynamic stability changes resulting from a single-site mutation were predicted using the PoPMuSIC webserver (14). Finally, at each mutated position, the conservation of wild-type residues and the occurrence of mutant residues were evaluated on an alignment of 279 thymidylate synthase sequences.

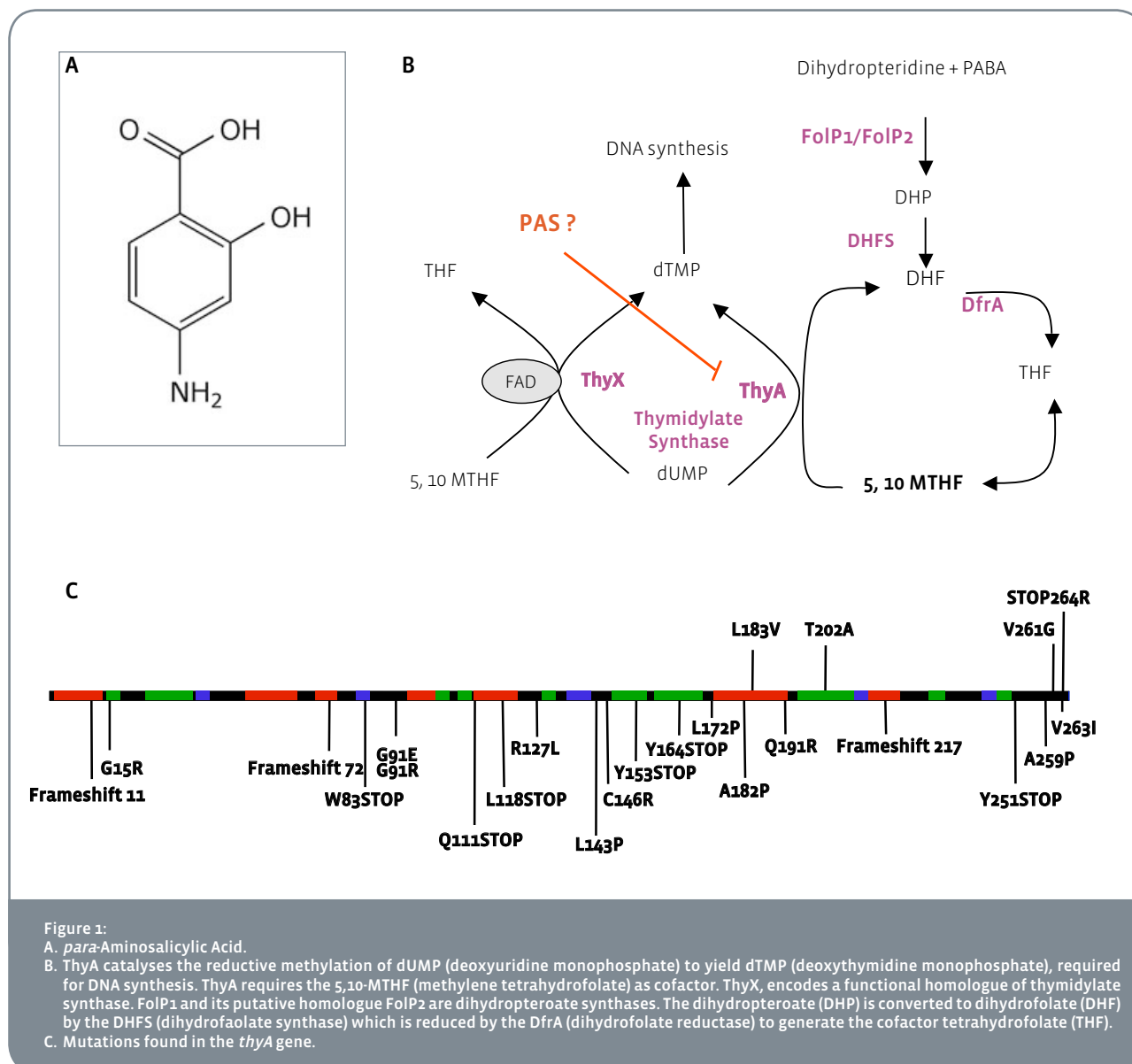


Figure 1:

A. *para*-Aminosalicylic Acid.

B. *ThyA* catalyses the reductive methylation of dUMP (deoxyuridine monophosphate) to yield dTMP (deoxythymidine monophosphate), required for DNA synthesis. *ThyA* requires the 5,10-MTHF (methylene tetrahydrofolate) as cofactor. *ThyX*, encodes a functional homologue of thymidylate synthase. *FolP1* and its putative homologue *FolP2* are dihydropterolate synthases. The dihydropterolate (DHP) is converted to dihydrofolate (DHF) by the DHFS (dihydrofolate synthase) which is reduced by the *DfrA* (dihydrofolate reductase) to generate the cofactor tetrahydrofolate (THF).

C. Mutations found in the *thyA* gene.

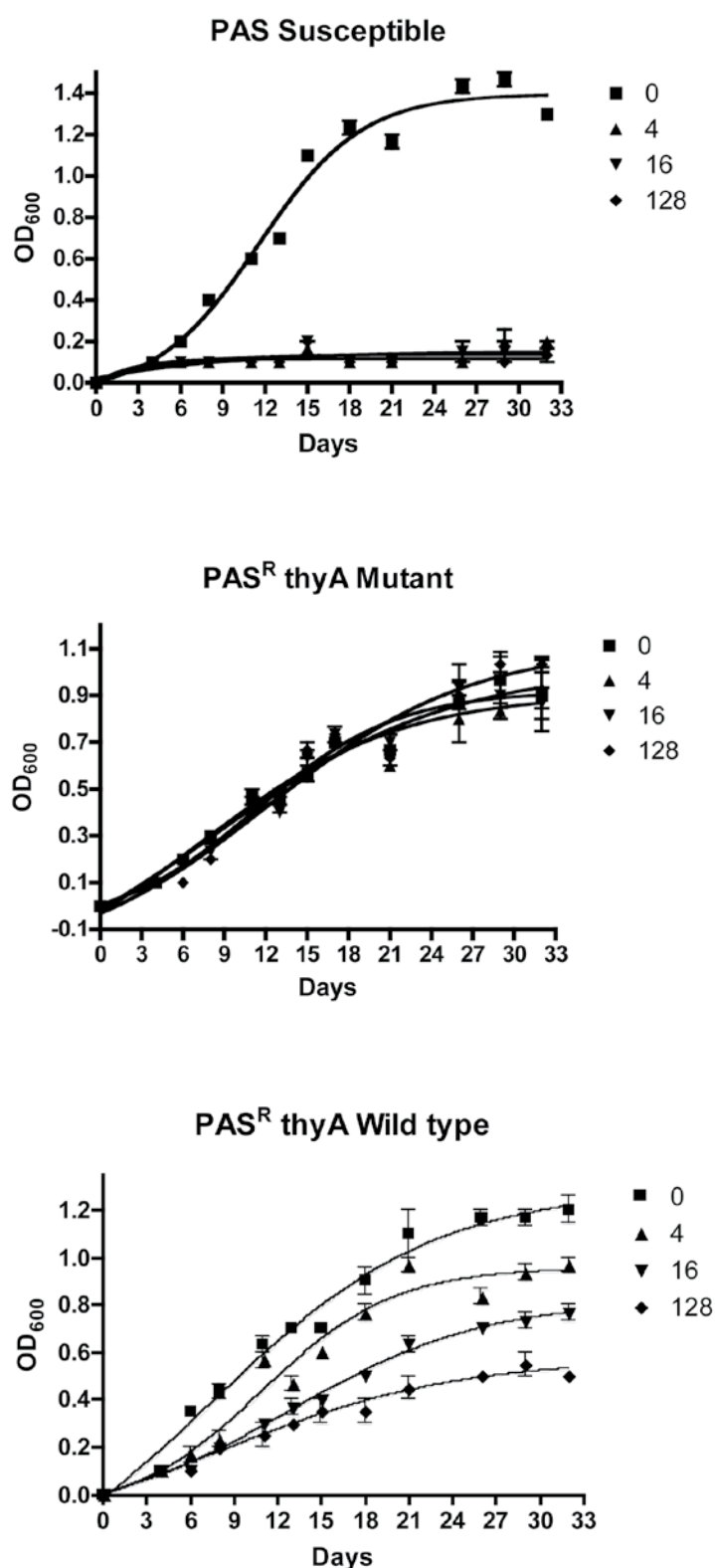


Figure 2: Representative growth curves of PAS-susceptible and PAS-resistant isolates, possessing a *thyA* mutation or not, in the presence of various concentrations of PAS (in µg/ml).

RESULTS AND CONCLUSION

Mutations in the *dfrA*, *folP1/folP2*, and *thyX* genes are not associated with PAS resistance

Three mutations in the *dfrA* gene were identified in two clinical isolates that, in addition, already bore a mutation in the *thyA* gene. No PAS-resistant isolate with a polymorphism only within the *dfrA* gene was found; consequently, it is not known whether the *dfrA* mutations alone contribute to a PAS-resistance. No mutations were found within the *thyX* gene or flanking regions in either the clinical isolates or the spontaneous mutants. Likewise, the *folP1* gene and flanking sequences were conserved, while some polymorphisms were noted in the upstream region of the *folP2* gene (-19A→G). This single-nucleotide substitution was found in both PAS-susceptible and PAS-resistant isolates, suggesting that this mutation is not associated with PAS-resistance, but rather, is a molecular characteristic of some related strains. Indeed, all isolates characterised by this mutation were correlated with a phylogenetic lineage, rather than PAS resistance (15). Additionally, no polymorphism was identified in *folC* and three *N*-acetyltransferase genes, including *nhoA*, *aac1*, and *aac2*. Thus, no mutations within the genes encoding enzymes in the folate and thymine biosynthetic pathway, other than *thyA*, were correlated with a PAS-resistant phenotype.

Thirty-seven percent of PAS-resistant strains have a mutation within the thymidylate synthase A (*thyA*) gene

To our surprise, only 37% of the PAS-resistant clinical isolates or spontaneous mutants encoded mutations in enzymes of the folate pathway. Overall, 24 distinct mutations were identified in the *thyA* gene, including 4 polymorphisms found uniquely in clinical isolates and 20 observed in the spontaneous mutants. It is noteworthy that most polymorphisms were distinct (Figure 1C). Protein structural predictions

indicate that all the mutations recorded reside within essential functional or structural sites.

Only the mutation (202ACC→GCC; 202T→A) most commonly found in clinical isolates was also identified in a single spontaneous mutant. The divergence in mutation type observed in clinical isolates and spontaneous mutants could result from experimental bias due to PAS-resistance selection at elevated concentrations of PAS or to the limited number of PAS-resistant isolates investigated. Alternatively, *ThyA* may not be an essential enzyme for survival *in vitro* while assuming a significant function *in vivo*.

PAS-resistant *M. tuberculosis* P strains have dual mutations in *thyA*

Sequence analysis of 15 PAS-resistant strains belonging to the P strain family was realised. These isolates belong to a larger cluster of over 120 isolates associated with MDR outbreaks in New York City and neighbouring states (16). Their analysis revealed a characteristic mutation in codon 202 of *thyA*. Among these, 12 included a second mutation in the stop codon (stop264R), suggesting that the P strain acquired the 202 mutation prior to the one in the termination codon. Association of the deletion of the stop codon with PAS-resistance could not be confirmed as no isolate with the obliterated 264 stop codon alone was identified. Additionally, two of the double-*ThyA*-mutant P strains developed secondary mutations on the flanking *dfrA* gene, as described above.

Predicting the structural implications of the *thyA* mutations identified

To understand the impacts of the identified mutations on protein function, a 3D homodimer structure of *M. tuberculosis* *ThyA* was modelled. Analysis of the mutations positioned within the structure model revealed three groups of mutants:

1. Mutations that modify the length of the protein (stop mutations and insertion/deletion alterations introducing early frameshift). Such modifications are expect-

ed to render the enzyme non-functional due to the loss of its ternary structure;

2. Mutations of amino acids involved in the active catalytic site or within the cofactor or substrate binding site, and;

3. Mutations that could destabilise the ternary structure of the *ThyA* protein, rendering the enzyme dysfunctional or less active. The observation that most mutants reported here affect highly conserved positions otherwise rarely found in the protein family of thymidylate synthase is noteworthy.

These detrimental alterations to *ThyA* were permissible in *M. tuberculosis* due to the presence of the complementary functional homologue *ThyX*, which, per se, does not seem to be susceptible to PAS.

Elevated MICs in PAS-resistant spontaneous mutants and clinical isolates

The MICs of selected clinical isolates and spontaneous mutants were determined. Samples including mutations within the *thyA* gene proved to be highly resistant to PAS. In addition, the growth curves of representative PAS-resistant samples were followed in triplicate over a period of 32 days (Figure 2). Growth curve profiles of PAS^R *thyA*-mutated strains were all similar, independently of the PAS concentration.

However, the response of PAS-resistant mutants encoding wild-type *ThyA* proteins was found to be dose-dependent. For these isolates, growth was proportionally inhibited as the dose of PAS was increased, clearly indicating the presence of a different PAS resistance mechanism.

Conclusion

In conclusion, mutations in *thyA* were identified in only 37% of the PAS-resistant samples. The absence of mutation in the *thyA* gene and the 8 other genes considered in this study, in 63% of the PAS-resistant strains reveals that PAS-resistance in *M. tuberculosis* involves mechanisms or targets other than those pertaining to the biosynthesis of thymine nucleotides. The differences between the growth curve profiles of PAS-resistant mutants presenting mutations or not, indicates that the as yet unidentified alternative resistance mechanism(s) is PAS concentration dependent, in marked contrast to *thyA*-mutated PAS-resistant isolates.

The observation that PAS is active only in the presence of a functional *ThyA* enzyme supports the idea that, like other antimycobacterials (isoniazid, ethionamide, and pyrazinamide), PAS could be a prodrug whose activation somehow requires a viable mycobacterial enzyme.



IMPACT ON PUBLIC HEALTH

Understanding the mechanisms of resistance to anti-tuberculosis antibiotics may improve the molecules or lead to the development of new effective compounds. Moreover, identification of specific genetic markers associated with resistance may allow the rapid detection of resistant strains and thus permit the avoidance of applying ineffective treatments.

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SOURCE

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- during the “7^e journée des Doctorants” in ULB (Université Libre de Bruxelles-faculté de médecine) (18/12/2007; Brussels, Belgium) poster presentation;
- during the annual meeting of the Belgian Society for Microbiology (BSM) (24/11/2006; Brussels, Belgium) poster presentation.

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Surveillance of antibiotic susceptibility patterns among *Shigella sonnei* strains isolated in Belgium show that cotrimoxazol is no longer appropriate for the treatment of shigellosis

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INTRODUCTION

Shigella species are important pathogens responsible for 5-10% of diarrheal diseases and dysentery occurring all over the world (1). Human infections can usually be either foodborne, waterborne or are caused by human-to-human transmission. Shigellosis produces inflammatory reactions and ulceration on the intestinal epithelium followed by bloody diarrhoea and mucus in the stool. Infection may lead to dehydration and sometimes, most particularly in immunocompromised individuals, even death.

Despite the disease being self-limiting, antibiotic treatment is recommended because it reduces the duration of illness and the transmission rate of the disease by shortening the excretion period of the pathogen. Until 2007, fluoroquinolones, β -lactams and combination of trimethoprim-sulphamethoxazole (cotrimoxazole) represented the drugs of choice to treat shigellosis (2). However, the use of these drugs is becoming compromised by the emergence of strains resistant to these commonly used antibiotics.

The National Reference Centre for *Salmonella* and *Shigella* (NRCSS) has been conducting a continued surveillance programme for antimicrobial resistance of *Shigella* infections in Belgium since 1990. With approximately 400 cases of *Shigella* infections annually reported, shigellosis is the third most important bacterial gastrointestinal disease in Belgium. The majority of these infections (68%) are caused by the species *Shigella sonnei*. The current study presents the evolution of antimicrobial resistance in clinical *Shigella sonnei* strains isolated in Belgium over a 19-year period (between 1990 and 2009) (4).

METHODS

Identification, typing and antimicrobial susceptibility testing of *Shigella* isolates

Data were collected by the National Reference Centre for *Salmonella* and *Shigella*, which confirm and type the *Shigella* strains. Serotyping was performed by slide agglutination with commercial antisera from Denka Seiken Co (Derbyshire, United Kingdom). The susceptibility of thirteen antibiotics was determined by the disc diffusion method (Kirby-Bauer) following recommendations of the National Committee for Clinical and Laboratory Standards Institute (CLSI), formerly the National Committee for Clinical Laboratory Standards (NCCLS) (5).

RESULTS

Between 1990 and 2009, a total of 8,068 strains, mainly isolated from stools (98.2%) were diagnosed by peripheral laboratories

before serotyping confirmation as *Shigella* by the NRCSS. 5,458 were serotyped as *Shigella sonnei* (67.6%), 2,051 as *S. flexneri* (25.4%), 287 as *S. boydii* (3%) and 178 as *S. dysenteriae* (2.2%) (Figure 1).

Among the 5,458 *S. sonnei* strains isolated between 1990 and 2009, 3,413 (62.5%) were randomly selected for antimicrobial susceptibility testing. These *Shigella sonnei* isolates were resistant to streptomycin (Str) (82%), sulphonamide (Sul) (76%), trimethoprim (Tmp) (80.8%), sulfamethoxazole-trimethoprim (Sxt) (95.7%; only tested from 2000 to 2009) and tetracycline (Tet) (63.8%).

In 1990 the resistance to Str, Sul and Tmp was 74.4%, 70.8% and 61.1%, respectively. However in 2007 resistance increased and reached the respective levels of 90.4%, 79.8% and 90% ($P < 0.01$). Resistance to Sxt increased from 72.2% (2000) to 83.8% (2007) with a peak of 97.2% in 2005 ($P < 0.05$) (Figure 2). A significant rise in resistance to nalidixic acid (Nal) has also been observed since 1998 ($P < 0.01$), from values $\leq 2.5\%$ in the

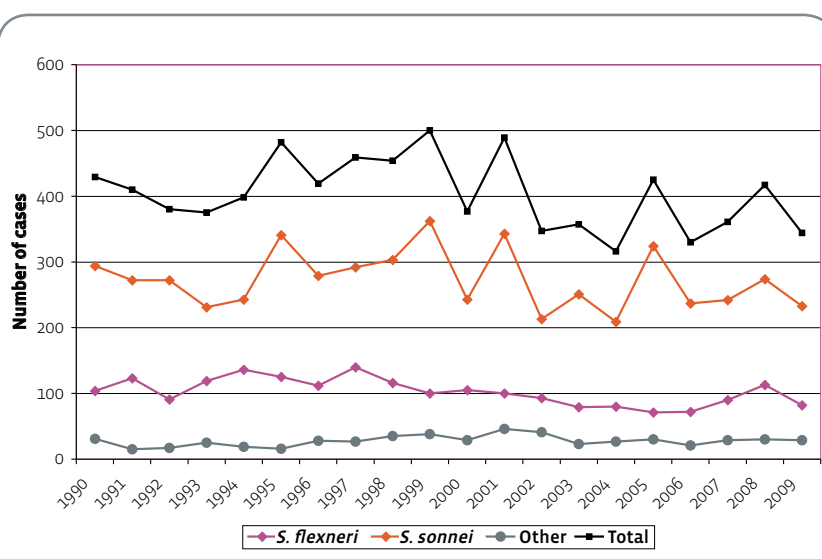


Figure 1: Prevalence of *S. sonnei* and *S. flexneri* in Belgium from 1990 to 2009.

nineties to 13.8% in 2009. Moreover, all these strains exhibited reduced susceptibility to ciprofloxacin (Cip) (MICs between 0.25 and 0.5 µg/ml).

Multidrug-resistance (MDR), defined as resistance to at least 4 antibiotics, was common among *S. sonnei* strains with an average of 68.3% between 1990 and 2009. The MDR frequency increased from 55.9% (1990) to 80.8% (2009) and reached a peak of 89% ($P < 0.01$) between 2004 and 2005 (Figure 2). Predominant MDR profiles observed in *S. sonnei* were Tet^RStr^RSul^RTmp^R(Sxt^R) (45.1%), Amp^RTet^RStr^RSul^RTmp^R(Sxt^R) (6.36%), Amp^RStr^RSul^RTmp^R(Sxt^R) (5.6%), and Tet^RNaI^RStr^RSul^RTmp^R(Sxt^R) (3.7%). Taken together these 4 MDR profiles were responsible for 93.3% of the multidrug-resistances in *S. sonnei* between 1990 and 2009. The incidence of these 4 predominant MDR profiles, already present in 1990 (47.6%), rose to 75.3% in 2007 with a peak of 87.2% observed in 2004 ($P < 0.01$).



Figure 2: Percentage of Sul, Tmp, Stx and MDR profile of *S. sonnei* strains in Belgium from 1990 to 2009.



CONCLUSION

This study represents the first report of long term surveillance concerning antimicrobial resistances in clinical *S. sonnei* strains conducted in Belgium since 1978 (3).

As observed in other countries, the NRCSS of Belgium also recorded an increase in antibiotic resistances in *S. sonnei* during their surveillance programme from 1990 to 2009. In particular, resistance to streptomycin and trimethoprim/cotrimoxazole (trimethoprim + sulfamethoxazole) substantially increased during this period to reach resistance levels of 80% of resistant isolates, and 90%, respectively. The high prevalence of resistance to cotrimoxazole could be explained by the prolonged use of this antibiotic to treat shigellosis, thereby ensuring a continuous selection pressure and thus the maintenance of the resistance profile. Indeed, in the 1970s, the use of cotrimoxazole for the treatment of shigellosis was highly recommended due to the capacity of this antibiotic to be absorbed from the gastrointestinal tract. Furthermore, it was proved that using cotrimoxazole signifi-

cantly reduced the duration of the illness. Although the Sanford guide to antimicrobial therapy (Belgium/Luxembourg version) still recommended in 2007 the use of cotrimoxazole in case of treatment failure with fluoroquinolones (2), our study has demonstrated that this antimicrobial is no longer appropriate for the treatment of shigellosis in Belgium.

Together with the increase of cotrimoxazole resistance, the frequency of MDR isolates increased from approximately 50% in 1990 to 80.8% in 2009. This increase was clearly associated with the emergence of MDR profile Tet^RStr^RSul^RTmp^RSxt^R (45.1%) and to a lesser extent with MDR profiles Amp^RStr^RSul^RTmp^RSxt^R, Amp^RTet^RStr^RSul^RTmp^RSxt^R and Tet^RNal^RStr^RSul^RTmp^RSxt^R. Interestingly, all these MDR profiles harboured resistance to streptomycin and cotrimoxazole combined or not with ampicillin, tetracycline and naladixic acid resistance.

In 2007, the Sanford guide to antimicrobial therapy (Belgium/Luxembourg version) recommended the use of second generation fluoroquinolones or norfloxacin to treat Shigellosis. It must be noted that, since 1998, the NRCSS recorded an increased resistance rate to Nal, while no full

resistance to ciprofloxacin (MICs between 0.25 and 0.5 µg/ml) has been detected so far in *S. sonnei* isolated in Belgium.

At the present time, ciprofloxacin is still one of the most potent antimicrobials available for medical treatment of invasive gastro-intestinal infections in adults (6). However, a decreased susceptibility to fluoroquinolones has become a major problem in medical practice. Aarestrup *et al.* (1) reported several treatment failures in patients infected with *Salmonella* isolates due to a decreased susceptibility to fluoroquinolones. Therefore, in Belgium, the emergence of naladixic acid resistance and the possible decrease in the susceptibility to fluoroquinolones must be closely monitored in *Enterobacteriaceae*.

In conclusion, when antibiotic treatment of shigellosis is indicated, cotrimoxazole should no longer be considered as an appropriate empirical therapy for shigellosis in Belgium. Resistance rates to nalidixic acid should also be carefully monitored to detect any shift in fluoroquinolone resistance because it represents the first line of antibiotics used in the treatment of shigellosis.

IMPACT ON PUBLIC HEALTH

Analysis and surveillance of antibiotic susceptibility of *Shigella* have a direct influence on the guidelines for antibiotics prescription in cases of shigellosis in Belgium. The 2009-2010 edition of the Sanford guide to antimicrobial therapy will be amended on the basis of the recommendations included in this publication.

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SOURCE

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Unusual *Aspergillus* species in patients with cystic fibrosis: interest in accurate identification and characterisation

INTRODUCTION

Patients with cystic fibrosis (CF) are at high risk of colonisation of the airways by various microorganisms, mainly bacteria, but also several filamentous fungal species, particularly some *Aspergillus* or *Scedosporium* species, *Exophiala dermatitidis* and the more recently described fungus *Geosmithia argillacea*. Among the *Aspergillus* species associated with CF, *A. fumigatus* is the most common agent of chronic colonisation of the airways with a high prevalence rate, ranging up to 56% (1). Other *Aspergillus* species recovered from respiratory specimens of CF, which may be responsible for chronic colonisation, are *A. terreus*, *A. flavus*, *A. niger*, *A. nidulans* and *A. versicolor*, with a varying prevalence rate within the different European countries. In the routine laboratory, other unusual or poorly sporulating *Aspergillus* species are generally identified as *Aspergillus* sp. However, identification of clinical *Aspergillus* isolates at the species level may be important as species may differ in their antifungal susceptibility patterns (2). For instance, some unusual *Aspergillus* species are *in vitro* resistant to some of the antifungal drugs commonly used for treatment of *A. fumigatus* infections, *i.e.* itraconazole (ITZ) and voriconazole (VRZ). The prevalence rate of these “uncommon” species is unknown, possibly low, but probably underestimated as molecular methods are required to ascertain reliable identification at the species level. Moreover, their clinical significance may be important. Here, we report four cases of colonisation of the airways in CF patients by unusual *Aspergillus* species belonging to the section *Fumigati*, but initially identified as *Aspergillus* sp. due to discrepancies with key features of

Fumigati section species. Detailed analyses confirm that these isolates belonged to *A. lentulus* or *Neosartorya pseudofischeri*, two species which can be isolated from the environment and show a worldwide distribution (3).

Case reports

• Patient 1

Male, born in December 1979, homozygous for the F508 Del mutation, he was diagnosed for CF at the age of 5 years. The patient was colonised by *Staphylococcus aureus* at the age of 8 years, by *A. fumigatus* and *S. apiospermum* at 11 years, and by *Pseudomonas aeruginosa* and *Candida albicans* at 12 years respectively. At this time, antibody response for *A. fumigatus* and *S. apiospermum* started to be positive and the patient received discontinuously ITZ from eight years. Sixty-one multiple and sequential isolates of *A. fumigatus* were collected for molecular typing during a 9-year period (from 1991 to 2000) (4). During morphological reassessment of these isolates, one “atypical” poorly sporulating isolate was observed among the 5 isolates collected from one sputum sample (13/01/1999).

• Patient 2

Male, born in 1982, and homozygous for the F508 Del mutation, he was diagnosed for CF at the age of 5 months. He was colonised by *S. aureus* since the diagnosis of CF, and later by *A. fumigatus* and *Achromobacter xylosoxidans* subsp. *xylosoxidans* at the age of 17 and 22 years, respectively. Serology for *A. fumigatus* always remained negative. Nevertheless, one year after the beginning of the colonisation by *A. fumigatus*, the patient received discontinuously and successively ITZ and VRZ. In November

2008, an “atypical” poorly sporulating isolate was recovered and the same observation was made 5 months later (April 2009).

• Patient 3

This male patient also homozygous for the F508 Del mutation was diagnosed prenatally in 1998. The patient was colonised by methicillin-resistant *S. aureus* and developed *Pseudomonas aeruginosa* infection at the age of two and five years, respectively. The patient was then colonised by several hyphomycetes: *Aspergillus fumigatus* since he was 7 years old, *Scedosporium apiospermum* and *Geosmithia argillacea* at 8 years, and *S. prolificans* at 9 years respectively. Two years after colonisation by *A. fumigatus*, the patient developed *Aspergillus* derived asthma and he was treated by ITZ and corticoids. Two years later (18/06/2009), *A. fumigatus* was isolated together with an “atypical” poorly sporulating isolate. Isolates with the identical morphology were isolated 2, 4 and 6 months later (25/08/2009, 05/10/2009 and 14/12/2009).

• Patient 4

For this male patient born in 1984 and colonised by *S. aureus* since early childhood (exact date unknown), CF was diagnosed at the age of 3 years with homozygosity for the F508 Del mutation with presence of IVS8-T tract 7T variant. When starting medical attendance in November 2003, the patient was diagnosed with *S. aureus*, *Achromobacter xylosoxidans* subsp. *xylosoxidans* and an “atypical” poorly sporulating *Aspergillus* isolate. The patient was in continuous medical attendance until November 2006 and was then lost for further observation but of note, he was still alive in November 2009. Isolates with the same atypical morphology were collected

on 27/07/2004, 04/10/2004 and 19/07/2005, whereas two typical isolates of *A. fumigatus* were recovered from a sputum sample collected on 04/09/2006. During the 3 years of attendance, there were no signs for APBA, especially no specific anti-*A. fumigatus* antibodies detectable. Likewise, there were no pancreatic insufficiency or signs of diabetes mellitus (body mass index 17 and FEV₁ 1.38 l (33%) in November 2006). Despite intermittent antibiotic treatment with ceftazidime and tobramycine, *A. xylosoxidans* could not be eradicated. Due to the relative good condition of the patient, no attempt was done to eradicate this atypical *Aspergillus* during his medical attendance.

MATERIALS AND METHODS

Fungal isolates

All the isolates presented in this study (Table 1) were recovered from sputum samples. They were preserved and referenced in the BCCM/IHEM Collection (Scientific Institute of Public Health, Brussels, Belgium (http://bccm.belspo.be/db/ihem_search_form.php). The reference strains of *A. lentulus* and *N. pseudofischeri* (Table 1) were obtained from the CBS (Centraal bureau voor schimmelculturen) Culture Collection (Utrecht, The Netherlands) and the reference strain of *A. fumigatus* (Af293) from the National Collection from Pathogenic Fungi (NCPF, Bristol, UK).

Molecular identification

Molecular identification was performed by sequencing of β -tubulin gene (5). The obtained sequences were assembled using the SeqMan II software (Lasergene 8.0, DNASTar, Inc., Madison, WI, USA) and finally compared to available sequences in the GenBank database using the BLASTn program of the National Centre for Biotechnology Information.

Table 1: Isolates of *Aspergillus lentulus* and *Neosartorya pseudofischeri*: sources, isolation dates, conidiation rate and antifungal susceptibility.

Source	IHEM or CBS number	Molecular identification	Isolation date	Conidiation rate	MIC 95 (µg/ml)		
					AmB	VRZ	ITZ
Reference	CBS 175.97	<i>A. lentulus</i>		++	8	8	1
Reference	CBS 116883	<i>A. lentulus</i>		+	2	4	1
Reference	CBS 208.92	<i>N. pseudofischeri</i>		++	0.5	4	2
Reference	NCPF 7367	<i>A. fumigatus</i>		+++++	2	1	0.5
Patient 1	IHEM 22112	<i>A. lentulus</i>	13/01/1999	++	4	8	2
	IHEM 22113	<i>A. fumigatus</i>	13/01/1999	+++++	1	2	1
	IHEM 22114	<i>A. fumigatus</i>	13/01/1999	+++++	1	1	0.5
	IHEM 22115	<i>A. fumigatus</i>	13/01/1999	+++++	2	0.5	1
	IHEM 22116	<i>A. fumigatus</i>	13/01/1999	+++++	2	1	0.5
Patient 2	IHEM 22905	<i>N. pseudofischeri</i>	Nov 2008	++	0.5	4	1
	IHEM 23044	<i>N. pseudofischeri</i>	Apr 2009	++	0.5	4	1
	IHEM 23045	<i>A. fumigatus</i>	Apr 2009	+++++	2	1	> 16
Patient 3	IHEM 23161	<i>N. pseudofischeri</i>	18/06/2009	+++	0.25	8	1
	IHEM 23434	<i>N. pseudofischeri</i>	25/08/2009	+++	0.5	8	0.5
	IHEM 23435	<i>N. pseudofischeri</i>	05/10/2009	+++	1	8	0.5
	IHEM 23463	<i>N. pseudofischeri</i>	14/12/2009	+++	1	8	1
Patient 4	IHEM 23146	<i>N. pseudofischeri</i>	Nov 2003	++	0.25	8	1
	IHEM 23147	<i>N. pseudofischeri</i>	27/07/2004	++	0.25	4	1
	IHEM 23148	<i>N. pseudofischeri</i>	04/10/2004	++	0.5	8	2
	IHEM 23149	<i>N. pseudofischeri</i>	19/07/2005	++	0.5	8	1
	IHEM 23150	<i>A. fumigatus</i>	04/09/2006	+++++	2	0.5	0.5
	IHEM 23151	<i>A. fumigatus</i>	04/09/2006	+++++	2	0.5	0.25

Morphological characteristics and growth rate

Morphological criteria to discriminate *A. fumigatus*, *A. lentulus* and *N. pseudofischeri* are used according to data accumulated by Samson *et al.* (3). All isolates were cultured on Malt Extract Agar (MEA) with incubation at 37°C. Texture and colour of the colonies, conidiation and its importance, vesicle form and size, and conidia form and size were recorded. The isolates of *N. pseudofischeri* were also grown on medium with straw in order to stimulate the sexual reproduction.

