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Sharing and promoting Reference Laboratory activities
in Animal and Plant Health, Food and Drinking Water Safety

No.2 - *Trends in Reference at the European Union level*

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Editorial

Reference activities: what's cooking ?

On 25 March 2017, Rome hosted the celebrations for the 60th anniversary of the signing of the Treaty of Rome, a crucial step of the European integration process. This decision brought remarkable benefits to the citizens of Member States, *e.g.* in terms of peace, freedom of movement, trade opportunities, health protection and social rights. Common policies for animal and plant health as well as for food, feed and drinking water safety were established, aiming to assure the same level of consumer protection and health promotion in all Member States. The European reference structure, organised into networks of official control laboratories sharing information and good practices with their respective National and European Union Reference Laboratories, was a key aspect of this goal. As time goes by, opportunities arise to review the progress made and address the remaining gaps to be filled, as well as to take into account scientific developments providing both innovative solutions and new challenges.

EuroReference is fully involved in sharing and disseminating information in order to promote continuous development and harmonisation, as this second issue of the journal, focusing on "Trends in Reference Activities at the European Union level", aims to show. Two articles look at combining biotechnology and digital sciences to support European Union public health: one reports on the building of a database for molecular typing data on foodborne pathogens, and the other describes a multidisciplinary research network supporting an integrated joint platform for the detection and analysis of emerging infectious diseases and foodborne outbreaks in Europe. Harmonisation and standardisation, as key factors for uniform implementation of European Union legislation, have also been addressed in four other papers. Thus, a harmonised surveillance programme is presented that provides an overall picture of honeybee colony mortality within the European Union. The adoption of a national conversion system has led to a positive effect on the reproducibility of total bacterial counts in milk via automated instruments. A report on quality assessment procedures for biobank biological materials and reference specimens underpinned the importance of ensuring standard features and appropriate documentation. The experience of the French Plant Health Laboratory in developing methods within an accreditation framework with flexible scope was shared. Finally, the changes in regulations for the network of official, national and European Union reference laboratories with the release of the new regulation on official controls is also presented. We hope you will find these topics interesting and continue to be a reader and contributor to this journal.

Marina Patriarca & Umberto Agrimi

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The ECDC-EFSA molecular typing database for European Union public health protection

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§ Disclaimer: The positions and opinions presented in this paper are those of the authors alone and are not intended to represent the views or scientific works of their employers.

Abstract

Molecular typing or microbial DNA fingerprinting has developed rapidly in recent years. Data on the molecular typing of foodborne pathogens such as *Salmonella*, *Listeria monocytogenes* and Shiga toxin-producing *Escherichia coli* (STEC) could substantially contribute to the epidemiological investigations of foodborne outbreaks and to the identification of emerging health threats. Following the STEC O104:H4 outbreak in 2011, the European Commission asked EFSA and ECDC in January 2013 to provide technical support for the EU/EEA-wide collection of molecular typing data on foodborne pathogens from food, feed, animal, environmental and human samples. At that time point, ECDC had already been collecting equivalent data for human isolates since 2012. In addition, the European Commission asked EFSA and ECDC to perform regular joint analysis of these molecular typing data, which required the establishment of a joint database. This paper describes the architectural and procedural characteristics of the joint ECDC-EFSA molecular typing database. Rules regarding data sharing and confidentiality in the context of the data collection system are also presented. This database represents a firm basis that will, in the future, be upgraded to other typing methods such as whole genome sequencing.

Keywords

- Foodborne outbreak
- Foodborne pathogen
- Molecular typing
- Multiple Loci Variable-number tandem repeat Analysis (MLVA)
- Pulsed-Field Gel Electrophoresis (PFGE)

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Introduction

Molecular typing or microbial DNA fingerprinting has developed rapidly in recent years. Many typing methods, like polymerase chain reaction (PCR) techniques, pulsed-field gel electrophoresis (PFGE) and sequencing, have become part of routine strain characterisation in many laboratories. Molecular typing provides essential tools for different surveillance purposes such as monitoring spread of clones and strains, early detection of dispersed (international) outbreaks, and prediction of epidemic potential.

PFGE is the current standard method for *Salmonella*, *Listeria monocytogenes* (*L. monocytogenes*) and STEC typing. In addition, multiple loci variable-number tandem repeat (VNTR) analysis (MLVA) is the current standard method for further subtyping of *Salmonella* Typhimurium (*S.* Typhimurium) [Larsson *et al.*, 2013]. They are invaluable methods for routine surveillance of circulation of food and clinical strains.

Molecular typing data of foodborne pathogens such as *Salmonella*, *L. monocytogenes* and STEC could substantially contribute to the epidemiological investigations of foodborne outbreaks and to the identification of emerging health threats. For the three pathogens cited above, national and cross-border outbreak investigations in Europe are regularly supported by molecular typing information from Member States [*e.g.*, Fretz *et al.*, 2010; Friesema *et al.*, 2008; Inns *et al.*, 2015; Kinross *et al.*, 2014; Yde *et al.*, 2012]. In addition, molecular typing data make it possible to assess the molecular diversity and circulation of strains within the food chain and could be useful for source attribution studies when estimating the contributions of different food categories or animal species as sources of human infections.

At present, the circulation of food- and waterborne pathogens in the food chain and the occurrence of human clusters and outbreaks in the EU/EEA are monitored with various systems and tools. The European Food Safety Authority (EFSA) coordinates a network of nominated experts on zoonoses and zoonotic agents (Zoonoses Monitoring Data Network) and collects from Member States data on zoonoses, zoonotic agents, antimicrobial resistance and outbreaks according to the Directive 2003/99/EC on the monitoring of zoonoses and zoonotic agents (EC, 2003). The European Centre for Disease Prevention and Control (ECDC) manages a network of nominated epidemiologists and microbiologists, with special expertise in food- and waterborne diseases, under the Food- and Waterborne Diseases and Zoonoses Disease Programme (FWD DP). This network helps to provide human data to the European Surveillance System (TESSy), which is a highly flexible metadata-driven system for collection, validation, analysis and dissemination of human communicable disease data. The separately collected human and non-human data are analysed jointly by EFSA and ECDC and published annually in two European Union Summary Reports: one report on zoonoses, zoonotic agents and foodborne outbreaks, and another on antimicrobial resistance [EFSA and ECDC, 2015; 2016].

