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Editorial

In this issue, two articles are devoted to the new French regulations on MTs, microorganisms and toxins which may be used for purposes of bioterrorism or agroterrorism. The first article has been published in the *Focus* section and presents the general rules and principles for implementing these new regulations in ANSES's laboratories in France; the second article, published in the *Methods* section, is a practical guide for setting up these new biosafety and security requirements. It is possible, perhaps even probable, that other European countries will adopt similar regulations, and so we felt that these guides might be a way to share experience that may be useful to all.

Also on the topic of new regulations, another article in the *Focus* section comments on the amendment of Regulation (EC) No. 882/2004 on the organisation of official controls in EU Member States.

So alongside articles on methodology and research (screening for neonicotinoids in nectar; modelling *Listeria monocytogenes* contamination; monophasic *Salmonella* strains), this issue also focuses extensively on French and European regulatory issues.

And to conclude, the *Point of view* section provides a discussion of the role of reference laboratories in surveillance, in an article written from a European point of view.

We hope you enjoy reading further.

The editorial team

Summary

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Point of view

What role do NRLs and NRCs play in disease surveillance?

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The core functions of NRLs and NRCs

In their technical report for 2010 (ECDC, 2010) "Core functions of microbiology reference laboratories for communicable diseases", the authors provide a clear and concise definition for five types of activity, the "core-functions", for National Reference Laboratories and Centres in Europe:

- function 1: Reference diagnostics;
- function 2: Reference material resources;
- function 3: Scientific advice;
- function 4: Collaboration and research;
- function 5: Monitoring, alert and response.

The document is the result of discussions between representatives of National Microbiology Focal Points (NMFPs) in the different countries of the EU. It is designed to encourage cooperation between "experts" and reference laboratories and to serve as a core document for future discussions concerning the European system of reference laboratories. "National Reference Laboratory (NRL)" and "National Reference Centre (NRC)" are both commonly-used terms. However, usage is often country-specific and different interpretations exist. To avoid confusion and to ensure that the report establishes a common reference point, we shall here use the term "microbiology reference laboratory" in this context (see ECDC, 2010).

Here we will discuss *Core Function 5*, which we see as an essential aspect of the work of microbiology reference laboratories: monitoring, alert and response. These are the activities on which hinge the interactions between a microbiology reference laboratory and the body in charge of epidemiological disease surveillance at national level (or regional level depending on the degree of decentralisation of this responsibility in each country).

The goals of Function 5 can be summarised as follows for a given pathogen:

1 – to measure at specific intervals (yearly, half yearly, monthly, etc.) spatio-temporal changes in the presence and number of identifications of the pathogen and its key characteristics (resistance to antibiotics, antivirals and antiparasitics, new serotypes, etc.);

2 – to alert the public health authorities of any unusual or unexpected event concerning this pathogen: appearance of any new resistance to antibiotics, emergence of a new serotype, shift in serotype, new virulence factor, unusual cluster of cases, etc.;

3 – in the event of an outbreak or a real epidemic or epizootic, to participate actively, in close collaboration with the body responsible for epidemiological surveillance of this disease, in documenting isolates of the implicated pathogens, in order to confirm that outbreak cases have a single aetiology and if necessary to differentiate them from endemic cases, to monitor any possible microbiological changes (for example the acquisition of resistance to antivirals, antibiotics, etc.) and especially to characterise them with sufficient precision to enable the source of the outbreak to be identified with certainty. This last point is especially important in the case of foodborne human illnesses, for which it is essential to identify the source of the outbreak in order to implement the appropriate public health measures. This last aspect, which is particularly important in terms of public health, is in fact intensely operational. It therefore requires a sound working relationship and mutual confidence, often on a daily basis, between the microbiology reference laboratory (or laboratories if several are involved, sometimes reporting to different ministries such as those responsible for health, agriculture, the environment, etc.) and the body responsible for epidemiological surveillance. The participation of microbiology reference laboratories in epidemiological investigations (which in France, for example, is inscribed in the mission of the NRCs) is one way of developing a common approach to this work.

The interactions characterising the relationships between the two types of investigator concerned with epidemiological disease surveillance – microbiologists and epidemiologists – are ***regular*** (for spatio-temporal tendencies, the adoption of new laboratory techniques or epidemiological methods, etc.), ***intense*** (during health emergencies, outbreaks, etc.) and ***organised*** (in order to have a clear view of the role of each participant, particularly during investigations of outbreaks). A sound relationship between these two types of partner with scientific cultural backgrounds that are different but necessarily complementary facilitates and vastly improves the results achieved in terms of public health.

Molecular diagnostics, a challenge to the role of microbiology reference laboratories in the monitoring of the way strains circulate

There can no longer be any doubt about the importance of molecular epidemiology in the activities of microbiology reference laboratories, whether for finding the source of contamination and the incriminated foodstuff in foodborne illnesses or, in animal or human health, for determining the origin of the clone of a pathogen implicated in a nosocomial infection, for finding the source of an emerging viral disease in Europe, or for determining the virulence of a given population of pathogenic bacteria. In all such cases, it is essential for microbiologists to work side-by-side with epidemiologists.

For microbiology reference laboratories, molecular diagnostics, especially if performed as a first-line response, which is the case increasingly often, will become a considerable challenge in the future. For most pathogens, molecular diagnostics seems bound to replace traditional methods involving the culture and isolation of strains of bacteria, viruses and fungi, thus bringing about a considerable change in the range of tools available to us for characterising the phenotype and genotype of pathogen isolates, while also progressively reducing the nature and memory of our collections and limiting the possibilities for retrospective historical analysis. This is important not only on an epidemiological and clinical level but also more fundamentally, especially as it will limit the possibility of studying the evolution of pathogens.

However, this is not particularly new. For several years now we have been faced with pathogenic microorganisms that

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were impossible or difficult to cultivate. The hepatitis viruses are an example of this, and especially hepatitis E virus (HEV). For this latter case, which cannot be cultivated routinely, microbiologists have nonetheless developed a comprehensive system of diagnosis and molecular typing (Baylis, 2011) performed directly on biological samples supplied by clinics (faeces, serum) or even from water samples. Targeted PCR followed by sequencing of the amplified strand enables the virus to be classified in one of four described genotypes, and then subtyped and located in the phylogenetic tree of HEVs. The same type of approach can now be extended to other genera of virus, irrespective of whether they are difficult to cultivate (Kroneman, 2011; Ren, 2013).

Molecular typing has also often been used on bacteria in place of traditional serotyping, which can be long and laborious (Doumith, 2004). Although certain techniques of molecular typing can theoretically be used on bacteria without requiring the traditional bacterial culture phase, at least when a sample is potentially mono-microbial, such as Variable Number Tandem Repeat (VNTR) typing, Single Locus Sequence Typing (SLST), typing the gene of the A protein of *Staphylococcus aureus*, or even Multi-Locus Sequence Typing (MLST), in practice techniques for the molecular typing of bacteria are carried out after traditional cultivation and isolation. This is the case of the most widely used typing techniques such as MLST and VNTR typing, and of course macro-restriction of DNA by pulsed-field gel electrophoresis (PFGE) for which considerable quantities of DNA are necessary. Easier access to whole sequences of bacterial genomes (Whole Genome Sequence, WGS) or viral genomes for molecular epidemiology by Next Generation Sequencing (NGS) will provide information of such quality and quantity, of use to both epidemiologists and physicians specialising in infectious diseases, that we may very well see these techniques becoming mainstream in the not too distant future. The knowledge that Whole Gene Sequencing will bring to the virulome, the "toxome" (the full set of all genes encoding toxins) and the resistome (Wright, 2007) of one or more clinical isolates could be essential for providing the patient with appropriate care, and also for decision-making in matters of public health. In addition, Whole Gene Sequencing, which currently requires DNA obtained from a pure culture, could also be performed, at least theoretically, by Whole Genome Amplification (WGA) based on Multiple Displacement Amplification (MDA) using DNA-polymerase of the phage Phi29 and random primers (Lasken, 2003). WGA kits are already on the market and can be used to obtain between 40 and 50 µg of DNA after reaction from 10 ng of DNA, which is enough from which to obtain a whole sequence. The method has also been adapted to enable the detection and amplification of very small quantities of DNA in pathological samples, such as for bacteria of the species *Chlamydia trachomatis* (Seth-Smith, 2013).

When molecular diagnostics is carried out in clinical microbiology laboratories it cannot be done in a single step: before the actual amplification phase, the phases involving the dilution of potential inhibitors and the concentration of DNA and RNA also provide essential sources of biological matter. In fact, only a few µL are generally used for diagnostic PCR, the remainder being stored for at least a few weeks and used at the request of reference laboratories to characterise the genotype or for molecular epidemiology, or alternatively for research purposes.

Lastly, the TYPENED experiment in the Netherlands (Niesters, 2013) provides another response to this challenge as a way of encouraging clinical microbiologists and infection specialists to take an interest in data from molecular epidemiology. The concept exploits a shared database which compiles clinical, microbiological (sequences) and epidemiological data. All participating laboratories, whether clinical or reference, have access to all the data in the base, thus allowing real-time comparison between the data obtained by a diagnostics laboratory and those obtained by other laboratories at the same period for example, or having the same clinical expression, the same therapeutic response, etc. Clinicians, public health epidemiologists and microbiologists from reference laboratories thus all benefit.

The outlook for the development of these systems seems very promising, as they open the door to real improvements in the monitoring of infectious diseases at a global level, both for clinicians specialising in infectious diseases and for microbiologists, epidemiologists and risk managers. With or without the traditional pathogen cultivation stage and after a few technical improvements in instrumentation, it will be possible to obtain complete sequences for each pathogen implicated in a disease at reasonable cost. Apart from the improved therapies that molecular microbiology will provide, we will achieve faster real-time integration of all the available information on the patient or patients, the pathogens and the epidemiological data. After all, molecular data can be transmitted and exchanged with incomparably greater ease than the isolates of bacterial, viral, fungal or parasitic pathogens. As long as these data are shared, we have an opportunity to create a global system of interlinked databases for the genetic characterisation of microorganisms isolated from patients, both human and animal, and the potential sources of contamination (hospital samples, foods, drinking water, etc.). Such integrated monitoring (Aarestrup, 2012) will enable public health authorities to provide better-coordinated responses, including across borders when necessary, which are also better adapted to real threats to public health.

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Focus

Implementation of the new French regulations on microorganisms and toxins: ANSES's experience

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The new French regulatory measures for operations involving microorganisms and toxins (MTs) are making lasting changes to the sector of microbiology laboratories. This new regulatory framework reinforces the control measures in this area to improve biological safety and security. In practice, it results in increased administrative and operating requirements that call for greater vigilance on the part of operators. To fulfil these new requirements, the French Agency for Food, Environmental and Occupational Health & Safety (ANSES) has implemented an in-house methodology for risk assessment, taking into account the specificities of its reference and research laboratories.

Introduction

The French Public Health Code in Chapter IX, Article L.5139-1 defines microorganisms and toxins (MTs) as biological agents that are pathogenic to humans and toxins whose use may involve a risk for public health, as well as products that contain these agents. In practical terms, these MTs are likely to pose a real public health risk in the event of accidental exposure (*biosafety*) or intentional exposure (*biosecurity*) outside their containment area. The list of microorganisms and toxins is determined by the Minister of Health. The amendment of Article L.5139-2 of the Public Health Code resulted in the publication of Decree No. 2010-736 of 30 June 2010 concerning MTs, which came into effect on 1 July 2012. The new regulatory framework, which includes seven implementation orders and one decision, is applicable to all French laboratories involved in any operation using MTs for diagnostic, research, development or teaching purposes.

The regulations primarily aim to protect workers, the environment and the population from accidental or intentional dissemination of a hazardous biological agent by establishing appropriate rules for safety and security to effectively reduce risks for public health. Control of the appropriate implementation of these rules is the responsibility of the French National Agency for Medicines and Health Products Safety (ANSM), which issues authorisations, and administers and monitors all operations involving MTs, including production, manufacture, transport, import, export, retention, supply, sale, purchase and use.

An exemption regime has been established for:

- certain proprietary medicinal products and investigational medicinal products containing MTs that have been inactivated or attenuated, ensuring a satisfactory safety level for public health;
- reagents intended for analyses in the veterinary and plant protection fields;
- operations carried out by establishments that receive biological samples purely for analysis (with a storage duration of less than 30 days);
- operations carried out by the establishments within the Ministry of Defence (except operations for import and export).

Another exception to these regulations is worth mentioning, even though the general notion of all or part of an MT remains valid. It involves fragments of genetic material (DNA or RNA) that are no longer considered part of MTs if they are less than 500 nucleotides in length.

Concerning toxins, protein toxin fragments containing fewer than 167 amino acids are also excluded from the regulations.

To demonstrate compliance with the requirements of the Decree of 30 June 2010, the authorisation application dossier is now made up of two separate parts:

- a technical dossier intended to describe in detail the facilities, procedures, and safety and security systems implemented by the laboratory to ensure protection of its personnel, the population, and the environment. In this dossier, the applicant must justify the utility of using MTs;
- a risk assessment concerning safety and security taking into account existing protection measures.