Growth at different temperatures of incubation 25°C, 37°C, 45°C, 48°C and 50°C was evaluated for the 22 isolates listed in Table 1 (*A. fumigatus*, n = 8; *A. lentulus*, n = 3; *N. pseudofischeri*, n = 11).

Antifungal susceptibility testing

Broth microdilution antifungal susceptibility testing of reference strains and patients' isolates of *A. lentulus*, *N. pseudofischeri* and *A. fumigatus* was performed according to CLSI 38-A2 protocol with slight modifications using amphotericin B (Bristol Myers Squibb, Braine l'Alleud, Belgium), itraconazole (Janssen Research Foundation, Beerse, Belgium) and voriconazole (Pfizer, Brussels, Belgium). All experiments were repeated twice on different days.

RESULTS

Molecular identification

For species identification of the poorly sporulating isolates, molecular identification was required. Sequences analyses of the β -tubulin gene allowed us to identify the isolate IHEM 22112 from patient 1 as *A. lentulus* (99% identity with the corresponding sequence EF669825.1 from strain CBS 117885, ex type strain of *A. lentulus*). Sequences obtained for the poorly sporulating isolates from patients 2, 3 and 4 ex-

hibited 99%, 100% and 99% identity with the β -tubulin sequence AY870742 from the *N. pseudofischeri* strain CBS 208.92 (ex type strain of *N. pseudofischeri*), respectively.

Morphological characteristics and growth rate

A further morphological investigation was performed for all the isolates in order to substantiate the results from β -tubulin sequence analysis. Isolate IHEM 22112 from patient 1, identified as *A. lentulus*, was poorly sporulating. The sporulation rate of the 11 isolates from patients 2, 3 and 4, identified as *N. pseudofischeri*, were variable from one patient to another (Table 1), but all isolates recovered from a given patient exhibited the same sporulation rate. After several subcultures, particularly on a medium containing straw, conidiation was enhanced, except for the reference strain (type strain) which showed a very low sporulation rate. All attempts to obtain the full sexual state, including several subcultures on straw-containing medium, were unsuccessful for all the isolates studied including the reference strain.

Figure 1 shows the mean diameter of the colonies for the three species measured

at 72 hours. Growth was enhanced for all isolates and reference strains at 37°C. *N. pseudofischeri* grew a little slower than *A. fumigatus*, and the smallest colonies were observed for *A. lentulus*. Increasing the incubation temperature markedly affected the growth for *A. lentulus* and *N. pseudofischeri*. Conversely to *A. fumigatus* which gave colonies covering more than half of the plates after 72 hours of incubation at 45°C, small colonies were obtained in the case of *A. lentulus* and *N. pseudofischeri*. A very restricted growth was observed at 48°C for *A. lentulus* and *N. pseudofischeri* isolates respectively, and none of them grew at 50°C.

Antifungal susceptibility testing

Antifungal susceptibility of patients' isolates and reference strains is shown in Table 1. The isolate of *A. lentulus* from patient 1 was resistant to AmB and VRZ, but it was strikingly susceptible to ITZ. The MIC values were similar to those obtained for the reference strains of *A. lentulus*. The 11 isolates of *N. pseudofischeri* from patients 2, 3 and 4 exhibited quite the same antifungal susceptibility pattern: they were all resistant to VRZ (MIC values ranging from 4 to 8 μ g/

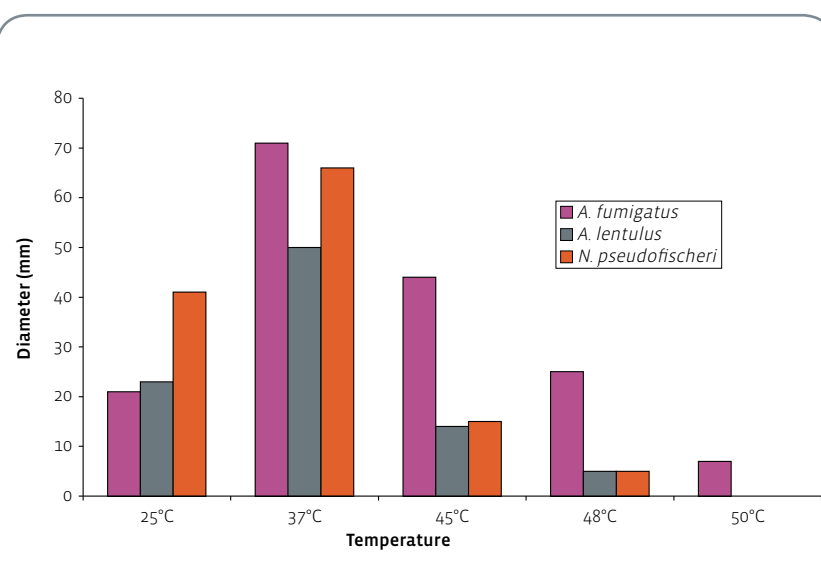


Figure 1: Mean diameter of the colonies of *A. fumigatus* (n = 8), *A. lentulus* (n = 3) or *N. pseudofischeri* (n = 11) clinical isolates or reference strains after 72 hours of incubation.

ml, which were very close to the MICs obtained for the reference strain). Conversely, they were all susceptible to AmB (MIC values: 0.25-0.5 µg/ml) and ITZ (MIC values: 0.5-2 µg/ml). Additionally, only one out of the 7 clinical isolates of *A. fumigatus* (IHEM 23045) recovered from these patients was resistant to ITZ.

IMPACT ON PUBLIC HEALTH

This report underlines the role of the National Reference Centre in Medical Mycology for identification at the species level of unusual fungi and for determination of their antifungal susceptibility profile.

CONCLUSION

This study underlines the difficulties in identifying poorly sporulating *Aspergillus* species from the section *Fumigati* only on the basis of morphological criteria. *Aspergillus fumigatus*, *A. lentulus* and *N. pseudofischeri* have overlapping morphological characteristics (3) and therefore use of sequence based methods is required for correct identification at the species level. Moreover, it is not uncommon that clinical isolates of *A. fumigatus* exhibit atypical phenotypes, with white or grey colonies instead of the typical dark green colour and poor sporulation. A very useful

and important physiological property of *A. fumigatus* for its differentiation from the other species of the section *Fumigati* is its ability to grow at 50°C. Both species *A. lentulus* and *N. pseudofischeri* here recovered from patients with CF are poorly susceptible to current antifungal drugs. *A. lentulus* showed resistance to AmB and VRZ whereas *N. pseudofischeri* isolates were found to be resistant to VRZ. The obtained MIC values were in agreement with the literature (2, 5-7). We also showed that *N. pseudofischeri* may coexist with ITZ-resistant isolates of *A. fumigatus*. The fact that *A. fumigatus* was reported to colonise bronchial mucus of CF in the form of biofilms may

explain higher MICs. Additionally, one may speculate that repeated therapies with antifungals in CF patients colonised by *A. fumigatus* may account for the ITZ resistance of the isolates studied. Antifungal treatments may also lead to elimination of susceptible *A. fumigatus* isolates and favour persistence of resistant species such as *A. lentulus* and *N. pseudofischeri*.

In conclusion, this study underlines the importance of identification at the species level of unusual or poorly sporulating *Aspergillus* isolates since some species within the *Fumigati* section may be poorly susceptible to some of the current antifungal drugs.

SOURCE

This work was presented in part during the 1st Meeting of the ISHAM Working group on Fungal respiratory infections in Cystic Fibrosis that was held in Angers, France, on June 7-8, 2009.

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Virological surveillance of Influenza A(H1N1)2009 pandemic: the role of the Belgian National Influenza Centre

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INTRODUCTION

On 24 April 2009, the World Health Organisation (WHO) first reported the emergence of a new influenza virus in the United States of America and Mexico (1). This novel influenza virus was identified as a new A(H1N1) subtype resulting from a re-assortment of avian, human and swine influenza viruses. This virus, which was later referred to as A(H1N1)2009, rapidly demonstrated its capacity to transmit among humans (2). On 11 June 2009, the WHO decided to raise the pandemic alert to the maximum phase 6 level (3).

Influenza in humans is caused by one of three types of influenza viruses A, B and rarely C, which all belong to the orthomyxoviridae family, and have two glycoproteins on the surface of their virions, the Hemagglutinin (HA) and the Neuraminidase (NA). These proteins elicit antibody responses to the influenza virus. So far sixteen different HAs (H1 to H16) and 9 different NAs (N1 to N9) have been recognised. Several combinations of HA and NA proteins are possible, each combination representing a different subtype (for example A/H1N1, A/H3N2, A/H1N2...). An important characteristic of influenza viruses is their ability to evolve continuously to escape the immune response. The mechanisms behind this evolution are either antigenic drift (point mutations in the HA and NA genes) or antigenic shift (re-assortment between different Influenza viruses).

For a virus to cause a pandemic, two major criteria must be met: the virus must be new (antigenic shift), as such a large proportion of the population is susceptible to infection, and the virus must be transmissible from person to person (4, 5). In this context, the initial reporting of this novel transmis-

sible influenza variant to the WHO and the continuous surveillance of this virus is of the highest importance. It enables virus evolution to be monitored and the risk to public health worldwide to be reduced (6). In Belgium, Influenza virus surveillance is ensured by the National Centre for Influenza (NIC). According to the WHO (7, 8), the responsibilities of a NIC during a pandemic period are: 1. to provide laboratory diagnosis for monitoring the geographical spread of the pandemic in the country; 2. as the pandemic intensifies and becomes widespread, adjust virological surveillance in order to monitor the progress of the pandemic in the country; 3. maintain adequate virological surveillance to assist the WHO in monitoring for example antigenic and genetic changes in the pandemic virus, pathogenicity, and susceptibility to antiviral treatment.

METHODS

Sampling during the containment phase

From 26 April to 13 July 2009, a **containment** strategy was implemented according to the WHO and CDC recommendations, in order to limit the extension of the epidemic (9). The Interministerial Influenza Coordination Committee established a series of measures and protocols to manage all suspect cases and contacts regarding notification, sampling, prophylaxis, treatment and isolation (10). An active surveillance system was then established, to detect all suspect cases of A(H1N1)2009 and to trace their contacts. In collaboration with the community health inspectors, physicians were asked to take a sample from each patient showing flu symptoms and returning from infected countries or regions. Due

to the rapid geographic extension of the pandemic, the list of such countries was updated regularly.

During this period, more than 3,000 sampling kits were sent to physicians, laboratories and hospitals all around Belgium and additional kits were provided on request. Each kit contained 4 swabs, transport medium, FFP2 masks, an information form and a detailed sampling procedure to take respiratory secretions from nose and throat. Samples needed to be sent to the NIC on the same day, in order to give a diagnostic result within 5-6 hours. The WIV-ISP laboratory was operational 24/24 hours, 7/7 days, in collaboration with all involved public health authorities.

Sampling during the mitigation phase

From 13 July 2009 onwards, because of the sustained community spread, Belgium's strategy shifted to a **mitigation** phase (week 29 to recent), where systematic laboratory confirmation testing was no longer required. Only high risk and hospitalised patients were to be tested for A(H1N1)2009 if suspected.

During this phase, the NIC strengthened the nationwide surveillance system to study the A(H1N1)2009 virus circulation in the Belgian population and to help reduce influenza morbidity and mortality. The influenza virological sentinel surveillance network, previously composed of 98 general practitioners (GPs) distributed across the whole country (11), was expanded to 139 GPs (78 GPs located in Flanders, 49 in Wallonia and 12 in the Brussels-Capital Region). The latter GPs were all part of Sentinel General Practitioners composed of approximately 160 GPs, who are also involved in clinical influenza surveillance (11). The decision to enlarge the virological sentinel

surveillance network was taken when the first three positive A(H1N1)2009 cases in Belgium were diagnosed via this network during week 27.

Laboratory testing

At the start of the pandemic, there was still a lack of information about the pathogenicity and transmission of the virus. Therefore, and to avoid any possible re-assortment between strains from swine and human origins, manipulation and RNA extraction of suspect specimens were performed in the biosafety level 3 laboratory (BSL3) of the WIV-ISP instead of BSL2 facilities where similar samples are routinely tested within the framework of monitoring seasonal flu. Viral RNA from clinical samples was extracted in duplicate with the QIAamp® Viral RNA Kit from QIAGEN (Hilden, Germany). The extracted RNA was submitted to a series of real time PCR tests in a BSL2 environment. The real time PCR reactions were performed on a Stratagene Mx3000P™ thermal cycler using the SuperScript® III Platinum® One-Step qRT-PCR System from Invitrogen (Carlsbad, California, USA). At first, there was no specific test avail-

able for the new pandemic influenza virus. Therefore the suspect samples were tested for influenza A and B, and subtyped for seasonal influenza A/H1 and A/H3. Primers and probes for each type (A/B) and subtype (H1/H3) were designed in a conserved gene region, selected either from the literature or after multiple-sequence alignment with a large amount of recent human and animal strains available at GenBank. Regions of the matrix gene (12) and of the Hemagglutinin gene (13) were amplified for typing A and B respectively. For the subtyping, a region in the HA gene was amplified. A sample which was positive for influenza A and negative for both seasonal subtypes (H1N1 and H3N2) was considered as a probable case.

From 3 May 2009, specific primers and probes for A(H1N1)2009 virus were sent by the Centers for Disease Control and Prevention (CDC) of Atlanta (14) and used as standard tests in our laboratory. The workflow of the tests is shown in Figure 1.

A series of randomly selected samples from the start, the peak and the end of the pandemic, as well as samples from more severely affected patients, were also sequenced, in order to detect any mutations

in the hemagglutinin or neuraminidase gene, including mutations associated with resistance to neuraminidase inhibitors. A selection of approximately 200 samples was sent to London (WHO Collaborating Centre) for antigenic characterisation and monitoring of antiviral resistance.

RESULTS

On 12 May 2009, the NIC confirmed the first case of influenza A(H1N1)2009 in a patient returning from the United States. In total, 614 patient samples were tested during the containment phase (week 18 to 28) in Belgium, of which 123 cases were confirmed positive for A(H1N1)2009, the majority of them being imported cases. One sample out of the 614 analysed samples was positive for influenza B, 15 samples were confirmed to be A/H3N2 positive and 13 others were found to be positive for influenza A but could not be subtyped. This may be due to the lower sensitivity of the PCR test for subtyping H1 in comparison with the PCR for typing A or due to evolution of the virus leading to mismatches with the primers or probes.

During the mitigation phase, 729 additional suspect cases were tested, with 285 confirmed cases for A(H1N1)2009, 3 cases positive for influenza A/H3N2 and 47 cases positive for influenza A which could not be subtyped.

In the first phase of the pandemic, a high number of suspected samples received from Belgian physicians, laboratories and hospitals (non-sentinel network), was analysed but revealed a relatively low number of A(H1N1)2009 confirmed cases (Figure 2A). This is especially true for week 28. Several factors could be implicated: the low incidence of influenza A(H1N1)2009 at that time, the high media attention to the flu crisis, the subsequent psychological impact on the population and also the first people returning from holidays from countries where the A(H1N1)2009 strain was already detected. A second peak in the number of analysed samples was observed in week 46, coinciding more or less with the

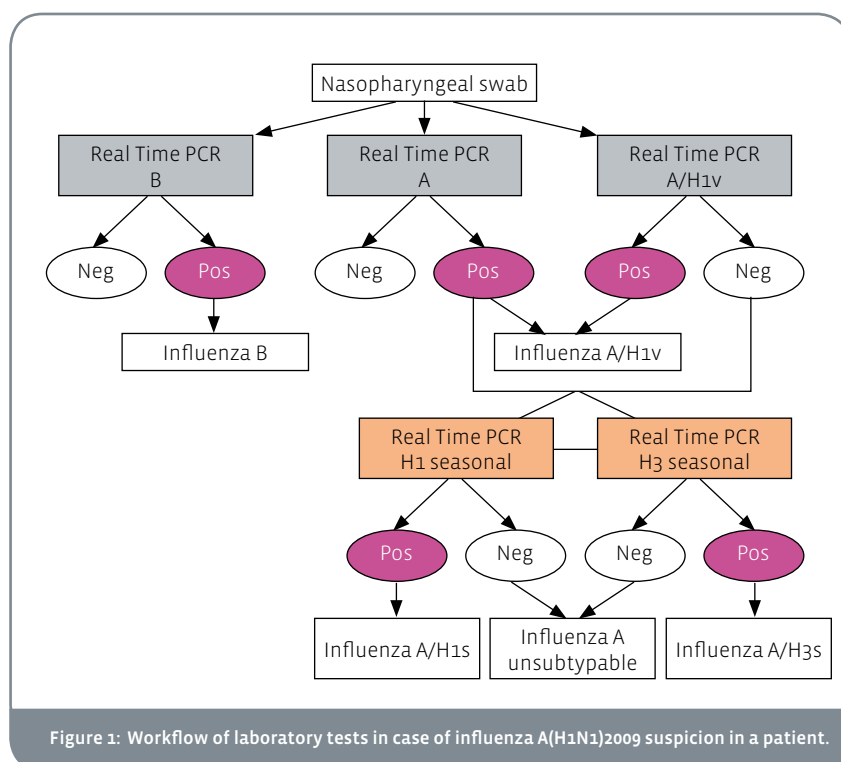


Figure 1: Workflow of laboratory tests in case of influenza A(H1N1)2009 suspicion in a patient.

epidemic peak, characterised by a high percentage of confirmed A(H1N1)2009 cases. A low number of influenza A/H3N2 could still be detected until week 34, probably corresponding to the end of the seasonal influenza epidemic 2008-2009 which was characterised by a high prevalence of the A/H3N2 subtype. In week 21, an increase in the percentage of A(H1N1)2009 positive samples was observed, resulting from a higher number of confirmation tests (samples already tested positive for influenza A but still untyped by other laboratories) at this stage of the epidemic.

Figure 2B shows the weekly evolution of the number of analysed samples and of influenza A(H1N1)2009 positive samples in the sentinel network. In Belgium, the proportion of positive samples increased from 28% in week 40 to 73% in week 44 (corresponding to the epidemic peak) (11). The proportion then decreased slowly from week 46 (51%) to week 52 (25%). From week 43 to 44, the graph shows a drastic decrease in the number of analysed samples. This is due to a change in the sampling strategy. Until that time, GPs were asked to take a sample from each patient who

presented Influenza Like Illness (ILI) symptoms. But from then on, they were asked to take a maximum of 2 samples a week, in order to decrease the number of analyses. Still, until week 50, the epidemiological criteria were respected: the rate of doctor visits was above the epidemic threshold (established at 141 doctor visits per 100,000 people) and among patients with ILIs, more than 20% people were positive for influenza A(H1N1)2009.

The genetic and antigenic analysis of a series of A(H1N1)2009 strains isolated in Belgium revealed no significant difference

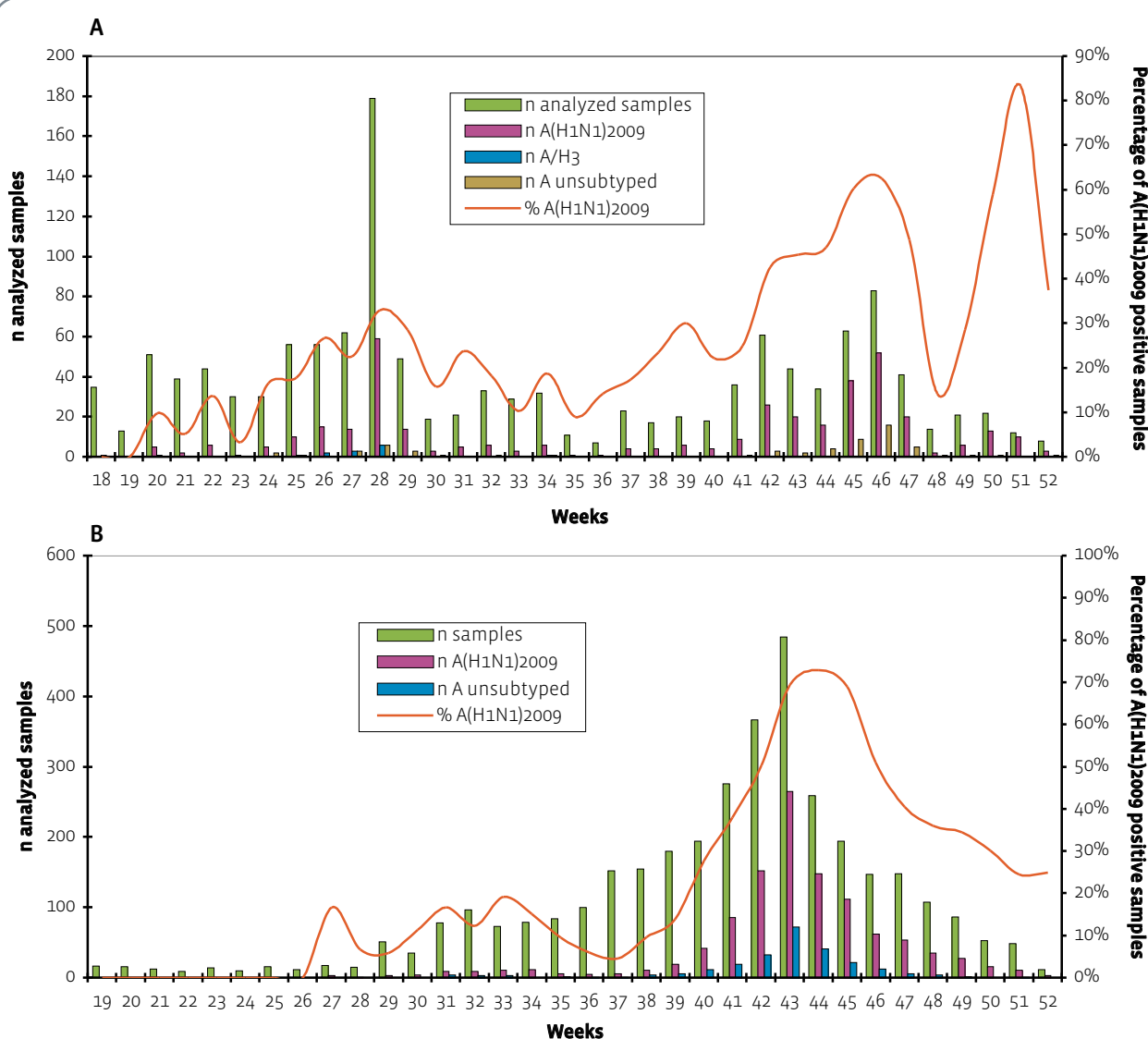


Figure 2:
A. Weekly evolution of the number of analysed samples and influenza A positive samples from week 2009-18 until week 2009-52 in the non-sentinel network.
B. Weekly evolution of the number of analysed samples and influenza A positive samples from week 2009-19 until week 2009-52, by the virological sentinel surveillance network.

with pandemic strains found worldwide. All corresponded to the reference A/Cali-fornia/4/09 strain. Out of the 48 samples analysed by the WHO Collaborating Centre, one strain isolated from an immuno-suppressed patient showed resistance to Oseltamivir (neuraminidase inhibitor) and presented the mutation H275Y in the neu-raminidase gene.

surveillance system worked in accordance with the NIC public health mission, demon-strating effective collaborations between the different partners and national author-ities. Until the end of 2009, the pandemic appeared to be less severe than initially predicted, with milder symptoms and a limited number of lethal cases. Finally its evolution was comparable with that of a classical seasonal epidemic.

Genotypically, the pandemic influenza strains isolated in Belgium up to now cor-respond to those isolated worldwide. Nev-ertheless, the A(H1N1)2009 pandemic virus will probably circulate for the coming seasons and its future evolution remains uncertain. Therefore a close and sustained monitoring of the evolution of the virus is required to provide an adequate response and surveillance system for the near future.

CONCLUSION

The introduction of the influenza A(H1N1)2009 virus in Belgium occurred similarly to other European countries, ini-tially causing a small but rapidly increas-ing number of cases (10). In this emergency context, the laboratory optimised a bat-tery of real-time RT-PCR assays, allowing it to perform a single-day diagnosis. The aim was to identify positive cases as soon as possible and to limit the propagation of the virus in the Belgian population. The

IMPACT ON PUBLIC HEALTH

Influenza virus may cause high morbidity and lethality and present a major risk to public health and economy. Surveillance of epidemic and pandemic influenza is crucial. Continuous effort to update our diagnostic methods, to follow the genetic variability of influenza viruses and to highlight poten-tial resistance to antivirals, is necessary.

ACKNOWLEDGEMENTS

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VHH-based Nanobodies® selected against the viral spike protein can protect mice against lethal rabies virus challenge

INTRODUCTION

Rabies causes 55,000 human deaths per year (1). The virus is present in the saliva of infected animals and transmitted by bites. From the infected wound, the virus travels through the peripheral nerves to the central nervous system and causes lethal brain infection. Once symptoms appear, the case-fatality rate is nearly 100%.

Lives can be saved if the person receives post-exposure prophylaxis (PEP) promptly after exposure (2). Once the first, often non-specific, symptoms occur, PEP is no longer effective. PEP involves active immunisation with vaccine and, for high-risk exposures, immediate passive immunisation treatment with anti-rabies antibodies. These antibodies are infiltrated the wound and neutralise viral particles before they enter the central nervous system. An important portion of the antibodies is retained at the infiltration site for at least 24 hours (3). Thus, passive immunisation provides immediate protection against the virus in attendance of vaccine-induced antibodies, which take at least a week to mount.

Approximately 10 million people receive PEP each year, of which 2-5% are also treated with anti-rabies antibodies (2). Most deaths occur in developing countries, where people do not receive appropriate PEP, due to poor availability, high cost or simply ignorance. Even Western countries report a shortage of anti-rabies antibodies for PEP (4).

The antibody products available on the market are polyclonal IgG preparations derived from pooled plasmas of vaccinated humans or horses (5). Since large scale production is not feasible, these are very ex-

pensive and have limited availability. Batch to batch variations are inevitable and the risk of transmission of (unknown) blood-borne pathogens or contaminants cannot be excluded. Allergic reactions can occur, especially with the equine variant. They have a relative low neutralising potency requiring infiltration of a large volume in the wound. In small body parts, such as a finger, this can cause the compartment syndrome. Part of the immunoglobulin content may interfere with live vaccines, e.g. against measles and rubella and thus vaccination against these diseases should be postponed for at least 4 months.

For this, the World Health Organisation urges the development of alternatives (6). Cocktails of human or (humanised) mouse monoclonal antibodies (Mabs) are being examined as alternatives for blood-derived antibodies (7-9). VHH-based Nanobodies® may be another alternative.

VHH are polypeptides derived from heavy-chain antibodies of camels and llamas (10). These animals possess naturally occurring single heavy-chain antibodies lacking light chains. They are composed of a single variable domain (VHH) and two constant domains (CH2 and CH3). Recombinant and isolated VHH are stable polypeptides (15 kDa, IgG = 150 kDa) harbouring the full antigen-binding capacity of the original antibody. Nanobodies® are VHH-based therapeutics for which treatment applications for different types of disease are currently being explored. They can be composed of a single VHH or be multimeric constructs of several VHH.

Recombinant VHH present different advantages over antibodies. They have a low production cost, can not be contaminated

by blood-borne pathogenic agents and are less likely to evoke allergic or immunopathological reactions. They have good thermal stability, which is an advantage in developing countries, where the cold chain for distribution and preservation cannot always be guaranteed.

We have found that VHH, directed against the viral surface glycoprotein G, are capable of neutralising rabies virus in an infectivity assay on baby hamster kidney (BHK-21) cells (11). Since VHH lack the functional Fc region of antibodies, which activates complement and facilitates uptake by phagocytes or activation of natural killer cells, it was not clear whether this is sufficient to provide protection in an *in vivo* system. Experimental rabies virus infection of mice provides an excellent tool to test the *in vivo* antiviral properties. This model requires only little virus to induce a lethal brain infection and the read out is exceptionally straightforward with mostly two outcomes: no clinical signs (protection) or lethal disease (no protection) (12).

Therefore the aims of this study were to examine whether (i) VHH, in single or bimeric conformations, are capable of neutralising rabies virus *in vivo* and (ii) treatment before or after virus exposure can protect against lethal infection.

MATERIALS AND METHODS

VHH and antibody

Llamas were kept in the animalarium of the WIV-ISP and vaccinated repeatedly with HDCV rabies vaccine (Sanofi). Peripheral blood lymphocytes were isolated, RNA was extracted and VHH genes were amplified from a cDNA library. Anti-rabies virus VHH were selected by panning phage libraries in 96-well plates coated with the native glycoprotein G (12). G forms the spikes on the viral surface. Most of the anti-G VHH were capable of neutralising rabies virus in a cell infectivity assay using BHK-21 cells. Anti-G VHH were also fused with glycine-serine (GS) linkers of variable length to obtain bimeric constructs directed against identical (bivalent) or different antigenic epitopes (biparatopic). Negative control mice were treated with phosphate-buffered saline (PBS) while positive controls received the potent rabies-virus neutralising Mab RV1C5 (Santa Cruz).

Virus

Challenge Virus Standard (CVS)-11 is a virulent genotype 1 lyssavirus obtained from the American Type Culture Collection (ATCC reference VR959). Chien Beersel-1 (CB-1) is a wild-type canine genotype 1 strain isolated from a rabid dog in Belgium in 2007. CVS-11 was grown in BHK-21 cells and CB-1 was grown in mouse brain by intracerebral inoculation. For intracerebral and intranasal virus challenge, CVS-11 was used at a dose of 101.5 and 102.0 TCID₅₀ respectively. For intramuscular virus challenge, CB-1 was used in a 1/10 dilution of the supernatant of centrifuged brain lysate.

Inoculations

Eight to ten female Swiss outbred mice (Harlan, The Netherlands) at the age of 6 to 8 weeks were used per group. The experimental procedure was approved by the local ethical committee of the institute (advice nr. 070515-05). Mice were anesthe-

tised by isoflurane and inoculated with VHH and/or virus directly into the brain by transcranial injection (20 µl), the muscle (50 µl) or the nose (25 µl) (12).

Clinical follow up

Mice were observed daily throughout the experiment for signs of disease until 35 days after inoculation. Scores ranged from 0 (no disease) to 7 (severe nervous disease) (12). According to our experience, mice with disease scores of 6 to 7 die within one day. Therefore, mice were euthanised by cervical dislocation when they reached that disease score. Results were expressed as Kaplan-Meier survival curves.

RESULTS

Proof of concept that VHH can neutralise virus particles *in vivo*

Contrary to irrelevant control VHH, pre-incubation of the virus with rabies virus-specific VHH protected mice against brain infection, disease and mortality upon inoculation of the virus-VHH mix in the nose, muscle or brain (Table 1). Excellent protection was obtained when bimeric VHH and virus were introduced simultaneously in the same body compartment. Similar results were obtained with Mab RV1C5. Monovalent VHH were less performant in the brain. This agrees with the fact that bimeric constructs show a massive increase in neutralising potency and cross-neutralisation of variable genotypes *in vitro*, compared to the monovalent building blocks (11, 13).