Three platforms exist for rapid and secure online exchange of information on detected foodborne threats in humans and hazards in food or feed: 1) the Epidemic Intelligence Information System for Food- and Waterborne Diseases (EPIS-FWD): an ECDC-hosted platform for communication and exchange of information about emerging clusters and outbreaks as well as unusual increases in human cases detected at the national level; 2) the Early Warning and Response System (EWRS): an official notification system of the European Commission (EC) and competent Public Health Authorities in Member States regarding events of cross-border relevance due to communicable diseases at the European Union (EU) level; and 3) the Rapid Alert System for Food and Feed (RASFF): an official system for sharing information on hazards found in food and feed and trade of (potentially) contaminated batches between Member States, and for tracing these batches back and forward.

The European Union Reference Laboratories (EURLs), established in accordance with Article 12 of Regulation (EC) No 882/2004 [EC, 2004], coordinate the implementation of the analytical methods in their respective networks of veterinary National Reference Laboratories

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(NRLs). In particular, they (i) provide NRLs with details of analytical methods, including reference methods, (ii) coordinate the application by the NRLs of the methods referred to in (1), in particular by organising comparative testing and by ensuring appropriate follow-up of such comparative testing in accordance with internationally accepted protocols, when available, (iii) coordinate, within their area of competence, practical arrangements needed to apply new analytical methods and inform NRLs of advances in this field, and (iv) conduct initial and further training courses for the benefit of staff from NRLs and of experts from developing countries. For this purpose, the EURLs conduct regular training sessions, annual workshops, and typing proficiency testing trials (PT trials).

The development of databases of molecular typing data represents a tool to support and enhance surveillance and monitoring of foodborne pathogens by allowing the linkage of genetic profiles of isolates from human cases of disease to similar genetic profiles of respective strains isolated from food, feed, animals and their environment. Being able to query such a repository makes it possible to improve preparedness for outbreak investigations. For the purpose of collecting usable typing data of pathogens isolated from food, feed, animals and the related environment as well as from humans, the standardisation of processes for typing data production, analysis and storage is essential.

Aim of the database

Following the outbreak of STEC O104:H4 infections in 2011 [EFSA, 2011; Frank *et al.*, 2011], a vision paper on the development of databases for molecular typing of foodborne pathogens with a view to outbreak preparedness was prepared by the EC [EC, 2012], in consultation with EFSA, ECDC and the EURLs for *Salmonella*, *L. monocytogenes* and *Escherichia coli*. The vision paper was endorsed by the Member States' food and veterinary competent authorities at the Standing Committee on Plants, Animals, Food and Feed (PAFF) (former Standing Committee on the Food Chain and Animal Health (SCFCAH)) meeting in December 2012. Soon after this, the EC asked EFSA and ECDC to provide technical support regarding the collection of molecular typing data on foodborne pathogens, namely *Salmonella*, *L. monocytogenes*, STEC and possibly others such as *Campylobacter*, from food, feed, animal, environmental and human samples. At that time point, the ECDC had already established an equivalent molecular typing data collection system for human isolates, which was operational since 2012. In addition, the EC asked EFSA and ECDC to perform regular joint analysis of the molecular typing data on these pathogens, which required the establishment of a joint database.

The purpose of the joint ECDC-EFSA molecular typing database (referred to as 'the joint database') is to share comparable typing data in a common repository so that microbiological data from humans can be linked to similar data from the food chain. This will enable and support early detection and investigation of cross-border foodborne outbreaks, will contribute to source attribution studies, and will enhance better understanding of the epidemiology of foodborne pathogens. At present, the molecular typing data collection covers PFGE for *Salmonella*, *L. monocytogenes* and STEC, together with MLVA for *S.* Typhimurium and *S.* Enteritidis. In addition, other typing data will be collected, including serotype and serogroup, when available.

Architecture of the database

The joint database is physically hosted at, developed and maintained by ECDC, and more specifically in the European Surveillance System (TESSy) [Van Walle, 2013]. Since 2012, typing data of strains isolated from human *Salmonella*, *L. monocytogenes*, and STEC infections are submitted to ECDC by public health authorities and laboratories of the Member States. Typing data on respective bacterial isolates from food/feed and animals and their environment (non-human data) are reported to EFSA (through the EFSA molecular typing data collection system) by the food and veterinary authorities and laboratories of the Member States. These

data are then submitted by EFSA to the joint database within 48 hours.

Figure 1 presents an overview of the overall logical architecture of the data collection system and describes the main entities (systems or user groups) involved in the context of the joint database, as well as the data flow of the shared information.

For each bacterial isolate from non-human samples, the data providers at Member State level generate the molecular typing results, PFGE and MLVA data [Caprioli *et al.*, 2014; Jacobs *et al.*, 2014; Peters *et al.*, 2017; Roussel *et al.*, 2014], and any other microbiological results together with the epidemiological data of the sample from which the isolate was obtained, according to EFSA requirements [EFSA, 2014]. In particular, data providers are required to structure their data according to a specific data model based on the Standard Sample Description ver. 2 (SSD2) [EFSA, 2013]. Data are then submitted to the EFSA's molecular typing database via machine-to-machine communication (*i.e.*, web service).

Data on human samples are collected through the TESSy that allows Member States to upload and analyse molecular typing data from isolates of *Salmonella*, *L. monocytogenes*, and STEC, including a minimum set of epidemiological data [Van Walle, 2013]. Standardisation of molecular typing results of *Salmonella*, *L. monocytogenes* and STEC across the participating laboratories is ensured by standard operating procedures (SOPs) developed by ECDC.

The whole process of non-human data collection, as well as the characteristics of EFSA's molecular typing database have been harmonised with the standards of the TESSy database, in order to support joint integrated analysis of molecular typing data from non-human and human isolates. The EFSA molecular typing data collection system interfaces with and submits data to the joint database through TESSy. To guarantee the confidentiality of non-human data for the respective data owners, the microbiological information (PFGE and MLVA typing data as well as serotype/serogroup) will be accompanied by a minimum subset of epidemiological data stored in the EFSA database for the purpose of sharing it in the joint database (Table 1). Additionally, the different user groups that are allowed to query the joint database have specific access rights, limiting their access to the information (Table 2). In particular, restrictions apply to 'sensitive' data that are visible only to the respective data providers and to all nominated authorised users from the same country. Data managers and data curators have access to all data present in the joint database.