The ANSES reference and research laboratories are particularly affected by these new measures since they are called on to work with all types of MTs, including bacteria, viruses, proteins, DNA, and toxins. As a result, to respond to the new requirements, ANSES quickly set up an action plan to avoid some of its activities being called into question. Faced with the relative complexity of the new regulations and the wide range of MTs it studied and used, the Agency needed to set up an in-house working group with the task of analysing the regulatory requirements, proposing a joint method to harmonise the applications and to help and support the ANSES laboratories through the procedure. The Committee for control of biological risks in the laboratory (CMRBL) was therefore established and included the expertise needed to fulfil the requirements of the new regulations. In particular, the committee worked on the basis of the methodology for risk assessment provided by the ANSM, and proposed to ANSES laboratories a methodological guide for risk assessment adapted to the issues specific to research and reference activities. We felt that it could be useful to make this methodology accessible to laboratories that have the same specificities as those within ANSES. In addition, the CMRBL was to play the role of sole contact for forwarding questions to ANSM from the various laboratories within ANSES, which enabled constructive exchanges to be set up with ANSM and helped to find answers to most of the questions posed.

The ANSES methodology

Of the eleven laboratories within ANSES, six work with microorganisms and toxins. These laboratories are located in various regions in France and, depending on the laboratory, have level 2 or 3 containment facilities and/or animal housing. Since

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June 2012, 13 renewal applications and three authorisation applications have been submitted to ANSM.
The CMRBL is made up of 14 members of staff with complementary expertise: laboratory head, scientist, engineer, technician, quality manager, biosafety manager, head of animal housing, health and safety officer, and security-defence representative. Two committee members participated in a three-day training course on risk assessment, based on the Failure Mode Effects and Criticality Analysis (FMECA) method.
Given the significant delay in the schedule for publication of implementation orders related to the MT regulation, the CMRBL was only able to start its work at the beginning of 2012, leaving the laboratories with very little time to finalise their applications, with the deadline stipulated in the 2010 Decree of 30 June 2012.
To begin with, the CMRBL analysed the technical dossier and asked ANSM for clarification on points that seemed unclear. ANSM always replied clearly to each question, by email or by telephone, and indicated as a general rule that the applicant can give a wide range of responses provided that they are well substantiated. The main answers provided to ANSES's questions are shown in **Table 1**. Further to this analysis, the CMRBL issued a template of the technical dossier for ANSES laboratories, along with an explanatory text and suggested responses.
The second phase involved the development of a "Methodological guide for risk assessments concerning biological safety and security" [http://www.ansespro.fr/euroreference/], drawing on the model proposed by ANSM ("Risk management method in biological safety and security", version dated 3 May 2011), available on request. However, this model proved to be relatively unsuitable for the issues faced by ANSES laboratories, both in terms of description and semantics. As a result, the hazard identification questionnaires were adapted to ANSES specificities (reference and research). The rating scales for risks related to biological safety and security initially proposed were amended qualitatively and quantitatively. The limits defining "low", "average" and "unacceptable" risk levels were also changed. Concerning the biological safety aspect, the methodology for calculating risk was completely revised, with introduction of the concept of extrinsic severity and a change in calculation of the criticality index. These calculation methods were tested in several ANSES laboratories and then adjusted, before being adopted by the CMRBL.
Furthermore, ANSES chose to integrate the biological risk management system into its overall risk management policy, and then to apply the policy depending on the specificities of each entity.

Implementation of the regulation: impact on the laboratories

Personnel training

The Ministerial Order of 17 March 2011 defines a minimum level of competence and qualifications required for the authorisation holder, and for the persons whom he/she duly authorises. In addition, the requirements of the Ministerial Order of 23 January 2013 are very clear concerning authorisations, and initial and continuing training of personnel before they can be granted access to facilities and MTs. Clearly, each laboratory will need to implement an individual training plan, suitable for each activity. Certain universities or private organisations already offer specific training programmes on biological risks, which can be adapted to the area of MTs. It is interesting to note that a working group, sponsored by the French Society for Microbiology, is working on the development of a national reference standard on training concerning biological risks to harmonise knowledge and practices, and to provide a formal framework so that personnel do not need to start training again, if they change laboratories. In effect, these training and authorisation requirements for personnel working on MTs exclude short-duration interns from working on projects involving all or part of a microorganism or toxin. This could have significant consequences for some research laboratories.

Facilities, equipment and materials

The design and use of facilities and equipment are based on the process of risk management, which involves a number of requirements in terms of resources that have to be provided for in the budget, before working on MTs. The operating capacity of the facilities must be documented in normal and limit conditions, depending on the volume of activity of the laboratory, in order to avoid any overuse. Moreover, operations intended for the validation, qualification, maintenance and monitoring of safety and security equipment will account for a large proportion of the running costs of a laboratory. "Older" laboratories should expect to incur significant costs to upgrade their facilities.

Subcontracting

Faced with such constraints, some laboratories may be tempted to outsource certain tasks. Here again, the regulations define very clearly the roles and responsibilities of each party, and require contracts to be established for all operations related to study or use of MTs. In this way, the responsibility of the client is clearly emphasised.

Document management

As in any quality system, document management should enable tracking of all operations carried out and secure storage of documentation certifying implementation of biological safety and security measures. All of these documents must be made available, requiring implementation of a specific document management system.

Specific requirements

The Ministerial Order of 23 January 2013 related to good practice rules to ensure biological safety and security defines "specific requirements" in Chapter 7 concerning the use of vertebrates and invertebrates (arthropods) exposed to MTs and genetically modified MTs. These requirements are additional and without prejudice to the regulations concerning animal experimentation (Decree of 1 February 2013 and corresponding orders) and genetically modified microorganisms (GMMs) (Directive 2009/41/EC of the European Parliament).
For animal testing facilities, these requirements now include new constraints that were previously not mandatory. For example, vertebrates must have individual and lasting marking in order to ensure their traceability. This requirement is not difficult or expensive to fulfil for medium or large sized animals such as lagomorphs, dogs, cats, primates, and production livestock, etc. which are already identified individually before they enter animal testing facilities (Articles L.212 and R.214 of the Rural Code).
However, for small laboratory rodents such as mice and rats, it is more complex to identify animals individually and this involves significant additional costs depending on the

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technique used (tattooing, banding, or electronic chips). The most simple and above all safest identification method for small animals is subcutaneous implant of electronic transponders. This technology does however have some disadvantages: 1) it cannot be used systematically because of changes to the immune system related to a local inflammatory response at the transponder's implant site that could interfere with experimental findings; 2) its cost can reach €3 to €4 before tax per animal, depending on the size and quality of the transponder, for an animal that has a commercial value of €2 to €3 before tax (for instance in the case of OFI or Swiss mice). A biological safety risk assessment, depending on the type of MT and the specific animal model, will help in selecting the most suitable technique for individual identification.

Concerning arthropods, the regulations require that a biological safety risk assessment be performed before MTs are used in invertebrates to avoid dissemination of arthropods outside the chosen containment systems. This risk assessment should take account of whether the arthropods can fly, e.g. mosquitoes, or not, e.g. fleas, lice or ticks. Additional precautions must also be taken to avoid manipulation of free arthropods or those attached to vertebrates in class I or II biosafety cabinets. There are two main types of precautions: 1) protection of personnel with personal protective equipment that must cover the skin entirely to avoid a risk of bites by arthropods; 2) installation of a cold airlock or sticky mats in front of exit doors in facilities housing arthropods, to prevent the risk of insects escaping to the outside. Finally, the regulations require systematic careful counting of all individuals before and after manipulation, with all the constraints in terms of working time that this implies.

It should be noted that during development of the risk assessment methodology prepared by the CMRBL within ANSES, these specific points concerning animal testing were integrated both in terms of biological safety and biological security.

Emergency plans and restricted access areas

Importantly, laboratories will be required to implement an internal emergency plan to address any situations that may endanger its personnel, the public, or the environment. This emergency plan includes a clear description of the internal alert circuits and the information exchanges with external emergency services and administrative authorities. It must also include periodic simulation exercises. To develop this plan, the laboratory will necessarily need to work with external services (local authorities, fire-fighters, paramedics, police, etc.). Finally, in addition to these safety measures, laboratories will also have to implement security measures aimed at limiting the risk of malicious use of microorganisms and toxins. To avoid weighing down the system, these measures will need to comply with the requirements of the Decree of 2 November 2011 regarding the protection of the scientific and technical potential of the nation, which requires the creation of restricted access areas (ZRRs) for material and immaterial assets with dual use, that could be misappropriated or diverted.

Furthermore, a specific intervention plan must be implemented for the microorganisms and toxins included in Annex I of the Ministerial Order of 30 April 2012. This plan defines the assistance measures implemented and the way in which they are managed in the event of an accident with consequences that extend beyond the installation at risk. This includes the arming, alert and intervention phases, but also the emergency services exercises carried out periodically to ensure adoption of the system. The specific intervention plan is part of the system for the organisation of emergency services (ORSEC) in each *Département*.

Conclusion

Although these regulations are clearly part of the movement to protect public health that is gradually being implemented at the European level, it is also true that the administrative burden of this regulatory framework, and the significant time constraints imposed by the public authorities, have led to difficulties in implementation for certain laboratories. Moreover, implementation of the new regulations leads to a disparity between the laboratories that work with MTs and level 3 containment laboratories that do not work with MTs, since the latter are not subject to systematic control or inspections to verify the implementation of the Ministerial Order of 16 July 2007 stipulating the preventive measures required for workers who may be exposed to pathogenic biological agents. As a reminder, this order concerns the recommendations that are to be implemented in a laboratory to ensure compliance with biological safety requirements, and to a lesser extent biological security measures. It is therefore surprising that laboratories handling class 3 agents, though they are not MTs, are not subject to controls. On the contrary, laboratories working with MTs, whether in class 2 or 3, are subject to very strict regulatory constraints. For some laboratories, MT regulations will overlap with ZRR regulations, or even with those concerning sectors of vital importance, and those indicated in the Defence Code concerning toxins which are considered chemical products included in **Table 1** of the Chemical Weapons Convention (CWC), and those on dual-use items (Regulation (EU) No 388/2012 of 19 April 2012). Finally, even though the set of constraints imposed by MTs enabled some clarification for the actors involved in the MT area, the withdrawal of certain laboratories from such activities could lead to gaps in the health network in France for microorganisms that are highly regulated in the laboratory, but present in the natural environment in the country (ultra-resistant *Mycobacterium tuberculosis* in hospitals, *Francisella tularensis* regularly isolated in wildlife, etc.).

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Arrêté du 17 mars 2011 relatif aux compétences et qualifications dont le titulaire de l'autorisation mentionnée à l'article R. 5139-1 du code de

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la santé publique justifie pour lui-même ainsi que pour les personnes qu'il habilite pour contribuer sous sa responsabilité aux opérations faisant l'objet de cette autorisation. [http://www.legifrance.gouv.fr/affichTexte.do?cidTexte=JORFTEXT000023776935&dateTexte=&categorieLien=id]

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Arrêté du 30 avril 2012 fixant la liste des micro-organismes et toxines prévue à l'article R. 5139-1 du code de la santé publique. [http://www.legifrance.gouv.fr/affichTexte.do?cidTexte=JORFTEXT000025837146&dateTexte=&categorieLien=id]

Arrêté du 11 juin 2013 modifiant l'arrêté du 23 janvier 2013 relatif aux règles de bonnes pratiques tendant à garantir la sécurité et la sûreté biologiques mentionnés à l'article R. 5139-18 du code de la santé publique. [http://legifrance.gouv.fr/affichTexte.do?cidTexte=JORFTEXT000027047902&categorieLien=id]

Décision du 20 octobre 2010 fixant le contenu du dossier technique mentionné à l'article R. 5139-3 du code de la santé publique et accompagnant la demande d'autorisation prévue à à l'article R. 5139-1 du code de la santé publique. http://www.legifrance.gouv.fr/affichTexte.do?cidTexte=JORFTEXT000022993011

Regulatory texts concerning containment measures

Arrêté du 16 juillet 2007 fixant les mesures techniques de prévention, notamment de confinement, à mettre en œuvre dans les laboratoires de recherche, d'enseignement, d'analyse, d'anatomie et cytologie pathologiques, les salles d'autopsie et les établissement industriels et agricoles où les travailleurs sont susceptibles d'être exposés à des agents biologiques pathogènes. http://www.legifrance.gouv.fr/affichTexte.do?cidTexte=JORFTEXT000000465273&dateTexte=&categorieLien=id

Directive 2009/41/EC of the European Parliament and of the Council of 6 May 2009 on the contained use of genetically modified micro-organisms. http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=CELEX:32009L0041:EN:NOT

Regulatory texts concerning the protection of laboratory animals

Décret n° 2013-118 du 1er février 2013 relatif à la protection des animaux utilisés à des fins scientifiques. http://legifrance.gouv.fr/affichTexte.do?cidTexte=JORFTEXT000027037840&categorieLien=id