Still, the *in vitro* neutralising potency was not correlated with the level of *in vivo* protection. Rab-C12 was the most potent monovalent VHH *in vitro*, but proved less protective than Rab-E6 in the brain. These VHH recognise different epitopes (11). This discordance is also known for antibodies. For further studies, the biparatopic VHH Rab-E8(15GS)H7 was selected because of its high neutralising potency *in vitro* and



good protective effect in all challenged body compartments.

Effect of preventive treatment with VHH on subsequent virus challenge

Preventive administration of VHH Rab-E8(15GS)H7 or Mab RV1C5 in the nose, 24 hours prior to intranasal virus challenge, almost completely prevented disease and lethal infection (Figure 1A). This suggests that VHH remain active for at least 24 hours in the nose and are capable of neutralising

at least part of the invading virus. VHH may therefore be suitable for passive mucosal immunisation against entry or local infection with viruses in the nose.

Effect of post exposure treatment with VHH after intramuscular virus challenge

PEP by injection of the biparatopic VHH Rab-E8(15GS)H7 in the contralateral muscle 10 minutes after intramuscular virus challenge reduced mortality by 50% (Figure 1B). Treatment 24 hours after virus

challenge was no longer effective, albeit survival time was prolonged by one day. At this late time point, PEP with the Mab RV1C5 was still able to reduce mortality by 30%. Results thus depended on the interval between the virus inoculation and the VHH treatment. Treatment at 10 minutes was able to protect part of the mice, whereas treatment at 24 hours was no longer effective. For both VHH and Mab, the same *in vitro* neutralising dose of one IU was used, which proved equally effective when applied prior to or simultaneous with the virus challenge.

CONCLUSION

Our results show that anti-G VHH can neutralise rabies virus in an Fc-independent way. VHH probably hinder the recognition of cellular receptors or interfere with conformational changes of the G protein necessary to fuse viral and cellular membranes. It remains unclear to what extent complement- or effector cell-mediated lyses of infected cells are also involved in the protective effect of antibodies. Such a cytotoxic effect might however not be desirable in humans. Optimal treatment of rabies favours a healthy balance between maximal virus neutralisation and minimal immunological cell damage. Indeed, intravenous administration of immunoglobulins to symptomatic patients has been associated with sudden onset of muscle paralysis (15). Presumably, paralysis involved the activation of complement by IgG deposits on axons containing rabies virus antigens (16). Some experts advise against the use of antibodies for treatment of symptomatic patients, because (i) it might interfere with the ontogeny of the natural immune response (17) and (ii) it might shorten the survival time and cause “early death” (18). Early death might be due to immunopathological damage to infected neurons or, as proposed by one study, enhancement of infectivity by uptake of virus in macrophages through Fc receptor-mediated endocytosis (19). The importance of this phenomenon in rabies is controver-

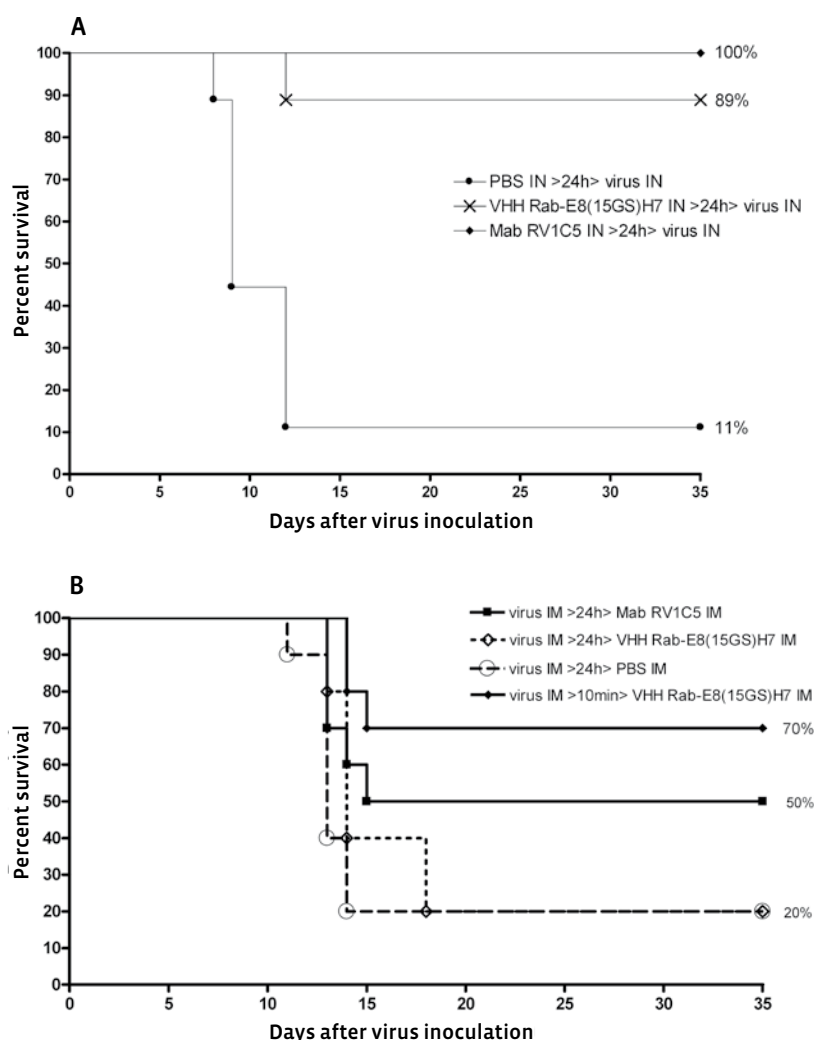


Figure 1:
A. Survival proportions of mice treated with the biparatopic VHH Rab-E8(15GS)H7 in the nose and challenged with virulent virus one day later through the nasal route (IN) (preventive treatment model).
B. Survival proportions of mice treated with the biparatopic VHH Rab-E8(15GS)H7 at 10 minutes or 24 hours after intramuscular (IM) virus inoculation (post exposure treatment model). Positive and negative control mice were treated with Mab RV1C5 and phosphate-buffered saline (PBS), respectively.

sial, but it has clinical relevance for other viruses, such as dengue virus (20).

VHH offer a neutralising potency per weight unit which is best in its class. For one rabies virus-neutralising unit, only 122 µg of VHH Rab-E8(15GS)H7 is required, whereas for Mab RV1C5 this is 606 µg and for blood-derived human immunoglobulins (Imogam, Sanofi) 1111 µg. This reduces the antigenic content and volume of the administration, which is beneficial for infiltration in small body compartments.

Still, the protective effect in the PEP experiment was limited especially at the longer time interval of 24 hours between virus exposure and subsequent treatment. In this experiment, the virus had the occasion to infect cells which in turn can release new virus. In such a set up, it becomes important to neutralise the newly produced virus over a prolonged period, and not only

the challenge virus introduced by the inoculation. Once absorbed in the blood, VHH are however quickly cleared by the kidneys resulting in a short systemic half-life of 30 min. The half-life of VHH can easily be extended to the range of an antibody (weeks) by conjugation with an anti-albumin VHH (14). Most likely, this would improve performance in the PEP model.

In conclusion, we proved that llama VHH can neutralise rabies virus *in vivo* in an Fc-independent way. Especially the bimeric constructs were highly protective in different challenge models. The relative weak protection in the PEP model might be due to the short half-life of the VHH used. Considering that prolongation of the half-life of VHH is feasible by different approaches, VHH technology may offer perspectives as an alternative to antibodies for PEP.



IMPACT ON PUBLIC HEALTH

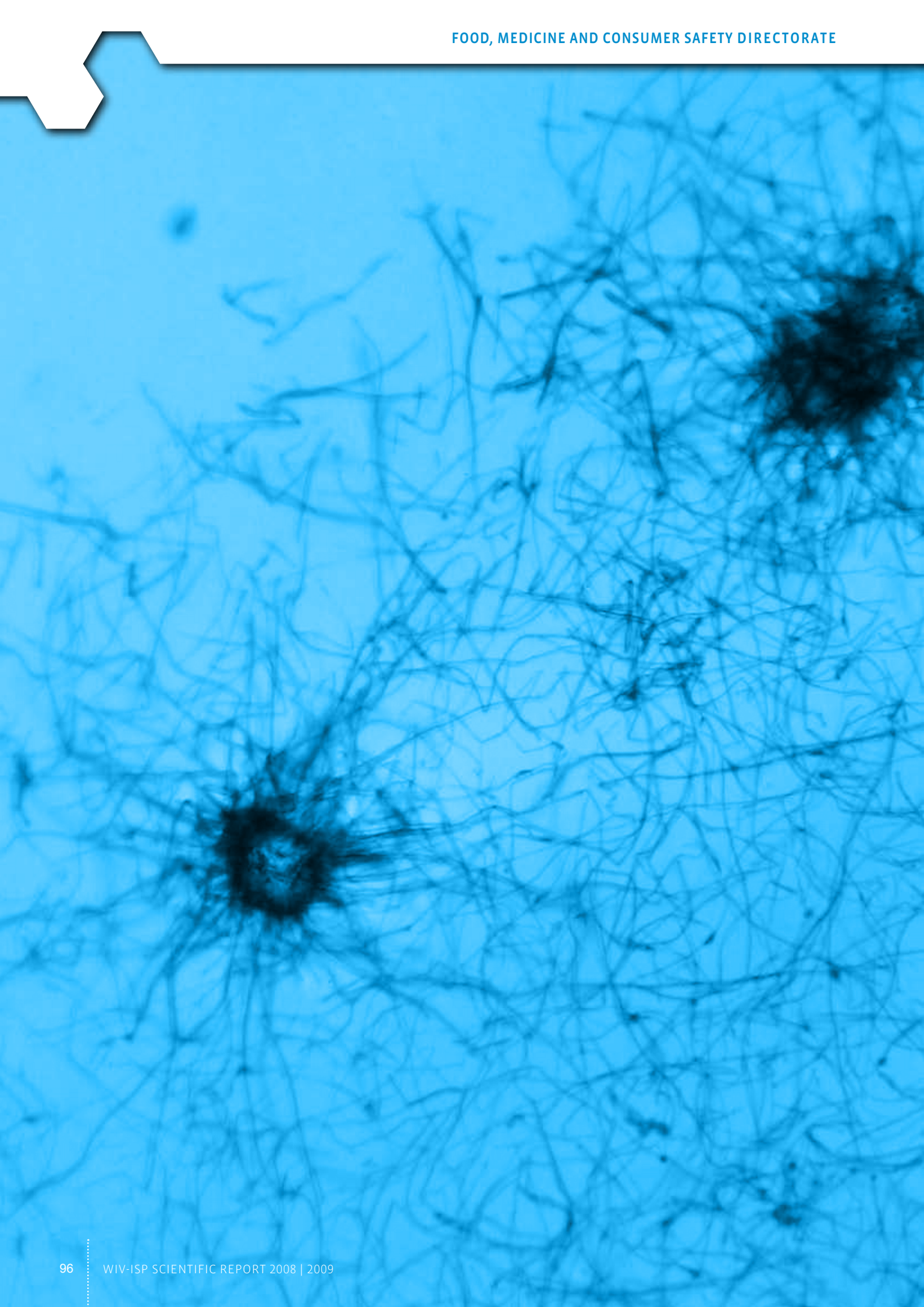
Failure to rapidly obtain treatment with anti-rabies antibodies can be lethal in severe cases of exposure. The current post-exposure therapy requires blood-derived human anti-rabies antibodies which are increasingly difficult to obtain worldwide. Our study demonstrates that recombinant VHH may offer a safe, cheap and easy-to-manufacture alternative for rabies prophylaxis.

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FOOD, MEDICINE AND CONSUMER SAFETY DIRECTORATE

Introduction by Ir. Joris Van Loco

The Food, Medicine and Consumer Safety Directorate was established by Ministerial Order on 26 August 2008. The directorate's main focus is to perform policy-supportive scientific research and to provide services to federal and regional partners including the Federal Agency for Food Chain Safety (AFSCA-FAVV), the Federal Agency for Medicines and Health Products (AFMPS-FAGG) and the Federal Agency for Nuclear Control (AFCN-FANC). The directorate has modern analysis equipment and highly trained personnel. The quality of the performed laboratory analyses is highly regarded and the scientific directorate has a quality system accredited according to the ISO 17025 standard.

The directorate works closely with Belgian and international universities as a partner or coordinator in various externally financed scientific research projects.

Medicines

Description:

The Medicines division specialises in analysing medicines, including illegal and counterfeit medicines), medicine ingredients, magistral preparations, medicinal plant extracts, drugs and vitamin preparations. The section is ISO 17025 accredited and periodically participates in ring tests, quality control of centrally authorised products and other related activities. It represents Belgium in the European network of Official Medicines Control Laboratories (OMCL), which was established as part of the mission of EDQM (Strasbourg) and EMEA (office: London).

Activities:

- To support the Federal Agency for Medicines with laboratory activities in order to maintain the quality of medicines. These laboratory activities include inspecting medicine ingredients, magistral preparations and specialities, detecting counterfeit medicines and analysing illegal preparations;
- To actively participate in the creation of monographs for the Belgian and European Pharmacopoeia Commissions and the Therapeutic Magistral Formulary (FTM-TMF);
- To perform quality control on vitamin nutrients on behalf of AFSCA-FAVV and AFMPS-FAGG;
- To analyse drugs as part of a national monitoring plan on drug use.

Food & Consumables

Description:

The Food & Consumables division specialises in analysing residues and contaminants in food and consumables (food contact materials, piercings, cosmetics...). The division uses a quality system and is ISO 17025 accredited. The division is also the National Reference Laboratory (NRL) for detecting residues of veterinary medicines, hormonal products, pesticides, dioxins, polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs) and other environmental contaminants, marine toxins and food contact materials. It works closely with AFSCA-FAVV and Belgian laboratories in this respect. The division is also active in the European and National Reference Laboratories working group. The division harbours several ongoing scientific projects in cooperation with Belgian universities on environmental contaminants, toxins, pesticides and food contact materials.

Activities:

The division's reference tasks are to research and develop methods of analysis, participate in collaborative trials, organise interlaboratory tests and communicate and distribute results and methods on behalf of AFSCA-FAVV;

- To perform routine analyses;
- To carry out intake and exposure studies;
- To provide scientific and technical support to the administration in case of a crisis and to perform urgent analyses.

Radioactivity

Description:

The National Reference Laboratory for Monitoring Radioactivity in the Environment works closely with the Federal Agency for Nuclear Control (AFCN-FANC).

Activities:

- To monitor radioactivity levels in the environment on Belgian soil with a specific focus on areas near nuclear industry;
- To measure the radioactivity of food, air, precipitation, ground- and surface waters, plants, sea water, sediment and biological indicators;
- To make analysis and confirmatory tests of food samples and some other products;
- To be involved in the implementation of emergency plans in case of nuclear accidents.

Analysis of the photoinitiators benzophenone and 4-methylbenzophenone in breakfast cereals

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INTRODUCTION

Photoinitiators are widely used as catalysts for photopolymerisation. They are generally used in a mixture and their role is to start the polymerisation process, which aims to harden the ink film on the substrate, for example cardboard. Traditionally, inks were cured thermally and their formulations included organic solvents, which then had to be eliminated with a drying process. Consequently, organic solvent residues could contaminate the food surrounded by the packaging material. Therefore, UV-curing seemed to be a good alternative. However, the photoinitiators are not always completely utilised or removed after the printing process. As a result, low molecular weight photoinitiators such as benzophenone (BP), which are applied to the outside of the packaging material, may permeate through the rather open structure of cardboard and subsequently migrate into the food. There is also the possibility that, if benzophenone residues are not completely removed from recycled paper and cardboard, benzophenone may persist in any packaging made from these recycled materials even if that packaging itself has not been printed with UV-cured inks.

Recently, 4-methylbenzophenone (4MBP) was detected in breakfast cereals, leading to notifications in the Rapid Alert System for Food and Feed (RASFF). In March 2009, the European Food Safety Authority (EFSA) declared that “4-methylbenzophenone is used as a photoinitiator of UV-cured printing inks and lacquers applied on the surface of packages, mainly cardboard boxes”. This press statement indicated that 4-methylbenzophenone may be used in combination with or as replacement of benzophenone and that, given the volatility of both benzophenone and 4-methyl-

benzophenone, both substances may migrate into the package and contaminate foodstuffs. Like benzophenone, 4-methylbenzophenone is expected to be a non-genotoxic carcinogen.

At present, there is no specific standardised EU regulatory framework for the control of inks applied to the outer surface of food packaging and the corresponding possible migration of these inks into the foodstuff. However, there is a resolution of the Council of Europe ReSap (2005) 2 on the use of ink, which includes 3 technical documents and a list of authorised substances. It should be noted that neither benzophenone nor 4-methylbenzophenone are listed. Moreover, all materials and articles intended to come into contact with food should comply with the general criteria laid down in the relevant EC framework regulation 1935/2004, i.e. they should not transfer their constituents into food in quantities that could endanger human health or bring about unacceptable changes in composition or characteristics of foodstuffs. In addition to all specific directives related to Food Contact Materials, the European Commission Regulation 2023/2006 lays down the rules on Good Manufacturing Practice (GMP) for groups of materials and articles, intended to come into contact with food and details processes involving printing inks. For the use of benzophenone as an additive in plastic, a specific migration limit (SML) of 0.6 mg kg⁻¹ has been set in the European Commission Directive 2002/72/EC.

As a result, the optimisation and validation of methods to determine potential low-weight substances used in packaging formulations for food are essential to ensure the safety of packaged foods. Nevertheless, this is not easy to achieve since food samples are complex matrices and

moreover, there exists a vast range of possible migrant substances, packaging materials and applications. In the specific case of photoinitiators, there is little information available regarding the determination of these substances in food items. Most studies evaluated the packaging material or performed an extraction using a food simulant. Most relevant studies for the determination of photoinitiators in food have involved the determination of isopropylthioxanthone (ITX) (1-4). Up to now, only a few methods have been developed for the determination of BP in food and no methods have been published for the determination of 4MBP in food.

In this contribution, a fast and reliable method for the determination of BP and 4MBP in cereals is described. The sample was extracted ultrasonically using a mixture of dichloromethane and acetonitrile (1:1), followed by purification of the extract by solid phase extraction. Finally, the extract was analysed by GC-MSⁿ. The presented method is in-house validated.

METHOD AND MATERIALS

Chemicals

Neat certified standards of benzophenone (BP) (purity 99%), 4-methylbenzophenone (4MBP) (99%) and benzophenone-d₁₀ (BP-d₁₀) (99%) were purchased from Sigma-Aldrich (Bornem, Belgium). Hexane, acetonitrile, methanol and ethyl acetate were supplied by Merck (Darmstadt, Germany). Dichloromethane was purchased from Biosolve (Valkenswaard, The Netherlands). All solvents were HPLC grade. Stock solutions of each individual compound and all the working solutions were prepared in hexane. These solutions were stored at 4°C.

Sample preparation

Method development was done using 5 g of cereal sample (Kellogg's extra, Kellogg's, Manchester, UK) spiked with BP, 4MBP and the internal standard BP-d₁₀ at 600 µg kg⁻¹ (ppb) (which is a sample spiked at the specific migration limit (SLM)). Next, 10 mL of an organic solvent (hexane, dichloromethane:acetonitrile (1:1), acetonitrile and methanol) was added and this mixture was placed in the ultrasonic bath (Transsonic T780, Elma, Mettler Toledo, Zaventem, Belgium) for 10 min. Then, the cereal was further extracted by shaking the Falcon tube for 30 min. in a rotary agitator (Orbital Reax 2, Heidolph instrument, VWR, Leuven, Belgium). After the extraction, the sample was centrifuged (8 min, 800 rpm, -20°C) (Jouan BR4i, DJB Labcare, UK).

The liquid phase, obtained after centrifugation, was then evaporated to dryness under a gentle stream of nitrogen at 30°C (Turbovap, Caliper Life Science, Terafene, Belgium). The residue was re-dissolved in 2 mL hexane before purification by solid phase extraction on a silica cartridge (1 g, 6 mL, Si) (Varian, Lake forest, CA, USA). This cartridge was previously conditioned with 3 mL hexane. Then, the extract was loaded and elution of the photoinitiators was evaluated using 2 mL of different solvents (acetonitrile, hexane:ethyl acetate (30:70), ethyl acetate). Finally, the eluate was directly analysed by GC/MSⁿ.

Instrumental

Analyses were carried out on a Polaris Q mass spectroscopic detector combined

with a Trace GC Ultra gas chromatograph equipped with AS 3000 autosampler (Thermo Finnigan, Austin, TX, USA). The injections were carried out in a split/splitless injector at 250°C. An injection volume of 2 µL in splitless mode was used. The carrier gas helium was supplied in constant flow mode at 1.2 mL min⁻¹. The analysis were carried out on a non-polar 5% phenyl-polysilphenylene-siloxane SGE BPX-5 column of 25 m L, 0.22 mm ID and a phase thickness of 0.25 µm (SGE Incorporated, Austin, TX). The temperature was programmed from 50°C (1 min) to 280°C at rate of 25°C/min. This temperature was then maintained for 5 min. Detection was carried out in MSⁿ. For each analyte, two transitions were followed.



RESULTS AND DISCUSSION

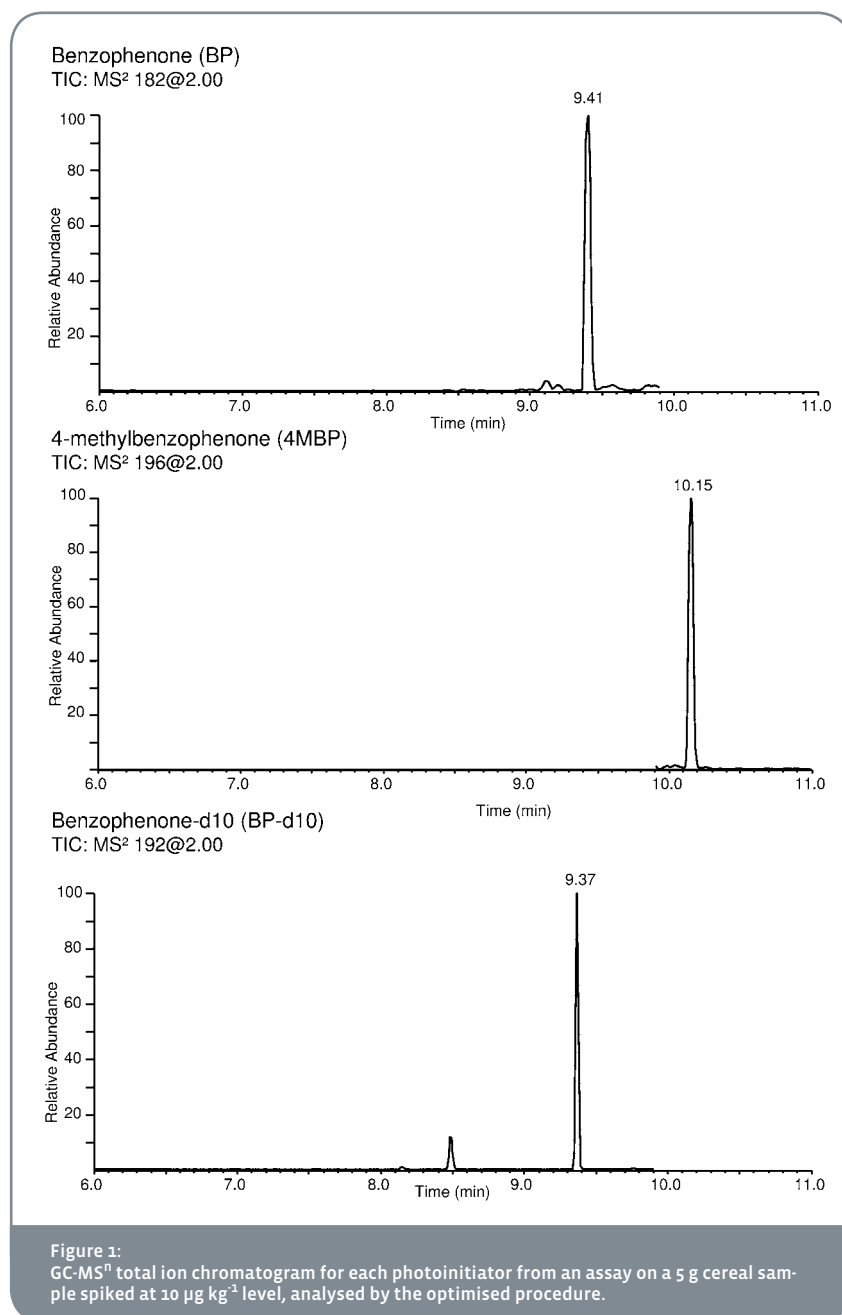
Optimisation of the sample preparation

Most methods for the determination of BP in food utilise conventional solid-liquid extraction with an organic solvent, followed by a purification step using solid phase extraction. These types of methods are very time-consuming and/or consume a lot of toxic organic solvents. Furthermore, no methods are available for the determination of 4MBP. Therefore, in this contribution a method is developed for the simultaneous determination of BP and 4MBP in food using ultrasonic extraction.

Optimisation of the extraction procedure was done using 5 g of breakfast cereal spiked with BP and 4MBP at $600 \mu\text{g kg}^{-1}$. Different solvents (acetonitrile, methanol, dichloromethane:acetonitrile (1:1) and hexane) were evaluated for the ultrasonic extraction of BP and 4MBP in cereals. The best results were obtained when the 1:1 mixture of acetonitrile and dichloromethane was used as extraction solvent. Therefore, this solvent was selected for further experiments.

The purification step with the Si SPE cartridge was also optimised using 5 g of cereal spiked at $600 \mu\text{g kg}^{-1}$ of both compounds and 10 ml acetonitrile:dichloromethane (1:1) as extraction solvent. Different elution solvents (acetonitrile, ethyl acetate:hexane (7:3) and ethyl acetate) were evaluated. From these experiments, it can be concluded that acetonitrile was the solvent of choice for the elution of the SPE cartridge and consequently, this solvent was used for further experiments.

The presence of a matrix effect was evaluated by comparing the slopes of both solution and matrix-matched calibration lines by means of a Student's t-test (5). The calibration curves were obtained by injecting a solution of standards in parallel with matrix-matched standards (corresponding to blank cereal samples fortified at the end of the sample preparation). Since BP-d₁₀ is used as internal standard, no matrix effect will be observed for BP. For 4MBP on the other hand, a matrix-effect is present for



4MBP. Therefore, calibration curves in matrix should be used for the quantification of all the photoinitiators.

Performance of the method

The performance of the method was evaluated by a full in-house validation of the method. The following performance characteristics were assessed: linearity, recovery, precision, specificity, limit of detection and limit of quantification.

Figure 1 shows the chromatograms from a blank cereal sample spiked with BP, 4MBP and BP-d₁₀ at a concentration of $10 \mu\text{g kg}^{-1}$. The cereal sample was analysed in accordance with the method described above. The different photoinitiators can very easily be detected, thus the presented method is very specific for these photoinitiators. The linearity was evaluated using the Mandel's Fitting test (6). This test evaluates whether a quadratic model better fits the data than a straight line regression model.

This was done by extracting cereal samples spiked at 5 concentration levels (100-300-600-900-1200 $\mu\text{g kg}^{-1}$). The calibration curves were obtained by plotting the peak ratios (BP/BP- d_{10} or 4MBP/BP- d_{10}) versus the concentrations. According to the Mandel's fitting test, the straight-line regression model is preferred for both BP and 4MBP. The two photoinitiators showed good linearity ($R^2 = 0.97$ for BP and 0.96 for 4MBP) in the investigated range.

The recoveries obtained under optimised conditions, i.e. 5 g cereal extracted ultrasonically with 10 mL acetonitrile:dichloromethane (1:1), purification using Si SPE cartridge, acetonitrile as elution solvent were measured by comparison of the peak area of the solutes for a 600 $\mu\text{g kg}^{-1}$ spiked cereal sample with those obtained by direct liquid injection of 2 μL of a 1.5 mg mL^{-1} . The obtained values were 74% for BP and 98% for 4MBP.

The repeatability (r) and the intralaboratory reproducibility (R_w) were evaluated by calculating the coefficients of variation (CV) for these two parameters (CVR and CVR_w) according to the ISO-5725-2 guidelines (7). From these results, it can be concluded that the method shows good precision at the levels of interest.

The sensitivity, expressed as limits of detection (LOD) at S/N 3, are 2 $\mu\text{g kg}^{-1}$ for both BP and 4MBP, showing excellent sensitivity of the method. The limits of quantification (LOQ) at S/N 10 were 6 $\mu\text{g kg}^{-1}$ for BP and 8 $\mu\text{g kg}^{-1}$ for 4MBP.

The performed analyses are first qualitatively evaluated in terms of relative retention time, relative abundances of the diagnostic ions and the signal to noise ratio. The relative retention time (retention time of the analyte divided by the retention time of the internal standard) must be within the tolerance of $\pm 2.5\%$, when compared with the results of a cereal sample spiked at 300 $\mu\text{g kg}^{-1}$ for each photoinitiator. The relative abundances of the diagnostic ions and the signal to noise ratios were within the acceptance criteria of the Commission

decision 2002/657/EC (8). For the signal to noise criterion, a minimum ratio of 3 on the less diagnostic ion was considered as acceptable. If an 'unknown' sample is suspected to be non-compliant (concentration of BP and 4MBP $> 600 \mu\text{g kg}^{-1}$), the analysis is confirmed including a calibration curve in matrix for a correct quantification of 4MBP.

CONCLUSION

Ultrasonic extraction, followed by solid phase extraction in combination with GC-MSⁿ is a versatile method for the determination of photoinitiators in breakfast cereal and may be applicable to other foodstuffs.

IMPACT ON PUBLIC HEALTH

A fast and reliable method for the determination of the photoinitiators benzophenone and 4-methylbenzophenone in breakfast cereals has been developed. Consequently, all food can now be screened for the presence of photoinitiators, thus protecting consumers from these dangerous chemicals.

ACKNOWLEDGEMENTS

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SOURCE

Van Hoeck E, De Schaetzen T, Paquet C, Bolle F, Boxus L, Van Loco J. Analysis of benzophenone and 4-methylbenzophenone in breakfast cereals using ultrasonic extraction in combination with gas chromatography-tandem mass spectrometry. *Anal Chim Acta* 2010;663:55-59.

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Determination of inorganic arsenic in seafood by HG-ICP-OES after conventional wet digestion

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INTRODUCTION

Chronic arsenic (As) exposure places people at risk to multiple adverse health effects. As is a recognised human carcinogen (1, 2) and, moreover, several recent studies have shown As to be a potent endocrine disruptor, altering hormone-mediated cell signalling at very low concentrations (3, 4).

The majority of harmful exposure comes from drinking water that contains primarily inorganic As. Other major sources are food, soil and air, with most people actually being exposed to dietary organic As forms. The inorganic As species arsenite or As (III) and arsenate or As (V) are generally held to be more acutely toxic. Moreover, As has a rich organic chemistry and this is reflected in the large diversity of identified organocompounds (5). Methylated As species, such as monomethylarsonic acid (MMA) and dimethylarsenic acid (DMA), are relatively non-toxic and are believed to contribute little to the overall risk associated with As exposure. Some researchers argue, however, that the organic metabolites may be the ultimate carcinogens. DMA has been shown to induce bladder cancer and to promote tumour development in other organs in rodents for example (6).

Human dietary exposure to As is very often related to the consumption of seafood. It is known that marine organisms exhibit highly variable As profiles (7, 8). Arsenosugars are present in algae, whereas arsenobetaine (AB) is absent (9). However, AB is the predominant species in fish and crustaceans (10) and bivalves contain both AB and arsenosugars (11). Mostly, total As concentrations in marine food items are high and are therefore considered to be the principal public health concern.

Speciation analyses have showed that merely a small fraction was in the inorganic form and several laboratory approaches to distinguish the inorganic As fraction or to specify the different As species have been published in the scientific literature. As a safeguard for public health, maximum permissible concentrations of metals in food were set to limit the dietary exposure. Since it is nowadays well accepted that the chemical forms must also be considered when assessing health effects, the separation, identification and quantification of different fractions become more important (5, 12). The present study focuses on the distinction between the inorganic and total As concentrations. Its aim is the optimisation and validation of a simple and economic determination of the inorganic As fraction in marine matrices and to illustrate its feasibility through a local market survey.