Users and their role in the database

The actors involved in the process of molecular typing data collection and analysis in the joint database have different roles. Besides the responsibility of both ECDC and EFSA for the management of the database, two main roles are identified: data provider and curator.

The data providers (national public health reference laboratories for human data, and NRLs and other official laboratories in the Member States for non-human data):

- are nominated by the relevant Competent Authority at Member State level;

- are responsible for uploading microbiological data, including molecular typing data, and epidemiological data to ECDC and EFSA, respectively;

- can query the joint database for matching isolates for instance, and visualise and/or download the data depending on data accessibility rights.

The curators (*i.e.*, the relevant EURLs and ECDC's curators) have the responsibility to:

- assess the quality of data (when applicable) submitted by data providers;

- support data providers in correctly implementing the SOPs for molecular typing methods and provide suggestions for improving image quality in case of PFGE typing;

- assign molecular typing specific nomenclature (*e.g.*, reference types).

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In addition, EFSA, ECDC, EURLs and ECDC curators are in charge of the following tasks:

- perform regular scientific analyses of the data.

- provide technical support to internal and external users.

Curation

The integrated analysis of data stored in the joint database requires validation of the PFGE molecular typing data, namely the curation process, and assignment of reference types to the isolate profiles. This activity is carried out in the joint database by the EURLs (in their role as curators) for the non-human strains according to the relevant SOPs for curation [Caprioli *et al.*, 2014; Jacobs *et al.*, 2014; Roussel *et al.*, 2014], and by the laboratories specifically appointed for this task by ECDC (ECDC curators; currently Statens Serum Institute in Denmark for all three pathogens) for the human isolates. Briefly, the new PFGE profiles submitted are validated for their quality and are classified as either 'accepted' or 'rejected'. If the profile is accepted, a standardised sequential reference type is assigned to each indistinguishable PFGE pattern. The nomenclature of the reference types follows the TESSy nomenclature - short text codes analogous to *e.g.*, a serotype (*e.g.*, '*Asc*I.0001': *Asc*I for the restriction enzyme *Asc*I and the reference type number). The system offers the possibility for the data provider to consult, through the TESSy web interface, the joint database according to the differentiated access rights previously mentioned and which are described in a specific agreement. The system also offers data providers the possibility of downloading the results of the curation process, *i.e.*, whether their molecular typing data were accepted and what reference types were assigned and, in this way, to synchronise their database with the joint database at the EU level. This functionality is similar to what was set up in the EURL for the *L. monocytogenes* molecular database [Felix *et al.*, 2014].

The curation process forms an important quality step for PFGE so that any scientific analyses are performed (*i.e.*, cluster detection) on only those isolates meeting the minimum requirements for PFGE quality.

Analysis of data in the database

The joint analysis of human and non-human molecular typing data aims at finding joint microbiological clusters, based on the reference type, time, and geographical localisation of the strain profiles submitted, and identifying those that merit further attention and investigation at EU/EEA level because they may be part of a cross-border foodborne outbreak. This analysis is carried out in the joint database by ECDC and EFSA, with the support of the relevant curators, and the clusters are notified through the EPIS-FWD to the affected countries' public health and food safety and veterinary contact points. In case of specific public health threats, such as cross-border foodborne outbreaks or the emergence or re-emergence of specific clones of foodborne pathogens of particular concern, EFSA could search the EFSA molecular typing database to retrieve additional epidemiological information for the purpose of generating or testing hypotheses that could explain the clusters identified. The analysis of data and the investigation of an event are also supported by information shared by Member States through the EPIS-FWD, EWRS and RASFF.

Data confidentiality

Different rights for data accessibility are associated with each role. Moreover, to further protect the confidentiality of data, a collaboration agreement has been signed between the main actors in the database (ECDC, EFSA and EURLs). In addition, to avoid any improper or non-authorised use of the data, all data providers are asked to sign an agreement with EFSA or ECDC, based on their area of competence, before any data submission or access to the database.

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Participation in data collection

The Member States participate in data collection as data providers on a voluntary basis. The data providers are invited to upload their molecular typing data as soon as these become available, in order to maximise the usefulness of molecular typing data collection for disease prevention and control, and food and feed safety. The upload of historical data from the participating laboratories is also encouraged. This would support source-hypothesis generation when a multi-country outbreak is under investigation, but would also contribute to a better understanding of the epidemiology and transmission routes of foodborne pathogens.

Discussion

The joint ECDC-EFSA molecular typing database has been designed to allow the timely identification of microbiological clusters of public health relevance and support epidemiological investigation.

This represents the first attempt to implement a fully integrated enhanced surveillance/monitoring system for foodborne pathogens and related human infections according to the 'One Health' principle at the EU level. The system was designed to be compliant with the legal requirements and the official role of relevant institutions under the legislation of the public health and the food safety/veterinary area. In addition to the legal aspects and the technical challenges in developing a system able to support outbreak detection and investigation for public health purposes at the European level, major efforts were made to ensure that the system is attractive to data providers and in particular is able to support their surveillance activities at the national level, while respecting the sensitivity of the data. This is achieved by offering the data providers the possibility of retrieving the results of the curation process from the joint database and the assignment of the reference type. In addition, data providers have the possibility of searching, based on their access privileges, the joint database in order to perform their own analysis in the EU/EEA context, while respecting confidentiality of data.

Within the international community, the importance of sharing data is increasingly recognised. This provides numerous benefits including a more efficient and contextualised analysis of data and information. However, its adoption also entails overcoming a number of barriers with respect to legislation, data quality, data completeness, data timeliness and participation. In particular, there are concerns about the ultimate use of the data provided by data producers, generators and collectors without data owner's explicit permission. For these reasons, a compromise has been found between the policies for data accessibility and data protection, and this compromise has subsequently been agreed with both public health and food safety and veterinary Competent Authorities representing all Member States. The sharing of a limited set of descriptive data and the differentiated rules for data accessibility for the users of the joint database guarantee that the relevant scientific information is shared between all official actors in the process, but limits the possibility of tracing back restricted information, thereby ensuring compliance with the obligation to protect confidentiality.