Arrêté du 1er février 2013 fixant les conditions d'agrément, d'aménagement et de fonctionnement des établissements utilisateurs, éleveurs ou fournisseurs d'animaux utilisés à des fins scientifiques et leurs contrôles. http://www.legifrance.gouv.fr/affichTexte.do?cidTexte=JORFTEXT000027037983&dateTexte=&categorieLien=id

Arrêté du 1er février 2013 relatif à l'acquisition des compétences des personnels des établissements utilisateurs, éleveurs et fournisseurs d'animaux utilisés à des fins scientifiques. http://www.legifrance.gouv.fr/affichTexte.do?cidTexte=JORFTEXT000027037960&dateTexte=&categorieLien=id

Arrêté du 1er février 2013 fixant les conditions de fourniture de certaines espèces animales utilisées à des fins scientifiques aux établissements utilisateurs agréés. http://www.legifrance.gouv.fr/affichTexte.do?cidTexte=JORFTEXT000027037949

Arrêté du 1er février 2013 relatif à l'évaluation éthique et à l'autorisation des projets impliquant l'utilisation d'animaux dans des procédures expérimentales.

http://www.legifrance.gouv.fr/affichTexte.do?cidTexte=JORFTEXT000027038013&dateTexte=&categorieLien=id

Regulatory texts concerning safety and security plans

Regulation (EU) No 388/2012 of the European Parliament and of the Council of 19 April 2012 amending Council Regulation (EC) No 428/2009 setting up a Community regime for the control of exports, transfer, brokering and transit of dual-use items.http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2012:129:0012:0280:EN:PDF

Décret n°2005-1158 du 13 septembre 2005 relatif aux plans particuliers d'intervention concernant certains ouvrages ou installations fixes et pris en application de l'article 15 de la loi n° 2004-811 du 13 août 2004 relative à la modernisation de la sécurité civile. http://www.legifrance.gouv.fr/affichTexte.do?cidTexte=JORFTEXT000000786335&dateTexte=&categorieLien=id

Décret du 2 novembre 2011 relatif à la protection du potentiel scientifique et technique de la nation. http://www.legifrance.gouv.fr/affichTexte.do?cidTexte=JORFTEXT000024749915&dateTexte=&categorieLien=id

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Table 1. Overview of the questions posed and responses from ANSM.

Question	ANSM response
What does the term "fate" of MTs refer to, used in Part 2.2 of the technical dossier?	The authorisation applicant must indicate the "fate" of the MT: destruction after handling, possible storage by freezing, destruction of the batch at the end of the project, etc.
What is meant by the term "operation" in Part 3.4 "Description of operations" in the technical dossier?	"Operation" is a general term that can be defined by the applicant depending on the specific activities of the laboratory.
In Chapter 3.4.4 "Description of implementation", it is difficult to respond from the outset, before the protocols are effectively implemented: maximum number of animals used for the experiment; maximum inoculated infectious dose per animal; duration and frequency of animal experiment; maximum culture volume and surface area; duration and frequency of cultures, etc.	The description of implementation must be drafted using average figures in the case of research laboratories that often change their protocols.
Risk management system: must a risk assessment be performed for each protocol or can risk operations be organised into groups?	The aim is to evaluate the risks concerning "general" hazards (risk of bites, risk of theft, escape of an animal, etc.) encountered when implementing the protocols.
In the particular area of these specificity validations, can an NRL keep DNA extracted from strains of organisms classed as MTs and use the DNA for a period not exceeding 30 days, thereby enabling an exemption from authorisation?	- either the DNA contains fewer than 500 base pairs, making it exempt from the MT regulation (Ministerial Order of 30 April 2012); - or you consider the DNA fragment to be a veterinary reagent, also rendering it exempt.
Most of the available methods are PCR protocols which require a positive control. How can Departmental veterinary laboratories have a positive reference control that they keep for less than 30 days? What criteria are used to determine whether a DNA fragment is a veterinary reagent?	Article R.5139-2 of the Public Health Code provides for an exemption from authorisation specifically for reagents containing MTs, when they are reagents intended for analyses carried out in the veterinary and plant protection fields, as defined in Article L.202-6 and in paragraph 1 of Article R.203-1 of the Rural and Maritime Fishing Code (CRPM). The only exempt veterinary reagents are those validated by the NRL.
For avian flu viruses, how should we interpret the term "causing human infection"? Should we only consider the availability of effectively reported cases in humans, or the suspected zoonotic potential given certain documented viral characteristics, or in the absence of this data, a default classification in this category in line with the principle of precaution?	The regulation is based on the availability of effectively reported human cases. The Ministerial Order of 30 April 2012 stipulates for Orthomyxoviridae: - Type A avian influenza virus and H5N1 subtype, causing human infection; - Type A avian influenza virus and H7N7 and H7N3 subtypes, causing human infection This list may change if other cases are reported.
Some of the data requested in the dossier are security-defence related: must this information be submitted?	The documents can be classified security-defence confidential if necessary before submission to ANSM, which has authorised personnel to handle this type of document.
How can we evaluate the physical and psychological capacity of persons who work with MTs?	During the occupational medical assessment, the physician attempts to identify fears related to handling MT agents or working in a confined space (claustrophobia). The decision "able" is sufficient if these questions were asked.
What are the training requirements for MT auditors?	No specific requirements, only a need to validate their competence in auditing and knowledge of MTs.

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Revision of regulation (EC) No. 882/2004 on the organisation of official controls

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The European Commission recently adopted (6 May 2013) a set of four proposed regulations concerning animal health, plant health, plant reproductive material, and official controls[1]. This last text will replace Regulation (EC) No. 882/2004 on official controls, drawn up as a part of the "Hygiene Package" to cover a broader scope, especially concerning the plant sector. It will provide a basic text governing the organisation and quality of official controls, both as regards production in the various Member States and the importing of products and animals from outside the EU. The next stage is for the European legislative bodies, the Parliament and the Council, to examine the proposals and bring them into law in their final form.

Background

Although it was drawn up as part of the "Hygiene Package"[2], the current Regulation (EC) No. 882/2004, known as the "Official Controls" Regulation, already covers animal health and welfare as well as food legislation. The three other Regulations will replace a series of Directives, consisting of more than 60 texts on animal health, Directive (EC) No. 2000/29 on plant health and 12 directives concerning plant reproductive material, with provisions directly applicable to all Member States.
The revision of Regulation (EC) No. 882/2004 is a part of this process and addresses the need to adapt the rules governing official controls for all the sectors concerned. It also provides an opportunity to improve the current provisions and clarify certain points in light of acquired experience. The proposed Regulation is therefore broader in scope than the current version and has been drafted with a view to bringing greater legal consistency to all the texts covering the sector. Before adoption, it will be supplemented by about 40 delegated acts adopted by the European Commission (EC) and a similar number of implementing acts approved in technical committees.

Broader coverage and qualitative criteria for the control services

The proposal clarifies and broadens its field, which, beyond the areas of foodstuffs, plant health, animal health and welfare, and plant reproductive material, will cover fields closely related to the food chain such as animal by-products, GMOs, and plant protection products. Indications of quality and origin will now be mentioned explicitly.
In addition to official controls for verifying compliance with regulations, the proposal introduces the notion of "other official activities" to cover such activities as epidemiological surveillance, or combating animal diseases or pests.
The qualitative criteria, which will apply to the competent authorities and the organisation of controls, continue to follow the current principles embodied in Regulation (EC) No. 882/2004:

- among other requirements, the competent authorities, designated by each Member State, must be staffed by personnel with no conflicts of interest, qualified and of sufficient number, and have a sufficient number of laboratories and the legal power to carry out their missions. They must set up "internal" audits, their officials must have an obligation of discretion, etc.;
- controls must be carried out in accordance with risk, especially the risk of non-compliance, and depending on the nature of the hazard. The transparency of the results of controls is clarified and a programme for the verification and efficacy of controls and procedures must be set up.

One of the new items is the requirement for operators to grant control services access to IT units and systems, and to cooperate with them in carrying out the controls.

Coordination with control procedures in specific fields

In order to make the legislative package as consistent as possible, a series of ten articles provides a legal connection with the provisions to be adopted by delegated acts in different areas, in order to harmonise certain control procedures at European level and retain some of the existing provisions.
For example, the provisions concerning controls on residues of veterinary drugs and prohibited substances will be retained in a text based on the new "Official controls" Regulation, and Directive 96/23/EC will be repealed.

Delegation of activities adapted for better application in different sectors

Under the proposals, it will remain possible to delegate control activities or other official activities, as long as delegatees satisfy certain strict quality criteria, which may even include certification. To facilitate the protection of animal health, an individual, such as a veterinarian, can also be delegated to carry out missions on behalf of the competent authorities.

An entire chapter dedicated to analyses and laboratories

The choice of analytical methods to be used (Article 33)[3] favours harmonisation at European level and gives priority to the methods stipulated in European texts. If no clear choice

1. http://www.ansespro.fr/euroreference/Documents/ER10-Actu3.pdf
2. The "Hygiene Package" includes Regulations (EC) Nos. 178/2002, 882/2004, 852/2004, 853/2004, 854/2004, 183/2005 and several supplementary acts of application.
3. Article numbers are those of the provisional document of December 2013 but may differ in the final version.

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emerges, the method to be used must be selected according to a cascade approach: (i) internationally recognised methods, accepted by the European Committee for Standardization (CEN), or (ii) methods validated in terms of scientific protocols accepted internationally, developed or recommended by European Union Reference Laboratories (EURLs), or (iii) methods stipulated in national regulations, or (iv) methods validated, developed or recommended by National Reference Laboratories (NRLs), or lastly (v) validated *ad hoc* methods.
A section has been added to cover cases of analyses needing to be performed urgently, in the absence of available methods and satisfying the above criteria: NRLs or other competent laboratories are granted the possibility of using non-validated methods if necessary.
Article 34 maintains the right for operators whose animals or goods are subject to controls to benefit from a second expert opinion, but this process may be limited by application texts (implementing acts) in the future.
As regards sampling (Article 35), a new provision has been added enabling samples to be collected for controls via the Internet without identifying a "controller".
Regarding official laboratories responsible for analysis in the context of official controls (Articles 36 to 41), these are to be designated on the basis of the laboratory's certification, although temporary exemptions are possible to take account of new or changing methods and/or emergency situations, as well as the inspection of meat for *Trichinella*. Laboratories specialising in the analysis of seeds and plants are also exempt from the requirement to be certified. The text also makes it possible for the requirement to be certified to be relaxed at a later date in certain cases. The proposal adds the requirement, for official laboratories, to participate in inter-laboratory proficiency tests organised by the NRL or the EURL.
The competent authorities must ensure that the conditions for designating laboratories are satisfied, via audits and inspections.

Controls on imported animals and goods

With a view to simplifying and harmonising procedures between sectors, the proposal modifies the provisions of Regulation (EC) No. 882/2004 and the procedures for controlling products and animals entering the EU. It contributes to the prioritising of controls in accordance with risk. A new title, "border inspection post", replaces the various titles specific to each sector concerning the mandatory controls required for customs clearance. Different instruments, such as the Common Health Entry Document, will be created. Control procedures and the measures to be taken are defined on the basis of a common foundation, and cooperation with the other authorities such as the Customs Services will be strengthened. There is no question of inspecting a living animal in the same way as one would a can of food, but of using the same procedures and a common vocabulary.

Funding for controls and other official activities

The Member States remain entirely responsible for funding controls and "other activities" but the issue of the financial participation of operators is considerably modified compared to the current rules, under which fees are mandatory for certain sectors and allow those Member States who so wish to impose fees in the other sectors.
The basic principle that the entire cost of controls should be covered by the operators via "fees" charged by the competent authorities is considerably moderated by an exemption for micro-enterprises (those with turnover below €2 million and employing fewer than 10 people). This means that only a very small number of operators would be involved in financing controls via a system of fees, which would therefore apply only to companies above a certain size.
This is no doubt the section which will give rise to the most discussions and debates when the text comes up for examination.

Official certification

The Commission has taken on a considerable challenge, as different sectors use the terms "certification" and "certificate" with different meanings. For example, "certification" can cover both official signed certificates, such as health certificates for exports or certificates for the trade of living animals between Member States, and official declarations by professionals, as is the case in the seed and plant sector, with express authorisation from the control authorities.

Laboratories and reference centres

Reference activities are not limited to laboratory analyses, and the new provisions (Articles 91 to 97) enable the European Commission to designate EU reference centres in the plant reproductive material sector and for animal welfare.
The European Union Reference Laboratories (EURLs) are of course retained, and the conditions for their designation remain essentially unchanged.
The missions of the EURLs are clarified and extended, in coordination with those of the national reference laboratories (NRLs) designated by each Member State. Their principal mission is to improve and harmonise methods for analysis, testing and diagnosis, as well as to contribute to the quality and uniformity of the analytical data generated. This mission was not quite so explicitly defined in the current regulations and the proposed text confirms it as one of the core missions of an EURL. Other missions are added: (i) to collaborate with EFSA and the ECDC, (ii) to provide active support in the diagnosis of outbreaks of foodborne, zoonotic or animal diseases or plant pests, by examining pathogens sent to them for confirmation, characterisation and taxonomic or epizootic studies, and (iii) to set up and maintain reference collections of pathogens or plant pests, as relevant to their field of competence.
EURLs must publish lists of NRLs.
With the extended scope of the Regulation, the field of plant health is set to benefit the most from the EURL/NRL system across the EU, which already operates in the food and animal health sectors.
The conditions governing the designation and missions of NRLs (Articles 98 to 99) are essentially retained, with a few minor changes.