MATERIALS AND METHODS

Basically, the determination of both total and inorganic As relies on the reduction of As (V) into As (III), the subsequent conversion of As into AsH₃ and atomisation in the argon plasma (13). The instrument used is a Perkin-Elmer ICP Optima 4300 DV inductively coupled Ar plasma optical emission spectrometer (Norwalk, CT, USA), equipped with an autosampler (AS 93 plus, PE) and with a hydride generation (HG) system (FIAS 400 PE) with two peristaltic pumps and regulated gas supply. The gas flow rate was set to 40 – 250 mL/min.

Prior to HG, samples for total arsenic determination were mineralised in an open digestion system (2040 Digestor, Foss Tecator) for 40 test tubes (quartz, 100 mL), with air cooled condensers and built-in electronic temperature and time control (13).



Samples for inorganic arsenic determination were digested in a drying oven (Binder FD 115, Tuttlingen, Germany), equipped with a thermostat and an integrated timer. Ultrasonic extractions were performed using a Branson 5510 E-DTA ultrasonic cleaner (Danbury, CT, USA) and extracts were isolated using an Eppendorf 5810 R centrifuge (Hamburg, Germany).

All reagents and standards were prepared as before (13). The certified reference materials (CRM) that were used for this study included a dogfish muscle (DORM-2), dogfish liver (DOLT-3), lobster hepatopancreas (TORT-2), and non-defatted lobster hepatopancreas (LUTS-1), purchased from the National Research Council of Canada (NRCC, Ottawa, Ontario). BCR 278R (mussel tissue) and BCR 422 (cod muscle) were purchased from the Institute for Reference Materials and Measurement (IRMM, EC, Geel, Belgium).

Additionally, canned fish from a proficiency testing exercise (fresh crab meat, FAPAS Series 7, Round 55, CSL, York, UK) was also used.

Several natural seafood samples were purchased at a local market. All CRMs and samples were homogenised by mechanical mixing in a high speed Blender and then frozen at -20°C.

RESULTS AND DISCUSSION

Optimisation of the inorganic As extraction

A variety of methods from the literature (14-18) was tested for the extraction and solubilisation of total As. Extraction with different methods: water or methanol:acetic acid mixture proved often insufficient. Only the methanol: water mixture (1:9,v:v) showed sufficient extraction efficiency, with 74% of total arsenic being extracted from TORT-2.

More aggressive methods to solubilise the arsenic of TORT-2 were investigated using

nitric and hydrochloric acid with variable concentration and by sonication for 1h at 40°C. The extraction efficiency ranged from 76-83% of the total arsenic content in TORT-2, with maximal extraction efficiency being obtained with HCl (0.5 M). The remaining arsenic may be bound to thiol groups of proteins or may correspond to species such as arsenolipids that are not soluble (19). Solubilisation with nitric acid produces much foam when analysing inorganic As with HG-ICP-AES and this generates technical problems. For concentrations ranging from 1-5 M, an efficiency of 80% was observed. Therefore, HCl was extended to other CRMs and optimisation of several parameters was performed such as the acid concentration, the sonicating time and temperature of the solubilisation. The extraction efficiencies in the six investigated fish species (CRMs) ranged from 74-98% of the total arsenic.

As a result, hydrochloric acid extraction (0.5 M) is better than methanol or nitric

acid solubilisation, however, the extraction of inorganic arsenic is not quantitative especially for fatty matrices such as FAPAS S7R55 (crab meat). These low efficiencies are in accordance with the findings reported by other authors (11, 20).

Optimisation of the inorganic As digestion method

In order to facilitate the dissolution of fatty and proteinaceous material, the development work was directed towards the use of acid digestion to improve the extraction efficiency of As. For this purpose, digestion with nitric acid was performed for six CRMs of different matrices. The effect of increasing the digestion temperature in the range of 80-180°C was investigated for the Canadian CRMs. The response behaviour depends on the CRM matrices. For the same species (dogfish), we observed a difference in behaviour with temperature between muscle matrix (DORM-2) and liver

Table 1: Inorganic arsenic content reported in the literature and in this study for some investigated CRMs.

CRMs	Reported result in mg/kg dry weight	References
DORM-2	0.088	(27)
	0.10 ± 0.02 ^a	(10)
	0.102 ± 0.029	(28)
	0.145 ± 0.011	(23)
	0.15 ± 0.01	(24)
	0.19 ± 0.03	(29)
	0.132 ± 0.007^c	Present work
TORT-2	0.41 ± 0.03 ^a	(30)
	0.46 ± 0.07	(24)
	0.506 ± 0.031	(28)
	0.581 ± 0.055	(23)
	0.680 ± 0.405	(31)
	1.268 ± 0.080^c	Present work
BCR 422	< 0.002	(29)
	< 0.026	(24)
	0.043 ± 0.013^c	Present work
FAPAS S7R55	0.0851 ± 0.0176 ^b 0.083 ± 0.005^c	FAPAS Present work
DOLT-3	Not available 0.091 ± 0.006^c	Not available Present work
LUTS-1	Not available 0.105 ± 0.006^c	Not available Present work

^a Sum of As (III) and As (V)

^b Assigned value; wet weight

^c Standard deviation

matrix (DOLT-3). The same goes for the defatted lobster hepatopancreas matrix (TORT-2) and the non-defatted matrix (LUTS-1). These results can explain the difficulties in fully extracting the inorganic arsenic by using conventional methods (sonication and centrifugation) except for the TORT-2, which seems to release inorganic arsenic easily both for extraction and digestion methods.

For temperatures below 60°C, the digested sample produces a lot of foam in the hydride generation due to non-destroyed proteins, which disturb the plasma and causes analytical errors.

For higher temperatures, over 200°C, there is a risk of converting the organoarsenic compounds into inorganic arsenic. It was reported that the behaviour of cationic arsenic as AC and AB, in a microwave system with nitric acid and hydrogen peroxide, shows a conversion to trimethylarsine oxide (TMAO) at 207°C and the latter remain unaltered (21). The same goes for MMA, DMA and As (V), in close pressurised digestion systems. This is more aggressive than the conventional oven digestion used in our work. These compounds remain intact up to 160°C. Then at 190°C, MMA was partially decomposed to arsenate whereas DMA and As (V) were unaltered (22). In our work, the optimised digestion procedure was applied to AB, MMA and DMA and no conversion to arsenate has been observed but unfortunately, if MMA is significantly present in the sample, such as seaweed, the proposed method can overestimate the inorganic As value. For the arsenosugars, we have no data about their behaviour in acid digestion but these are not the predominant compounds in seafood.

Determination of inorganic arsenic in reference samples

The two proposed methods (extraction and digestion) were applied to the following reference materials: TORT-2, DORM-2, DOLT-3, LUTS-1, BCR 422 and the FAPAS S7R55. The content of inorganic arsenic in these CRMs

Table 2: Determination of inorganic and total As in real samples.

Seafood products	Total As found (mg/kg)	Inorganic As found (mg/kg)	% of Inorg. As
Mussel	1.99	0.151	7.6
Oyster	1.85	0.073	3.9
Scallop	1.29	0.007	0.5
Prawn	0.75	0.023	3.1
Scampi	0.75	0.011	1.5
Cuttle fish	6.56	0.020	0.3
Squid	0.27	0.004	1.5
Ray	11.67	0.014	0.1
Cod	2.54	0.010	0.4
Shark	8.98	0.006	0.1
Trout	1.34	0.004	0.3
Plaice	6.55	0.004	0.1
Sole	2.44	0.004	0.2
Tuna	2.09	< L _D ^a	< L _D ^a

^a Detection limit

is not certified and there are few previous references in the literature for its quantification. Table 1 shows the results from the analysis of marine CRMs and also includes published results for inorganic arsenic in these samples (if data available). A large variability exists between the reported values. The results of inorganic arsenic for the acid extraction method are generally among the lowest reported for all CRMs with the exception for BCR 422, which is equal to that found by acid digestion and higher than that published.

The values obtained in the present study for the acid digestion method are within the range given in the literature except for TORT-2 which gave higher results with the two proposed methods.

The discrepancies between the values reported in the literature are probably due to the different sample preparation methods and analysis techniques used. Indeed, after examining several publications, it seems that the HG-AAS technique gives higher values for the inorganic arsenic than a coupled technique such as HPLC-ICP-MS used for the speciation of As (V) and As (III), where the latter is often not detected.

Determination of inorganic arsenic in real samples

Inorganic As levels were analysed in frozen seafood samples of various matrices such as fish, crustaceans and bivalves using the acid wet digestion method. The results are given in Table 2. Bivalves (mussels, oysters) show high levels of inorganic As compared to the other species but we cannot generalise this observation because only one sample of each species was analysed. The values found varied between 0.004 and 0.151 mg/kg fresh matter. Expressed as a percentage of total As, the inorganic As varied between 0.1-7.6%, which agrees with results reported by other authors (23-26).

CONCLUSION

Despite the high levels of total arsenic found in seafood samples, inorganic arsenic, the highly toxic form, was present at less than 10% of total As. Thus, there is no direct hazard for human health due to the consumption of these seafood products. Comparing the two proposed methods, acid extraction and acid digestion, the latter shows better efficiencies and the

Evaluation of risk of consuming shellfish contaminated with domoic acid in Belgium

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INTRODUCTION

Domoic acid (DA) is a marine biotoxin causing amnesic shellfish poisoning (ASP) in humans. Symptoms of ASP include gastrointestinal effects (nausea, vomiting, diarrhoea or abdominal cramps) and/or neurological signs (confusion, loss of memory, or other serious signs such as seizure or coma) occurring within 24 and 48 hours after ingestion, respectively. This toxin was detected by monitoring services in the United States, Canada and in a number of European countries. While the evaluation of the DA concentration is performed in many countries on a regular basis, the exposure assessment of consumers to DA, which relies both on reported occurrences and consumption data, is only done upon request and the limited consumption data poses a considerable breach in evaluating the exposure risk of toxins present in shellfish products.

Several types of shellfish are consumed in Belgium. Mussels are most frequently eaten, followed by scallops and oysters. However, these shellfish may sometimes be contaminated with ASP toxins, thus posing a risk to consumers. The risk was evaluated by linking the data of DA concentrations detected in shellfish samples collected during a limited period and consumption data obtained from the National Belgian Food Consumption Survey in adults (2004).

MATERIALS AND METHODS

Samples

In this study, 42 mussel samples, 7 oyster samples, and 61 scallop samples were collected and analysed in 2007. The samples originated both from National Food Control Programme and various Belgian supermarkets were collected in the scope of the ALTEVAL 1 (RT project 06/5).

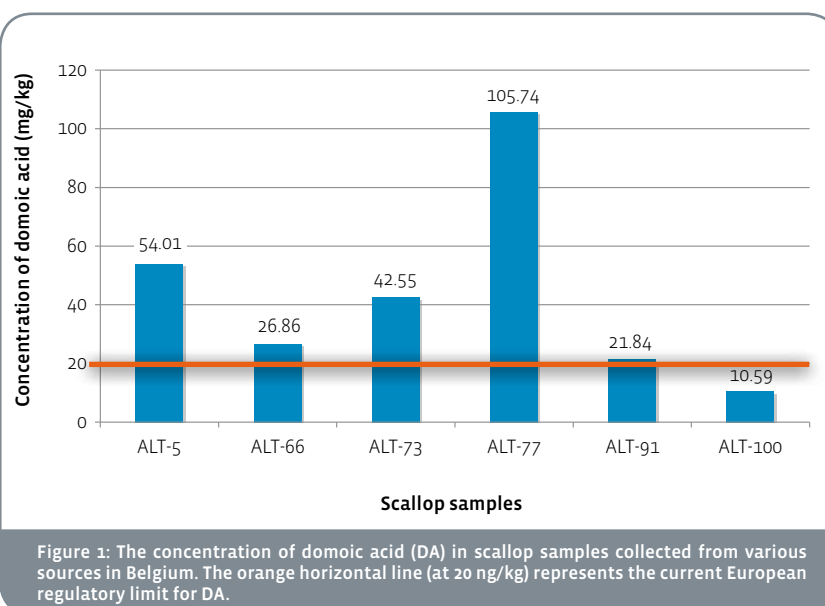
Analytical part

The analytical part of the study included the development and validation of the LC-MS/MS method for detection of domoic acid. The quantification of DA was done by collecting the data with LCQ DECA XPplus mass detector (Thermo Finnigan) and plotting the calibration curve in the range of 2-25 µg/ml of methanol. HPLC separation was done on HPLC XTerra C18 (5 µm 2.1 X 250 mm) column with isocratic gradient

of the mobile phase containing: 58% acetonitrile and 42% water with 0.05 mM CH₃COONH₄, both with 0.05% trifluoroacetic acid (250 µl/min). MS conditions: electrospray ionisation and detection in positive MS² mode; capillary temperature: 275°C; capillary voltage: 19 V; collision energy: 45; detecting parent ion (312 m/z) and transition ion (266.25 m/z).

Consumption data

The consumption data were obtained from the Belgian National Food Consumption Survey (VCP-2004). Aims, design and methods used in this survey are described elsewhere (1). The survey describes the consumption pattern of the Belgian population (all Belgian inhabitants of 15 years or older) and includes information on the usual consumption of nutrients and foods, and information on age, sex and body weight of 3083 individuals. The individuals were selected randomly from the National Register.





Statistical analyses of data

Computer-based Monte Carlo simulations (2) for probabilistic modelling were used to calculate percentile intakes for domoic acid. These simulations generate results for models in which one or more inputs can be defined by a distribution of values (food intakes and toxin concentrations). The detected concentrations of domoic

acid in the scallop samples were combined with the consumption data to generate a new distribution of intake values. The output from the Monte Carlo simulation was used to assess the risk-intake by estimating percentiles of the intake distribution and toxin concentration. In this study, the 95th percentile values were used as high-end estimates.

RESULTS

Domoic acid (DA) concentration

In 6 out of 61 scallop samples DA was detected. This represents around 10% prevalence. Figure 1 illustrates 5 scallop samples where DA level exceeded the current European regulatory limits of 20 mg/kg. None of these samples entered the market. No toxin was detected in mussel and oyster samples.

Consumption

Mussels were the dominant shellfish products eaten by the Belgian population as illustrated in Table 1. Out of 3,083 individuals who took part in the VCP-2004, 73 consumed mussels (2.4%), 9 consumed oysters (0.3%), and 14 consumed scallops (0.5%).

A distinction was made between raw (total quantity) and cooked (edible part) shellfish. The reported quantity of raw mussels used for preparation of an average portion was around 400 g but only 100 g of mussel meat was consumed. The ratio between raw and edible samples was estimated to be around 5 for mussels while both oysters and scallops did not have noticeable inedible parts.

Table 1: Shellfish consumption data in Belgium (Belgian food consumption survey, 2004), maximum permitted level and estimated concentrations of domoic acid (DA) in shellfish.

	No of consumers per food item	% of consumers per food item	average (g)	standard deviation	p0	p25	p50	p75	p95	p97,5
raw										
mussels			386.47	476.83	5.00	60.00	102.00	1000.00	1000.00	1500.30
oysters			103.67	101.72	5.00	21.00	52.00	180.00	252.00	252.00
scallops			64.22	55.99	4.86	30.00	50.00	87.57	200.00	200.00
cooked										
mussels	73	0.04	107.93	87.81	2.90	30.00	102.00	167.50	250.00	320.00
oysters	9	0.00	103.24	102.12	5.00	21.00	52.00	180.00	252.00	252.00
scallops	14	0.01	48.39	46.86	3.40	21.00	35.00	61.30	160.00	160.00
DA concentration	*20 mg/kg		43.54	34.15	10.59	22.83	34.71	51.15	92.81	99.27

* EU regulatory limit

CONCLUSION

It was estimated that on average people in Belgium eat per dish 108 g (2.9-320 g) of mussels, 103 g (5-252 g) of oysters, and 48 g (3.4-160 g) of scallops. However, higher portion sizes occasionally eaten in some cases (maximum 320 g mussel portion) are lower than 400 g portion size suggested by CONTAM Panel of EFSA to be used in the acute exposure assessment of marine biotoxins (3).

Consumption of 160 g of edible part of scallops (95th percentile of the portion size) containing 95th percentile of DA concentration (92.8 mg/kg shellfish meat) would result in a dietary exposure of 14.85 mg DA per person. This is equivalent to an exposure of 0.25 mg/kg bw (estimated for a person of 60 kg) which is around eight times higher than the provisional acute reference dose (ARfD) for domoic acid (0.3 mg/kg bw). The high levels of DA could pose a risk to some population groups. This study was based on a limited number of samples and therefore further risk assessment of exposure to domoic acid is necessary on a larger number of samples.

IMPACT ON PUBLIC HEALTH

This study shed light on the risk of domoic acid in shellfish, in particular in scallops handled by the Belgian national food control programme. The estimations of risk and portion size offer new insights into the protective effect of the current European Union regulatory limits of some marine toxins.

ACKNOWLEDGEMENTS

The authors are grateful for financial support from the FPS Health, Food Chain Safety and Environment for the contract research ALTEVAL 1 (RT 06/5): Validation of the Elisa technique and the LC-NS/MS method for the detection of phycotoxins as an alternative to the biological methods with mice.

SOURCE

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Method development for the analysis of cereulide, the emetic toxin produced by *Bacillus cereus*, in food

INTRODUCTION

Food-borne intoxications due to bacterial toxins are frequently reported in many countries. Several lethal cases caused by cereulide, the emetic toxin of *Bacillus cereus*, have even been reported (1, 2). This emetic toxin is pre-formed in food and is resistant to heat and usual food preparation procedures. The existing detection methods for cereulide are non-specific and/or only semi-quantitative. Therefore the development of a method for sensitive quantification of the emetic toxin is needed. The use of a LC-MS (Liquid Chromatography Mass Spectrometry) may be a powerful analytical tool for the specific determination of cereulide in food. This study details the development and validation of an adequate analytical method in order to monitor the toxin in food samples like rice or pasta, allowing determination of critical conditions for food consumption.

METHOD

Experimental set-up

The previously described method by L. Delbrassinne and collaborators (3) combines a simple extraction step from bacterial biomass with LC-MS analysis. Validation experiments were performed in freshly cooked rice inoculated with a cereulide-producing strain; the protocol was thus slightly modified. Briefly, 3 g of the inoculated rice was weighed in stomacher bags (International Medical) and 6 ml of pure methanol was added to each replicate (n = 3). The homogenised liquid was collected in glass tubes and placed in boiling water for 15 min, followed by evaporation until dryness. The residue was diluted in 3 ml methanol, vortexed and centrifuged. The supernatant was collected and stored at -20°C prior to analysis. Valinomycin (Sigma, Fluka, Germany) was used as an external standard.

The following validation parameters were determined on the basis of valinomycin spiking: linearity, matrix effect, recovery, repeatability and reproducibility. The validation parameters were evaluated in accordance with the performance criteria suggested in 2002/657/EC (4).

Method analysis

Cereulide and valinomycin contents of each extract were analysed by a LC-MS modified method as described by Haggblom *et al.* (5). The LC-MS analysis was performed on a LCQ Deca-XP Plus ion trap mass analyser (ThermoFinnigan, USA). Chromatographic separation was done on the Symmetry C₈ column, 2.1 mm x 150 mm, 5 µm (Waters, USA). The mobile phase of the isocratic method was a mixture of 95% acetonitrile, 4.9% water and 0.1% trifluoroacetic acid at a flow rate of 0.2 ml min⁻¹ with a sample injection volume of 5 µl. A full mass spectrum was



recorded from 500 to 1300 m/z in positive electrospray mode (ESI+). The total ion chromatogram was smoothed with a Gaussian function. For detection of valinomycin and cereulide, the monitored m/z values were respectively valinomycin adduct ions 1128.5 (NH_4^+ adduct) and 1149.5 (K^+ adduct), and cereulide adduct ions 1170.4 (NH_4^+ adduct) and 1191.5 (K^+ adduct).

Applications

As a direct application, the influence of different parameters (temperature, strains, incubation time and inoculation level) on cereulide production was tested in rice and pasta. Importance of temperature on cereulide production was tested in two experimental settings (in inoculated rice incubated at 23°C and 30°C and in inoculated pasta at 4°C, 8°C and 25°C). Whereas in the first set of experiments in rice, the impact of inoculation level of *B. cereus* (10^3 or 10^6

CFU g^{-1}) on the cereulide production was evaluated, in a second set of experiments the ability of two cereulide-producing strains (TIAC303 and *Kinrooi* k5975c), isolated from different lethal food poisonings, was observed in pasta over time. Cereulide production was analysed as described above. The described application in rice imitated a naturally occurring emetic food poisoning, where a healthy child died 6 hours after consuming leftovers of fried rice, left at room temperature for a whole day (2).

RESULTS

Validation

The linear range for the calibration curve of valinomycin in Full MS extended from 1 to 1000 ng/ml. Almost no matrix effect was observed, making additional clean-up of the extract unnecessary. The analytical

performance was evaluated with the analysis of spiked rice samples at 100 $\mu\text{g}/\text{kg}$. The recoveries were between 51 and 55% with RSDs in repeatability and reproducibility conditions below 5%. Representative MS chromatograms of valinomycin and cereulide are illustrated in Figure 1.

Applications

Regarding the effectiveness of temperature to prevent/postpone cereulide production, 4°C and 8°C were observed to be safe food storage conditions, while a clear increase of toxin amount was noticed in both pasta and rice subject to temperature abuse. In these conditions, strains k5975c and TIAC303 reached the induced emesis dose of cereulide after only one day of pasta incubation. Regarding the initial inoculum level, much greater quantities of cereulide were produced at all incubation temperatures when the inoculation level

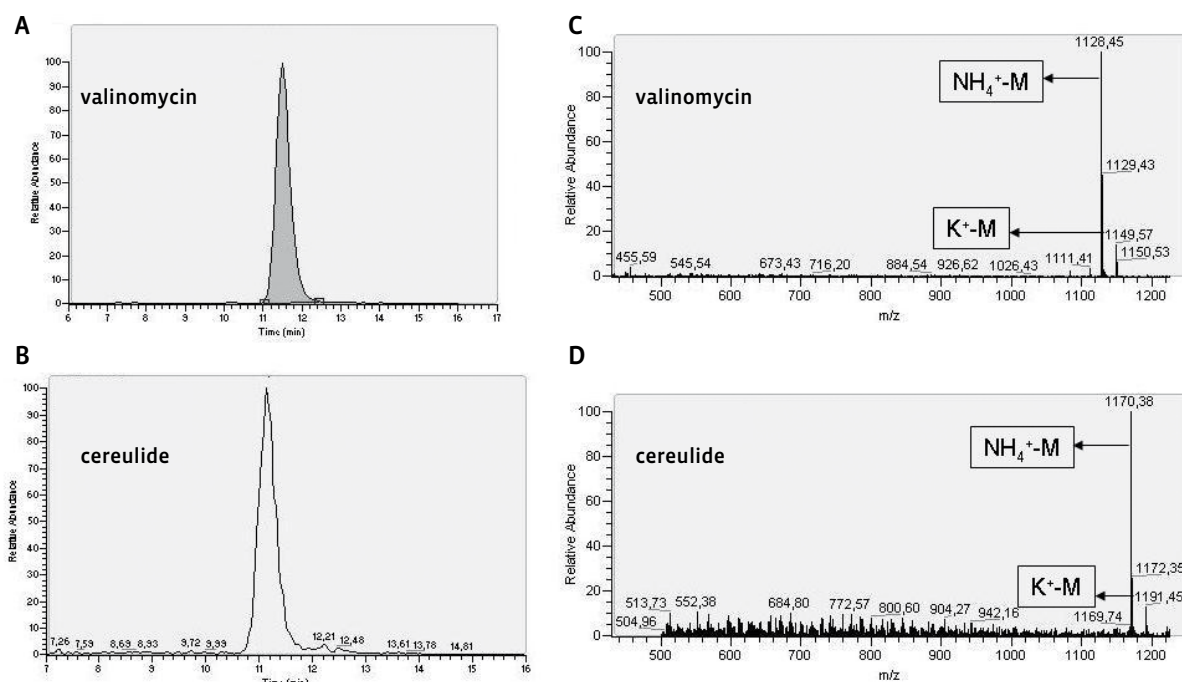


Figure 1: Valinomycin and cereulide representative chromatograms and mass spectra.

For detection of valinomycin and cereulide, the monitored m/z values were respectively valinomycin adduct ions 1128.5 (NH_4^+ adduct) and 1149.5 (K^+ adduct), and cereulide adduct ions 1170.4 (NH_4^+ adduct) and 1191.5 (K^+ adduct).

was 10^6 CFU g⁻¹. The optimum for cereulide production in rice was observed at 23°C; far exceeding the levels inducing emesis (0.01-1.6 µg cereulide/g food) in humans after only 24 hours (6). Cereulide was not detected in the negative controls.

shown to be strain-dependant and some cereulide-producing strains, such as *Kin-rooi* K5975C, may present a serious risk for the consumer if present in prepared meals left at room temperature. Temperature is thus an essential parameter to control and react to prevent/ postpone cereulide production in food.

CONCLUSION

The developed LC-MS method is easily performed, sensitive and capable of successfully analysing the impact of different factors on the production of cereulide in food samples. However, a better suited standard (¹³C labelled, chemically synthesised or purified cereulide) is required to improve current moderate recoveries. Although a wide range of foods can be contaminated by *Bacillus cereus*, rice was shown to contain the highest cereulide concentration when artificially inoculated with emetic strain (6). Nevertheless, pasta as a food matrix also favours easy and fast cereulide production. Cereulide production was



IMPACT ON PUBLIC HEALTH

The emetic toxin of *Bacillus cereus* has caused an important number of severe food-borne intoxications, occasionally leading to the death of young healthy people. As the current detection methods for cereulide are non-specific and only semi-quantitative, this project delivers a more sensitive alternative for its quantification in food and allows prompt reaction for consumer protection.

ACKNOWLEDGEMENTS

This project was financially supported by the Federal Public Service, Belgian Science Policy.

The application part of the rice experiment was performed in collaboration with E. N'Guessan, PhD student in the Laboratory of Food and Environmental Microbiology at the Université Catholique de Louvain. The authors thank Jacques Lhermitte for excellent technical assistance.

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Pesticide programme under continuous challenge

V. Hanot

S. Goscinny

INTRODUCTION

Pesticides are chemical compounds designed to control or minimize crop damage. This chemical input makes a considerable contribution to agricultural yield but inevitably leaves traces on food crops. The pesticides that remain in or on foodstuffs are called “residue”. The tolerated levels of these residues (MRL = Maximum Residue Level expressed in mg/kg of product) are set in Europe by the European Commission to ensure that they do not constitute an unacceptable health risk for consumers. To guarantee that the food on the market is safe for consumption, official national monitoring programmes have been established on a yearly basis, with sampling targeting different commodities (*items*) of the diet for evaluation. Laboratories that want to participate in the monitoring programme must demonstrate a certain level of performance (accreditation and participation in different proficiency or

interlaboratory tests) with respect to EU guidelines for quality control procedures (SANCO/10684/2009). A review of a decade of monitoring programme results shows a considerable increase in the number of pesticides being sought in a broader range of commodities and a tendency towards reduced time analyses. In 1997, only 13 molecules were mandatory for the monitoring programme, 11 years later the number is 101. This overall trend is a challenge for any laboratory involved in pesticide analysis. The Scientific Institute of Public Health is the National Reference Laboratory (role defined in Regulation 882/04) for the following areas:

- Pesticides Residues in Food of Plant Origin;
- Pesticides Residues in Food of Animal Origin;
- Pesticides Residues in Cereals;
- Pesticides Residues, Single Methods of Analysis.

Our laboratory scope covers more than 300 compounds for all the above matrices. However, the EU regulation has to adapt to new active compounds and continuous changes to pesticide-foodstuff combinations. Consequently, official laboratories have to continuously upgrade their validation with the objective of gradually extending their accreditation. The present article provides a brief account of our major laboratory activities with emphasis on the analytical tools set-up for pesticide residue analysis.

METHODS

Analytical methods are used to generate data on the basis of which the European Commission will assess dietary exposure and enforce MRLs. To fulfil this objective, two major steps were taken by the EC:

1. A new regulation came into force from 1 September 2008 which completes the harmonisation and simplification of pesticides MRLs for all EU countries and facilitates commerce with non EU-partners.
2. To ensure the quality and comparability of the data generated by laboratories, a guidance document on method validation and quality control procedure was drawn up for all control laboratories (latest version SANCO/10684/2009). This document is complementary to and in synergy with ISO/IEC 17025 requirements. This guidance document favours the “criteria approach” in contrast to rigid reference methods as standard. The criteria approach provides remarkable versatility, allowing laboratories to implement their own methods. In our laboratory and in general, with over 300 compounds to be monitored, a multi-residue method is the

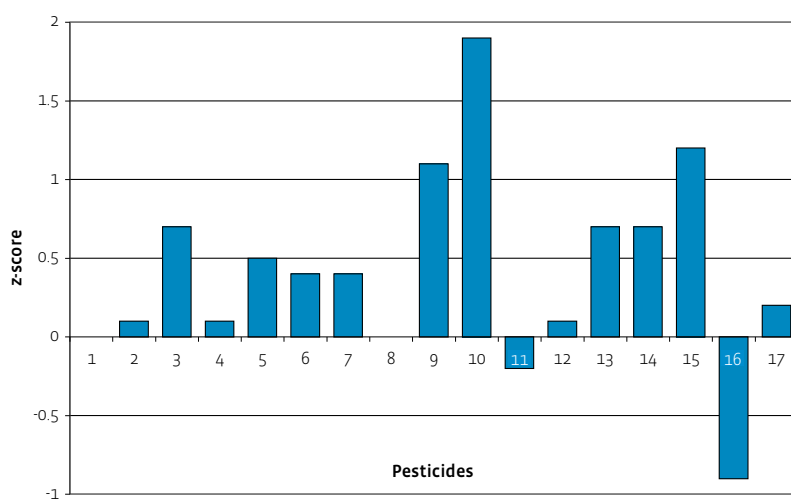


Figure 1: Z-score plot for the EUPT 10.

best choice in terms of cost and throughput. This approach enables coverage of pesticides from different classes in a single analysis. Single-residue methods in pesticide analysis are exceptional and are developed when the compound is not amenable to multi-residue methods (e.g. dithiocarbamates) or needs specific separation and/or detection conditions (e.g. bromide and chlormequats). These specific cases will not be discussed in this report as the intention is to give an overview of the most important activities of our laboratory; the application of multi-residue methods for fruit and vegetables.

In general, analytical methodology includes several steps: sample preparation, extraction, cleanup, and determination. For multi-residue methods each of these steps are crucial.

Sample preparation and pesticide extraction

Sample preparation is dictated by Regulation 396/2005 Annex 1. The extraction step has to take into account the polarity, solubility, thermolability and the detection limits required for all the pesticides sought, but also the nature of the matrix (acidic, high protein content...). We homogenise in a stirrer a representative portion of the sample with an adequate solvent (methanol or ethyl acetate). These solvents are able to extract a wide range of analytes with different polarity but with the drawback of co-extracting matrix components.

Cleanup

This step should discard interfering co-extractives. Nevertheless, all the different available cleanup possibilities tested hamper the analysis of many compounds. To overcome this problem we choose to skip this step and just dilute the crude extract. This approach can be applied only to LC system which is less sensitive to complex matrices resulting in faster analysis and reduced costs. For GC-MS/MS methods a cleanup by liquid-liquid partitioning was necessary and proved to be less deleterious to extracted pesticides compared to other cleanup procedures like SPE.

Determination

The measurement is a combination of separation (chromatography techniques) and detection. In our laboratory, we use two complementary separation techniques: liquid chromatography (LC) and gas chromatography (GC). The latter has been the most widely used technique for routine pesticide analysis since the 1970s in combination with selective detectors such as electron capture and nitrogen-phosphorus. To confirm positive results with these types of detectors, a second analysis was required with a different column. With the era of tandem mass spectrometers, simultaneous detection and confirmation is possible in one analytical run.