The strength of the system itself is the clear definition of rules, procedures and actors involved in a cross-sectoral environment, increasing the potential for protection of public health from widely spread and dispersed foodborne infections across countries, which are otherwise hard for Member States to control in a sustainable manner. This approach guarantees the assignment of specific roles to the actors involved in the process, the clear understanding of what data will be shared and how data will be used, the harmonisation and high quality of the information received, and the comparability of results between the public health and food safety/veterinary sectors.

The data collection system, designed according to the mandate received, works based on the voluntary participation of the Member States. The data collection system will have real added value only if a substantial number of Member States submit a consistent volume of data. Real-

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time data will support the detection and investigation of ongoing foodborne outbreaks, and historical data will contribute to source attribution studies and will enhance better understanding of the epidemiology of foodborne pathogens.

The database can be extended to other pathogens and methods, following the agreement between the relevant actors. This database represents a firm basis that will, in the future, be upgraded to other typing methods such as whole genome sequencing (WGS) as the technology and capacity at the EU level improves, while at the same time benefitting from an existing structure of rights/responsibilities in line with EU regulations.

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APPENDIX

FIGURE 1 / Structure of the joint ECDC-EFSA database

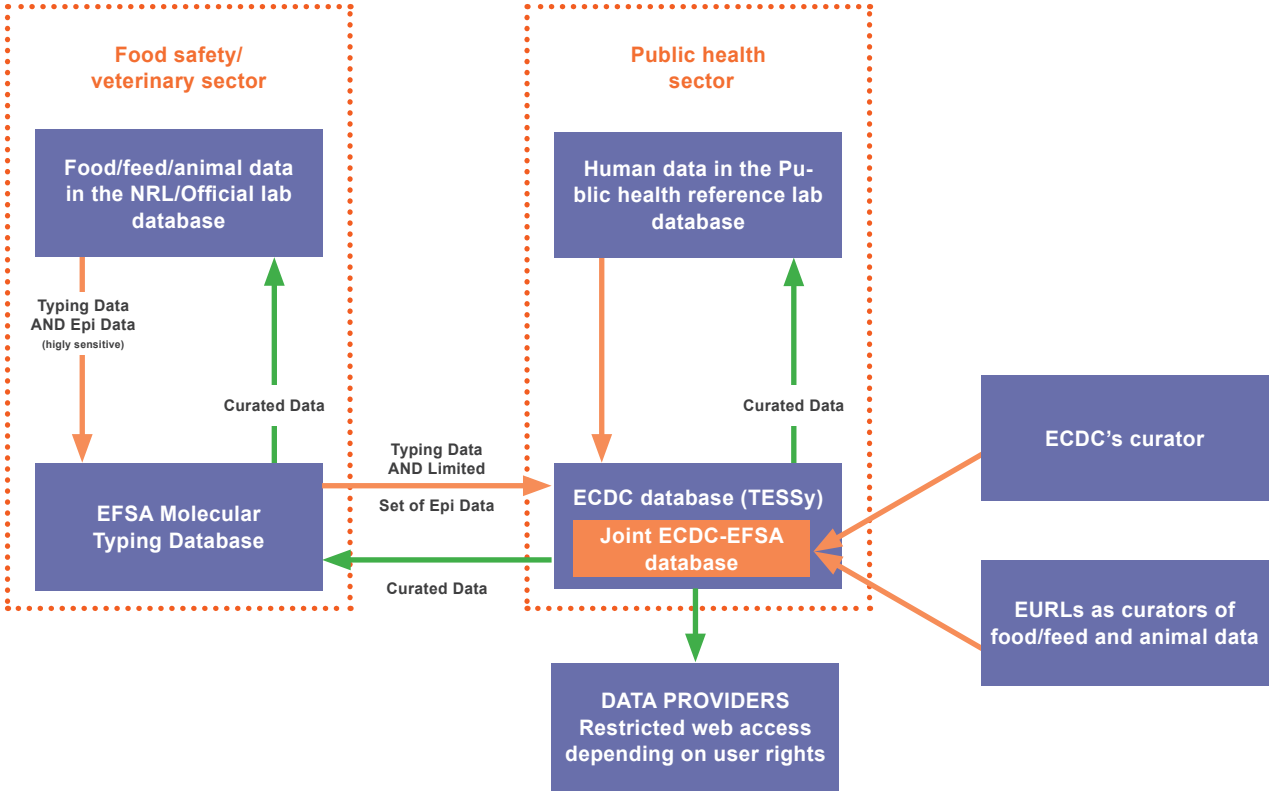

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TABLE 1 / Type of data stored in the Joint Database

Food safety/veterinary sector	
Non-sensitive data	Microbiological data, limited to • Molecular typing data: PFGE and MLVA. • Other typing data: *Salmonella* serotype, *Listeria* serotype and STEC serogroup. EFSA Isolate Id Date of sampling Date of receipt of isolate in the reference laboratory Type of sample: defines the source of the isolate, *e.g.*, 'animal', 'food', 'feed', 'environment'.
Sensitive data	Country of sampling Laboratory identification code
Human sector[1]	
Non-sensitive data	Microbiological data, limited to • Molecular typing data: PFGE and MLVA. • Other typing data: *Salmonella* serotype, *Listeria* serotype and STEC serogroup. ECDC Isolate Id Date of sampling Date of receipt of isolate in the reference laboratory
Sensitive data	Reporting country

1. All other human descriptive data such as age and gender are physically stored in the same system (TESSy) but are not part of the Joint Database.