General responsibilities incumbent upon the competent authorities

The proposed text clarifies the relationship between the competent authorities of the different Member States and with the European Commission, with a view to improving application of the regulations. The procedures for exchanging information and for cooperation are an extension of the procedures for emergencies, such as those covered by the Rapid Alert System for Feed and Food (RSAFF).

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Member States are still required to prepare a multiannual control plan and to furnish an annual report. One new feature should be noted however: the European Commission has retained the option of using delegated acts to determine such elements as criteria for categorising risks according to the activities of operators, control priorities, and performance indicators, which the Member States will be obliged to incorporate in their control programmes. Emergency plans concerning foodstuffs and animal feed must be drawn up. In animal and plant health, these emergency plans are governed by the regulations for each sector.
A specific section is dedicated to "coercive" measures and sanctions that must be set up by the competent authorities.

The Commission retains an important role with several new initiatives

Commission controls are maintained with the principal objective of inspecting the control system implemented by the competent authorities of the Member States. These inspections also concern non-member countries regarding the procedure governing the conditions for entry into the EU of animals and goods covered by the text.
The European training program for the officials of the competent authorities (entitled Better Training for Safer Food – BTSF) is extended to cover the entire field of the Regulation.
An overall system for information management is planned, incorporating the current TRACES information system on imports and trade and enabling data to be transmitted to the Commission.

Phasing in

The new regulation will be phased in between one and three (or even five) years after the final version passing into law comes into force, and its timing will be coordinated with the entry into force of the three other sector-based texts.

The legislative procedure

The European Parliament and Council of Ministers began examining the text and the three other proposals at the end of the first half of 2013. It is important that the "legislative package" remain internally consistent and the work can be expected to take several months. The French positions have been prepared by all the departments concerned, coordinated by the Secretary-General for European Affairs.

Conclusion

The proposed Commission Regulation for "official controls and other official activities" retains most of the principles of Regulation (EC) No. 882/2004 as regards the "technical" organisation of controls, while attempting to harmonise the way they are applied in the different sectors. Harmonised procedures for import controls will be common to all types of products and animals. Reference activities will be given greater importance by the creation of reference centres for animal welfare and in the area of seeds and plants. Concerning analytical laboratories, European Union Reference Laboratories and National Reference Laboratories will continue to contribute to the quality of the system, with clearly-defined and broadened missions. The issue of financing, however, will considerably modify the current system.

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Methods

Methodological guide to the assessment of biological safety and security risks

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Laboratory activities using pathogenic micro-organisms or toxins pose potentially significant risks of harm to humans and the environment. ANSES's Committee for the Control of Biological Risks in Laboratories (CMRBL) offers a **general method for identifying hazards and analysing and assessing risks related to the use of micro-organisms and toxins (MOTs),** as defined by the Decree of 30 June 2010[1], and in the rules for good practice drawn up by the French National Agency for Medicines and Health Products Safety (ANSM)[2]. This method is derived from the Failure Mode and Effects Analysis (FMEA) method. It is based on a model proposed by the ANSM. However, the method presented in this guide takes into account the particularities of ANSES's reference and research laboratories. The hazard identification questionnaires have been adapted accordingly, as well as the scales for ranking biological safety and security risks. These calculation methods were tested with various pathogens used in ANSES's laboratories and then adjusted before being definitively adopted by the CMRBL.

This guide includes four separate sections:

- Presentation of the risk assessment model
- Presentation of the micro-organism or toxin
- Booklet 1: Analysis of biological safety risks
- Booklet 2: Analysis of biological security risks

The complete version of the methodological guide is available at the following address:
http://www.ansespro.fr/euroreference/Documents/ER11-MethodologicalGuideEN.pdf

1. Decree no. 2010-736 of 30 June 2010 on micro-organisms and toxins.
2. Ministerial Order on rules of good practice tending to guarantee biological safety and security mentioned in Art. R.5139-18 of the French Public Health Code.

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Molecular confirmation method for monophasic and non-motile variant strains of *Salmonella* serovar Typhimurium

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***Salmonella* remains the primary cause of confirmed foodborne illness outbreaks in France. Among the 2600 serovars identified in the *Salmonella* genus, some are isolated more frequently in human health, food hygiene and/or animal health.**
Over the past five years, salmonellae known as "Typhimurium-like variants" have emerged in humans and are found in many areas of the food chain and livestock sector.
This article presents a molecular characterisation method developed and applied since 2010 for surveillance purposes. The method meets the on-going need to change laboratory analyses to comply with regulatory requirements and to implement control measures for the prevention of the microbiological hazards associated with the possible presence of salmonellae in food.

Abstract
Since 2008, the French Reference Laboratories tasked with monitoring salmonellae in human health or in the food and veterinary sectors have observed the emergence of strains with the *S.* 1,4,[5],12:i:- antigenic formula, known as "monophasic Typhimurium variants". Emergence of these strains has also been demonstrated at the European level and, in 2010, led the European Food Safety Authority (EFSA) to issue recommendations concerning characterisation and surveillance of these isolates throughout the food chain. Detection of these variants in regulated poultry sectors has led Europe to implement control measures identical to those required for *S.* Typhimurium.
French regulations are more stringent and cover two types of monophasic variants, *S.* 1,4,[5],12:i:- and *S.* 1,4,[5],12:-:1,2, and the non-motile variant *S.* 1,4,[5],12:-:-.
In order to confirm the presence of variants of the serovar Typhimurium, a conventional multiplex polymerase chain reaction (PCR) method has been developed. This makes it possible to monitor changes in isolation trends for these variants throughout the food chain.
Overall analysis of the range of strains collected by the *Salmonella* network for the 2011-2012 period has demonstrated the emergence of strains with the *S.* 1,4,[5],12:i:- antigenic formula, confirmed as monophasic variants of the Typhimurium serovar, within several animal production sectors.
This PCR method can be used in conjunction with the conventional serotyping method by slide agglutination and provides rapid confirmation of the identity of these variants. It is also a useful tool in determining the epidemiological picture, in monitoring trends related to strain isolation, and in assessing risks and adjusting control measures in the various sectors.

Background
Internationally, monitoring data from recent years have shown a considerable increase in the occurrence of strains with an antigenic formula (*S.* 1,4,[5],12:i:-) very similar to that of *Salmonella* Typhimurium (*S.* 1,4,[5],12:i:1,2) (EFSA, 2010; ANSES, 2013; Mulvey, 2013). These strains are flagellar variants of the serovar Typhimurium, called monophasic because they lack expression of the second flagellar phase, encoded by the *fljB* gene. Strains that have lost antigen expression of the first flagellar phase or of both phases (*S.* 1,4,[5],12:-:1,2 and *S.* 1,4,[5],12:-:-, respectively), are also found but far less commonly (EFSA, 2010; ANSES, 2013; Mulvey, 2013).
Considering, on the one hand, the emergence of monophasic variant strains of *S.* Typhimurium at the European level, and on the other, the risk that they pose to public health, thought to be similar to serovar Typhimurium, EFSA recommended full serotyping of all strains suspected of being salmonellae, followed by PCR confirmation of absence of the *fljB* gene for strains with the *S.* 1,4,[5],12:i:- antigenic formula (EFSA, 2010).
In France, given that there have been several foodborne illness outbreaks associated with *Salmonella* strains known as "variants of serovar Typhimurium", the scope of Ministerial Orders has been extended beyond European regulations to include the three existing flagellar variants of serovar Typhimurium (*S.* 1,4,[5],12:i:-, *S.* 1,4,[5],12:-:1,2 and *S.* 1,4,[5],12:-:-). These Orders[1] stipulate that flocks contaminated with a variant of serovar *S.* Typhimurium are now to be treated as positive flocks for *S.* Typhimurium.
Depending on the type of farm involved, these measures require slaughter of the contaminated flock, transfer of eggs to establishments producing egg products, or heat treatment of positive meat following tests in muscle.
In view of emergence of these strains and the associated regulations, since 2010, the Directorate General for Food[2] has required that first-line veterinary and agro-food analysis laboratories forward the strain without delay to the *Salmonella* network of the Laboratory for Food Safety, along with the specific identification sheet of the network, whenever they isolate a variant with one of the above-mentioned antigenic

1. Both Ministerial Orders of 26 February 2008 concerning control of *Salmonella* infections (in the egg-laying and broiler sectors); Ministerial Order of 4 December 2009 concerning control of *Salmonella* infections in breeding turkey flocks; Ministerial Order of 22 December 2009 concerning control of *Salmonella* infections in flocks of broiler chickens and meat turkeys.
2. Guidance note DGAL/SDSSA/N2010-8059 of 04 March 2010, amending Guidance note DGAL/SDSSA/N2010-8026 of 27 January 2010.

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formulas. The *Salmonella* network ensures surveillance after confirming the identity of the so-called "Typhimurium-like variants" using an in-house method based on molecular tests defined earlier by EFSA and described below.
Strains suspected of being variants of serovar Typhimurium may in fact be found to be variants of other less frequently identified serovars, given the antigenic formula detected. In this way, for the *S.* 1,4,[5],12:i:- antigenic formula, it is possible to identify 6 serovars. For *S.* 1,4,[5],12:-:1,2, and *S.* 1,4,[5],12:-:-, 16 and 148 serovars, respectively, can be identified (ANSES, 2013).

Principle of the method

The method used to confirm the identity of the variants of serovar Typhimurium is based on EFSA recommendations (2010) concerning solely confirmation of the emerging monophasic *S.* 1,4,[5],12:i:- variant, and on studies carried out by Bugarel *et al.* (2012). French regulations concern all monophasic and non-motile variants. As a result, additional markers were included in this method to cover all the confirmation needs for these variants.
The method uses the principle of polymerase chain reaction (PCR) and is applied after conventional serotyping detection of a strain with one of the following antigenic formulas: *S.* 1,4,[5],12:i:-, *S.* 1,4,[5],12:-:1,2 or *S.* 1,4,[5],12:-:-. It aims to amplify four genes through two multiplex PCRs. The first targets the *fljB* gene, coding for the second flagellar phase, and the *fliA-fliB* intergenic region. The presence of an *IS200* sequence of 1000 bp in this region is specific to the serovar Typhimurium and its variants, since it is not detected in the other serovars for which the corresponding amplicon is 250 bp in size. The second PCR targets the *mdh* gene, marker of the serovar Typhimurium and the *fliC* gene coding for the first flagellar phase. The sequences of the primers used to detect these markers are listed in **Table 1**.

Analytical procedure

The molecular method described in this article is applied using a pure culture of a *Salmonella* strain for which the antigenic formula has been determined by slide agglutination serotyping. This conventional serotyping method uses specific antisera against cell wall ("O") or flagellar ("H") antigens (Danan, 2009). The steps in the molecular confirmation method for variants are as follows:

- Culture of strains on TSAYE agar, 18 - 24h at 37°C;
- Extraction of DNA from isolated colonies on TSAYE agar using a standard kit;
- Measurement of the concentration of DNA extract using a spectrophotometer at 260 nm;
- Dilution of the extract to adjust its concentration to 50 - 100 ng/µl;
- Two multiplex PCRs for fliA-fliB + fljB and mdh + fliC, as per the conditions presented in **Table 2**;
- Migration of the amplification products on 2% agarose gel;
- Visualisation of EtBr-labelled amplicons by fluorescence under a UV lamp;
- Reading of the gel (see **Figure 1**) and interpretation of results.

The method requires use of control strains: *Salmonella* Typhimurium LT2 reference strain (positive control) and a *Salmonella* Brandenburg strain (field strain and negative control). A negative control without DNA is also included in each experiment.