In 2008, with the acquisition of a GC coupled with a triple-quadrupole mass spectrometer, we developed and validated a multi-residue method for 116 pesticides in fruit and vegetables. This was an intense exercise, for example in terms of number of peak integrations (we have around 20,000 for the entire validation). For LC method, the laboratory has been accredited to use this technique for routine analysis since 2006. The separation is done on an ultra-performance liquid chromatography (UPLC). This technology has the advantage

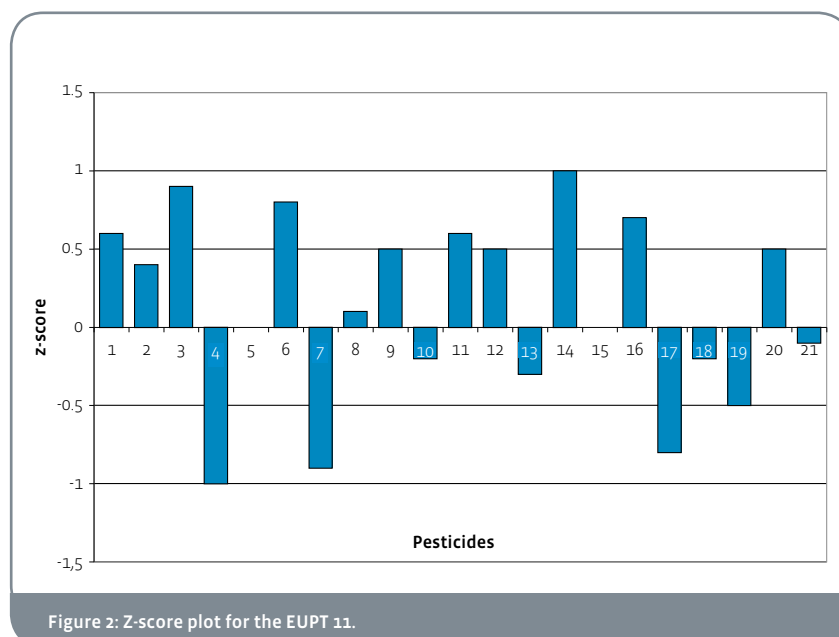


Figure 2: Z-score plot for the EUPT 11.



of generating narrow peaks and uses faster run times. In 12 minutes 120 pesticides are eluted and determined. During 2008, we extended our scope from 63 pesticides to 120 and accreditation was obtained after validation. This UPLC-MS/MS has proved to be easily upgraded to determine more compounds, nevertheless validation is still a laborious and complex step for multi-residue methods (7,560 peak integrations were necessary for this accreditation extension).

RESULTS

The method is validated, meaning that it has successfully passed all the performance criteria detailed in the SANCO document. Accuracy is a key parameter for pesticide residue analysis in fruit and vegetables, and can only be assessed by participation in the European Commission proficiency tests (EUP). This particularity is due to the fact that no certified pesticides in reference materials for fruit and vegetables are available. As NRL we have been participating in this annual EUP from the beginning in 1996, and it was very interesting to evaluate our performance with the new implemented technologies. Our results for EUP 2008 and EUP 2009 are represented in Figure 1 and Figure 2 respectively. No false positives nor false negatives were reported, and from the 2008 results only one z-score was close to 2, but for 2009 all the results were between -1 and 1. This performance demonstrates both excellent accuracy and that the methods are ready for routine determination of regulatory samples.

CONCLUSIONS AND PERSPECTIVES FOR MULTI-RESIDUE METHODS

The combination of LC-MS/MS and GC-MS/MS is a very powerful and complementary technique which covers the majority of active compounds in the pesticide field. At this point, the implementation to routine analysis was successful. However, to be

able to continue our participation in the EU monitoring plan, we are aware that it is essential to widen our scope of target molecules. This is not an easy task because the methods used have some limitations in terms of number of analytes covered in one LC-MS/MS and GC-MS/MS run. To overcome this problem and still have high-throughput methods we are currently trying new instrumental technologies.

IMPACT ON PUBLIC HEALTH

Pesticides are used to guarantee agricultural yield by controlling pests and protecting crops during post-harvest storage. The consequence of their use is their inevitable presence in food, thereby enabling them to reach the population. To ensure food safety the European Union has established maximum residue limits. The analysis of pesticide residues is a crucial tool for assessing the level of contamination and enabling enforcement of legislation.

MORE INFORMATION

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Method validation and quality control procedures for pesticide residues analysis in food and feed. Document N°. SANCO/10684/2009.
http://ec.europa.eu/food/plant/protection/resources/qualcontrol_en.pdf

ACKNOWLEDGEMENT

This work would not have been possible without the devotion and motivation of the Pesticide unit staff.

Rapid analysis of melamine residue in milk, milk products, bakery goods and flour by ultra-performance liquid chromatography/tandem mass spectrometry: From food crisis to accreditation

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INTRODUCTION

Melamine (2,4,6-triamino-s-triazine) is a chemical compound that was first considered as a potential crop fertiliser and as a dietary non-protein nitrogen source for cattle because of its high nitrogen content (66.6% nitrogen by mass). However, these applications were discontinued due to the slow and incomplete hydrolysis of melamine. Since then, melamine has been successfully used in various industrial applications such as the production of glues, laminates, plastic resins, tableware and flame retardants. However, in March 2007 (first crisis), melamine, in combination with cyanuric acid, was found to be responsible for the death of numerous pets in the United States due to renal failure. After investigation, the wheat gluten used in the pet food was identified as the source of contamination which was imported from China.

In 2008 (second crisis), powdered infant formula in China was contaminated with melamine resulting in more than 290,000 children being affected and 6 reported deaths of infants (1). Soon after, other products such as milk powder and related goods, egg powder, raising agents, protein powders and animal feed were also found to be contaminated. Due to the globalisation of trade, the crisis became an international scare which triggered the development of various analytical methods for the determination of melamine in different target food matrices and feed. Lin (2) recently published a review on all the different available techniques for melamine detection in food and feed. Briefly, different instrumental methods were reported which offer a panel of possibilities. For example, gas chromatography-mass spectrometry (GC/MS) has been reported despite the fact

that it requires a time-consuming derivatisation step. On the other hand, liquid chromatography can be used with different detectors such as diode array detection, ultraviolet absorption, or coupled with mass spectrometry and tandem mass spectrometry (MS/MS) with the benefit of qualitative and quantitative results. Screening methods (NMR spectroscopy, infrared spectroscopy and ELISA) are also considered as potential solutions for fast analysis with the drawback of being only qualitative.

An analytical method that delivers high-throughput analysis during crisis situation, with good identification and quantification to enable regulatory action is thus much needed. Moreover, sensitivity should be compliant with the maximum allowed level of 2.5 mg/kg set out in the EU Decision 2008/921/EC. In this respect, we propose a rapid validated method for the determination of melamine in a wide range of food matrices for regulatory control.

METHOD

Sample treatment

Samples were homogenised and 0.5 g was accurately weighted. Then, 10 mL of acetonitrile and water (v/v 1:1) was added to the sample and vortex-mixed for 10 seconds. The samples were placed in an ultrasonic bath for 25 minutes and centrifuged for 10 minutes. The extract was transferred for a liquid-liquid cleanup with dichloromethane and HCl was added. The cleaned extract was diluted (factor 5) with water prior to injection.

Chromatographic and mass spectrometric settings

LC analysis was performed with an UPLC™ (Waters, Milford, MA) equipped with a mass spectrometer Quatro Premier™ (Waters). A HILIC Acquity™ column (1.7 µm; 2.1 x



100 mm) was used. Only 1 µl was injected on the column. The sample was ionised with an electrospray probe in the positive mode. The spectrometer was programmed in MRM mode allowing 126.9 > 85 (quantification) and 127 > 68 (confirmation) transitions for melamine and 129.9 > 87 and 129 > 70 transitions for ¹³C₃-labelled melamine.

RESULTS

Sample preparation optimisation

The first objective of the extraction-purification step was to keep it as simple as possible such as a dilute and inject. Nevertheless, the various targeted food products are complex matrices with at least 3% lipid content. Direct injection of the crude extract would generate non-replicable quantitation results, matrix effect and loss of sensitivity. To avoid these drawbacks, we included a simple liquid-liquid extraction of fat with dichloromethane and diluted the extract as much as possible with water to lower the concentrations of potential interferences. To enhance protonation of melamine, concentrated acid was added to the crude extract prior to fat removal. Although vortex-mixing was found sufficient for extraction of milk samples, an ultrasonic process was included to accelerate the dissolution of melamine from the other matrices. In order to harmonise the protocol for all matrices, this latter step was also extended to the milk samples. The time in the ultrasonic bath was also optimised and extraction efficiency peaked at 20 minutes. For sonication beyond that time, no significant improvement in the extraction yield was noticed.

LC and MS/MS method optimisation

Initially, HILIC mode was directly chosen because of the high polarity and hydrophilic properties of melamine. An UPLC BEH HILIC[®] was found to produce good chromatographic peak shapes, sufficient retention of melamine and did not demand a long reconditioning time because only a 2 minute total run was sufficient to have stable and repeatable injections with no sign of column deterioration. Melamine was tuned in ESI⁺ mode with *m/z* 127 as the protonated molecular ion. Only two major product ions were observed *m/z* 85 and *m/z* 68. As the fragment *m/z* 85 was the dominant ion, the transition 127 > 85 was chosen for quantification and the transition 127 > 68 for confirmation. The surrogate ¹³C₃ was monitored by the transitions 129 > 89 for quantification and transition 129 > 70 for confirmation. The combination of a simple sample treatment and fast UPLC analysis offers the advantage of less reagent consumption as can be seen in Table 1.

Validation

The method was validated in-house (3) and included tests on specificity, linearity, matrix effect, precision and accuracy. The choice of sample material for validation was based on the ingredient composition. Industrial waffles were chosen because they contained 3 products reported to be contaminated: milk, egg powder and raising agent. Blank waffle samples were spiked at the beginning of the extraction protocol at 3 concentration levels corresponding to 1 mg/kg, 10 mg/kg and 30 mg/kg and with a constant amount of surrogate

of 2.5 mg. Analyses were performed in triplicate over three different days, with at least one day in between.

• Specificity

Due to the lack of SPE in the extraction step, it was very important to investigate if the specificity of MRM with two transitions was sufficient for the overall method. Therefore, sample blanks of different matrices were performed to check for interferences. No coelution was observed from the chromatograms of the sample blanks at the retention time of melamine (Figure 1).

• Linearity

Linearity was assessed at concentrations ranging from 0.5 mg/kg to 40 mg/kg matrix equivalent. Each calibration level was injected in triplicate and responses were submitted to a Mandel's Fitting test (4). This test evaluates whether an alternative regression model, in our case a quadratic model, fits the response better than a linear regression model. In our case a linear model was preferred. Different weighting schemes (i.e. 1/X, 1/X², 1/Y and 1/Y²) were tested and the choice for a 1/X selection was guided by the evaluation of the correlation coefficient and percent of recovery of back calculated quality controls.

• Matrix effect

A blank matrix was extracted 7 times in order to construct a matrix-based calibration curve. Each level of matrix-match and solvent calibration solutions were injected in triplicate. Then, two calibration lines (solution and matrix-matched) were built and their slopes were compared by means of a Student's t-test. There was no significant difference between the slopes of the matrix-matched standard curve and the solvent standard curve, meaning that no matrix effect is present and the calibration curve was done with the analytes dissolved in solvent (water).

• Limit of Quantification

The limit of quantification (LOQ) for melamine was established as the concentration

Table 1: The method solvent consumption.

Organic solvent consumption	Acetonitrile (mL)	Dichloromethane (mL)
Extraction	5	0
Purification	0	10
Liquid chromatography	1.26	0
Standard	0	0

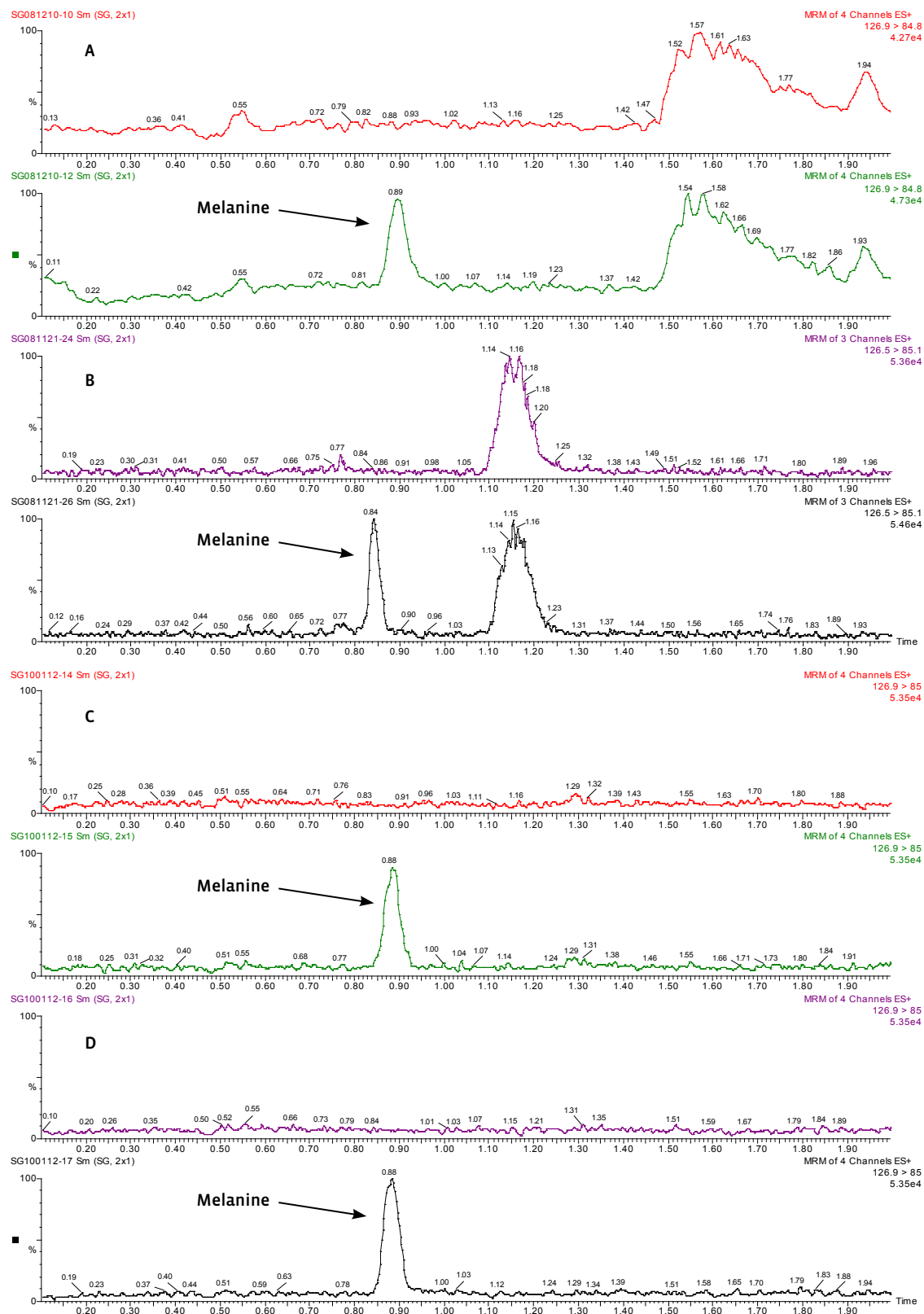


Figure 1: MRM chromatograms for
 A: (upper panel) Blank waffle and (lower panel) Spiked waffle at 1 mg/kg;
 B: (upper panel) Blank milk powder and (lower panel) Spiked milk powder at 1 mg/kg;
 C: (upper panel) Blank rice flour and (lower panel) Spiked rice flour at 1 mg/kg;
 D: (upper panel) Blank raising agent and (lower panel) Spiked raising agent at 1 mg/kg.

with a signal to noise ratio of 6. This limit of quantification was determined to be 0.5 mg/kg based on processed samples (9 replicates) spiked at 1 mg/kg which gave signal to noise ratios of more than 13. These samples had recoveries of between 93.3% and 103.6% and a good relative standard deviation (< 5%).

• Precision, recovery and accuracy

Precision was assessed by analysing waffle samples spiked at 3 different concentrations in triplicate for each level on 3 different days. The CV_i and CVR_w were calculated as described in the ISO 5725-2 guidelines and ranged from 0.9% to 3.2% and from 1.2% to 3.3% respectively. All CVs were below the maximum allowed values from the Horwitz equation of 10.7% for repeatability and 16.1% for reproducibility.

Apparent recovery was calculated for each spiked sample and ranged from 93.3% to 103.6%. No reference material was available at the time, thus, the accuracy of the method was evaluated by means of participation in two Proficiency Tests (PT). The first one was organised by the Dienstleistung Lebensmittel Analytik GbR (DLA), in which 51 laboratories participated worldwide, while the second one was coordinated by the European Commission Joint Research Centre (JRC) and the Institute for Reference

Materials and Measurements (IRMM), in which 120 laboratories participated worldwide. Milk powder samples were proposed by both PTs but the JRC/IRMM PT also had a sample of baking mix. The method performed well for the two PTs with z-scores between 0 and 1.07.

CONCLUSION

In summary, a simple and rapid method for determining melamine levels in food matrices has been developed. The validation results demonstrate that the method is specific with good sensitivity and precision. The use of UPLC allows short chro-

matographic runs compared to traditional LC-MS/MS and is also a good alternative to the time-consuming derivatisation step prior to GC. From sample preparation to instrumental analysis, the protocol is easily performed and many samples can be analysed in parallel with a reduction in organic solvent consumption. Isotopic dilution method is particularly convenient allowing fast recovery with the benefit of reliable results, which is supported by the IRMM proficiency test results.

This method has successfully passed accreditation and is destined to be applied in the framework of the national control plan in response to the Commission Decision 2008/798/EC.

IMPACT ON PUBLIC HEALTH

Melamine was implicated in a major food crisis in 2008. Due to the globalisation of trade, the crisis became an international scare, which triggered the development of various analytical methods for the determination of melamine in different target food matrices and feed. After risk assessment studies, the EU has set a limit of 2.5 mg/kg for melamine in food. In this respect, we propose a rapid validated method for the determination of melamine in a wide range of food matrices for regulatory control.

ACKNOWLEDGEMENTS

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Spectroscopic detection of counterfeit Cialis®

INTRODUCTION

Counterfeit medicines have become increasingly prevalent over the past decade. This is mostly due to the growth of the internet and the appearance of numerous fraudulent websites where anyone can easily and anonymously buy prescription only medicines (1, 2). In developed countries, the most popular counterfeit drugs are lifestyle medicines such as the phosphodiesterase type 5 (PDE-5) inhibitor drugs: sildenafil citrate (Viagra®), tadalafil (Cialis®) and, more recently, vardenafil hydrochloride (Levitra®) (3).

The internationally recognised definition of a counterfeit medicine is that of the World Health Organisation (WHO) (4):

“A counterfeit medicine is one which is deliberately and fraudulently mislabelled with respect to identity and/or source. Counterfeiting can apply to both branded and generic products and counterfeit products may include products with the correct ingredients or with the wrong in-

gredients, without active ingredients, with insufficient active ingredient or with fake packaging.”

In this study, 39 counterfeit and imitations of Cialis® and 4 genuine Cialis® were analysed by Raman-, NIR- and FT-IR-spectroscopy. Investigations have been carried out into which technique or combination of these techniques was the best to 1. Detect counterfeit Cialis® and 2. To determine clusters among illegal medicines which can be of interest for forensic investigations by the authorities.

MATERIALS AND METHODS

Samples

The counterfeit and imitation tablets of Cialis® were donated by the Federal Agency for Medicines and Health Products in Belgium (AFMPS/FAGG). They all come from postal packs ordered by individuals via internet sites. All samples were delivered in

blisters with or without packaging. All samples, once received, were stored at ambient temperature and protected from light. The samples have been divided into groups according to their visual appearance. Table 1 shows the groups of Cialis®-like samples.

Eli Lilly SA/NV (Benelux) kindly provided one batch of each commercial packaging of Cialis® (5 mg, 10 mg and 20 mg). Two other batches of Cialis® 20 mg were purchased in a local pharmacy in Belgium.

All references were delivered in sealed blisters with packaging and were stored protected from light at ambient temperature.










Instrumental

• Raman spectroscopy

A RamanRxn1 spectrometer (Kaiser Optical Systems, Ann Arbor, MI, USA), equipped with an air-cooled charge coupled device (CCD) detector (back-illuminated deep depletion design) was used in combination with a fibre-optic non-contact probe



Table 1: Visual classes of Cialis®-like samples (For RIVM classes see Ref. 3).

Group number	Sample photo	Symbol in plots	RIVM Class	Number of samples
1		●	Mixed counterfeits	5
2		X	Professional imitation	4
3		□	Professional imitation	5
4		✱	Non-professional imitation	3
5		△	Professional imitation	13
6		☆	Non-professional imitation and two mixed imitations	4
7		✱	Non-professional imitation	2
other		◇	Professional imitation and mixed imitations	3
genuine		○		4

to collect Raman spectra from the core of the tablets. The laser wavelength during the experiments was the 785 nm line from a 785 nm Invictus NIR diode laser. All spectra were recorded at a resolution of 4 cm⁻¹ using a laser power of 400 mW. Data collection, data transfer, and data analysis were automated using the HoloGRAMS™ (Kaiser Optical Systems, USA, version 2.3.5) data collection software, the HoloREACT™ (Kaiser Optical Systems, USA, version 2.3.5) reaction analysis and profiling software, the Matlab software (The Mathworks, Natick, MA, USA, version 7.7), and the Grams/AI-PLSplusIQ software (Thermo Fisher Scientific, Waltham, MA, USA, version 7.02). Ten second exposures were used for spectral acquisition. Spectra were collected at 3 locations per tablet. Spectra were pre-processed by baseline correction [Pear-

son's method, (5)], mean centred and averaged before data-analysis.

• NIR spectroscopy

Diffuse reflectance NIR spectra were collected per tablet using a Fourier-Transform NIR spectrometer (Thermo Fisher Scientific, Nicolet Antaris II near-IR analyser) equipped with an InGaAs detector, a quartz halogen lamp and an integrating sphere, which was used for NIR spectra collection from the tablets. Data analysis was done using Thermo Fisher Scientific's Result software, SIMCA-P (Umetrics AB, Kinneelon, NJ, USA, version 11) and Matlab (The Mathworks, Natick, MA, USA, version 7.7). Each spectrum was collected in the 10,000 – 4,000 cm⁻¹ region with a resolution of 16 cm⁻¹ and averaged over 16 scans. All spectra were pre-processed using standard

normal variate transformation (SNV) and mean centred before data-analysis. Each spectrum was performed on the core of the tablet.

• FT-IR spectroscopy

A Spectrum 1000 (Perkin Elmer, Waltham, MA, USA) FT-IR spectrometer with a DTGS detector was used. All spectra were recorded from the accumulation of 16 scans in 4,000-400 cm⁻¹ ranges with a 4 cm⁻¹ resolution. Samples were prepared by compressing a 0.3% mixture of pulverised tablet with spectral grade KBr (Merck, Germany). Three spectra of each sample were obtained, normalised and averaged.

Once recorded, the spectra were normalised with the Spectrum software (Perkin Elmer, Waltham, MA, USA, version 5.0.1.).

Data processing

The data pre-processing was performed using HoloREACT™ software. For NIR and FT-IR spectroscopy, the three spectra of a sample were normalised and averaged. For Raman spectroscopy, the three spectra of a sample were baseline corrected using the Pearson's method. All calculations were done with Matlab (The Mathworks, Natick, MA, version 7.9.0). The Principal component analysis (PCA) of the data has been performed with algorithms based on Kernel PCA (6). The Partial Least Squares (PLS) analysis of the data has been performed with the algorithms described by de Jong (7). The algorithms are part of the ChemoAC toolbox (Freeware, ChemoAC Consortium, Brussels, Belgium, version 4.0).

RESULTS AND DISCUSSION

Measurements

All IR measurements were performed in triplicate on the pulverised tablet. All NIR measurements were performed once on the core of three different tablets of each sample and all Raman measurements were performed on three different locations of the core of one tablet of each sample. Only

the fingerprint region of the IR spectra ($1,800\text{--}400\text{ cm}^{-1}$) and the $7,000\text{--}4,000\text{ cm}^{-1}$ region of the NIR spectra were used because of their high variability and their richness of information. The Raman spectra were taken with an exposure time of ten seconds on the core of the tablets at three different locations per tablet.

Case study two: Cialis®

• PCA

PCA analysis of Raman spectroscopy did not enable the genuine sample to be distinguished from the imitations or counterfeits. Therefore this technique was abandoned in preference of NIR and FT-IR.

PCA analysis of the Cialis® FT-IR dataset enabled clear identification of the counterfeit samples from the imitations and from the genuine samples. However, one group of imitations was not distinguished from the genuine samples. These imitations (group 5 in Table 1) have different brand names but are visually similar and can be easily identified as belonging to a single group. Analysis by HPLC and dissolution of some of them indicated that they are of good quality. This may be a result of a chemical composition and a manufacturing process very similar to that of the genuine Cialis®. So, PLS analysis was needed in order to distinguish these imitations from the genuine samples.

• PLS

1. FT-IR spectroscopy

PLS analysis enabled very good identification of the imitations and the counterfeits from the genuine samples. FT-IR also permitted 5 clusters of samples to be observed. One cluster shows the imitation samples that were not distinguished from the genuine samples by PCA. It contains imitations that were visually similar: oval shape with E20 embossed; without distinction between conventional tablets and chewable tablets. It can therefore be postulated that only few chemical differences are present (flavouring agents such as menthol).

A second cluster contains the counterfeit samples. It was very clearly separate from the other samples. HPLC analysis showed that they contain both sildenafil and tadalafil. This combination of API may explain this clear separation. Another cluster contains samples from the same manufacturer (according to the packaging) that are visually similar: round, orange and without film-coating. In this case, the chewable tablets were separated from other samples.

One cluster contains samples that were neither counterfeit nor imitation samples. Once again, the chewable version of these tablets was not included in the cluster.

A final cluster contains the other samples, except for three of them that were widespread in the plot. They were not similar but were quite close to each other. They probably have the same chemical composition and the same manufacturer.

The examination of the loadings scores did not permit the identification of which component was correlated with this separation.

2. NIR spectroscopy

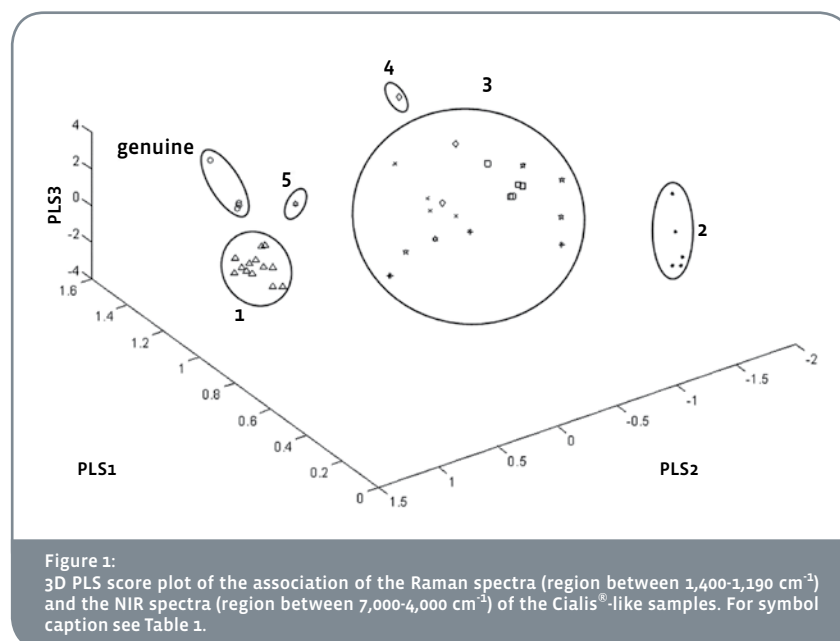
NIR spectroscopy shows the same separation as FT-IR but the different clusters are closer to each other. For this reason, NIR spectroscopy alone has been considered to be less efficient than FT-IR.

3. Raman spectroscopy

Raman spectroscopy enables the distinction between genuine and counterfeit samples. This distinction was greater when the region between $1,400\text{--}1,190\text{ cm}^{-1}$ was studied. Therefore this region has been selected for the rest of the analysis with Raman spectroscopy on Cialis®-like samples. One cluster contains the imitations from group 5 (Table 1) and one sample from group 7. This sample wasn't classified in any cluster by both NIR and FT-IR spectroscopy. A second cluster contains two samples from the same group but no reason has been found for their separation from the other ones. A final cluster contains the remaining samples. The loadings scores didn't permit the identification of which component was correlated with these clusters.

4. Combination of techniques

When the NIR and the FT-IR data were combined, the plot obtained shows a separate cluster of the genuine samples and four other clusters. Another cluster contains the professional imitations identified by both FT-IR and NIR (group 5 of Table 1) and it also contains a sample that was not included in a cluster by each technique separately, except by Raman spectroscopy. A third cluster contains the counterfeit samples and a final cluster contains the remaining



samples. Only one sample stands alone but it has not been possible to explain why the association of the techniques isolated it. Figure 1 shows the combination of the NIR and Raman spectra. Three clusters are observed. These clusters are the same as for the association of NIR and FT-IR spectroscopy except that the sample from group 7 that was in cluster 1 is now separated in cluster 4. The sample standing alone from the combination of FT-IR and NIR spectroscopy remains alone. The association of the three techniques permitted exactly the same separation as the association of NIR and Raman spectroscopy.

CONCLUSIONS

The aim of this study was to establish which technique or combination of techniques was the most powerful in distinguishing counterfeits from genuine samples of Cialis®. The spectroscopic techniques investigated comprised FT-IR, NIR and Raman spectroscopy.

FT-IR is a widely used and relatively low cost technique, used for decades and present in each analytical laboratory. The main drawback of this technique is its destructive character.

NIR and Raman techniques are increasingly used in the pharmaceutical industry because of their ease of use, their speed and the fact that they are non-destructive. Therefore further analysis can later be carried out on the tablets analysed by NIR or Raman spectroscopy, which is very important for an official analytical laboratory.

The ability of each technique separately to make the distinction between genuine and counterfeit or imitation samples has already been demonstrated. PCA analysis of the data was insufficient to achieve complete separation of the samples. Hence, PLS analysis was preferred because it is a powerful tool for a discrimination study with reference samples.

For the Cialis®-like samples, each technique separately enabled classification of the samples and distinction between genuine and illegal samples. However, this classification was insufficient by Raman spectroscopy or incomplete by FT-IR and NIR spectroscopy. It is concluded that the association of NIR spectroscopy (region between 7,000-4,000 cm⁻¹) and Raman spectroscopy (region between 1,400-1,190 cm⁻¹) is the most useful association of techniques. This association permitted a very

good separation between genuine and counterfeit or imitation samples. The classification performed allows the distinction between very bad counterfeits, very good imitations and other samples from genuine samples. This is useful for its application in a control laboratory.

The use of spectroscopic tools allows an objective distinction between legal and illegal tablets based on chemical and physical information of the tablets. This distinction sometimes confirms the visual classification of the samples but most of the time it completes this classification. As has been demonstrated, it is often the case that many visually similar samples are finally classified into the same cluster, which indicates that they have similar physico-chemical properties. This kind of objective classification is the most useful in any further investigation.

IMPACT ON PUBLIC HEALTH

This rapid detection of counterfeit medicines ensures the reliability of products present in the legal supply chain. It allows the population to have greater confidence in the fact that they are taking the exact drug that their physician has prescribed and not a substandard imitation of it.

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EXPERTISE, SERVICE PROVISION AND CUSTOMER RELATIONS

Introduction by Dr Ir. Myriam Sneyers

The Expertise, Service Provision and Customer Relations Directorate was officially created on 26 August 2008 by Ministerial Order. It came about following the merger of the Clinical Biology division of the former Epidemiology-Toxicology Department and the Biological Standardisation and Biosafety & Biotechnology divisions of the Microbiology Department.

The main tasks of this scientific directorate are:

- Managing services geared towards the monitoring of its three functional strands: Clinical Biology, Biological Standardisation, Biosafety & Biotechnology;
- Conducting scientific research projects essentially aimed at improving the services provided by the operational divisions;
- Coordinating the preparatory tasks as regards services for the entire WIV-ISP (dispatching unit, sample preparation);
- Formulating expert opinions for the international, European, federal and/or regional authorities on matters that come under its functional programmes;
- Coordinating relations with the Institute's main customers and partners, in particular the FPS Public Health, Food Chain Safety and Environment, the Federal Agency for Safety of the Food Chain (FASFC), the Federal Agency for Medicines and Health Products (FAMHP), INAMI-RIZIV (National Health & Disability Insurance Institute), the Communities and Regions;
- Providing effective assistance to the other scientific divisions in the event of crises affecting public health.