TABLE 2 / Access to the Joint Database

User group[1]	**Non-human data (food, feed, animal, environmental data)**				**Human data**			
	Country of sampling, Laboratory identification code	**Date of sampling/ sample type**	**Microbiological data**[2]	**Food, feed, animal or environmental descriptive data**[3]	**Country of sampling**	**Date of sampling/ sample type**	**Microbiological data**[2]	**Human descriptive data**[4]
EFSA	Yes	Yes	Yes	No (not in Joint Database)	Yes	Yes	Yes	No
ECDC	Yes	Yes	Yes	No (not in Joint Database)	Yes	Yes	Yes	Yes[5]
Users from Member State food/ veterinary side	Only if isolate is from the same country as the user	Yes	Yes	No (not in Joint Database)	Only if isolate is from the same country as the user	Yes	Yes	No
Users from Member State human side	Only if isolate is from the same country as the user	Yes	Yes	No (not in Joint Database)	Yes	Yes	Yes	Yes[5]
Curators non-human data	Yes	Yes	Yes	No (not in Joint Database)	Yes	Yes	Yes	Yes
Curators human data	Yes	Yes	Yes	No (not in Joint Database)	Yes	Yes	Yes	Yes[5]

1. EC has the right upon request to receive any data related to a specific event.
2. PFGE and MLVA typing as well as serotype/serogroup
3. Detailed description of the sample, *e.g.*, food category/animal population, origin. These are considered sensitive data and are not part of the Joint Database.
4. More information on the patient, *e.g.*, age, gender. These are considered sensitive data.
5. These data are stored physically in the same system (TESSy), but are conceptually not part of the Joint Database.

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The first pan-European epidemiological study on honeybee colony losses (2012-2014) revealed winter colony losses up to 32.4% and seasonal colony losses up to 11.1%

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Abstract

For the first time, a harmonised active epidemiological surveillance programme on honeybee colony mortality (EPILOBEE) was set up in 17 European Union Member States for two consecutive years. The national protocols were based on guidelines issued by the European Union Reference Laboratory for Honeybee Health (EURL). The objective of the two-year programme was to obtain an overall picture of honeybee colony losses on a harmonised basis in each of the participating Member States.

Winter colony mortality rates ranged from 3.2% to 32.4% and from 2.4% to 15.4% during the first and the second year of the programme. Rates of seasonal colony mortality (2013) ranging from 0.02% to 10.2% did not drastically change during the second year of the programme in 15 of the 16 Member States taking part in EPILOBEE for two years.

This programme was a descriptive epidemiological study enabling the collection of official and comparable data on honeybee health over two years with a methodology that was feasible and repeatable. The outcomes of EPILOBEE are an essential prerequisite to the implementation of future explanatory studies investigating the potential causes of honeybee colony losses such as pesticides and their possible interactions with pathogens or other stress factors.

Keywords

- Epidemiological data
- European Union
- Honey bee
- mortality
- surveillance programme

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** see Table 6.

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Introduction

Over the years, honeybee health has become a major concern. Many publications that have looked into colony losses in any part of the world have reported that several biological and environmental factors acting alone or in combination have the potential to cause colony mortality (Genersch *et al.*, 2010, Henry *et al.*, 2012, Vanengelsdorp *et al.*, 2013). In the United States and Canada, alarming losses of honeybee colonies were reported (Vanengelsdorp *et al.*, 2007, Vanengelsdorp *et al.*, 2009). In Europe, the decrease in honeybee colonies was estimated at 16% between 1985 and 2005, and the reduction of beekeepers at 31% (Potts *et al.*, 2010). European beekeeping reports have also provided worrying insights on the difficulties facing honeybee hive health, sometimes accompanied by colony losses (Hendrikx *et al.*, 2010). However, it has also been described that standardised surveillance systems are needed to accurately assess bee health in Europe (Hendrikx *et al.*, 2010).

To document this phenomenon, a consortium was set up in 2009 following a call launched from EFSA to assess existing surveillance systems and to collate and analyse the data related to honeybee colony mortality across Europe. In the conclusions of the report “Bee mortality and bee surveillance in Europe”, the weakness of the surveillance systems implemented in the European Union was highlighted as well as the lack of comparable data on colony losses. It was concluded that a common operational system to assess honeybee colony mortality at the European level was needed. The recommendations of the report pointed out the need to develop and enhance standardised EU surveillance systems to accurately assess bee health in Europe (Hendrikx *et al.*, 2010).

In this context, the European Commission requested harmonised and comparable data at the European level. A call was launched following the guidelines issued by the EURL. The first harmonised active epidemiological surveillance programme on honeybee colony mortality (EPILOBEE) was set up for two years in September 2012 with 17 and 16 European Union Member States participating for the first and second year, respectively. The objective of the two-year programme was to quantify the mortality of honeybee colonies on a harmonised basis in each participating Member State. Simultaneously, the main honeybee infectious and parasitic diseases were investigated based on case definitions and a sampling protocol provided by the EURL to assess honeybee colony health. Information related to beekeeping practices (treatments administered, livestock management), the beekeeper (training, experience in beekeeping), and the environment around the apiaries was also recorded.

Methods

Study design

The EPILOBEE surveillance programme was implemented over two consecutive years (September 2012 to September 2014). It was designed to collect data on a representative sample of apiaries and colonies in each participating Member State through harmonised onsite investigations and a sampling framework. The sampling framework was based on two-stage random sampling with apiaries as primary units and bee colonies as secondary units. Representativeness was reached through a random sampling of apiaries implemented by each Member State either in the entire Member State or in some regions of the Member State considered as representative of the Member State’s situation. Beekeepers and apiaries were randomly selected in each Member State from a national list of beekeepers that was as complete as possible. Within each apiary, the number of tested colonies was randomly selected according to the probability of detection of mortality and bee diseases. A total of 17 Member States participated in the programme during the first year, and 16 in the second year (Table 1). About one third of the beekeepers were renewed during the second year, to avoid the population under study being different from the general population. New beekeepers were selected with the same methodology as the one selected during the previous year.

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TABLE 1 / Number of randomly selected apiaries and colonies during the first visits of the two years of the programme in the Member States taking part in EPILOBEE.

England and Wales are reported as one Member State, taking part in the 2012-2013 project only.