Table 1: Sequences of PCR primers used

Target gene	Function	Name of primer	Sequence (5'-3')	Reference
mdh	Malate dehydrogenase	MDH F MDH R	TGCCAACGGAAGTTGAAGTG CGCATTCCACCACGCCCTTC	[Amavisit, 2005]
fliC	Phase 1 flagellar antigen	Anti-sense-i Sense-60	ATAGCCATCTTTACCAGTTCC ACTCAGGCTTCCCGTAACGC	[Herrera-Leon, 2004] [Bugarel, 2012]
fljB	Phase 2 flagellar antigen	Sense-59 Anti-sense-83	CAACAACAACCTGCAGCGTGTGCG GCCATATTTCAGCCTCTCGCCCG	[EFSA, 2010]
fliA-fliB	Intergenic region of variable size depending on whether it contains an IS200 insertion sequence	FFLIB RFLIA	CTGGCGACGATCTGTCGATG GCGGTATACAGTGAATTCAC	[EFSA, 2010]

Table 2: Description of operating conditions for the two multiplex PCRs (*fliA-fliB* + *fljB* and *mdh* + *fliC*)

PCR 1 Preparation of mix		PCR 2 Preparation of mix	
Buffer without MgCl2	1 X	Buffer without MgCl2	1 X
MgCl2	2 mM	MgCl2	2 mM
dNTPs	0.2 mM	dNTPs	0.2 mM
Anti-sense 83 primer	0.8 µM	MDH-F primer	0.4 µM
Sense 59 primer	0.8 µM	MDH-R primer	0.4 µM
FFLIB primer	0.4 µM	Anti-sense I primer	0.4 µM
RFLIA primer	0.4 µM	Sense-60 primer	0.4 µM
Taq polymerase	1 unit	Taq polymerase	1 unit
Total PCR reaction volume 25 µl (24 µl or 23 µl of reaction mix / tube + 1 µl of DNA at a concentration of 100 ng/µl or 2 µl of DNA at 50 ng/µl)			
Amplification conditions		**Amplification conditions**	
3 min	94°C	3 min	94°C
35 cycles:		35 cycles:	
30 sec	94°C	30 sec	94°C
40 sec	64°C	40 sec	58°C
1 min 30 sec	72°C	1 min 30 sec	72°C
7 min	72°C	7 min	72°C

Expression of results

Interpretation of results is carried out according to predefined rules presented in **Table 3**. The strain is considered to be a non-motile or monophasic variant if the amplicons corresponding to the *fliC* and/or *fljB* genes are absent.
A variant of serovar Typhimurium is confirmed if the amplicons corresponding to the *mdh* gene and to the *fliA-fliB* intergenic region are detected and if the amplicon of the intergenic region has the expected length of 1000 bp.
A variant of a serovar other than Typhimurium is confirmed if the amplicon corresponding to the *mdh* gene is absent, and if that of the *fliA-fliB* intergenic region is 250 bp in length.

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Inconsistent variants are also identified (as per Hopkins *et al.* (2010)) for *S.* 1,4,[5],12:i:- variants, and this term can be extrapolated to the two other antigenic formulas if the genes coding for the flagellar phases (*fliC* and *fljB*) are detected but not expressed (non-detection of the antigens by conventional agglutination serotyping).

Table 3: Interpretation of results obtained by the method of confirmation for *Salmonella* strains, variants of serovar Typhimurium.
[+: detection of the specific amplicon for the marker of expected length; -: absence of detection of the specific amplicons for the marker; bp: DNA base pairs]

Serovar by agglutination	Target markers				Interpretation
	fliC	*fliA-fliB*	*fljB*	*mdh*	
S. 1,4,[5],12:i:-	+	1000 bp	–	+	Confirmed monophasic variant of Typhimurium
	+	1000 bp	+	+	Inconsistent monophasic variant of Typhimurium
	+	250 bp	–	–	Monophasic variant of a serovar other than Typhimurium
S. 1,4,[5],12:-:1,2	-	1000 bp	+	+	Confirmed monophasic variant of Typhimurium
	+	1000 bp	+	+	Inconsistent monophasic variant of Typhimurium
	-	250 bp	+	–	Monophasic variant of a serovar other than Typhimurium
S. 1,4,[5],12:-:-	-	1000 bp	-	+	Non-motile variant of Typhimurium
	+	1000 bp	+	+	Inconsistent non-motile variant of Typhimurium
	-	250 bp	-	–	Non-motile variant of a serovar other than Typhimurium
S. 1,4,[5],12:i:1,2	+	1000 bp	+	+	Typhimurium

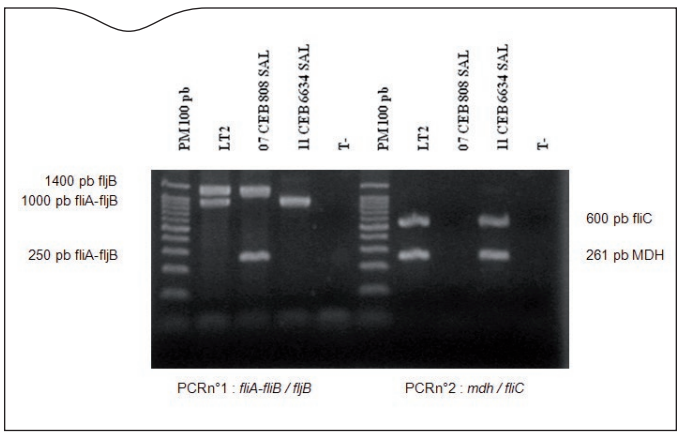

Figure 1: Illustration of the results of amplification obtained for confirmation of *Salmonella* strains, variants of serovar Typhimurium.
[PM100bp: molecular mass marker; LT2: serovar Typhimurium; 07CEB808SAL: *S.* Brandenburg; 11CEB6634SAL: monophasic variant *S.* 1,4,[5],12:i:- of serovar Typhimurium; T-: negative control without DNA]

Summary of results using the confirmation method

During the 2011 to 2012 period, a total of 703 "*Salmonella* Typhimurium-like" strains of various origins (see **Table 4**) were analysed using this method (Lailler, 2013). Within this group, 690 strains had the *S.* 1,4,[5],12:i:- antigenic formula, of which 650 strains (94.2%) were identified as monophasic variants of serovar Typhimurium, 38 strains as "inconsistent variants" by the presence of the *fljB* gene, and only two strains as monophasic variants of serovars other than Typhimurium (see **Table 5**).
Analysis of the eight strains with the *S.* 1,4,[5],12:-:1,2 antigenic formula showed the presence of the *fliC* gene and confirmed their status as monophasic variants of serovar Typhimurium.
Of the five non-motile strains with the *S.* 1,4,[5],12:-:- antigenic formula, only one strain was confirmed as a monophasic variant, the others were variant strains of other serovars.

Table 4: Distribution of sources for the 703 "*Salmonella* Typhimurium-like" strains collected in 2011 and 2012 by the *Salmonella* network, coordinated by the Maisons-Alfort Laboratory for Food Safety (ANSES).

Serovars	*S.* 1,4,[5],12:i:-	*S.* 1,4,[5],12:-:1,2	*S.* 1,4,[5],12:-:-
Feed	23	/	2
Ecosystem	27	4	1
Animal health and production… *including* *cattle* *poultry* *swine*	233 *48* *160* *13*	4 *4* 	2 *1*
Food … *including* *beef* *Poultry meat ?* *pork*	407 *39* *13* *133*	/	/
Total	**690**	**8**	**5**

Table 5: Results obtained by multiplex PCR on the range of strains collected in 2011 (n=312) and in 2012 (n=391) by the *Salmonella* network, coordinated by the Maisons-Alfort Laboratory for Food Safety (ANSES).

markers \ serovar				*S.* 1,4,[5],12:i:-	*S.* 1,4,[5],12:-:1,2	*S.* 1,4,[5],12:-:-
fliC	*fliA-fliB*	*fljB*	*mdh*			
+	1000 bp	–	+	650	/	1
+	1000 bp	+	+	38	8	/
+	250 bp	–	–	2	/	4
Total				**690**	**8**	**5**

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Discussion / Conclusion

The confirmation method for monophasic and non-motile variants presented in this article is a qualitative test based on the absence or presence of the amplicon of expected length, detected by multiplex PCR for genotyping, as described in Chapter 8 of French Standard XP U47-600-2.

This method is based on the protocol recommended by EFSA, and on studies carried out by Bugarel *et al* (2012). As part of these studies, three different markers, known to be specific for the serovar Typhimurium, were tested in a series of known strains belonging to the serovar Typhimurium or confirmed variant of *S.* Typhimurium.

The markers are the *fliA-fliB* intergenic sequence proposed in EFSA recommendations and the *mdh* gene. *Mdh* was systematically detected in all strains of serovar Typhimurium and variants. This marker, known to be present in many *Salmonella* strains, was also tested for in a series of 937 strains of various serovars (more than 230 different serovars), enabling determination of its extrinsic specificity. No cross-reaction was detected, with the exception of one strain of serovar Kibusi (*S.* 28:r:e,n,x) and one of serovar Newmexico (*S.* 9,12:g,z_{51}:1,5) (Bugarel, 2012). These two serovars do not belong to the O:4 group, unlike Typhimurium and its variants.

Inclusion of the *mdh* gene in the series of tested markers makes it possible to exclude any false positive or false negative result (100% detection in strains expected to be positive).

As EFSA recommends in its opinion (EFSA, 2010), confirmation of the identity of these "*Salmonella* Typhimurium-like" strains by accurate and complete characterisation is important in terms of surveillance. Regular updates will be used to assess the suitability of regulatory measures in view of public health objectives in France and in Europe.

Concerning variants classified as inconsistent (*fljB+, fliC+, fliA-fliB+ at 1000 bp and mdh+)*, these strains have all the genetic material required to be identified as belonging to the serovar Typhimurium. When considering only the results of the PCR tests applied, these strains cannot be distinguished from strains of *S.* Typhimurium. Only characterisation by conventional serotyping can demonstrate the absence of expression of one or both flagellar phases. This lack of expression could also be reversible (Soyer, 2009). Identification of these inconsistent strains by the method described here could also be useful in detecting new genes involved in the inversion mechanism of the flagellar phase.

This method cannot be used for complete identification of variants of other serovars. Additional geno-serotyping methods could help to counter this limitation. One of the currently available methods, that can be used for this molecular serotyping, was used in part to complement molecular confirmation with the described method. This sometimes enabled identification of other serovars such as *S.* Coeln and *S.* Schwarzengrund, which were the sources of the monophasic and non-motile variants in the study carried out in 2011-2012. However, this approach to geno-serotyping is still experimental and needs to be validated more generally.

As part of surveillance carried out by laboratories, it is important that the epidemiological situation concerning salmonellae be evaluated regularly in order to adjust monitoring, and if necessary, control measures in the various sectors, to changes in serovars (particularly emerging ones such as the recently identified Kentucky serovar) and to changes in antibacterial resistance profiles.

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Methods

Determination of neonicotinoid residues in nectar by liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS)

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Nectar is a sweet liquid produced by the nectaries of plants. It is the primary source of energy for bees. Melliferous plants visited by pollinators can contain pesticide residues as the result of plant protection treatment or environmental contamination (soil, water or air). Bees can thus come into contact with these residues via the contaminated nectar that they take back to the colony. The laboratory has therefore developed a method for assaying residues of neonicotinoids in nectar to help establish the implication of these insecticides in cases of the weakening of bee colonies.

Principle of the method

The pesticides studied (imidacloprid, clothianidin, acetamiprid, thiacloprid, thiamethoxam and dinotefuran) belong to the neonicotinoid family (**Figure 1**), which are chemical substances used in agriculture (**Table 1**) either for coating seeds or as a foliar spray on crops. They are systemic molecules which can subsequently be found in the plants and the different environmental compartments. It should be noted that these substances have sufficient remanence in soil (Goulson, 2013) for that plants grown the following year even without treatment, including weeds, assimilate them. The nectar secreted by plants can therefore be a good indicator of contamination by these residues (Dively and Kamel, 2012; Stoner and Eitzer, 2012) as it is one of the main vectors for the contamination of foraging bees and their colonies. When a forager returns to its hive, it regurgitates the nectar from its honey stomach into cells in the comb. A bee can transport up to 75 mg of nectar in its honey stomach.

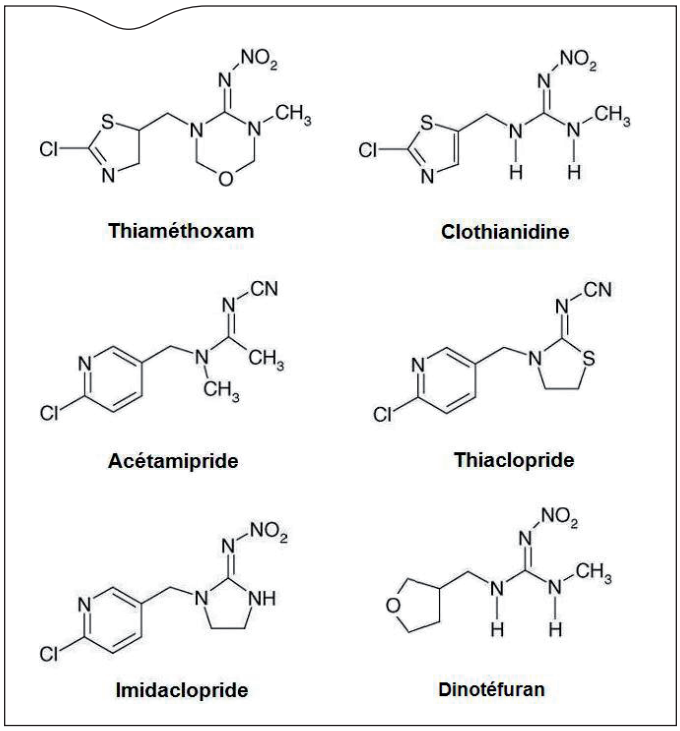

Figure 1: Formulae of the pesticides studied.