Biological standardisation

Description:

The division has the task of monitoring the quality of biological products. It is responsible for expert appraisals in the area of vaccines for human use and blood products, in conjunction with the Belgian and European authorities, as well as internationally. This appraisal consists specifically in assessing the quality part of registration files, participating in Good Manufacturing Practice (GMP) inspections, participating in various opinion groups and drafting regulatory texts on these matters.

Activities:

- Carrying out quality control of vaccines for human use;
- Carrying out quality control of blood and blood products;
- Verifying their compliance with international standards before placing on the market;
- Providing expertise in the area of vaccines for human use and blood products.

Biosafety & biotechnology

Description:

The directorate's tasks include the assessment of risks associated with the use of GM organisms and/or pathogens and with the monitoring thereof.

Activities:

- Providing scientific support to the competent federal and regional authorities on biosafety matters. This expert appraisal task covers the contained use of GMOs and/or pathogens, deliberate release and placing GMOs on the market. Essentially, it is aimed at the risk assessment for human health and the environment posed by such organisms;
- Acting as the secretariat of the Belgian Consultative Biosafety Council;
- Also coordinating the national Belgian GMO reference laboratory and completing an analytical and research mission within laboratories covering the detection, identification and quantification of GMOs, in particular regarding foodstuffs.

Clinical Biology

Description:

The directorate's main tasks include approving clinical biology laboratories on behalf of the Minister after verifying the official regulations, organising external assessments for clinical biology analyses and being the competent authority for in vitro diagnostic (IVD) medical devices within the framework of EU Directive 98/76.

Activities:

- Organises site visits to clinical biology laboratories;
- Acts as the secretariat of the Clinical Biology Commission and its various work groups;
- Manages authorisations issued to clinical biology laboratories;
- Alongside expert groups and the Clinical Biology Commission, organises a national compulsory programme for external quality assessment;
- Notification of IVDs in Belgium;
- Organises Post-market vigilance.

Development of a molecular detection platform for GMO detection in food based on “combinatory Q-PCR” technology

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INTRODUCTION

The number and diversity of genetically modified (GM) crops are exponentially increasing and are becoming a reality in agriculture as well in the food and feed market. 144 GM events, representing 24 crops have already received worldwide regulatory approval (1). In 2008, 25 countries have cultivated GM crops, whereas 30 countries have granted regulatory approvals for their import for food and feed use and release into the environment (1). With respect to the introduction on the market of genetically modified organisms (GMOs), many countries have implemented a specific government policy supporting a strong commitment to consumer protection and freedom of choice (2). For this, the traceability and labelling of GM products along the food chain is critical (2). In particular in Europe, testing food and feed materials for GMOs is regulated through EC regulations EC/2003/1829 and EC/2003/1830. Through such policies, two particular concepts have been elaborated: 1. The amount of GMO has to be calculated per ingredient (defined as a taxon, e.g. soya bean, maize, oilseed rape, etc.); 2. For authorised GMOs, product labelling is mandatory when exceeding a threshold above 0.9% GMO per ingredient.

Analytical GMO detection approaches basically consist of sampling, sample grinding, DNA extraction and target DNA analysis by applying Polymerase Chain Reaction (PCR) assays. Due to the broad range of authorised GMOs on the market, typically, the DNA analysis firstly comprises ‘screening’ in which a minimum set of PCR tests (targeting specific genetic elements) should allow conclusions to be made as to the absence/presence of as many GM events as possible. Only in the positive cases will

a second step specifically identify and/or accurately determine the content of the individual event(s) that is (are) present in the sample (Figure 1).

Our group (GMO-LAB in WIV-ISP) is the GMO-National Reference Laboratory coordinator for Belgium and the founding member of the European Network of GMO laboratories and has extensive experience in the development, improvement and application of molecular genetic methods to detect GMOs in food and feed. In this context, the WIV-ISP has developed a GMO detection approach by SYBR®Green PCR screening analysis. This new technology, designated “CoSYPS” (standing for “Combi-

natory SYBR®Green PCR Screening”) represents a novel tool facilitating the detection of material derived from genetically modified crops in Food/Feed matrices (Figure 1). The full system has been submitted for patent protection (3) and its methodological principle published in the literature (2). Here, we summarise and review all of the qPCR methods developed (targeting generic plant-DNA denominator, taxon-specific elements, generic recombinant DNA elements, recombinant trait-specific elements) and their integration in the CoSYPS for GMO routine analysis of food and feed samples in commonly applied 96-well plate qPCR format.

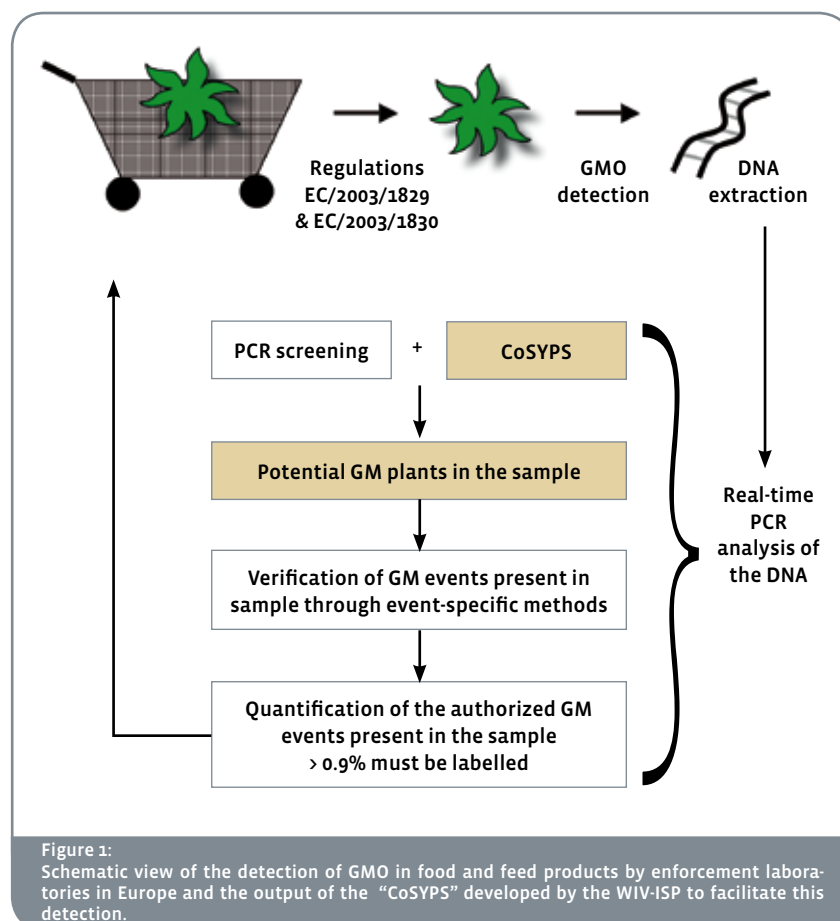


Figure 1: Schematic view of the detection of GMO in food and feed products by enforcement laboratories in Europe and the output of the “CoSYPS” developed by the WIV-ISP to facilitate this detection.

MATERIAL AND METHODS

Plant materials and genomic DNA extraction

Typical plant materials were Certified Reference Materials (CRM) from IRMM or AOCS. Genomic DNA (gDNA) was extracted from these plants using a CTAB based method as described by Barbau-Piednoir *et al.*, 2010 (4). The extracted gDNA was quantified using a VersaFluor™ Fluorometer (Biorad, Nazareth, BE) and the Quant-iT™ PicoGreen® dsDNA Assay Kit. The DNA was stored at -20°C.

Development of primer pairs

Real-Time PCR methods and the corresponding primers were chosen to target four different types of DNA elements (generic plant-DNA denominator, taxon-specific elements, generic recombinant DNA elements, recombinant trait-specific elements). The respective primer sets were either retrieved from the public domain or specifically developed in the context of the CoSYPS. The new designed primers bio-informatic analysis and *in silico* specificity of DNA sequences was performed according to Barbau-Piednoir *et al.*, 2010 (4).

Real Time PCR

All qPCR assays were standardised to be performed on an ABI 7300 PCR System (Applied Biosystems, Lennik, BE) in 25 µl reaction volume containing 1X SYBR®Green PCR Mastermix and 250 nM of each primer, except for the *RBCL* primers for which the primers concentration was set to 1 µM. The following thermal program was applied: a single cycle of DNA polymerase activation for 10 min at 95°C followed by 40 amplification cycles of 15 s at 95°C (denaturing step) and 1 min at 60°C (annealing-extension step). Subsequently, melting temperature analysis of the obtained amplification products is performed by gradually increasing the temperature from 60°C to 95°C over 20 min (±0.6°C/20 s).

Amplicon cloning, sequencing and plasmid deposit

PCR fragments obtained using the different qPCR methods was cloned and characterised by dideoxy-sequence analysis according to Barbau-Piednoir *et al.* (4). The so-called "SYBR®Green amplicons" (abbreviated as "Sybricons") and "pENGL plasmids" (= plasmid deposited in the context of the European Network of GMO Laboratories)

were registered under "Safe Deposit" or "Patent deposit" at the "Belgian Culture Collection for Micro-organisms" (BCCM/LMBP) (Gent, BE).

SYBR®Green qPCR specificity assessment

Primer pair specificity was tested by amplification of genomic DNA from various CRM. A sample is considered as positive and specific according the following criteria:

1. An (exponential) amplification is obtained with template DNA containing the target sequence(s) (and no amplification is obtained with the non target sequence),
2. A single peak upon melting analysis with a unique T_m value corresponding to the nominal T_m value obtained with the respective Sybricon or pENGL as template DNA,
3. A single band on agarose gel analysis with a molecular weight corresponding to the predicted size. Complete details concerning this assessment are described by Barbau-Piednoir *et al.* (4). Only specific qPCR methods considered were retained.

SYBR®Green qPCR sensitivity assessment

In this study, the sensitivity of the assays was estimated according to the former AFNOR Norm XP V03-020-2 as adapted by



Example 1: $GPN_{Bt176} = P_{maize} \times P_{p35S} \times 1 \times P_{PAT/pat} \times P_{CryIAB}$

Wherein,

GPN_{Bt176} , Gödel prime number associated to the GM plant Bt176
 P_{maize} , Prime number corresponding to qPCR method targeting the maize specie
 P_{p35S} , Prime number corresponding to qPCR method targeting *P35S*
 $P_{PAT/pat}$, Prime number corresponding to qPCR method targeting *PAT/pat*
 P_{CryIAB} , Prime number corresponding to qPCR method targeting *CryIAB*

Barbau-Piednoir *et al.* (4) and was tested in a dilution series of corresponding gDNA (20,000 haploid genome copies (HGE) to a single haploid genome copy). Each dilution analysis was performed in hexaplicate (n = 6). The so-called “LOD₆” of a qPCR method for detection of a particular target represents then the estimated haploid genome copies, at which level within a linear serial dilution analysis, each of the 6 repeats provides a specific signal (n = 6; 6/6 specific signals).

Only methods with a high sensitivity (LOD₆ < 30 HGE) were retained.

RESULTS

The qPCR methods developed for high coverage and discriminative detection of GM plants

The aim of our research was to develop a tool able to detect GM plants placed on the market. Therefore, on the one hand, the qPCR methods enabling detection of the presence of plants (method targeting a plant kingdom marker, the chloroplastic *rbcL* gene) and a number of crop-specific methods targeting the main GM commodity crops (soya bean, maize, oilseed rape, cotton and rice) (5) and, on the other hand, methods enabling detection of the presence of GM plants were developed. To detect as many GM plants as possible (high coverage power), methods targeting the most represented sequences in commercial GM plants (GM generic elements) were developed: the cauliflower mosaic virus promoter (*p35S*) and the *Agrobacterium* nopaline synthase terminator (*tnos*)

(4). Limiting the screening to those common targets has the disadvantage that the presence/absence of large numbers of GM plants needs to be confirmed via a second GM identification. Therefore, discriminative targets such as herbicide resistance genes and insect resistance genes (GM specific elements) were added to reduce the number of possible GM plants in the sample (high discriminative power).

The CoSYPS, a decision tool to conclude which GM plants is present in a sample

The aim of the CoSYPS is to integrate and combine the results given by each qPCR method to predict which GM plants may be present in a sample.

To construct this decision support system of the CoSYPS, a unique prime number was assigned to each particular screening qPCR method developed (the prime number have by definition only two dividers, the prime number itself and “1”). Each GM plant is characterised by the presence of several targets and consequently, a prime numbers product (named “Gödel prime number”) can be associated (Equation 1).

Equation 1: $GPN_{\alpha} = P_{Sp,\beta} \times P_{p35S} \times P_{tnos} \times \prod P_{event 1 to Z}$

Wherein,

GPN_{α} , Gödel prime number associated to the GM plant named α
 $P_{Sp,\beta}$, Prime number corresponding to qPCR method targeting the species β
 P_{p35S} , Prime number corresponding to qPCR method targeting *P35S*
 P_{tnos} , Prime number corresponding to qPCR method targeting *tnos*
 $\prod P_{event 1 to Z}$, Product of the prime numbers corresponding to qPCR method targeting GM specific elements

When the target associated to a qPCR method is present (positive “correct T_m & ≥ LOD₆” as described in materials and methods), the corresponding prime number is assigned to the relationship. While, when the target is absent, the number 1 is assigned to the relationship.

For example, the GM event Bt176 is transformed maize comprising the generic GM event sequence corresponding to *p35S* and two GM specific elements corresponding to the coding region of the phosphotransferase acetyl-transferase Liberty-link herbicide resistance (*PAT/pat*) and the coding region of the Bt insect resistance gene (*CryIAB*). Therefore its specific Gödel prime number is given by the product defined in Example 1.

In a similar way, when using multiple qPCR methods for GMO routine analysis of samples in commonly applied 96-well plate qPCR format, the product of the prime numbers of the positively scored screening outcomes for a sample can be represented by the “GPN_{sample}” product (Equation 2). This number comprises the targets that are detectable (≥ LOD₆) in the sample.

Equation 2: $GPN_{sample} = \prod GPN_{event 1 to \alpha}$

Moreover, the GPN_{sample} can be used as a divider to search for individual GPN (corresponding to specific GM plants) present in the sample. In the case where the result of the division is an integer, the specific GM plant may be theoretically present in the sample. Thus based on the GPN of the sample (determined experimentally as out-

comes of the screening analysis) and the GPN of the different GM plants (theoretically determined as a function of the DNA element present in the sample), the possible presence of a GM plant in a sample may be easily determined by a simple division function.

Next to this mathematical tool, for formal interpretation of the outcomes of the screening analysis, some experimental qPCR criteria (T_m and C_t) are used as decision values to give additional quantitative information on the possible GM present in a sample. These decision values and their integration in the CoSYPS are fully described in Van den Bulcke *et al.*, 2010 (2).

CONCLUSION

CoSYPS represent a novel real-time qPCR for GM plant analysis based on SYBR®Green technology. This decision support system is able to identify in a single qPCR run (96-well plates), the potential presence of the GM plants in a sample. Only the proposed GM plants (specific events) in the sample will be specifically identified in a second step. In a third step, the identified GM plants will be, if necessary, quantified to assess if their threshold is above 0.9% GMO per ingredient. This system, developed by the WIV-ISP, is considered a versatile, cost-effective, time efficient approach to assessing the presence of GM plants in a sample, particularly useful in routine analysis used for enforcement.



IMPACT ON PUBLIC HEALTH

Better screening for the presence of GMOs in food and feed products will improve traceability issues for commercially available GMO products and will reduce safety concerns related to GMOs, since unauthorised GMO products have not received a safety assessment and thus may present a potential threat to consumer health.

ACKNOWLEDGEMENTS

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Implementation of the 3Rs concept during batch release of a DTPa-IPV combined vaccine at the Belgian OMCL

A. Maes

INTRODUCTION

In general, regulations for medicinal products only require animal models for pre-clinical studies in order to test the toxicity of the drug substance. Laboratory animal testing is not usually required for routine batch release testing of these products. However, biological medicinal products in general and vaccines in particular are an exception as the quality of these products cannot usually be assessed by physico-chemical testing alone and tests measuring the biological characteristics on a routine batch to batch basis may be needed. Such testing includes laboratory animal tests for safety and potency of every batch (1). The necessity of developing some kind of efficacy assay was first seen in the late 19th century when it was observed that the therapeutic effect of diphtheria antiserum could differ from batch to batch from zero to 100%. Therefore the potency assay, which relies in general on *in vivo* testing is still today one of the main pillars for the control of sera and vaccines (2).

The three Rs concept in relation to laboratory animal experimentation was first introduced by Russell and Burch in 1959. This concept defined 'reduction' as lowering the number of animals required to obtain information of a given amount and precision, 'refinement' as any development leading to a decrease in the incidence or severity of inhumane procedures applied to those animals which have to be used and 'replacement' as any scientific method employing non-sentient material to replace methods which use conscious living vertebrates (3).

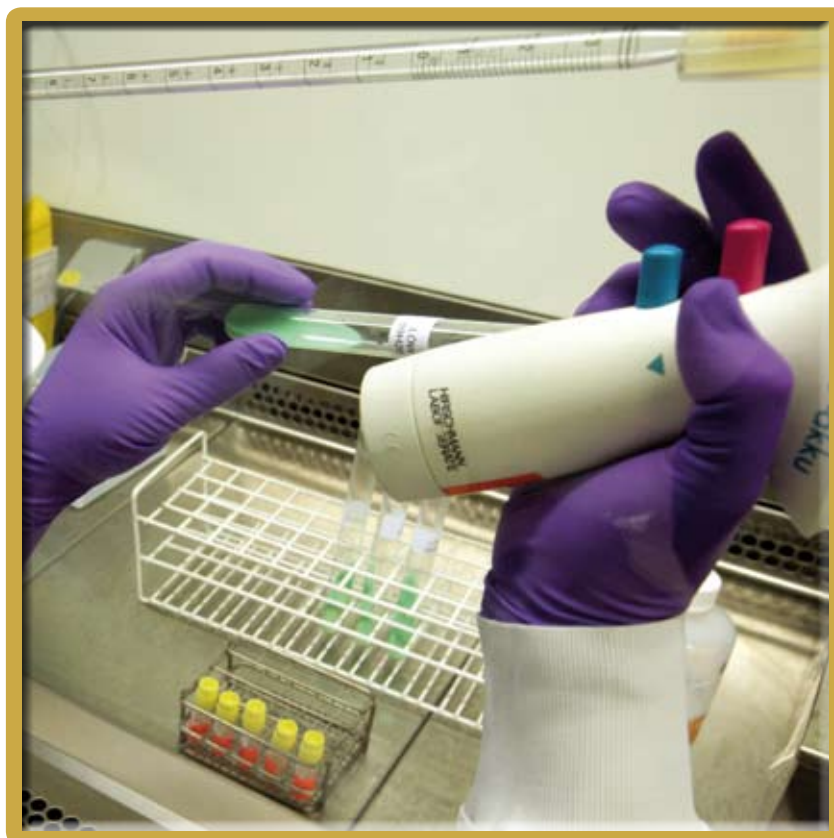
These principles were the basis of Council Directive (86/609/EEC) of 24 November 1986 on the approximation of laws, regula-

tions and administrative provisions of the Member States regarding the protection of animals used for experimental and other scientific purposes (4) and Convention ETS 123 of the Council of Europe (5).

In addition, European regulatory quality requirements for medicinal products including biological medicinal products such as vaccines, plasma derivatives and therapeutic proteins are laid down in Directive 2001/83/EC (medicinal products for human use), Directive 2001/82/EC (medicinal products for veterinary use) and the European Pharmacopoeia. Articles 114 and 82 of the respective Directives state that where it is considered necessary in the interests of public health, a Member State may require

the holder of a marketing authorisation to submit samples from each batch of the bulk and/or the medicinal product for examination by an OMCL (Official Medicines Control Laboratory) or a laboratory that a Member State has designated for that purpose. The European Directorate for the Quality of Medicines (EDQM) elaborated an EC Administrative procedure for OCABR which is based on Article 114 of Directive 2001/83/EC and the amended Directive 2004/27/EC (6).

The European Pharmacopoeia defines the assays to be performed on each type of product under the form of general and product specific monographs. These include *in vivo* assays for the quality control (QC) of biologicals.



METHOD

The European batch release procedure includes the retesting by an independent laboratory of each batch produced by the manufacturer before it may be released on the market. The tests to be performed by the OMCL are stipulated in the guidelines of the procedure and include *in vivo* potency and safety tests requiring the use of a large number of animals. However, since the mid-eighties, the European Pharmacopoeia Commission intensified the revision of all animal tests in monographs in order to apply the 3Rs concept. WIV-ISP performed validation activities and statistical analysis of historical data to implement the 3Rs measures.

RESULTS

WIV-ISP, Belgian OMCL, performed validation activities to reduce the number of animals used during testing of a DTPa-IPV combined vaccine (Diphtheria, Tetanus, acellular Pertussis, Inactivated Poliomyelitis vaccine).

Validation of the single dilution assay for potency testing of Diphtheria and Tetanus toxoid (7-9)

Potency testing of diphtheria and tetanus toxoid vaccines described in the Ph. Eur. is based on the multiple dilution assay, requiring the injection of multiple dilutions of the test and reference vaccine into different groups of animals. The number of surviving animals is counted 4 days after injection with the challenge toxin. The potency of the vaccine to be examined is calculated relative to the potency of the reference preparation on the basis of the proportion of animals surviving in each of the groups of vaccinated guinea pigs, using the usual statistical methods. The vaccine passes the test if it contains not less than an amount of international units (I.U.) as stipulated in the monographs for Diphtheria Vaccine (adsorbed) and Tetanus Vaccine (adsorbed).

The single dilution assay is based on immunising a group of animals with a single dilution of the test vaccine and a comparable group of animals with one dilution of the reference preparation after which the animals are challenged with toxin. The test vaccine passes the test if it is demonstrated that it is significantly more potent than the reference preparation.

The selection of the appropriate dilution for the reference preparation is based on historical data and a dilution containing a number of I.U. known to elicit an immune response in the lower part of the dose-response curve is considered acceptable. For the test vaccines, a dilution containing a same number of I.U. is selected based on the assumption that it contains exactly the required minimum per single human dose. Statistical evaluation should then demonstrate that the survival after challenge in the test vaccine group is significantly higher than in the reference vaccine group.

The single dilution assay for D and T potency testing was validated at WIV-ISP in 1991 using results from different kinds of combined vaccines (T, DT and DTPw) tested between 1986 and 1989. The reference preparation was a DT combined vaccine. Since the introduction of the single dilution assay for potency testing of diphtheria and tetanus toxoid containing vaccines, a threefold reduction in the number of animals used can be observed.

Validation of a single dilution immunogenicity test to monitor consistency of aP vaccine (10-12)

Testing the immunogenicity of aP vaccines was introduced in 1995 using a multiple dilution assay, consisting of the immunisation of groups of animals with 3 dilutions of the test vaccine and 3 dilutions of the reference preparation. The animals are bled 4-5 weeks after vaccination and the serum is analysed for specific antibodies against each aP component using enzyme-linked immunosorbent assay (ELISA). The antibody titres in the sera of mice immunised with reference and test vaccines are calculated and, from the values obtained,

the potency of the test vaccine in relation to the reference vaccine is calculated by usual statistical methods. The in-house procedure used three dilutions of vaccine and reference preparation for vaccination of the animals. The Ph. Eur. mentions that after validation, for routine testing, a single-dilution method may be used.

In 1998 the single dilution assay was validated by recalculating serum titres of 6 different vaccinations of the 1/3 dilution. Additional criteria were established in order for the assay to be valid.

Again, the introduction of the single dilution test for immunogenicity testing of aP containing vaccines reduced the number of animals significantly.

Validation of D and T potency testing on the first final bulk used in different vaccine batches

In the OMCL network, it is possible for an OMCL to submit a proposal in order to reduce the number of animals to be used during batch release. The validation was based on a draft document PA/PH/OMCL (98) 11 PROP: "Mechanism for reducing *in vivo* testing by OMCLs during batch release".

In 2000, a validation report was submitted proposing the reduction of diphtheria and tetanus potency testing of a DTPa-IPV vaccine. The validation was based on the demonstration of the production consistency by assessing the overall statistical homogeneity of final bulk lots produced between 1998 and 1999, the statistical homogeneity of the potency data from different final bulks in which the same batch of bulk antigen is used, the compliance of the potency data with the Ph. Eur. requirements and the marketing authorisation and the manufacturers data on Al³⁺ content as a parameter of the homogeneity of adsorption, formulation and filling. The above assessment is used to support the hypothesis that a valid potency assay performed on the first final bulk that is formulated with a new bulk antigen can be considered representative for the subsequent

Figures 1 and 2:

show a comparison of the number of animals used with and without the implementation of the reduction scheme for Diphtheria and Tetanus potency testing respectively.

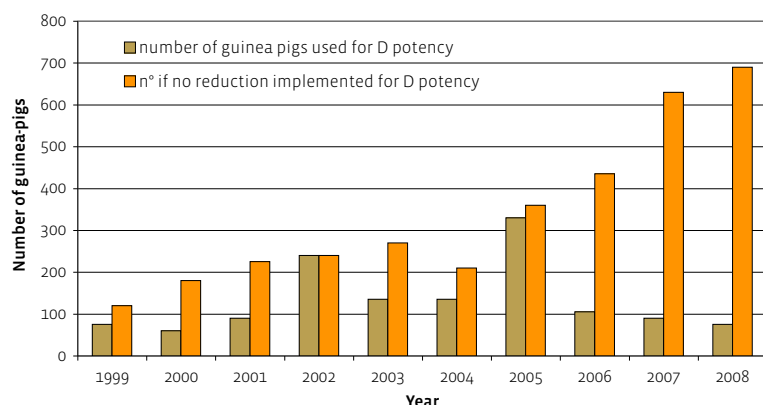


Figure 1:
Comparison of the number of animals used for D potency testing with and without implementation of reduction scheme.

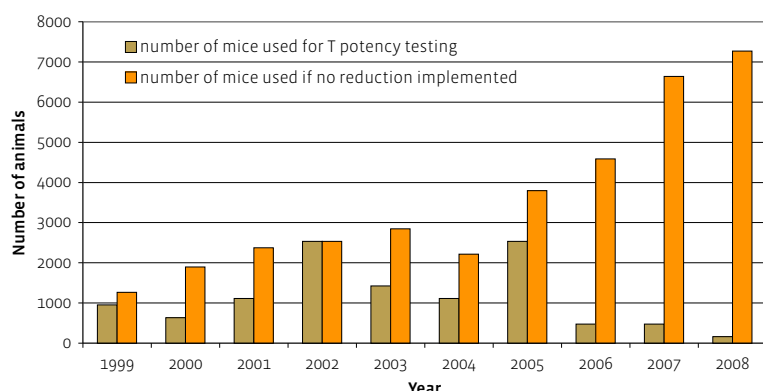


Figure 2:
Comparison of the number of animals used for D potency testing with and without implementation of reduction scheme.

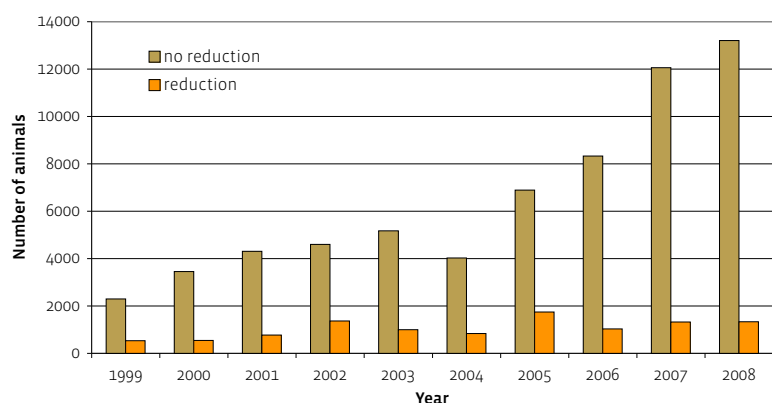


Figure 3:
Comparison of the number of animals used without and with reduction implementation.

final bulks formulated with the same batch of bulk antigen. The proposal was accepted in 2001 by the OMCL network and officially implemented in 2003 at WIV-ISP.

DISCUSSION AND CONCLUSION

Although the implementation of reduction, replacement and refinement should be one of the goals in quality control of vaccines, the implementation and/or validation of alternative methods should be based on a scientific rationale and without jeopardising the quality of OCABR. Public health remains the most important element when releasing vaccines for use in a healthy population consisting mainly of young children.

Since 1989, WIV-ISP has implemented several 3R measures, following Ph. Eur. principles, which are primarily based on the reduction of the number of animals to be used.

As an example, Figure 3 shows a simulation of the number of animals used for release of a DTPa-IPV combined vaccine in the case where WIV-ISP would not have implemented all the reductions discussed above.

As can be seen, a sevenfold reduction in the number of animals has been achieved for the release of one vaccine. When sufficient experience has been gained for a given assay and product, it can be concluded that the reduction in the number of animals used for batch release of vaccines can be easily implemented based on the review of historical data.

The 3R principle could be further achieved by implementing the principle of refinement. The validation of a humane-endpoint for the execution of the lethal challenge potency assays for tetanus and pertussis is currently in progress at WIV-ISP. One of the hurdles delaying this implementation has been the availability of qualified personnel. The implementation of humane endpoints requires the knowledge to interpret clinical signs of diseases in animals. In general, international recommendations do not explain in detail the clinical signs and

at which time-point the animals should be considered dead. The elaboration of such international guidelines would help the implementation of refinement measures.

The introduction of “refined” *in vivo* methods (e.g. the validation of the serological method for D and T potency for replacement of the lethal challenge assay) remains a great challenge for our laboratory. Validation of a new *in vivo* method requires the tests to be performed in parallel during a minimum required number of assays. For that purpose, one must move back from the established single dilution assay to a multiple dilution assay which – at least temporarily – increases the number of animals to be used. This means an increased burden on the animal facilities in terms of space and personnel. The replacement of *in vivo* test by *in vitro* methods could be considered easier as parallel testing would not increase the number of animals but, on the other hand, it may require investment in high technological and thus expensive equipment.

One additional hurdle to introduce alternative methods exists when manufacturers have not validated and introduced such methods. Indeed, in such a case, it would be difficult for the OMCL to use a different (alternative) method for the purpose of OCABR; the use of different methods by manufacturer and OMCL in general increases the risk of conflicting results and outcomes in terms of release and rejection of batches.

In conclusion, to encourage OMCLs to implement 3R principles, it would be desirable that the following conditions be fulfilled: acceptance, validation and implementation of alternative methods by the manufacturers, elaboration of international guidelines defining unambiguously humane endpoints, availability of qualified personnel at the level of animal caretakers, technicians and scientists, and the support of the management for investment in facilities and equipment.



IMPACT ON PUBLIC HEALTH

WIV-ISP has implemented the 3Rs concept via a significant reduction in the number of animals used for the quality control of human vaccines, without jeopardising the quality of those controls, a key element in authorising the administration of vaccines to a healthy population consisting mainly of young children.

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Methodology of the biological risk classification of animal pathogens in Belgium

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INTRODUCTION

Pathogenic micro-organisms receive much attention due to their potentially harmful effects on human, animal or plant health. Over the past few decades, this attention has grown due to the emergence of new (and known) infectious diseases inducing local epidemics as well as worldwide pandemics. Along with the research and diagnosis of those etiological agents, (bio)safety concerns have highlighted the biological risks associated with their deliberate use in laboratories, animal facilities and production plants, and their transboundary movements (import and export). This has led to their categorisation into risk groups and the elaboration of classification lists.

Current international classification systems rely on criteria defined by the World Health Organisation, which cover the severity of the disease the micro-organism might cause, its ability to spread and the availability of prophylaxis or efficient treatment (1). Animal pathogens are classified according to the definitions of the World Organisation for Animal Health, which also consider economic aspects of disease (2).