	Number of apiaries visited during		Size of the apiaries visited during autumn 2013 (%)[1]			Number of colonies inspected during[1]	
	Autumn 2012	Autumn 2013	<50 colonies	[50-150]	>150 colonies	Autumn 2012	Autumn 2013
Belgium	149	150	100	0	0	624	644
Denmark	203	212	100	0	0	1,393	1,243
Estonia	197	196	91.3	8.7	0	2,337	1,616
Finland	161	161	100	0	0	787	682
France	343	350	93.7[2]	6.0[2]	0.3[2]	2,265	2,331[6]
Germany	223	217	99.1[3]	0.9[3]	0[3]	1,971	1,879
Greece	162	67	40.3	46.3	13.4	2,639	1,060
Hungary	197	185	45.1[4]	40.8[4]	14.1[4]	3,936	3,810
Italy	184	166	79.4[5]	17.6[5]	3[5]	1,969	1,849[7]
Latvia	194	190	90	8.4	1.6	1,937	1,918
Lithuania	191	163	51.5	44.8	3.7	2,483	2,061
Poland	190	190	73.2	24.2	2.6	3,207	3,147
Portugal	147	145	95.2	4.8	0	778	865
Slovakia	190	198	88.4	11.1	0.5	3,199	3,036
Spain	204	190	43.7	54.7	1.6	2,325	2,157
Sweden	151	150	100	0	0	730	758
England and Wales	200	-	-	-	-	891	-
Total	**3 286**	**2 930**				**33 471**	**29 056**
Mean			80.7	16.8	2.5		

1. Unless otherwise stated below, the rates (%) and numbers of colonies inspected were calculated on the number of apiaries visited in autumn 2013
2. The calculation was based on 331 apiaries
3. The calculation was based on 210 apiaries
4. The calculation was based on 184 apiaries
5. The calculation was based on 165 apiaries
6. The calculation was based on 333 apiaries
7. The calculation was based on 163 apiaries

Surveillance protocol

Three visits were performed by bee inspectors each year: before winter (2012 and 2013), after winter (spring 2013 and 2014) and during the beekeeping season (summer 2013 and 2014). Farming practices, description of the environment and clinical signs of the main infectious and parasitic diseases were recorded through a detailed questionnaire. Samples were taken if necessary for further laboratory analyses. Each selected colony was thoroughly inspected and examined.

Each Member State organised the training of the bee inspectors on the basis of the documents provided by the EURL. Each Member State was also in charge of implementation of the visits in consistent periods of time for comparison purposes.

It is important to acknowledge that remarkable work involving many different stakeholders belonging to different levels, from the ministry to the field, was carried out during the two years of

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EPILOBEE, producing an extensive set of data that was as reliable as possible. Particularly, a huge effort was required regarding the data validation (for details see the report produced by Jacques *et al.* 2016).

Data collection and management

The overall information collected can be found in the EPILOBEE reports published on the European Commission website (Laurent *et al.*, 2015). The questionnaire filled in by the bee inspectors was refined and clarified for the second year of EPILOBEE thanks to feedback from the field. Some questions were added for the second year (*e.g.* the record of colony strength) whereas others were removed (*e.g.* location of the migration, name of all the treatments applied in colonies). These modifications improved the forms without compromising the data collected and their comparison throughout the two-year programme. Data were stored in a standardised way in an online European database via a website developed by the EURL and the French Platform for epidemiological surveillance in Animal Health.

The descriptive analyses were performed using R software (version 3.1.0). Due to the size of the database (9,566 apiary visits and 117,269 laboratory analyses the first year, and 8,580 apiary visits and 49,626 laboratory analyses the second year), a data cleaning step was necessary to identify recording errors. Dedicated R algorithms were used to identify duplicates or nonsense data and incorrect or missing data were discarded from the calculation (Chauzat *et al.*, 2016).

Calculation of mortality rates at the colony level

The calculation of mortality rates was related to the size of the apiaries. Hence, the rate of affected honeybee colonies (*i.e.* colony mortality θ) was a weighted average, by the apiary size, of the affected honeybee colony rate of each apiary, and calculated as follows:

$$\hat{\theta} = \frac{\sum_{i=1}^{n} (Mi\ \hat{P}i)}{\sum_{i=1}^{n} Mi}$$

where Pi was the proportion of colonies affected in the apiary (*i.e.* number of affected colonies divided by the number of observed colonies) and Mi was the size of the apiary (*i.e.* all the colonies of the apiary whether they were randomly selected or not).

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Results

Rates of winter colony mortality from EPILOBEE 2012 – 2013 ranged from 3.2% to 32.4% (Figure 1a and Table 2). In 12 Member States, this rate exceeded 10%. Most of the Northern European Member States had winter mortality rates higher than 10% with the highest rate in Belgium (32.4%). The lowest rate of colony mortalities (3.2%) was recorded in Lithuania.

FIGURE 1 / Winter colony mortality rates in the Member States of the European Union recorded in EPILOBEE 2012 – 2013 (a) and EPILOBEE 2013 – 2014 (b)

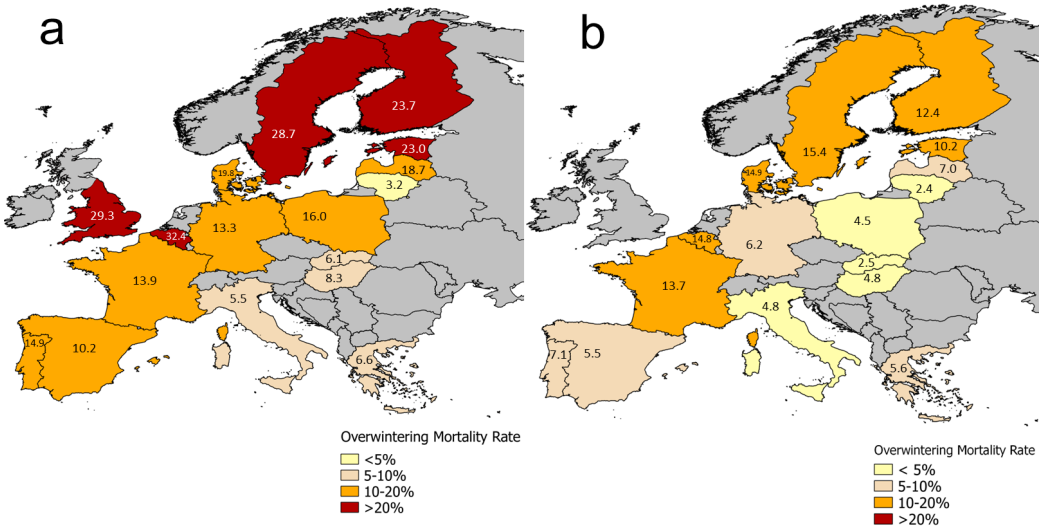

TABLE 2 / Winter mortality rates in the Member States of the European Union recorded in EPILOBEE 2012–2013