When analysed, nectar is found to be a matrix consisting essentially of water and sugars (fructose, glucose and, in much lower quantities, complex sugars such as sucrose). The water content of nectar varies considerably, from 20 to 95%, depending on the species of nectar-producing plant and on environmental, especially meteorological, factors (air humidity, temperature, etc.). The composition in sugars also varies, depending on plant species (Nicolson and Thornburg, 2007). It remains relatively stable for a given species or even for a given family. Depending on the nature and proportions of the sugars, plants can be divided into those where sucrose is dominant in the nectar, those where the quantity of sucrose equals that of glucose and fructose (white clover) and those in which glucose and fructose are dominant (colza) (Kevan and Shuel, 1991). The ratio between glucose and fructose is also usually stable in a given species. For example, in colza, there is a higher level of glucose than of fructose, which can cause a rapid crystallisation.

The method for assaying these six insecticides, which are toxic for bees (**Table 2**), is based on extraction by dissolution. The nectar sample obtained is diluted in ultra pure water for injection and analysis by liquid chromatography coupled with tandem mass spectrometry (LC-ESI-MS/MS). This method for multi-residue analysis enables quantification and identification of neonicotinoid residues in the “nectar” matrix. The limit of quantification (LOQ) is 0.3 pg/µl for all the pesticides with the exception of dinotefuran for which the LOQ is 0.6 pg/µl.

Equipment and reagents

The specific equipment consists of (1) a propipette for extracting nectar samples from micro-capillaries; (2) a centrifuge (Centrifuge 5810R, Eppendorf); (3) an HPLC instrument (liquid chromatography) with an autosampler and a column compartment thermostatted (UltiMate 3000, Thermo Scientific) coupled with a TSQ Vantage Triple Stage Quadrupole Mass Spectrometer (Thermo Scientific) equipped with HESI-II probe (Heated Electrospray Ionization Source).

For the analysis by LC-MS/MS, LC-MS grade methanol and formic acid (98%) were used. The measurement standards were prepared using certified active substances purchased from CIL Cluzeau Info Labo: imidacloprid (98% purity), clothianidin (99.5%), acetamiprid (99%), thiacloprid (99.5%), thiamethoxam

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Table 1: The uses of neonicotinoids in agriculture (AGRITOX, 2013; Index phytosanitaire, 2013; Mitsui Chemicals America, 2013)

Pesticide	Solubility in water (g/l)	Type of application	Crops treated	Commercial brand names
Imidacloprid*	0.613	Treating seeds and plants	Beetroot, oats, wheat, barley, rye	Ferial Gaucho 350 Imprimo Nuprid 70
		Treating aboveground parts	Apricot, peach, plum, rose, forest conifers	Confidor Merit Forest Nuprid 200
Clothianidin*	0.304	Treating aboveground parts	Maize, sorghum, apple	Cheyenne Dantop 50 WG
Acetamiprid	2.95	Treating aboveground parts	Fruit trees *(apricot, citrus, cherry, fig, peach, pear, apple, plum)*, field crops *(potato, oil-bearing crucifers, oats, wheat)*, vegetable crops *(asparagus, aubergine, cabbage, cucumber, courgette, lettuce, parsley, sweet pepper, tomato, beetroot)*, roses, various flower crops, crops grown for seed	Suprême Suprême 20SG Polysect Ultra
Thiacloprid	0.186	Treating aboveground parts	Fruit trees *(apricot, gooseberry, almond, black currant, cherry, chestnut, fig, raspberry and other Rubus, hazel, walnut, olive, peach, pear, apple, plum)*, field crops *(colza, mustard, potato)*, crops grown for seed	Biscaya Calypso Ecail Proteus
		Treating the soil	Ornamental trees and shrubs, various flower crops	Exemptor
Thiamethoxam*	4.1	Treating seed and plants	Beetroot, maize, pea	Cruiser 350 Cruiser FS Cruiser SB
		Treating aboveground parts	Potato, apple, aubergine, cucumber, lettuce, pepper, sweet pepper, tomato, ornamental trees and shrubs, chrysanthemum, various flower crops, rose, all floral species (under glass)	Actara Flagship Pro
		Treating the soil	Ornamental trees and shrubs, various flowering crops (under glass), rose (under glass)	Flagship Pro
Dinotefuran**	39.83	Treating aboveground parts	Rice, cabbage, lettuce, sweet pepper, tomato, cucumber, melon, celery, citrus, apple, peach, potato, cotton	Safari 20SG Safari 2G

* In April 2013, the European Union announced that it would suspend the use of imidacloprid, clothianidin and thiamethoxam on four field crops (maize, colza, sunflower and cotton) for two years, with effect from 1 December.
** Dinotefuran is prohibited in Europe on all crops.

Table 2: Toxicity of the pesticides studied on bees (AGRITOX, 2013, EPA, 2004)

Pesticide	LD_{50} (contact)	LD_{50} (oral)
Imidacloprid	81 ng/bee	3.7 ng/bee
Clothianidin	44.26 ng/bee	3.79 ng/bee
Acetamiprid	8.09 µg/bee	14.53 µg/bee
Thiacloprid	38.82 µg/bee	17.32 µg/bee
Thiamethoxam	24 ng/bee	5 ng/bee
Dinotefuran	47 ng/bee	23 ng/bee

(99%) and dinotefuran (99%). The certified dimethoate-D6 solution (99.8% purity, 100 mg/l in acetone) also came from CIL Cluzeau Info Labo.

Procedure

1. Extraction

Nectar samples were extracted from flowers by capillary action using a micro-capillary tube (5 µl). The samples were then extracted from the micro-capillary tube using a propipette. In a microvial were added ultra pure water, 10 µl of the internal standard (dimethoate-D6) and then, 10 µl of the nectar sample. The nectar sample was then homogenised using a vortex and centrifuged at 500 rpm for five minutes. The volume of the final extract was 100 µl.

2. Measurement

2.1. High-performance liquid chromatography (HPLC)

Chromatographic separation was carried out on a Pursuit PFP (pentafluorophenyl) analytical column 100 x 3 mm (3 µm) (Agilent). The mobile phase consisted of ultra pure water (A) and methanol (B), each solution being acidified with 0.02% of formic acid. The insecticides were separated by gradient elution, with the following protocol: linear gradient from 80% A (at t=0 min) to 0% (at t=13 min), then a linear gradient of 0% A (at t=13 min) to 80% (at t=13.5 min) and holding at 80% A for 4.5 min. The column and autosampler temperature was 25°C, the flow rate was 0.4 ml/min and the injection volume was 15 µl.

2.2. Mass spectrometry

Positive mode electrospray (HESI-II +) was used as the source of ionisation. The divert valve was set to allow the admission of the mobile phase in the source between 2.50 min and 12 min. The mass analyser was a TSQ Vantage triple stage quadrupole and the collision gas was argon. The acquisition mode used was the SRM mode (Selected Reaction Monitoring). Transitions and retention times (indicative only) are given in **Table 3**.

Results and conclusion

For assaying, calibration was performed using a range extracted from the "nectar" matrix (blank and fortified samples). Like blank nectar is not always available, a representative sugars solution of a nectar was prepared for this calibration. This

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Table 3: Transitions of the pesticides studied and retention times (indicative only)

Pesticide	Retention time (min)	Precursor ion (m/z)	Product ions (m/z)	Collision energy (V)	S-Lens
Dinotefuran	3.49	203.0	114.1 129.1	13 13	43 43
Thiamethoxam	4.92	292.0	211.0 181.0	13 24	57 57
Imidacloprid	6.09	256.0	209.1 175.1	18 20	65 65
Clothianidin	6.30	250.0	169.1 131.9	15 19	54 54
Dimethoate-D6	6.39	236.0	177.1 131.0	16 22	43 43
Acetamiprid	7.12	223.0	126.0 90.0	21 34	53 53
Thiacloprid	8.03	253.0	126.0 90.0	22 39	71 71

solution with 36% sugars (w/v) was made with a mixture of 6 g of glucose and 3 g of fructose in 25 ml of ultra pure water. The linear range is defined as being the calibration range and has been validated up to 15 pg/µl for each pesticide.

The limits of detection (LOD) and quantification (LOQ) were respectively 0.1 pg/µl and 0.3 pg/µl for imidacloprid, clothianidin, thiacloprid and thiamethoxam. For dinotefuran, LOD and LOQ were respectively 0.2 pg/µl and 0.6 pg/µl (**Figure 2**).

In the absence of reference material, accuracy was estimated by the rate of recovery, determined using a control sample (blank matrix) spiked with analytes assayed at three different concentrations (LOQ, 5LOQ and 10LOQ). For each concentration, three samples of sugars solutions were extracted and analysed. For the method validation, five series of three samples were processed for each spiking level. The mean recoveries obtained were satisfactory as they were between 98.9% and 110.2% at the LOQ, and between 93.0% and 96.6% and between 92.6% and 99.7% for the samples spiked at 5LOQ and 10LOQ respectively (V03-110 Normalisation). The method is repeatable because the relative standard deviation (RSDr) is ≤ 20% for each concentration. The method is also reproducible (RSDR ≤ 22%) for all the pesticides studied (**Table 4**).

It can therefore be stated that this method enables the quantification of residues at very low levels and can thus be applied to samples of nectar extracted directly from flowers (**Figure 3**) or from the honey stomachs of bees in order to monitor the exposure of foragers to environmental contaminants.

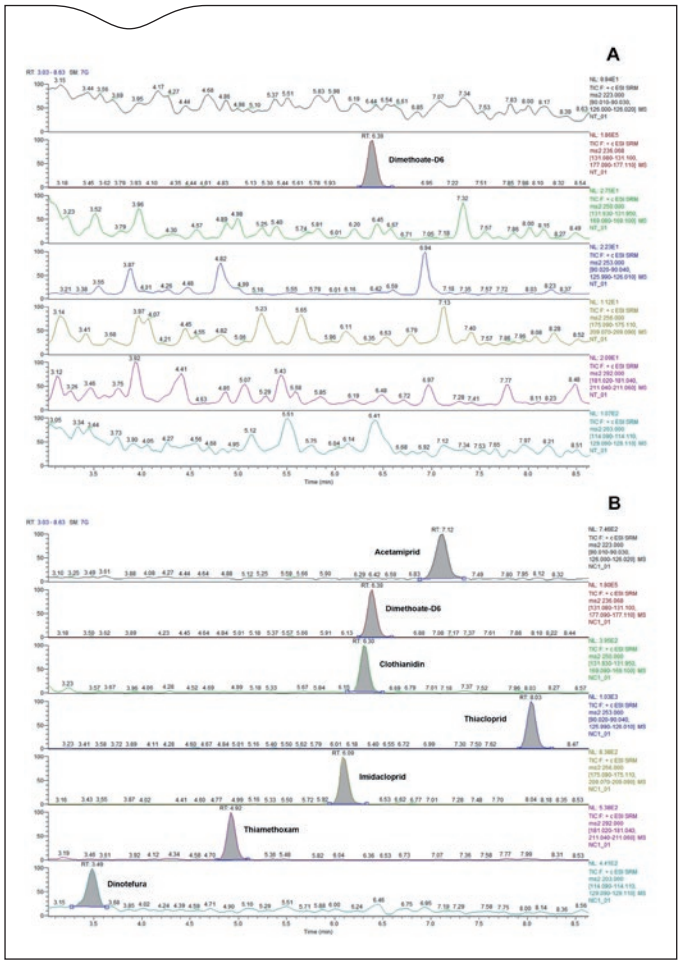

Figure 2: Chromatograms obtained by LC-MS/MS for (A) the blank sample (36% sugar solution) and for (B) the sample fortified with pesticides at the LOQ.