Evolution of scientific knowledge will demand regular updating of classification lists. This paper describes the Belgian risk classification system and the methodology that was used for its peer-reviewed revision (with a focus on animal pathogens).

METHODOLOGY

Belgian classification system

The Belgian classification system defines 4 risk groups, using the term “risk class”. The classification lists however, are limited to human, animal and plant pathogens, which are classified into three risk classes, as non-pathogenic organisms of risk class 1 are not included. Pathogenic micro-organisms for either humans or animals or for both are compiled in a single list, with risk classes assigned with regard to humans as well as with regard to animals (3).

The first Belgian classification lists were established in 1998, taking into account relevant European Community legislation, international and national classification schemes as well as relevant scientific publications. Since these lists reflect the state of knowledge at the time they were devised, they needed to be updated in terms of taxonomy and risk groups. The revised lists are not exhaustive but are intended to be representative in terms of the variety of pathogens that are prevalent and/or used (e.g. in research) in Belgium.

Procedure for revision

The revision of the classification lists with respect to taxonomy and biological risk class was conducted by the Division of Biosafety and Biology (SBB) of the Scientific Institute of Public Health. The lists were first revised taxonomically under the coordination of the BCCM (Belgian coordinated collections of micro-organisms) and the division of Mycology of the Scientific Institute of Public Health.

As a second step, an internationally recognised expert was chosen to coordinate Belgian animal health and biosafety experts in their review of the risk classes of animal pathogens in the taxonomically reviewed classification lists of human and/or animal pathogens. The experts were asked to focus on organisms that represented a risk to animal health without considering the risk to humans in the case of zoonotic pathogens (zoonotic characteristics of animal pathogens were assessed later, during the revision of human pathogens). Scientific knowledge was judged in the context of existing definitions of risk class in order to decide whether the assignment of a pathogenic organism should be modified or whether the lists should be extended. For each revision proposal, a revision form had to be completed that identified the given organism and documented the rationale for the proposed risk class revision. The revision form was also used to add organisms that were not previously included in the list. The revision forms were collated by the SBB and the coordinator. A compilation document was established and discussed in a plenary meeting where unanimity was reached with regard to the assignment of a given pathogenic organism to a given risk class.

Criteria for revision

In the assessment of the biological hazard of an organism, the following elements were considered:

- Impact of the disease or severity of the infection (pathogenicity);
- Infectivity (the virulence of the strain, the infective dose, the mode of transmission, natural route of infection);

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* contributed equally to this work

Table 1: List of revised animal pathogens.

Bacteria	Former risk class	Revised risk class
<i>Bordetella bronchiseptica</i>	3	2
<i>Campylobacter fetus</i>	3	2
<i>Clostridium chauvoei</i>	3	2
<i>Clostridium septicum</i>	3	2
<i>Francisella tularensis</i> type B ^(a)	3	3
<i>Leptospira interrogans</i> (all serotypes)	3	2
<i>Mannheimia haemolytica</i>	3	2
<i>Mycoplasma gallisepticum</i>	3	2
<i>Mycoplasma hyopneumoniae</i>	3	2
<i>Mycoplasma mycoides</i> subsp. <i>Mycoides</i>	4	3
<i>Mycoplasma suis</i>	3	2
<i>Pasteurella multocida</i> ^(a)	3	3
<i>Salmonella</i> (other than <i>Salmonella enterica</i> serovars)	3	2
<i>Streptococcus equi</i> subspecies <i>zooepidemicus</i>	3	2
<i>Taylorella equigenitalis</i>	3	2
Viruses	Former risk class	Revised risk class
Avian leukosis viruses (ALV)	3	2
Avian sarcoma viruses (Rous sarcoma virus (RSV))	3	2
Bluetongue virus (BTV)	4	3
Border disease virus	3	2
Bovine viral diarrhoea virus (BVDV)	3	2
Duck enteritis virus (DEV)	3	2
Duck hepatitis B virus	3	2
Equid herpesvirus 1	3	2
Equine arteritis virus	3	2
Feline infectious peritonitis virus (FIPV)	3	2
Fowlpox virus	3	2
Haemagglutinating encephalomyelitis virus	3	2
Infectious bronchitis virus (IBV)	3	2
Infectious bursal disease virus (IBDV)	3	2
Marek's disease virus (MDV)	3	2
Orf virus (Contagious ecthyma of sheep)	3	2
Porcine epidemic diarrhoea virus	3	2
Pseudocowpox viruses (bovine papular stomatitis, milker's nodes, paravaccinia)	3	2
Transmissible gastroenteritis virus	3	2

Parasites	Former risk class	Revised risk class
Ancylostomatidae (family) ^(a)	2	2
Anisakidae ^(c)	2	-
Ascarididae (family)	2	2
<i>Argas</i> spp. ^(b)	-	2
<i>Babesia gibsoni</i> ^(b)	-	3
Calliphoridae (family) ^(a)	3	3
<i>Cryptosporidium</i> spp. ^(b)	-	2
<i>Dermanyssus gallinae</i> ^(b)	-	2
<i>Dipylidium caninum</i> ^(b)	-	2
<i>Dirophilaria immitis</i> ^(b)	-	2
<i>Eimeria</i> spp.	3	2
<i>Giardia duodenalis</i> ^(b)	-	2
<i>Giardia</i> spp. ^(b)	-	2
<i>Histomonas meleagridis</i> ^(b)	-	2
Oestridae (family) ^(b)	-	2
Oxyuridae (family) ^(b)	-	2
<i>Neospora caninum</i> ^(b)	-	2
Psoroptidae (family) ^(b)	-	2
Sarcoptidae (family)	3	2
Strongylidae (family) ^(b)	-	2
<i>Taenia saginata</i>	3	2
Trichostrongylidae (family)	2	2
<i>Wohlfahrtia</i> (gender of the Sarcophagidae family) ^(b)	-	3

^(a) proposal for lowering the risk class was not validated by the expert group or risk class remained unchanged

^(b) added to the list

^(c) removed from the list

Table 2: Conclusive criteria for revising the classification of animal pathogens.

Risk Class (RC)	Criteria corresponding to Belgian definitions (in order of decreasing frequency)	Additional criteria
Bacteria		
RC 3 => RC 2	<ul style="list-style-type: none"> no control or eradication programs active in Belgium (e.g. <i>Mannheimia haemolytica</i>) no severe disease (e.g. <i>Mycoplasma hyopneumonia</i>) no epizootics (e.g. <i>Salmonella</i>) enzootics (part of normal microbiota) (e.g. <i>Bordetella bronchiseptica</i>) no interspecies transmission (e.g. <i>Mycoplasma hyopneumonia</i>) limited economic impact (e.g. <i>Taylorella equigenitalis</i>) 	<ul style="list-style-type: none"> similarity to other species of same genus with comparable biological risks (e.g. <i>Clostridium septicum</i>) poor persistence (survival) in the environment (e.g. <i>Mycoplasma gallisepticum</i>)
Viruses		
RC 4 => RC 3	dependence on multiple factors and the dissemination characteristics such as the serotypes and exclusively transmitted by an insect vector (e.g. BTV)	
RC 3 => RC 2	<ul style="list-style-type: none"> no severe disease (e.g. fowlpox virus) no epizootics (e.g. ALV) no interspecies transmission (e.g. Duck hepatitis B virus) prophylactic or therapeutic measures (vaccines available), controlled by isolation or eradication, quarantine (e.g. IBV) limited economic impact no control or eradication programs active in Belgium (e.g. TGV) enzootics (e.g. BVDV, Marek's disease virus) worldwide distribution (e.g. BDV) (latent) carriers (e.g. FIPV) 	<ul style="list-style-type: none"> no reservoir (e.g. IBV) low (or limited) concentration of host in Belgium (e.g. DEV) sporadic occurrence of the disease
Parasites		
Added to the list: assignment of RC 3 (for the parasite alone or family of parasites)	<ul style="list-style-type: none"> severe illness and discomfort (e.g. <i>Babesia gibsoni</i>, <i>Wohlfahrtia</i>) 	
Added to the list: assignment of RC 2 (for the parasite alone or family of parasites)	<ul style="list-style-type: none"> illness and serious discomfort economic impact by loss of productivity (e.g. <i>Psoroptidae</i>, <i>Dermanyssus gallinae</i>) or due to reduced growth (e.g. <i>Ascarididae</i>) Widespread (e.g. <i>Trichostrongylidae</i>, <i>Giardia duodenalis</i>) 	<ul style="list-style-type: none"> only a threat for young or immunocompromised animals (e.g. <i>Cryptosporidium</i> spp. <i>Giardia</i> spp.) abortions (e.g. <i>Neospora caninum</i>)
RC 3 => RC 2	<ul style="list-style-type: none"> illness and serious discomfort economic impact by loss of productivity (e.g. <i>Sarcoptidae</i>) 	<ul style="list-style-type: none"> asymptomatic disease (e.g. <i>Taenia saginata</i>) only a threat for young or immunocompromised animals (<i>Eimeria</i> spp.)
Withdrawn from the list	<ul style="list-style-type: none"> no symptoms in the host (e.g. <i>Anisakidae</i>) 	

- Host range (e.g. reservoir) and spectrum of specificity of target-species (age, sex);
- Genetic stability;
- Potential of survival outside host (e.g. ability to form resistant spores) and dissemination in the community or the environment (e.g. zoonosis, presence of vectors, reservoir);
- Availability and effectiveness of prophylactic or therapeutic measures (vaccination or antisera, antibiotics, chemotherapeutic agents, taking into consideration the possibility of emergence of resistant strains);
- Active control or eradication programs for the disease in Belgium;
- Production of allergens or toxins.

Based on the aforementioned elements, Belgian legislation defines criteria for classification of organisms into 4 biological risks classes, taking into account the theoretical maximum hazard incurred by immunocompetent humans, healthy animals and plants (4-6). They were used as a starting point for revision of the classification of animal pathogens.

At the start, it became evident that the criteria for a given risk class did not apply equally to all pathogens (due to their specific characteristics). It was therefore decided to introduce the following *additional specifications* for how to apply or weight the criteria:

- The characteristics of the pathogen should correspond as much as possible to the criteria considered for a given risk class.
- Though all criteria should be used, some criteria should be considered more important than others. This is the case for the epizootic, enzootic and exotic character of the pathogen (in order of importance).
- Although criteria that address the economic and/or sanitary importance of a pathogen should be taken into account, criteria that are inherent to the pathogen should be considered first and foremost.

- As the severity of the disease can vary with different strains of a given pathogen, the mean pathogenicity that is expected and/or observed is taken into consideration.

RESULTS AND DISCUSSION

Rationale for the chosen methodology

In the Belgian classification system, the assignment of a risk class depends on the inherent properties of the organism, independent of the activities (e.g. diagnosis, research, animal experiments) undertaken with it. This means that a clear distinction is made between the biological risk class of the pathogen and the risk class of the activity. In a risk assessment, both need to be considered in order to define the containment level and specific safety measures that should be adopted in order to protect human health and the environment. Hence the risk class of the activity may be equivalent to the risk class of the micro-organism or it may be higher or even lower. Consequently, work with the same pathogen can be undertaken under different containment levels, depending on the risk assessment of the activity. This approach ensures the resultant classification lists are not bound to containment levels. More than 70% of new and emerging infectious human diseases are known to be zoonotic (7). It was chosen for the Belgian classification lists to elaborate a common list for human and animal pathogens. In addition, and if necessary, two different classes of risk are assigned to the same pathogen with respect to its pathogenicity for humans and/or animals. This enables the consideration of the risk of animal pathogens within a larger context and also ensures harmonisation between different regulations concerning human and animal health.

Revision of animal pathogens

A compilation document with the peer-reviewed proposals for revision of risk class (57 in total, see *Table 1*) was discussed in a meeting with the expert group, the coordinator and the SBB and unanimity was obtained on the risk classes for the proposed pathogens.

For the majority of revised pathogenic bacteria and viruses, lower risk classes were proposed and accepted. In contrast, parasites were often assigned a higher risk class and many parasites were added to the list. *Table 2* gives an overview of the conclusive arguments that were used for revision of the risk classes for bacteria, viruses and parasites, illustrated by some examples.

The main arguments for reducing the risk classes for some bacteria and viruses were quite similar, although they did not appear in the same order of frequency. For viruses in particular, the reasons were linked to the situation in Belgium. For parasites, the main reason to either remove or add families to the list was the *degree* of discomfort and illness. Parasites causing only slight discomfort and no disease were withdrawn from the list. On the other hand, parasites causing very serious discomfort or severe illness or mortality, and causing significant economic impact, were added to the list. Depending on the severity of the symptoms, the host range, the geographical distribution and the economic impact, the parasites were either classified in risk class 2 or 3. The vector-borne nature of some pathogens was taken into account in the risk assignment of the pathogen itself. However, parasites that are only acting as carriers for pathogens were excluded from the list. In contrast, vectors producing toxins or serious allergic diseases were considered for inclusion on the list.

In general, the chosen set of criteria for assigning a risk class worked fairly well for the majority of micro-organisms. However, additional factors were considered in some cases, since a classification based on a single set of criteria was not always possible. A case by case evaluation was still needed for some specific pathogens, as is illustrated below.

For example, although all criteria were considered, some criteria constituted a conclusive argument for assignment to a given risk class in some cases. This was illustrated by the case of the Marek's disease virus, occurring worldwide and constituting a serious economic threat to poultry. The development of the disease is prevented by vaccination, but poultry still remain carriers of the virus. Due to its enzootic character it was decided to reclassify the virus to risk class 2.

Though the severity of the disease remained at the forefront of experts' minds, in some specific cases criteria addressing the economic impact or sanitary importance of a pathogen were taken into account. Infection by the Duck enteritis virus, for example, is known to be limited to anatidae (ducks, geese and swans). However, since the anatidae population in Belgium is rather small, the economic impact is limited, so a reduction from risk class 3 to risk class 2 was considered justified.

Another example of weighing up different factors against each other is illustrated in the case of the Bluetongue virus (BTV): although the characteristics of the virus meet all the criteria defined for risk class 4, the virus does not cause high mortality or important economic losses. The dissemination of the virus is strictly dependent of the presence of the *Culicoides* insect vector. Based on these factors, a reclassification of the virus to risk class 3 was agreed. Nevertheless, the actual risk class will also depend on the potential spread of the vector due to climatic changes (8).

CONCLUSIONS

The Belgian risk classification process categorises human and animal pathogens into a common list using the most comprehensive approach possible. The risk class of an organism is determined independently from the activities undertaken with it, allowing case-by case consideration of the activities carried out with pathogens and the determination of appropriate containment measures. The strength of methodology that was adopted during the revision of these classification lists lies in the peer-review process, supported by a multidisciplinary panel of scientists. As the methodology is based on unanimously accepted criteria, this will greatly facilitate future revisions of classification lists in response to new scientific knowledge and environmental changes in the broadest sense, with a particular emphasis on emerging infectious diseases.



IMPACT ON PUBLIC HEALTH

Classification lists provide an indispensable tool in the assessment of risks in order to define the specific safety measures to adopt when handling pathogenic organisms in research and diagnosis. Hence, regular revisions of these lists are crucial in the framework of increased scientific knowledge on new and known emerging diseases.

ACKNOWLEDGEMENTS

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A new statistical method for evaluating long term analytical performance of laboratories, applied to an external quality assessment scheme for flow cytometry

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INTRODUCTION

Flow cytometry is used for the quantification and identification of leukocyte subsets, particularly in the diagnosis and monitoring of leukemias, lymphomas and immunodeficiency diseases. The Belgian External Quality Assessment (EQA) programme surveys the performance of laboratories for several parameters determined by flow cytometry (1). Annually, the results obtained in the past three years are used to evaluate the long term analytical performance of the laboratories by assessing how well the laboratory's reported values relate to the target value, after it has been set for each sample. This is done by fitting a linear regression model to the data and comparing each laboratory's regression line with those obtained from other laboratories. In theory, each regression line should correspond to the 45°-line, yielding an intercept of zero and a slope equal to one. In practice, however, individual regression lines can be poorly estimated due to outlying observations, excessive residual variability, or gross systematic errors. As a consequence, the distribution of individual regression lines is overdispersed and laboratories with unacceptable quality levels may escape notice. The method proposed here is a 3-step approach which relies on robust statistics to combine the results of different samples collected over time.

MATERIALS AND METHODS

The linear model

Let N denote the number of laboratories participating in the EQA programme and n_i the number of EQA samples assayed by laboratory i ($i = 1, 2, \dots, N$). For each laboratory,

the relationship between the reported values and the target values of a given parameter can be described by the linear model:

$$y_{ij} = a_i + b_i x_j + e_{ij} \quad (\text{Equation 1})$$

where y_{ij} is the value reported by laboratory i ($i = 1, \dots, N$) for sample j ($j = 1, \dots, n_i$), a_i and b_i the intercept and slope of the regression line for laboratory i , x_j the target value for sample j and e_{ij} the residual or error term, assumed to be normally and independently distributed with mean 0 and variance σ_i^2 . The latter is a measure of the analytical variability for laboratory i , whereas the parameters a_i and b_i reflect the laboratory's bias from the 45°-line or from the mean regression line estimated from all laboratories. In this work, the target values have been taken as the medians of the values reported by all laboratories for each control sample.

The individual estimations of a_i , b_i and σ_i^2 and hence of their distribution over the population of laboratories can be adversely affected by outlying results. This prompted us to develop a 3-step method to obtain unbiased estimates of individual regression lines, discard participants with excessive variability, and unveil laboratories with exceeding bias.

The 3-step procedure

• Step 1: Excluding outliers from individual regression lines

The first step aims at excluding outliers from the laboratories' individual regression lines. To exclude potential outliers, we propose to use a least trimmed squares (LTS) regression (2, 3) to obtain a rough estimate of the regression line which holds for the majority of the data points, followed by the outlier searching algorithm described by Atkinson (4). LTS-regression

minimises the sum of the squared residuals of a predefined proportion of data, usually half of the data. Points satisfying the following inequality

$$|e_{ij} / s_i^*| \leq K$$

were subject to an ordinary least-squares (OLS) linear regression. In the above inequality, K is the upper α_1 -percentile of a Student t -distribution with $(n_i - 2)$ degrees of freedom,

$$s_i^* = 1.4826 \left(1 + \frac{5}{n_i - 2} \right) \sqrt{\text{median } e_j^2}$$

(Equation 2)

and α_1 is a predefined significance level. Let r_{ij} be the residuals of the OLS regression, s_i its residual error with m_i degrees of freedom, then outliers were defined as values for which $|r_{ij}| / s_i \sqrt{1 - h_{ij}} > t^*$ for the points included in the OLS regression and $|r_{ij}| / s_i \sqrt{1 + h_{ij}} > t^*$ for the points not included in the OLS regression. The critical value t^* is the upper- α_2 percentile from a Student t -distribution with $(m_i - 2)$ degrees of freedom and h_{ij} is the corresponding diagonal element of the hat matrix (5) from the LTS-regression. The h_{ij} -values are the so-called leverages, which measure the influence of the individual point on the position of the regression line.

• Step 2: Finding laboratories with high analytical variability

After having excluded outliers in the first step, a regression line is calculated for each laboratory separately and an estimate of the residual variability s_i^2 is obtained for each laboratory. The second step of the procedure is to depict laboratories with exceeding s_i^2 values. The method used has been described earlier (6, 7). It is based on the assumptions that both the theoretical residual variance δ_i^2 and the estimated residual variance s_i^2 are log-normally distributed. The following formula can be derived:

$$\log \sigma_{\alpha_i}^2 = \log M - 0.5 \log \left[1 + \frac{V - 2kM^2 / (2k + 1)}{M} \right] + Q_2(1 - \alpha_3) \sqrt{V - 2kM^2 / (2k + 1)}$$

(Equation 3)

where:

$k = \sum_{i=1}^N (n_i - 1) / N$, $M = \text{Mean } s_i^2$, $V = \text{Var } s_i^2$ and $Q_2(1 - \alpha_3)$ is the upper α_3 -percentile of the standard Normal distribution. Mean s_i^2 and Var s_i^2 are the mean and variance of the residual variances from the regression lines obtained in the first step. The value $\sigma_{\alpha_i}^2$ will be taken as the critical threshold above which a residual variance s_i^2 will be considered as exceedingly large. Since some of the N estimates s_i^2 may themselves be outliers, a trimming procedure is performed before calculation by disregarding an arbitrary proportion of the lowest and highest variance values.

• Step 3: Finding laboratories with exceeding bias

As before, let a_i and b_i be the estimated intercept and slope of the corresponding regression lines after having excluded outliers in the two previous steps. Literature about regression explains how the joint distribution of intercept and slope can be considered as bivariate Normal (5) with mean (a, b) , standard deviations (s_a, s_b) and correlation r .

When plotted on a two-dimensional graph, the points will be positioned in a cloud around which an ellipse-shaped tolerance region can be drawn. The centre of the ellipse is the intercept and slope of the mean regression line and the shape is characterised by their standard deviations (s_a, s_b) and correlation r . A point outside the ellipse corresponds to a laboratory with significant outlying bias.

Also here outliers may arise. The minimum covariance determinant (MCD) estimator (8, 9) is proposed as a robust measure of means, standard deviations and correlation of the bivariate distribution. Mahalanobis distances based on the MCD estimator are used as a measure of individual outliers. Regression lines for which the Mahalanobis distance is larger than the upper α_4 -percentile of a Chi-square distribution with two degrees of freedom correspond to laboratories exhibiting unacceptable bias.

RESULTS

Example data set

To illustrate the application of the 3-step procedure, we used data obtained from the Belgian External Quality Assessment scheme for CD4. Three times per year a survey is organised in which 3 fresh blood samples are sent to participating laboratories. Surveys included in the study spanned from 2005 to 2007.

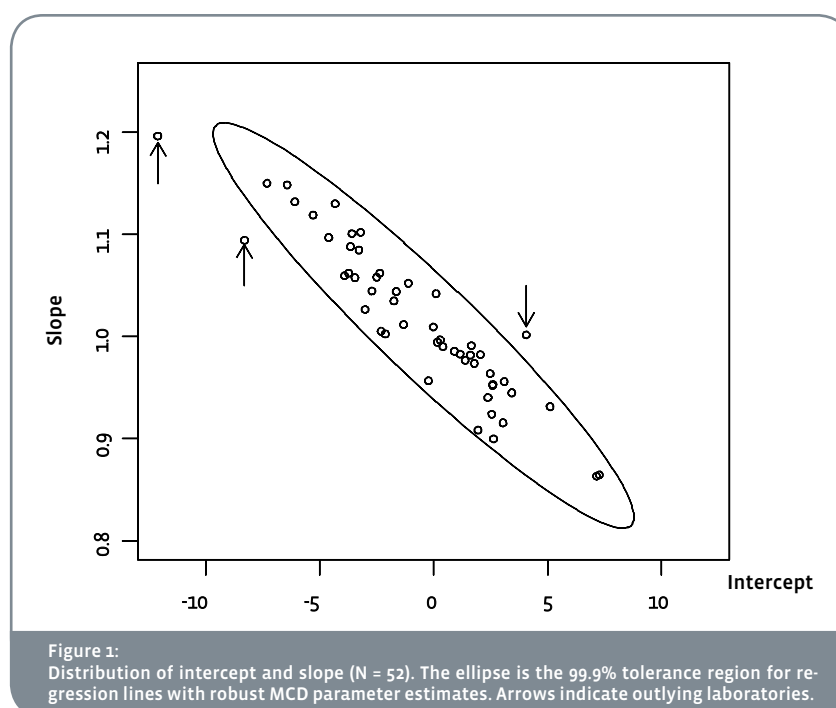
Application of the method

Step 1: Both α_1 and α_2 were set at 0.0005. The first step of the procedure revealed that 53 (4%) out of 1,340 points were too outlying to be included in the individual linear regressions according to the inequality given in the Materials and Methods section. Eighteen of them were eventually not considered as outliers. Thus, 35 data points were identified as outliers and not included in the ordinary linear regression lines. Scatter plots of residual against target values supported the assumption of linearity of the

regression model. Among the 35 outliers, 3 were clearly due to a mistake in unit reporting; 14 occurring together with at least one other outlier in the same survey may be caused by an accidental mistake which was not reproduced in other surveys. Among the other 18 outliers, 14 had very heavy deviation of at least 10 percent points between the reported and target value and 4 results occurred alone with smaller deviations.

Step 2: By selecting a value for α_3 of 0.01 and a trimming amount of the 5% most extreme variances, we obtained a threshold value for identification of residual variability outlyingness of 14.76. The median residual variance was 3.27 and the standard deviation was 3.87. One laboratory was found to have high residual variability. By excluding this laboratory, the standard deviation of the residual variances dropped to 3.00. If no trimming had been applied, the threshold value would have been 17.09 and the same laboratory would have been indicated for excessive analytical variability.

Step 3: The results of the third step are graphically presented in Figure 1. A value for α_4 of 0.001 was used. Three laboratories were found to have outlying bias. Two of them would have escaped notice if the first



and second steps had been omitted. The robust mean intercept and slope were 0.4529 and 1.0114, with standard deviations equal to 3.66 and 0.078, respectively, and the correlation was -0.94.

DISCUSSION

The proposed 3-step procedure evaluates single data points or regression lines as outliers using limits based upon statistical analysis of the performance attained. By this, the methodology is different from evaluation procedures using limits based upon experience or biological variability. The method is applicable whenever matrix effects can be excluded. Target values can be set by a reference laboratory or can be the consensus mean.

The method also helps laboratories better understand their results and is more informative than a single parameter performance characteristic like total error. Laboratories having exceeding values for the second step should be encouraged to control their random error. Laboratories having exceeding values for the third step should be encouraged to reduce their systematic error. Without excluding outliers in the first step, laboratories may have a high analytical or bias error, while this was attributable to only one or a few discordant results. Moreover, the method's ability to build up a hierarchy in the identification of mistakes in the laboratory is a major advantage and a distinction of searching different types of

outliers is, as far as we know, new and definitely useful in addressing the type of errors seen in EQA programs. While the first step evaluates single reported values and outliers may indicate accidental mistakes with a small chance of being reproduced, the second and third steps encompass a global laboratory evaluation and exceeding values found in these steps indicate poor overall laboratory performance.

Samples sent in different EQA surveys with different target values can be interpreted together and, as such, the second and third steps allow evaluation of laboratories for their analytical variability and bias over a longer period.

The method is flexible in terms of sensitivity and specificity of outlier findings by changing the values for the different α -values in the different steps. The purpose of the study should determine the choice of the different α -values. Here we have opted to detect solely heavily deviating values, which resulted in α -values of 0.01 or lower.

The results of the first step show that an indication of outliers based on one criterion, as pointed in the inequality of step 1 needs a closer look. We found, however, that the LTS-regression did not always fit the majority of data well enough. Changing the proportion for which the LTS-regression should minimise the sum of squared residuals is advisory in that case. The exclusion of outliers rather has influence on the variability estimate than on the position of the regression line, except when outliers differ with a magnitude of 10 or more, as is the case for unit mistakes. The results of the third step show a similar trend: leaving out outliers mainly affects the standard deviations and the correlation in lesser extent than their position.

Outliers in the first step indicate accidental mistakes, outliers in the second and third step provide evidence for high analytical variability or bias for all samples. In general, we found robust statistics as a useful solution for outlier searching in EQA testing.



IMPACT ON PUBLIC HEALTH

This method sheds an innovative light on the statistical processing of results obtained by External Quality Programs. External Quality of Clinical Laboratories is considered as a necessary tool for control and improvement of the quality of a basic process supporting patient's diagnosis and follow-up.

SOURCE

Coucke W, Van Blerk M, Libeer JC, Van Campenhout C, Albert A. A new statistical method for evaluating long-term analytical performance of laboratories applied to an external quality assessment scheme for flow cytometry. *Clinical Chemistry and Laboratory Medicine* 2010;48(5):645–50.

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New techniques of genetic modification and the GMO definition

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INTRODUCTION

In the European Union, genetically modified organisms (GMO) and genetically modified micro-organisms (GMM) are defined respectively according to Directives 2001/18/EC on deliberate release of GMO (1) and 2009/41/EC on the contained use of GMM (2). In accordance with the legislation, a GMO/GMM is defined as *“an organism/micro-organism ... in which the genetic material has been altered in a way that does not occur naturally by mating and/or natural recombination”*. This definition must be read together with a series of annexes that list techniques (i) that result in genetic modification, (ii) that are not considered to result in genetic modification, or (iii) that result in genetic modification but yield organisms that are excluded from the scope of the Directives.

A novel organism will therefore fall under the scope of the GMO regulation only if it has been developed with the use of defined techniques. With the advance of scientific knowledge, techniques which are applied in genetic modification of organisms have emerged that may challenge the current regulatory definition of a GMO because it is not always clear whether the products obtained through these techniques are subject to the prevailing European GMO legislation or not.

Several initiatives have been taken at the European level in order to evaluate some of these new techniques in the context of the existing legislative framework. The Division of Biosafety and Biotechnology (SBB) is actively contributing to this work.

NEW TECHNIQUES OF GENETIC MODIFICATION AND THE GMO LEGISLATION

The European GMO legislation was introduced in 1990. At that time, the legislation was mainly based on the principle that an organism is considered to be a GMO when new combinations of genetic material are formed through the use of certain techniques, in particular recombinant nucleic acid, injection or cell fusion techniques. However, with the advance of science and more particularly of biotechnology, new genetic modification techniques have been applied to research or commercial purposes since the introduction of the GMO legislation. There is now growing interest in using biotechnology in such a way that the resulting organism does not contain any added sequences, expresses new types of changes or bears changes similar to those achievable with conventional breeding techniques. For some of these techniques, questions have emerged as to whether they can be considered as resulting in genetic modification in the meaning of the Directives and/or whether the resulting products must be characterised and regulated as GMOs.

At European level, discussions on this issue started in 2007 within the Competent Authorities dealing with Directive 2001/18/EC. In a first step, the Competent Authorities raised questions concerning regulatory and safety issues associated with the use of oligonucleotide-mediated mutagenesis (OMM) as a technique of genetic modification.

OMM (also referred to in the literature under other names such as targeted nucleotide exchange, chimeraplasty or targeted gene repair) is a technique used to correct or to introduce specific mutations at defined sites of a target gene. OMM is mediated through the introduction of an oligonucleotide with homology to the target gene, except for the nucleotide(s) to be changed. The oligonucleotide hybridises at the targeted location in the genome to create a mismatched base-pair(s) which acts as a triggering signal for the cell's DNA repair enzymes. OMM has potential applications in fundamental research, medicine, agro-food and pest control. Mutations can target any nucleotide sequence for instance to inactivate a deleterious gene, to induce local modification in expression or to change an amino-acid in the corresponding protein resulting in a protein with possible new properties.

In April 2007, at the request of the Federal Public Service for Health, Food Chain Safety and the Environment, the Belgian Biosafety Advisory Council* published a scientific opinion on this issue followed, a few months later, by a scientific paper drafted by experts from the SBB, members of the Biosafety Council and scientists from Belgian universities. Both the Council's advice (3) and the scientific paper (4) provide scientific arguments for not having organisms developed through the OMM technique fall within the scope of the EU regulation of GMOs. Broadly speaking, it is considered that the technique does not pose biosafety questions other than those associated

* The Belgian Biosafety Advisory Council is one of the two pillars (the other one being the Division of Biosafety and Biotechnology - SBB) of the common scientific evaluation system that has been set up in Belgium to advise the competent authorities about the safety of activities involving genetically modified organisms (GMOs) and/or pathogens. The Council consists of representatives of the Regional and Federal authorities. It is assisted by experts in its scientific work. The secretariat of the Council is provided by the SBB.

with similar techniques already listed in the GMO Directives, and could be considered similar to mutagenesis, a technique currently excluded from the scope of the EU GMO regulatory framework. Another important point is that organisms developed through OMM could, in many cases, not be distinguished at the molecular level from those developed through “traditional” mutation techniques (using chemicals or ionizing radiation). It is important to realise that techniques such as OMM that do not involve the introduction into the genome of transgenes could pose challenges for unambiguous detection and testing of the resulting organisms, and ultimately enforcement of the European GMO regulatory system.

OMM is just one amongst several techniques that are challenging the approach followed in the EU to define a GMO. Therefore, at the request of the Competent Authorities, a working group of Member States experts was established in 2007 to evaluate a non-exhaustive list of techniques and to develop further guidance on how they should be considered in the context of the existing legislative framework. The SBB has been mandated by the federal and regional authorities to participate in this working group.