	Mortality rate (%)	95% CI[1] lower limit	95% CI[1] upper limit
Belgium	32.4	25.4	39.3
Denmark	19.8	15.6	23.9
Estonia	23.0	16.9	29.1
Finland	23.7	19.2	28.1
France	13.9	11.0	16.8
Germany	13.3	10.3	16.4
Greece	6.6	4.5	8.6
Hungary	8.3	5.8	10.8
Italy	5.5	3.6	7.5
Latvia	18.7	14.7	22.7
Lithuania	3.2	1.8	4.7
Poland	16.0	12.4	19.6
Portugal	14.9	10.0	19.7
Slovakia	6.1	3.5	8.8
Spain	10.2	7.8	12.5
Sweden	28.7	24.8	32.6
England & Wales	29.3	24.9	33.7

1. 95% CI = confidence interval at 95%

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Rates of winter colony mortality (2013-2014) ranged between the Member States from 2.4% to 15.4% (Figure 1b and Table 3). The winter colony mortality rates exceeded 10% in six Member States. In five of the 16 Member States, the winter colony mortality rates were lower than 5%. In each Member State, the winter 2013-2014 colony mortality rates were lower than the rates estimated during winter 2012-2013; none of the rates were over 20% (Figure 1).

TABLE 3 / Winter colony mortality rates in the Member States of the European Union recorded in EPILOBEE 2013–2014

	Mortality rate			
	%	Difference between the two years[2]	95% CI[1] lower limit	95% CI[1] upper limit
Belgium	14.8	↓	11.4	18.3
Denmark	14.9	→	10.9	18.8
Estonia	10.2	↓	7.4	13.0
Finland	12.4	↓	9.3	15.4
France	13.7	→	8.3	19.0
Germany	6.2	↓	3.2	9.1
Greece	5.6	→	0.3	10.9
Hungary	4.8	→	3.4	6.2
Italy	4.8	→	2.3	7.3
Latvia	7.0	↓	5.0	9.0
Lithuania	2.4	→	0.5	4.3
Poland	4.5	↓	2.8	6.1
Portugal	7.1	↓	4.5	9.6
Slovakia	2.5	→	1.4	3.5
Spain	5.5	↓	3.9	7.2
Sweden	15.4	↓	10.7	20.1

1. 95% CI = confidence interval at 95%
2. ↓: statistical difference between the two years towards a decrease;
   →: no statistical difference between the two years

However, it should be noticed that these rates were estimates of the real winter colony mortality rates based on representative samples of the honeybee population in each Member State. The confidence intervals in which the real colony mortality rates could be found with 95% probability were calculated (Table 2 and Table 3). For seven Member States (Denmark, France, Greece, Hungary, Italy, Lithuania and Slovakia), the winter colony mortality rates were not statistically different between the two consecutive years since confidence intervals overlapped. Conversely, winter colony mortality rates decreased statistically during the second year for nine Member States.

Rates of seasonal colony mortality (2013) ranged from 0.02% to 10.2% (Figure 2a and Table 4). The seasonal mortality rate was higher than 10% only in France. The seasonal mortality rates were lower than 5% for 12 of the 17 Member States. Rates were between 5 and 10% in Belgium, Finland, Spain and the United Kingdom (England and Wales).

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FIGURE 2 / Seasonal colony mortality rates in the Member States of the European Union recorded in EPILOBEE 2012 – 2013 (a) and EPILOBEE 2013 – 2014 (b)

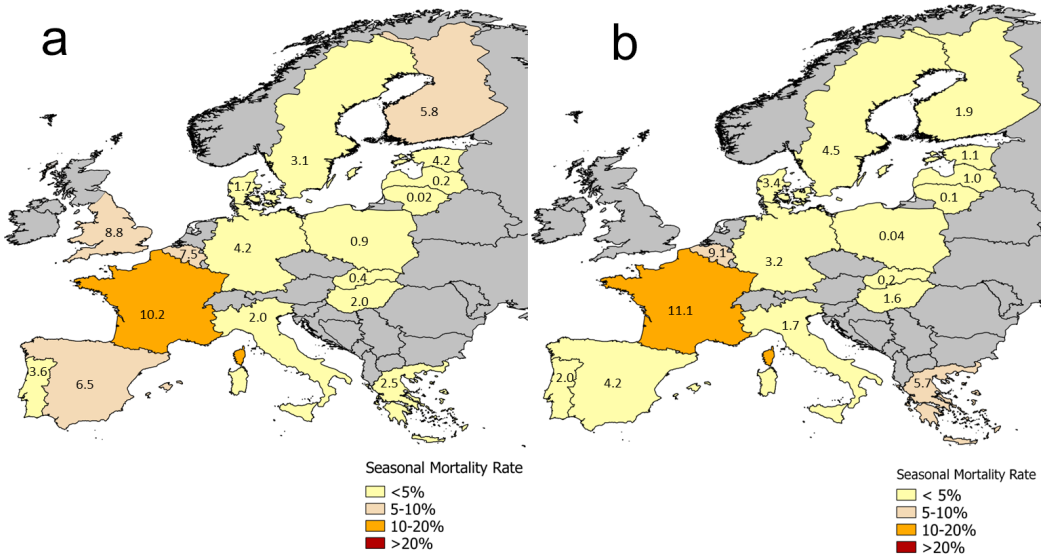

TABLE 4 / Seasonal mortality rates (2013) in the Member States of the European Union recorded in EPILOBEE 2012–2013