Table 4: Validation data of the method (AFNOR Normalisation V03-110)

Pesticide	Representative sugars solution of a nectar														
	1st fortification level (n=3, repeated 5 times)					2nd fortification level (n=3, repeated 5 times)					3rd fortification level (n=3, repeated 5 times)				
	C (pg/µl)	Mean recoveries (%)	RSD_r (%)	RSD_R (%)	Uncertainty (%)	C (pg/µl)	Mean recoveries (%)	RSD_r (%)	RSD_R (%)	Uncertainty (%)	C (pg/µl)	Mean recoveries (%)	RSD_r (%)	RSD_R (%)	Uncertainty (%)
Imidacloprid	0.3	106.7	8.6	12.5	26.6	1.5	95.8	6.6	8.2	17.4	3.0	98.3	4.5	6.6	14.0
Clothianidin	0.3	99.8	7.5	11.5	24.6	1.5	95.2	6.7	8.4	17.7	3.0	97.3	5.1	5.9	12.4
Acetamiprid	0.3	106.8	7.3	9.0	18.9	1.5	96.5	6.1	7.0	14.7	3.0	98.7	5.2	6.8	14.5
Thiacloprid	0.3	110.2	6.4	9.2	19.5	1.5	96.6	6.0	7.4	15.6	3.0	99.7	5.2	6.9	14.7
Thiamethoxam	0.3	98.9	9.4	16.3	35.1	1.5	93.0	7.4	10.4	22.2	3.0	92.6	5.5	9.6	20.7
Dinotefuran	0.6	105.5	7.4	14.0	30.2	3.0	93.5	6.5	9.7	20.7	6.0	94.7	5.1	7.0	15.0

C: pesticide concentration, RSD_r: repeatability, RSD_R: reproducibility

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Methods

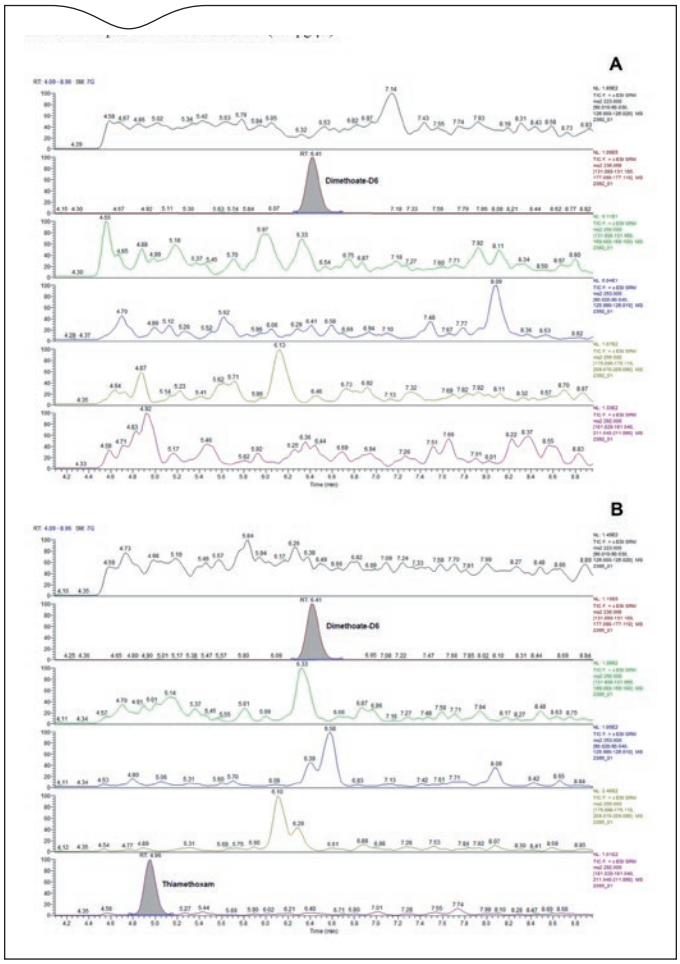

Figure 3: Chromatograms obtained by LC-MS/MS for (A) a blank sample of nectar of colza and for (B) a nectar of colza positive in thiamethoxam (0.5 pg/µl).

Acknowledgements

We thank the CETIOM and INRA Avignon (UMT PrADE) which supplied us nectars of colza.

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Research

Modelling *Listeria monocytogenes* contamination to improve surveillance in the agri-food industry

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Agri-food companies are accountable for the quality of the products that they place on the market. One way to check this quality is to determine how contamination is distributed. A sampling plan would be a useful decision-support tool. To determine the optimal batch sample size, we used an approach based on Bayesian decision theory for finished food products that minimises the average cost incurred by the manufacturer. Here, we used data on the presence of *Listeria monocytogenes* during the production of diced bacon. We built models to describe the *L. monocytogenes* concentration by taking into account various factors, we estimated parameters using Bayesian inference and then compared our models with real data. Finally, we developed a model to determine how to minimise the average costs incurred by a meat-processing company in the case of *L. monocytogenes* contamination in diced bacon.

Within- and between-batch sampling

Knowledge on contamination by pathogens in a food-processing plant is necessary for agri-food companies so that they can take appropriate actions to reduce contamination. To acquire this knowledge, analyses are necessary (e.g. counting or screening) on food products or surfaces. How should samples be taken at a given point in the production process? Should a sample be randomly chosen from the production line? Or should it be chosen randomly from each batch? These questions are not trivial because, according to the finished food product and the processing method, variability in contamination within and between batches can be very different. **Figure 1** shows two hypothetical cases of distribution of contamination among several batches. In Figure 1a, the between-batch variability is much lower than the within-batch variability. In this case, randomly choosing a sample from the entire production line without considering batch identity is sufficient. However, if the distribution of contamination resembles that shown in Figure 1b, sampling by batch is essential for determining whether a given batch is contaminated or not. Within- and between-batch variability has been studied recently (ILSI, 2010; Gonzales-Barron and Butler, 2011).

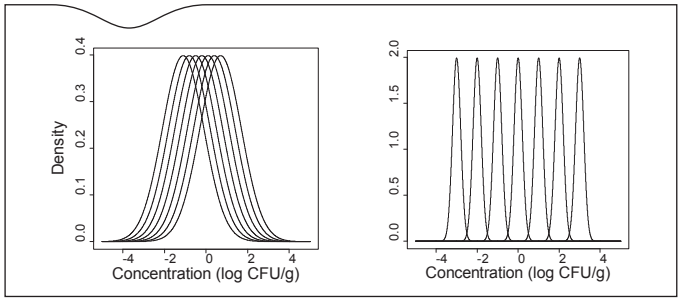

Figure 1: Representation of the variability within and between batches. Each curve shows the distribution of contamination in a given batch (log CFU/g). In Figure 1a (left), the standard deviation of contamination in a batch is 1 log CFU/g and the standard deviation between is 0.3 log CFU/g. For Figure 1b (right), the between-batches standard deviation is equal to 1 log CFU/g and the within-batch standard deviation is equal to 0.2 log CFU/g.

We begin by defining the term 'batch'. Although this term is used in everyday speech, it is not simple to define. According to European Commission Regulation (EC) No 2073/2005 (Article 2), a batch is "a group or set of identifiable products obtained from a given process under practically identical circumstances and produced in a given place within one defined production period." The definition given by the International Commission on Microbiological Specifications for Foods (ICMSF, 2002) begins by explaining that it is a quantity of food manufactured and handled in uniform conditions, but it goes further and indicates that this definition implies that the batch is homogeneous, e.g. the concentration of the contaminant follows a log-normal distribution. However, the ICMSF notes that batches do not always show homogeneous concentrations of microbial contaminants because microorganisms can be very heterogeneously distributed. The batch size should thus be adjusted according to the processing method. Nonetheless, statisticians modelling contamination must assume homogeneity to describe properly the distribution of contaminants in food production. Furthermore, the food business operator defines a 'batch' with respect to ensuring traceability and internal organisation.

Determining the structure of contamination in the production of diced bacon

To determine the structure of contamination, we sampled pork breast after the massaging process in a factory that produces fresh diced bacon and in which we analysed the presence and concentration of *Listeria monocytogenes*. A batch was defined as all the pork breasts contained in one tumbler, the first step in the production process. In total, eight or nine pork breasts were taken from 12 different batches. For each pork breast 100 cm² of meat was sampled and analysed to screen for detection and enumeration of *L. monocytogenes*. With the protocols used here, the limit of detection was 0.01 colony-forming units (CFU)/cm², while the limit of quantification was 0.2 CFU/cm². The raw data (presence or absence of detection, number of colonies counted) are shown in **Table 1**.

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Table 1: Raw data for the detection (0=absence, 1=presence) and the counts (number of CFUs counted on Petri dishes) of *L. monocytogenes* conducted on 100 cm² pork breast samples after tumbling, i.e. the first step in the diced-bacon production process, during which bellies are tumbled with brine for several hours in a tumbler. The same sample was used for both detecting and counting *L. monocytogenes*.

Batch number	Detection results	Counting results (CFUs)
1	0-1-1-1-0-1-1-1-1	0-0-0-0-0-0-0-0-0
2, 8, 9 & 10	0-0-0-0-0-0-0-0-0	0-0-0-0-0-0-0-0-0
3	0-0-0-0-1-0-0-0-1	0-0-0-0-1-0-0-0-0
4	0-1-0-0-1-0-0-0-0	0-0-0-0-0-0-0-0-0
5	0-0-0-0-1-0-0-0-0	0-0-0-0-0-0-0-0-0
6	0-0-0-0-0-0-0-0	0-0-0-0-0-0-0-0
7	0-0-0-1-1-0-0-0-1	0-0-0-0-0-0-0-0-0
11	1-1-1-1-1-1-1-1	11-9-6-5-12-29-16-3
12	0-0-1-0-0-0-0-0-0	0-0-0-0-0-0-0-0-0

The results were used in four contamination models:
- contamination structured by units and batches (model REF);
- contamination structured by batch (model B);
- contamination structured by food unit (model U);
- contamination with no structure (model NS).

The food unit was the individual pork breast because we wished to determine whether there was within- and between-unit variability along with within and between-batch variability. We used a Bayesian approach, which allows the incorporation of information other than raw data into the model.

All models include a combination of binomial, Poisson and normal distributions. Models NS, B and U are nested in model REF.

In model REF, x_{ijk} is the detection result (1 if positive and 0 otherwise) of batch *i*, pork breast *j* and test portion *k*; y_{ijkl} is the enumeration of batch *i*, pork breast *j*, test portion *k* and fraction *l*. A test portion is the sample of meat on which the experiments were carried out (here 100 cm²). A fraction is the volume of the solution composed of the test portion diluted in an appropriate culture broth that is poured onto a Petri dish to count *L. monocytogenes* colonies. Variable x_{ijk} follows a binomial distribution and variable y_{ijk} follow a Poisson distribution:

$$x_{ijk} \sim Bin(1, 1 - exp(-10^{\theta_{ij}} S_k))$$
$$y_{ijkl} \sim P(10^{\theta_{ij}} S_k d_l)$$

where θ_{ij} is the logarithm to base 10 of the concentration of *L. monocytogenes* in pork breast *j* belonging to batch *i*; S_k is the surface of test portion *k*, and *d* is the dilution of the fraction *l*. The log concentration θ_{ij} follows a normal distribution:

$$\theta_{ij} \sim N(z_i, \lambda^2)$$

where z_i is the log concentration of *L. monocytogenes* in batch *i* and λ is the standard deviation of the log concentration in the food units. The log concentration z_i also follows a normal distribution:

$$z_i \sim N(\mu, \sigma^2)$$

where μ is the mean log concentration and σ is the standard deviation of the log concentration in batches. For the priors, parameter μ follows a normal distribution and σ^2 and λ^2 both follow an inverse gamma distribution.

There is no unit effect in model B, so $\lambda=0$. Conversely, there is no batch effect in model U, so $\sigma=0$. Model NS has neither of these effects, so $\lambda=\sigma=0$. Models B, U and NS are described in **Table 2**.

Table 2: Description of models B, U and NS. Subscripts *i, j, k* and *l* refer to a batch, a food unit (i.e. pork breast), a test portion and a fraction, respectively.

Model B	Model U	Model NS
$x_{ik} \sim Bin(1, 1 - exp(-10_i^z S_k))$ $y_{ikl} \sim P(10_i^z S_k d_l)$ $z_i \sim N(\mu, \sigma^2)$	$x_{jk} \sim Bin(1, 1 - exp(-10_j^\theta S_k))$ $y_{jkl} \sim P(10_j^\theta S_k d_l)$ $\theta_j \sim N(\mu, \lambda^2)$	$x_k \sim Bin(1, 1 - exp(-10^\mu S_k))$ $y_{kl} \sim P(10^\mu S_k d_l)$

To determine the parameters of the prior distributions, we used the self-inspection results that various companies carry out in the meat-processing industry. The posterior distributions of the parameters in the models were estimated using OpenBugs software (Thomas *et al.* 2006). According to the experimental protocol we carried out, S_k=100 cm² and d_l=0.05. Quantiles of the posterior distributions of the four models are shown in **Table 3**.

Table 3: Descriptive statistics of the posterior distributions of models REF, B, U and NS.

Model	Parameter	Descriptive statistics of the posterior distributions				
		Mean	S.D.	2.5th perc.	50th perc.	97.5th perc.
REF	μ	-3.09	0.53	-4.25	-3.05	-2.15
	σ	1.55	0.49	0.89	1.45	2.77
	λ	0.38	0.08	0.25	0.36	0.57
B	μ	-3.12	0.51	-4.21	-3.09	-2.18
	σ	1.72	0.47	1.06	1.63	2.86
U	μ	-3.51	0.15	-3.81	-3.51	-3.21
	λ	1.99	0.24	1.59	1.97	2.51
NS	μ	-0.94	0.005	-0.95	-0.94	-0.93

S.D., standard deviation; perc., percentile

We investigated the ability of the models to replicate real data with a visual criterion based on data simulations: detection data were simulated using the posterior distributions of the parameters (same number of datasets per batch and same number of batches as for the observed data), then the proportions of batches with (1) only presences, (2) only absences, or (3) a mixture of presences and absences, were counted. This process was repeated *n* times to calculate the median and the credibility intervals at 50% and 95%. A credibility interval at *x*% indicates that there is an *x*% probability that a value is within the interval. The same process was then repeated for counting. The results are shown in **Figure 2**. The model that best replicated the data was model B. The model REF performed only slightly worse (not shown). Model B is the best of the four studied models. 3.