In addition to the OMM technique described above, the following techniques have been considered by the “New techniques working group”:

- *Cisgenesis*, consisting of the genetic modification of a recipient species (so far, plants) with a natural gene from a sexually compatible donor species (as compared to transgenesis where genes come from non-crossable organisms). To produce cisgenic organisms any suitable technique used for production of transgenic organisms may be used.
- *RNA-dependent DNA methylation via RNAi/siRNA*, a technique using small RNA to inhibit gene expression by methylation of the DNA. DNA methylation is one of the molecular mechanisms underlying epigenetic effects.
- *Grafting*, a technique that has been used for centuries in plant breeding in which the bud-bearing part (the scion) of a plant is grafted onto the rootbearing part (the rootstock) of another plant. Proteins and RNAs can be transported from the rootstock through the graft junction and into the scion. In the specific context of genetic modification, grafting can involve a non-GM scion onto a GM rootstock (the most common case) or a GM scion onto a non-GM rootstock.
- *Reverse Breeding*, a technique allowing plant breeders to derive homozygous parental lines from individual heterozygous plants selected for their elite qualities (non genetically modified). To obtain the homozygous parental lines, meiotic recombination is suppressed in the selected heterozygous line through RNAi-mediated downregulation of genes. One of the most important characteristics of the reverse breeding technique is that the offspring are not transgenic.
- *Agroinfiltration*, whereby plant tissues are infiltrated with *Agrobacterium* sp. containing a genetic construct in order to promote localised and transient expression of a protein. In this technique, there is no integration of the foreign genetic material into the genome. Instead, the aim is to use the ability of *Agrobacterium* to inject large numbers of copies of the foreign DNA into the plant cells where they are used to produce very high protein expression levels.
- *Synthetic Biology*, which refers to an area of biological research that combines different disciplines of science and engineering in order to design and build biological components and systems that do not already exist in the natural world, or to re-design and re-build existing biological systems (see e.g. <http://synthetic-biology.org/>).
- *Zinc Finger Nuclease (ZFN)*, a technique allowing site specific mutations to be generated in the host genome using ZFN, protein chimeras composed of a sequence specific zinc finger based DNA binding domain and a DNA cleavage domain. The genes encoding the ZFN complex are typically present and expressed transiently in the cell from an expression plasmid. In some applications of the technique, DNA fragments or gene cassettes can be inserted precisely at the level of the DNA break, thereby providing a powerful tool for targeted gene insertion.



Each technique has been evaluated in the context of the GMO/GMM definitions, the annexes of the Directives and the most recent scientific data. In this respect, familiarity with techniques already listed in the current Directives was considered. Experts also looked at the characteristics of the final product in determining whether a technique may be excluded or not (based on criteria such as transient presence of the transgenes or transient associated effect, heritability of the genetic modification, cases where offspring from a GMO no longer carry the genetic construct but continue to bear the effect). Other considerations included the extent to which the end-products of such techniques could be distinguished from products of traditional techniques. Safety issues were also taken into consideration where relevant.

In addition to and in coordination with the work carried out by the “New techniques working group”, the Joint Research Centre (JRC) of the European Commission has also been mandated to provide two reports dealing with the same issue:

- A report on the issues presented by these techniques for detection and monitoring of derived products. In this framework, an *ad hoc* Task Force on “New Techniques” has been established composed of selected members of the European Network of GMO Laboratories (ENGL). The SBB is participating in this Task Force;
- A report with regard to the socio-economic impacts of these techniques. This report will be the result of a global process including literature search, patent search, a workshop and a survey sent to companies applying these techniques. The report will, among others, reflect on the latest and future development, research and implementation of the techniques, taking into account different regulatory options (GMO or not). The SBB will also bring its expertise to this process by participating in the workshop and contributing to the literature survey.

CONCLUSIONS

The development of new techniques of genetic modification calls for greater clarity and perhaps also new interpretations of the current GMO legislation. The final decision as to whether organisms produced by a specific technique should fall under the scope of the EU regulation on GMOs is ultimately a matter of political and legal choices. These choices should be made on the basis of technical and scientific arguments. It seems obvious that societal and economic aspects might also be considered, notably given the complexity and associated costs of applying the GMO legislation for developers of novel organisms.

Moreover, without similar discussions at an international level, it is likely that the same products of emerging new techniques might be considered to be GMOs or not depending on the regulatory jurisdiction. Such discrepancies should be avoided as they would pose challenges for the international regulation of transboundary movement of GMOs.

IMPACT ON PUBLIC HEALTH

In the EU, the GMO legislation only applies to organisms obtained through certain techniques of genetic modification. Determining whether organisms produced by some new techniques should fall under the scope of this regulation is of utmost importance, as this will ultimately define whether these organisms are subject to case-by-case safety assessment.

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On state-of-the-art high biocontainment facilities in Belgium

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INTRODUCTION

The use of genetically modified organisms (GMO) and/or pathogens in laboratory facilities can have potential negative consequences for human health and the environment, if they are not correctly handled. It is thus necessary to perform a risk assessment prior to each use of these biological agents in order to take precautions to confine them and to minimise their negative effects.

For this purpose, in 1990, the European Commission adopted the Directive 90/219/CEE which is currently replaced by the Directive 2009/41/CEE (1). In Belgium, the transposition of the EU Directive was operated at the regional level as part of the environmental legislation. Consequently, all activities which are performed in a laboratory (but also in an animal housing area, greenhouse, hospital room or large scale production facility) and involving GMO or pathogenic organisms must have an authorisation delivered by the regional authorities (2-4). The Division of Biosafety and Biotechnology (SBB) acts as technical expert in this authorisation procedure and advisory body for the Regions. The presented data are based on the notification dossiers submitted for advice to the SBB.

This report presents an overview of the progress achieved on the high containment facilities in Belgium, covering the period from 1995 to 2008.

HIGH BIOCONTAINMENT FACILITIES

Laboratories, animal housing areas and production facilities are classified into four ascending levels of containment which are designated as biosafety levels 1 to 4. High

biocontainment facilities refer to the two highest biosafety levels, namely biosafety level 3 and 4 (BSL-3 and BSL-4). Since no BSL-4 is present in Belgium, this report only relates to BSL-3.

BSL-3 facilities are used for manipulating biological agents which may cause serious or potentially lethal diseases as a result of exposure by the inhalation route. In order to prevent any escape of infectious agents into the community and the environment, a BSL-3 laboratory should have special design features and protective equipment requirements, as well as advanced safety practices. The most important required measures are the following: 1. Negative air pressure relative to the immediate environment; 2. Airlock; 3. Room sealability for decontamination by fumigation; 4. Exhaust air HEPA filtered; 5. Inactivation of solid and liquid waste before evacuation.

In Belgium, since the implementation of the regulation imposing the notification of all contained uses of GMO and/or pathogenic organisms, 79 high level containment facilities have been declared.

BSL-3 can be distinguished into two categories: medical diagnostic laboratories and research laboratories. Most laboratories assigned to research and development are located in universities or pharmaceutical companies. BSL-3 dedicated to medical diagnosis are largely located in hospitals or other healthcare facilities and generally undertake the identification of airborne pathogens belonging to risk group 3 (for example *Mycobacterium tuberculosis*). We estimate that there are currently 15 BSL-3 diagnostic laboratories in operation. The activities carried out in governmental scientific institutes BSL-3 (WIV-ISP, CODA-CER-

VA) are mostly diagnostic but also include applied research.

The 8 Belgian Reference Laboratories for the diagnosis of AIDS are included in the 79 known BSL-3. In addition to BSL-3 containment measures, these laboratories must also meet very strict safety criteria defined by the royal decree of 8 October 1996 relating to AIDS Reference Laboratories (5). These laboratories are located in the following institutions: UCL Woluwe, Scientific Institute of Public Health, KUL-Universitair Ziekenhuis, ULB-Hopital Erasme, Instituut voor Tropische Geneeskunde, UGent-Universitair Ziekenhuis, ULg-CHU de Liège, and VUB-Universitair Ziekenhuis Brussel.

Geographical distribution of the high biocontainment facilities

The geographical distribution of BSL-3 is presented in Figure 1, which illustrates a rather homogeneous distribution of the high containment facilities in Belgium, except for the province of Luxembourg where no BSL-3 is established.

The high concentration of BSL-3 observed around Brussels is due to the presence of five academic hospitals (UZ University Brussel, Institut Jules Bordet, Hôpital Erasme, Cliniques Saint-Luc and CHU Saint-Pierre) as well as various universities and governmental scientific institutes (UCL, CODA-CERVA, and WIV-ISP).

A large number of the high biocontainment laboratories in the province of Walloon Brabant are explained by the presence of the big pharmaceutical company Glaxo-SmithKline Biologicals. In the province of Antwerp, where several pharmaceutical companies are established such as Jansen Pharmaceutica and Tibotec-Virco, there are also a large number of BSL-3 facilities.

In the province of Hainaut, all the high containment facilities are concentrated in the Gosselies aeropole (ULB, Henogen).

In the province of Liège, most BSL-3 are located on the site of Sart-Tilman, where the university (ULg) and its academic hospital (CHU Liège) are located.

In the provinces of Limburg, Eastern Flanders and Western Flanders, BSL-3 facilities mainly correspond to medical diagnosis laboratories. In the province of Namur, there is only one university laboratory (FUNDP-Namur), several production facilities from a pharmaceutical company (GlaxoSmithKline Biologicals) and one laboratory for diagnosis.

In summary, the 79 high containment facilities are distributed in Belgium as follows: 43 are located in the Walloon Region, 21 in the Flemish Region and 15 in the Brussels-Capital Region.

Type of installations

• Large scale containment level 3

The 11 large scale biosafety level 3 production units are all located within pharmaceutical companies. These institutions often

simultaneously perform various activities such as research and development of new products, manufacturing and production of drugs and vaccines, as well as quality control and environmental monitoring.

Large scale production that is taken into account in this report involves activities where large volumes of culture or strong concentrations of infectious agents are handled; although there is no strict definition of “large scale” activities, since the risks are more dependent on the agent, the procedures being used, and the frequency of activity.

• Laboratory animal facilities containment level 3 (ABSL-3)

Laboratory animal facilities represent a special type of laboratory because animals themselves may present unique hazards not found in standard microbiological laboratories. For example, animals may generate aerosols or shedding, may bite and scratch, or may be infected with zoonotic agents. ABSL-3 is suitable for work with animals infected by pathogenic agents of risk group 3.

Among the high containment laboratories

present in Belgium, 9 are animal facilities, which are located in the following institutions: CODA-CERVA, GlaxoSmithKline Biologicals, Jansen Pharmaceutica, KUL, Tibotec-Virco, ULg and ULB. ABSL-3 are primarily made up of housing areas for experimental animals but can also include food storage, cloakrooms, showers, autoclaves, etc.

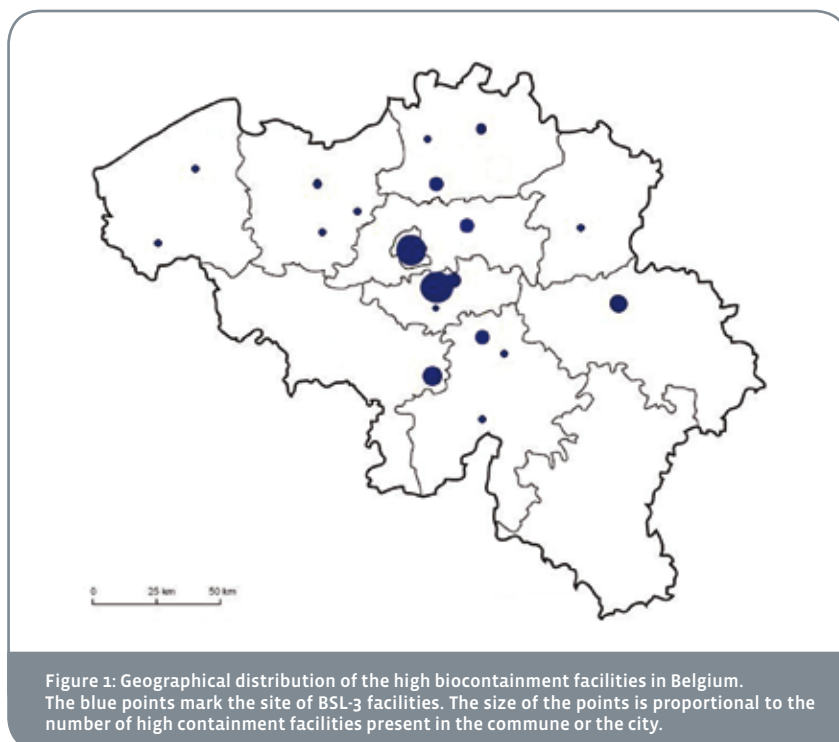
Manipulated organisms

The high containment activities may be categorised by the institutions in which they are performed: universities and governmental scientific institutes, pharmaceutical companies, hospitals and private clinics. Actually, the main differences that may be observed between these categories are related to the type of agents handled, the scale of the activity or the goal of the operation (research, diagnosis or production).

Only 10 of the high containment facilities are used to confine genetically modified micro-organisms whereas 41 of them are used for handling non-genetically modified pathogens. The 28 remaining facilities are utilised for activities that simultaneously handle GM and non-GM agents.

There is no direct correlation between the risk group of an organism and the level of containment to be adopted. A risk assessment must be carried out on a case-by-case basis in order to determine the appropriate containment measures and working practices by taking into account various factors such as the scale of the activity, the type of manipulation, the biological hazards inherent to the insert, etc. (6).

For example, the adoption of BSL-3 containment is mandatory for the production of recombinant adenoviral vectors carrying a potentially hazardous insert (oncogene, toxin, virulence gene, etc.). However, BSL-3 is not required for the same manipulation if the insert does not code for a dangerous component.



The diagnosis of tuberculosis constitutes another frequently encountered example: diagnosis activities including primary cultures of clinical specimens should be carried out under BSL-2 containment with reinforced safety equipment and level 3 working practices. On the other hand, any further manipulation involving opening of tubes containing *M. tuberculosis* positive cultures requires full BSL-3 containment (7).

History of the notifications

Since the transposition of the EU Directive into Belgian law, notifications of high containment laboratories did not simultaneously start in the 3 regions because the regional decrees did not come into effect at the same time. Indeed, the Region of Brussels-Capital transposed the Directive 90/219/CEE in December 1993, the Flemish Region in June 1995 and the Walloon Region in June 1996. Figure 2 shows the evolution of the notifications of BSL-3 contained use in Belgium since 1994.

The notifications of BSL-3 laboratories started in 1995 with two dossiers in the Region of Brussels-Capital. Although no notification for BSL-3 containment was introduced in 1996, 21 BSL-3 facilities were notified in 1997. This fact corresponds to the first notification by the company Glaxo-SmithKline Biologicals which, at that time, owned 16 BSL-3 including laboratories, animal housing and large scale facilities. Most universities introduced their dossiers from 1998 with two peaks in 2000 and 2002. In 2003 to 2004, BSL-3 notification stabilised with 8 new high containment facilities per year. Since then, the number of notifications has gradually reduced.

The evolution of notifications shows that after one period of high activity following the application of the Belgian decrees, the number of new notifications decreased appreciably. This is probably due to the fact that almost all of the high containment facilities were declared.

At the beginning of the notification process (1994 to 1998), several requests for authorisation of class 3 contained uses were rejected because of the lack of some containment criteria or even because of the absence of BSL-3 containment. The most common non-conformities which were observed were a lack of maintaining negative pressure in the area, rooms not sealable to permit fumigation, absence of sink with non-manual control, or inappropriate waste management. Except for one ABSL-3 animal facility, all of the laboratories concerned were in compliance following inspection visits and interventions of the competent authorities and the SBB.

In contrast, some activities that do not require a high level of containment are nevertheless performed in BSL-3 containment. For example, these activities correspond to the use of animal cell cultures for antibody production, the production of recombinant retroviral vectors for clinical trials

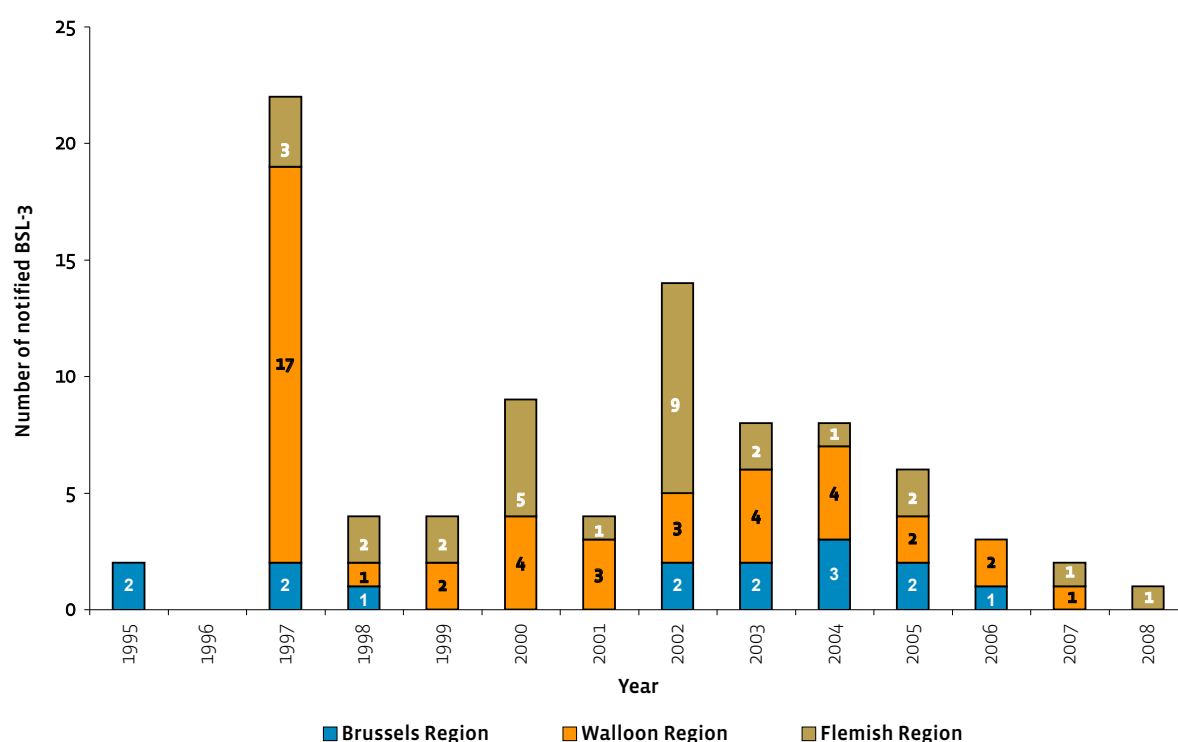


Figure 2: Number of BSL3 notifications per year and per region.

or the large scale culture of bacteria which are not pathogenic through aerosolisation (*Salmonella*). The adoption of more strict containment measures generally arises from the need to combine biosafety compliances with quality requirements (such as Good Manufacturing Practices). However, these facilities are listed in the number of BSL-3 in Belgium since they fill all required criteria for level 3 containment.

LABORATORIES FOR DETECTION OF BOVINE SPONGIFORM ENCEPHALOPATHY'S AGENTS (BSE)

Some high containment laboratories were not included in the 79 BSL-3 facilities previously mentioned. They correspond to laboratories performing BSE rapid test. These laboratories constitute a particular category because they do not have to meet all of the criteria of a conventional BSL-3 laboratory.

The agents responsible for BSE are prions or unconventional agents of risk group 3 for humans and animals. The diagnosis of prions by rapid test requires BSL-3 containment except for three criteria: the negative air pressure relative to immediate surroundings, the HEPA filtering of exhaust air and the room sealability for decontamination by a gaseous substance. However, special attention has to be paid to working practices and waste management because prions are very resistant to all traditional inactivation methods.

In Belgium, 25 laboratories for rapid detection of BSE are in operation.

CONCLUSION

Biosafety Level 3 containment facilities are used to handle airborne agents that may cause serious and potentially lethal infection and require special facility design, practices and safety equipment that protect laboratory workers, the environment, and the public from harmful exposure. This report reveals a high number of BSL-3 facilities present in Belgium, homogeneously located in the 3 regions. The evolution of BSL-3 contained use notifications indicates that almost all high biocontainment facilities are declared, authorised and controlled.

IMPACT ON PUBLIC HEALTH

BSL-3 containment is designed to minimise risks and provide secure conditions to protect workers, the public and the environment from exposure to infectious micro-organisms that are handled in the laboratory.

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Using a pharmacokinetic program improves the results of the Belgian antibiotics EQAS

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INTRODUCTION

The Belgian external quality assessment scheme (EQAS) for Therapeutic Drug Monitoring (TDM) has existed since 1991 for Digoxin and Theophyllin. Several parameters have been added over recent years. It currently comprises 12 parameters and is performed 4 times per year. The participation of Belgian laboratories is mandatory and integrated within the laboratory license. The EQAS' main objective is to evaluate the performance of laboratories on their ability to approach their peer group's median value, taking into account the overall variability. Another objective is to monitor the overall quality of particular measurement methods.

The recent use of case reports where laboratories are asked to interpret analytical results led us to conclude that many laboratories encounter problems interpreting results especially when unconventional blood collection intervals were used. Hence, the Belgian Health Authorities decided to supply laboratories with pharmacokinetic software, free of charge, to improve TDM.

MATERIALS AND METHODS

For each survey, carried out four times per year, participating laboratories receive lyophilised or liquid samples. The laboratories are surveyed for methodology and are asked to report the concentration of the drugs amikacin, carbamazepine, digoxin, gentamicin, lithium, paracetamol, phenobarbital, phenytoin, salicylate, theophyllin, valproic acid and vancomycin.

The same sample material and evaluation system are used for all laboratories. This assessment scheme, as well as other

schemes from the Belgian Health authorities, is used to monitor the performance of laboratories. The purpose of these EQAS is to be educational and there is no penalty associated with normal, non-repetitive errors.

After sending samples, laboratories have 15 days to submit their results either by returning the reply form or via a dedicated website (1). The data are statistically processed with the non-parametrical method of Tukey (2) and a draft report is written. After presentation and discussion with a committee of experts, the necessary corrections are made to the report. The final report is then sent to the participating laboratories (3). In addition to the survey reports, once a year, a special annual report (4) is written and sent to the participants, together with their individual annual overall performance.

In the global reports, for each survey and for each parameter, a table summarises the results obtained by the participating laboratories. They contain for each sample the following information:

- The median, standard deviation, CV and number of laboratories for each method (peer group);
- Fields that show the global results for all methods and all measuring systems combined (5, 6);
- Besides the results tables, a graphical representation (a "box and whisker" plot) is added.

From 2001, in addition to the analytical results, an interpretation for some parameters such as antibiotics has been added to the questionnaire.

The clinical interpretation for amikacin (aminoglycoside active against nosocomial Gram-negative infections but nephro-

and oto-toxic) was the first antibiotic to be introduced. Requests for interpreting vancomycin (glycopeptide active against Gram-positive infections but also nephro-toxic) began in 2004. From 2008, interpretations were also requested for gentamicin (aminoside used against Gram-negative and Gram-positive bacilli and methicillin sensitive staphylococci but nephro- and oto-toxic).

For the purposes of illustration, the following case studies for amikacin and for gentamicin were proposed in the 2009 EQAS.

Case 1

EQAS 2009/1 (February 2009): a man is treated with 1000 mg of amikacin on a "once a day" schedule. Two blood samples were collected to monitor the treatment: the first sample was taken 2h 25m and the second sample 6h 30m after the drug administration. The concentration of the first sample was the one used as quality control (= EQAS sample). The second sample contained a serum concentration of amikacin of 17.3 µmol/L.

Case 2

EQAS 2009/4 (November 2009): a 29 year old woman (55 kg) suffering from PID (Pelvic Inflammatory Disease) is treated with a dose of 240 mg gentamicin/24h (once daily). The intravenous perfusion was administered from 13:00 to 14:00. Blood was collected at 12:55 and at 20:30.

The concentration of the peak value is 0.4 µmol/L. The second value was the EQAS sample.

RESULTS

The analysis of the Belgian EQAS results for antibiotics show that many laboratories encounter difficulties in some of these surveys, specifically with the interpretation of the proposed case reports and mainly when interpretations of unconventional blood collection intervals are requested. In 2009, four surveys were conducted: in February, May, September and November. The two following results illustrate these interpretation difficulties.

Amikacin

105 laboratories participated in these surveys for amikacin in 2009.

Four lyophilised control samples were used, ranging from 23.77 to 49.76 µmol/L (medians for all methods and all measuring systems). As is often the case, the analytical results for this antibiotic were not a problem in 2009: the maximum observed CV for one measuring system was 8.1% for a median concentration of 47.7 µmol/L.

As shown in Table 1, for instance for the EQAS 2009/1 in February, analytical performance is satisfactory with a global CV at 6.4%.

On the other hand, many laboratories encountered problems interpreting the results for this EQA because a case report with unconventional sampling times was used (= case 1).

Despite the fact that guidelines for aminoglycosides monitoring have been published several years ago, the differences in interpretations are probably due to poor in-depth knowledge of clinical pharmacokinetics.

67.6% of participants regarded the results as being non-interpretable (*Table 1*).

In this case, the extrapolated peak value at time 0 will be around 85 µmol/L and the trough value far below 4.5 µmol/L. The final interpretation will depend on the target values recommended in the hospitals but could correspond to an appropriate treatment.

Gentamicin

The range of the 4 gentamicin control samples used in the 2009 EQAS lies between 9.2 and 18.9 µmol/L (medians for all methods and measuring systems).

The analytical results were, as usual, acceptable to satisfactory.

As a further illustration, the results for the lyophilised control sample used in the 2009/4 survey (November 2009) are shown in Table 2.

In addition to the analytical results, a case study for this aminoglycoside was proposed to the 49 participating laboratories (= case 2). 71.4% of the participants responded that the case was non-interpretable (*Table 2*). However, sufficient information was provided to allow calculation. The concentration found in the second sample is 9.4 µmol/L (*Table 2*) and may indeed appear weak.

Many other cases have shown the same difficulties in interpretation.

Table 1: Analytical results (lyophilised sample) and clinical interpretation of the 2009/1 Belgian EQAS amikacin case study (unconventional sampling time).

AMIKACIN Conversion factor: µmol/L / 1.71 = µg/mL		R/7534			
METHOD	Median µmol/L	SD µmol/L	CV %	Median µg/mL	N labs
001 Non-Isotopic Abbott TDx	49.42	2.54	5.1	28.90	26
003 Non-Isotopic Roche Integra	47.97	2.74	5.7	28.05	32
005 Non-Isotopic Roche Hit / Mod / cobas c	50.36	1.98	3.9	29.45	32
006 Non-Isotopic Syva Emit	44.76	3.45	7.7	26.17	12
011 Non-Isotopic Abbott – Architect/Aeroset	54.21 51.90				2
050 Home made	50.45				1
Global results (all methods and all measuring systems)	49.76	3.17	6.4	29.10	105
Interpretation	N	Median (µmol/L)		pct	
Inappropriate sampling/ result not interpretable	71	49.59		67.6%	
Appropriate treatment	17	49.76		16.2%	
Subtherapeutic	11	49.76		10.5%	
Toxic	3	51.47		2.9%	
No answer	3	45.83		2.9%	
Total	105				

Table 2: Analytical results and clinical interpretation of the 2009/4 EQAS gentamicin case study (unconventional sampling time).

GENTAMICIN Conversion factor : $\mu\text{mol/L} / 2.09 = \mu\text{g/mL}$		R/9243			
METHOD	Median $\mu\text{mol/L}$	SD $\mu\text{mol/L}$	CV %	Median $\mu\text{g/mL}$	N labs
021 Abbott - AxSym/TDx/TDxFlex - Fluorescence polarisation immunoassay	10.0	0.6	6.3	4.8	18
031 Coulter - Synchron LX/DxC - Particle enhanced immunoturbidimetry	11.9 9.6				2
040 Siemens - Dimension/ Vista - PETINIA	9.9	0.5	4.7	4.8	6
072 Roche - Cobas Integra - Fluorescence polarisation immunoassay	8.8	0.5	5.6	4.2	13
074 Roche - Hitachi/ Modular./ cobas c – KIMS immunoassay	8.6	1.2	13.5	4.1	8
084 Siemens Advia Centaur/CP - Immunochemiluminescence	9.2				1
090 Vitros - Enzyme immunoassay - spectrometry	10.0				1
Global results (all methods and all measuring systems)	9.4	0.9	9.9	4.5	49
Interpretation	N	Median ($\mu\text{mol/L}$)		pct	
Inappropriate sampling/ result not interpretable	35	9.4		71.4%	
Appropriate treatment	7	10.0		14.3%	
Subtherapeutic	4	8.7		8.2%	
No answer	3	10.0		6.1%	
Total	49				

However the interpretation is quite simple with the use of a pharmacokinetic program, even when the blood collection intervals are uncommon like in these 2 examples. Such a computer program, Pharmonitor, has been developed in St. Luc Hospital (Brussels, Belgium) to optimise aminoglycosides monitoring, responding to the demands of most daily clinical situations (7).

For example, for case 2, the Pharmonitor software shows a peak value of $44.66 \mu\text{mol/L}$ and a trough value of $0.42 \mu\text{mol/L}$. The clinical pharmacokinetic parameters ($t_{1/2}$, V_d , CL , etc.) appear to be normal. The proposed scheme can therefore be considered suitable for treatment. Only 15% of participants (without the use of software) considered the treatment proposed in this clinical case as appropriate and interpretable.

CONCLUSION

Therapeutic Drug Monitoring is an important field of clinical biology. The clinician expects not only a numerical result from the laboratory but also correct clinical interpretation in order to provide adequate treatment to patients adapted to individual pharmacological characteristics. For many years, TDM has been applied to monitor antibiotics, anti-epileptic and anti-arrhythmic treatments as well as those intended to treat cardiac decompensation. TDM has also been introduced for the monitoring of immunosuppressive drugs and more recently antiretroviral drugs. Many studies have emphasised the importance of the quality of the pharmacokinetic interpretation during the monitoring (8-11). A good interpretation results in an individualised regimen that improves the effectiveness of the treatment and reduces the incidence of toxic episodes, the duration of treatment and overall hospital costs (12).

EQAS monitors the overall quality of the laboratories' performance. Post-analytical error may be monitored by using case reports where laboratories are asked to interpret analytical results.

The examples, taken from the 2009 TDM Belgian EQAS and depicted above, show the usefulness of an interpretation program such as the Pharmonitor in answering correctly these case reports.

The difficulty of the first case report, for example amikacin (case 1), lies in the fact that the nursing staff failed to perform blood tests at regular sampling times (e.g. before the intravenous perfusion and one hour after the end of perfusion). In the second case report, for example (case 2 – gentamicin), the time of the second blood sample is very unusual (20:30 instead of 15:00, for example). Various reasons may explain this scheme: forgetfulness, overworked nursing staff, etc.

These examples clearly show that computerised approach to monitor antibiotics is mandatory.

Software for the interpretation of TDM exists in Europe and in the United States but these programs are expensive.

The Belgian Health Authorities decided to finance and support the development of an improved version of the former Pharmonitor and to supply the laboratories with this program free of charge (to be used for 4 aminoglycosides – amikacin, gentamicin, tobramycin, netilmycin- and 2 glycopeptides – vancomycin, teicoplanin) (13).

This software, based on a one-compartment open pharmacokinetics model, can calculate $t_{1/2}$, V_d , Cl , C_{pmax} and the theoretical optimal dose and interval. It can also evaluate the creatinine clearance.

In 2009, the Belgian Health Authorities organised several training sessions for the Belgian clinical laboratories and a free of charge copy of the pharmacokinetic software was distributed to the participants.

Further improvement and development of the program is planned such as adding a (blood) alcohol rate calculator and using formulas based on population pharmacokinetics and Bayesian estimation.

IMPACT ON PUBLIC HEALTH

Even when analytical performance is good, as shown by the Belgian EQAS, many laboratories encounter problems in clinical interpretation. Results illustrate the added value of the use of dedicated software to improve pharmacokinetic interpretation and to allow optimised dosage regimen avoiding unnecessary blood sampling and additional testing.

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