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Example illustrating the determination of the optimal sample size that minimises the costs for the company

Knowing the distribution of contamination helps to define a sampling strategy. However, sampling strategies must also consider the specific processing practices used in a given factory and the reasons for sampling. Several types of sampling plans are used in the agri-food industry. A widely used model is the two-class sampling plan: *n* products are sampled and screened (generally 25 g of finished product); if the number of positive results *y* exceeds a certain number *c*, then the batch is rejected (destroyed or sold for a different use); if not, the batch is delivered. A two-class plan assumes that the product is still in the factory when the results are made available, which is not always the case. To adapt this type of plan to bacon processing, we modified the definition of the sampling plan slightly. After discussion with an industry expert, we developed the following sampling plan:

- sampling is not based on a production batch but on a certain production time period (e.g. 1 week or 1 month);
- according to the number of positive results (x), three possible decisions are made by the processing plant: (1) do nothing; (2) take minor corrective actions because the prevalence of *L. monocytogenes* during the production period is intermediate; (3) take major corrective actions because contaminant prevalence is high.

Our goal was to determine the optimal sample size *n* as well as the thresholds c_1 and c_2, the values of *x* beyond which minor or major corrective actions, respectively, are taken. To achieve this goal, we used Bayesian decision theory. This theory was used to determine the best solution for an operator *in situ*ation of uncertainty. Application of this theory involves several steps:

- determine the set $\mathcal{D}$ of all the possible decisions (here, the three decisions described above);
- determine all the values $\mathcal{S}$ of the states of nature (here, contamination of pork breasts by *L. monocytogenes*) and the prior distributions;
- determine the set of all the observations $\mathcal{O}$ (here, bacterial detection and counts) and their distributions;
- define a so-called loss function *L* defined for $\mathcal{D} \times \mathcal{S} \times \mathcal{O}$ in $\mathbb{R}^+$ (see below);
- determine the best decision rule (function which associates a decision *d* with a set of observations), obtained by minimising the expected loss over the states of nature and the observables.

For more information on this theory, see Berger (1985), Parent (2007) or Robert (2006).

According to contaminant prevalence in the batches of finished product sampled during the chosen period, the customer (distributor) can apply a penalty for non-compliance with specifications and order additional tests over a given period of time. The cost of the penalties depends on the level of prevalence (i.e. the higher the prevalence, the higher the cost of the penalty), but can be adjusted according to any corrective actions taken by the meat-processing company (i.e. if the company applies a corrective action, the penalty decreases). To keep the model simple, prevalence was divided into three classes: low, intermediate and high. We asked our expert to estimate the cost of these penalties and the corrective actions. These are summarised in **Table 4**.

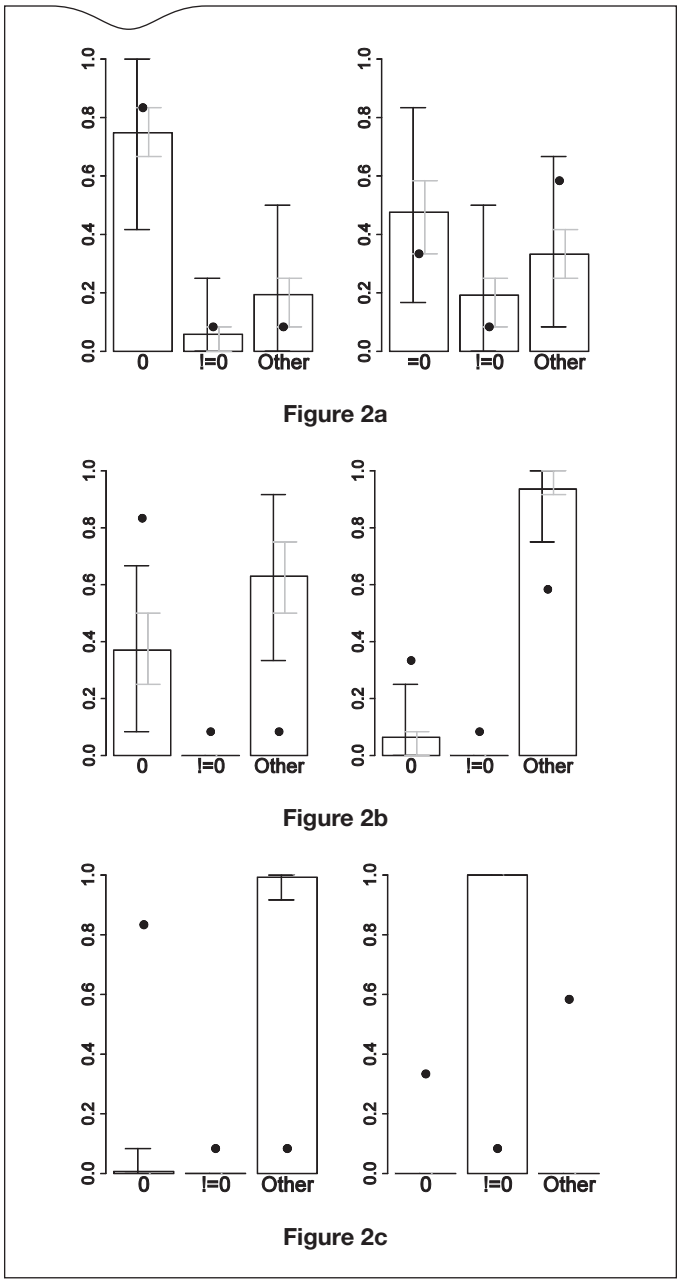

Figure 2a

Figure 2b

Figure 2c

Figure 2: Observed and simulated data for three models: (a) model B, (b) model U, and (c) model NS. The left-hand panel shows data for the detection method, and the right-hand panel data for the counting method. The histograms represent the average data for each group (0: proportion of batches with only null data, !=0: proportion of batches with only non-null data, and Other: all other batches). The grey error bar represents the credibility interval at 50% and the black error bar the credibility interval at 95%. The black dots indicate observed data.

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Table 4: Costs incurred (in euros) by the meat-processing company according to contaminant prevalence in the finished product and the decision made.

		Decision made		
		No action taken	Minor corrective action taken	Major corrective action taken
Actual results (Prevalence of contaminant)	Low prevalence	€0	€4 250	€14 000
	Intermediate prevalence	€6 200	€6 110	€14 930
	High prevalence	€92 050	€31 900	€27 800

The cost of sampling, estimated at €20 by the expert, must be added to each of these costs. To complete the calculation, we determined the thresholds of prevalence: below 0.2, prevalence is considered to be low and above 0.6, it is considered to be high. Finally, the beta distribution of parameters 2 and 3 was used to describe prevalence (see **Figure 3**). A beta distribution on parameters a and β has a probability distribution function equal to $\frac{\Gamma(\alpha+\beta)}{\Gamma\alpha+\Gamma(\beta)}x^{\alpha-1}(1-x)^{\beta}$, where $\Gamma t = \int_0^{\infty} z^{t-1}e^{-z}dz$. With this information, we can calculate the loss function which, for a given set of observations and value of contamination, there is an associated cost. The decision depends on the value of the observations; therefore knowing the observations automatically determines the decision to take.

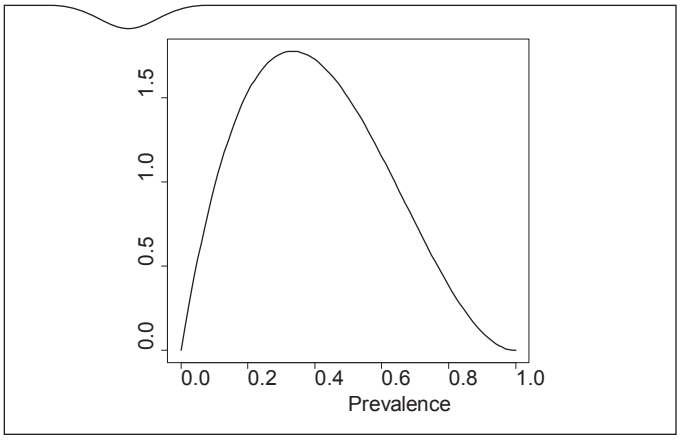

Figure 3: Distribution of prevalence between the different production periods.

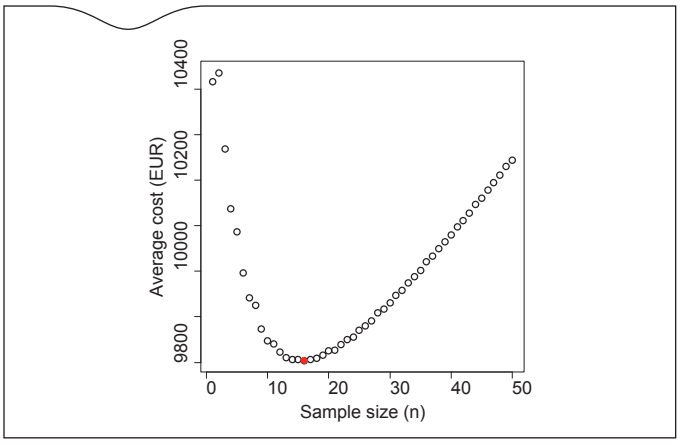

Figure 4: Average cost (in euros) incurred by the meat-processing company according to sample size n. The minimal cost is reached at n=16, c_1=4 and c_2=11 (red dot).

Based on this information and according to prevalence and analysis results, we calculated the average cost per production period for the meat-processing company with respect to sample size. By varying sample size, we can determine the value that minimises the average cost. The average costs based on the chosen numerical values are shown in Figure 4 and depend only on sample size. The minimum value was found for n=16, c_1=4 and c_2=11.

The distribution and thresholds of prevalence were set to complete the exercise. Obviously, when they vary this leads to a change in the optimal sampling plan: with a prevalence following a beta distribution of parameters 2 and 20 and thresholds of prevalence of 0.05 and 0.1, the average minimum cost for the company is reached at n=48, c_1=1 and c_2=6, which differs greatly from the previous result. Similarly, if costs vary then so does the sampling plan.

The application of Bayesian decision theory provides additional support for the decision-maker particularly *in situ*ations with many unknowns. This approach requires defining the population to which the method will be applied (e.g. here, we defined 'batch'), modelling prevalence, defining the set of decisions and their possible consequences, determining the costs, and, finally, carrying out probabilistic calculations. The final values depend strongly on the model used and current costs, which means that they must be defined carefully for each application. For more information on this work, see Commeau (2012).

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Agenda

EURL on Antimicrobial and Dye Residues in Food

Workshops

October 2013, EURL Fougères, France
Workshop organised by the ANSES Fougères Laboratory, EURL for Antibiotic and Dye Residues, and intended for analysts and managers from the EU's network of NRLs (32 participants): the workshop addressed issues on control of veterinary antibiotic drug residues in foods of animal origin and it also included a technical training session on the analysis by LC-MS/MS of antibiotic residues in honey products (Fougères, France).
Contact: Éric Verdon (eric.verdon@anses.fr)

Proficiency Testing Studies

December 2013, EURL Fougères, France
Provision of a Proficiency Testing Study to the attention of the EU-NRLs and to several Official Labs in Third Countries Worldwide.
Dedicated to the confirmatory control of Dye Residues in Aquaculture Products (Naturally Incurred Prawns)
Contact: Éric Verdon (eric.verdon@anses.fr) and Regine Fuselier (regine.fuselier@anses.fr)

March-May 2014, EURL Fougères, France
Provision of a Proficiency Testing Study to the attention of the EU-NRLs and to several Official Labs in Third Countries Worldwide is dedicated to the control of Chloramphenicol Residues, a banned substance in the EU, in Porcine Meat and Urine (naturally incurred testing materials)
Contact: Éric Verdon (eric.verdon@anses.fr) and Regine Fuselier (regine.fuselier@anses.fr)

EURL for Milk and Milk Products

Since October 3, 2013, the new website is open at https://eurl-milk.anses.fr

Workshops

October 2-3, 2013, Training session on AP determination in cheese (fluorimetric method)
October 3-4, 2013, Workshop dedicated to pasteurization tracers in milk & milk products
Contact:Bertrand Lombard (bertrand.lombard@anses.fr)
September/October, 2014, NRL-Workshop for milk and related products

Proficiency Testing Studies

October 7-18, 2013, PT trial on the counting of somatic cells in milk
November 27, 2013, PT trial on the determination of alkaline phosphatase in cheese

EURL for *Listeria monocytogenes*

Workshops

November 26-28, 2013, Training on *L. monocytogenes* PFGE-Typing
Contact: Adrien Assere (adrien.assere@anses.fr)
April 9-11, 2014, NRL-Workshop on *L. monocytogenes*, Teramo (Italy)

Proficiency Testing Studies

October 7, 2013, Proficiency Testing Trial dedicated to enumeration of *L. monocytogenes*

EURL for coagulase-positive Staphylococci

Workshops

November 5-7, 2013, Training session dedicated to CPS PFGE typing
June 4-6, 2014, NRL-Workshop on coagulase-positive Staphylococci, Maisons-Alfort (France)

Proficiency Testing Studies

November 19, 2013, detection of staphylococcal enterotoxins in food matrices
November 25-27, 2013, enumeration of coagulase-positive staphylococci in in powdered infant formulae.
Contact:Bertrand Lombard (bertrand.lombard@anses.fr)

EURL for equine diseases

Workshops

In 2014, two workshops will be organized, one on Glanders, another on Equine infectious anaemia

Proficiency Testing Studies

In 2014, two ring trials will be organized, one for CFT for Glanders, another for AGID test for Equine infectious anaemia
All detailed information will be given on http://www.ansespro.fr/eurl-equinediseases/

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