	Mortality rate (%)	95% CI[1] lower limit	95% CI[1] upper limit
Belgium	7.5	2.5	12.5
Denmark	1.7	0.2	3.1
Estonia	4.2	1.5	6.9
Finland	5.8	2.8	8.9
France	10.2	5.9	14.4
Germany	4.2	0.9	7.4
Greece	2.5	1.0	3.9
Hungary	2.0	0.6	3.5
Italy	2.0	0.5	3.5
Latvia	0.2	0	0.5
Lithuania	0.02	0	0.1
Poland	0.9	0.2	1.6
Portugal	3.6	0.2	7.0
Slovakia	0.4	0.1	0.8
Spain	6.5	4.4	8.5
Sweden	3.1	0.1	6.0
England & Wales	8.8	5.7	11.9

1. 95% CI = confidence interval at 95%

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TABLE 5 / Seasonal mortality rates (2014) in the Member States of the European Union recorded in EPILOBEE 2013–2014

	Mortality rate		95% CI[1] lower limit	95% CI[1] upper limit
	%	Difference between the two years[2]		
Belgium	9.1	→	4.6	13.6
Denmark	3.4	→	2.1	4.7
Estonia	1.1	→	0.2	1.9
Finland	1.9	→	0.8	3.0
France	11.1	→	4.7	17.6
Germany	3.2	→	1.7	4.7
Greece	5.7	→	0	12.9
Hungary	1.6	→	0.7	2.4
Italy	1.7	→	0.7	2.8
Latvia	1.0	→	0	2.1
Lithuania	0.1	→	0	0.3
Poland	0.04	↓	0	0.1
Portugal	2.0	→	0.9	3.2
Slovakia	0.2	→	0.1	0.4
Spain	4.2	→	2.9	5.5
Sweden	4.5	→	2.1	6.9

1. 95% CI = confidence interval at 95%
2. ↓: statistical difference between the two years towards a decrease;
   →: no statistical difference between the two years

Rates of seasonal colony mortality (2014) ranged from 0.04% to 11.1% (Figure 2b and Table 5). Seasonal colony mortality rates were below 5% in 13 Member States. The rate was over 10% only in France. The mortality rate during the 2014 beekeeping season was lower than the rate estimated during the 2013 beekeeping season for nine of the 16 Member States (Figure 2). Conversely, an increase in the seasonal colony mortality rate was observed during the second year for seven Member States (Belgium, Denmark, France, Greece, Latvia, Lithuania and Sweden).The confidence intervals in which the real seasonal colony mortality rates (2014) could be found with 95% probability overlapped with the confidence intervals calculated for the 2013 beekeeping season in 15 of the 16 Member States (Table 4 and Table 5). This means that seasonal colony mortality was statistically different from one year to the other in only one case (Poland), towards a decrease.

Discussion

Reliability and robustness of the protocol

This two-year active surveillance was implemented on a harmonised basis in 17 Member States for the first year and in 16 Member States for the second year, thus allowing comparisons between Member States and joint statistical analyses.

More than 90% of the apiaries randomly selected at the beginning of each year of the pro-

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gramme were monitored throughout each entire year. Given the scale of the programme, this high rate of follow-up shows the great involvement of all the stakeholders in each Member State and emphasises the feasibility and repeatability of EPILOBEE.

Winter colony mortality rates

As discussed previously (Chauzat *et al.*, 2014), no reference values are available for the acceptable level of colony losses during winter. Different winter colony losses have been reported in European countries (Charrière and Neumann 2010, Genersch *et al.*, 2010) and outside Europe (Vanengelsdorp *et al.*, 2008, Head *et al.*, 2010, Spleen *et al.*, 2013, Traynor *et al.*, 2016). For the purpose of the study, honeybee colony mortality of 10% during winter was empirically considered acceptable by the EURL. However, this threshold is debatable, since higher mortality rates can be considered as bearable by beekeepers and scientists.

During the second year of EPILOBEE, winter colony mortality rates were over the acceptable threshold of 10% in one third of the Member States (Belgium, Denmark, Estonia, Finland, France and Sweden). A south-north geographical pattern could be observed. Ten Member States had winter colony mortality rates lower than 10%, which correspond to 64.5% (8,931,600 colonies) of the total estimated number of colonies in the European Union in 2011 (Chauzat *et al.*, 2013). In contrast, Member States with winter colony mortality rates higher than 10% represented 13.2% (1,831,075 colonies) of the total estimated number of colonies in the European Union in 2011. The Member States that did not take part in EPILOBEE represented around 22.3% of the EU colonies (data from 2011).

The mortality rates for winter 2013 – 2014 showed a narrower range (2.4% to 15.4%) than the mortality rates observed during the winter 2012 – 2013. The decrease in winter colony mortality rates over these two years is noticeable. However, this should be interpreted with caution. The confidence intervals in which the real winter honeybee colony mortality rates can be found overlapped for Denmark, France, Greece, Hungary, Italy, Lithuania and Slovakia, meaning that the drop of the winter colony losses for 2013 – 2014 was not statistically significant for these Member States. Conversely, the winter colony mortality rates decreased statistically between the two years for nine Member States (Belgium, Estonia, Finland, Germany, Latvia, Poland, Portugal, Spain and Sweden).

The comparison of the confidence intervals for the seasonal mortality rates did not show any statistical difference between the two years for all Member States, with the exception of Poland for which the seasonal colony mortality rate decreased statistically during the 2014 beekeeping season.

It is known that climate strongly influences winter colony losses but other risk factors may also play a role. Specific statistical analyses have been conducted to explore statistical links between the colony losses and other information collected over the two years (health of the colonies, management of the apiary, use of veterinary treatments, environment) (Chauzat *et al.*, 2016, Jacques *et al.*, 2016). Therefore, there is a need for a holistic assessment of colony health, taking also the environment around the colony into account.

Sustainable outcomes

The first major outcome of this programme was the collection of representative and comparable data on honeybee colony mortality on a harmonised basis in the Member States taking part in EPILOBEE. In addition, this two-year programme enabled enhancement of the general European honeybee colony surveillance structure, methodology and capability of veterinary services, which most probably led, as a consequence, to better management of the European apiculture sector. EPILOBEE allowed the implementation of monitoring tools that did not exist to this extent in Europe prior to the programme. National surveillance systems also benefited from this experience in the field of bee health.

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Furthermore, it has been shown that communication, particularly between beekeepers and veterinary services, increased during EPILOBEE and was a positive outcome of the programme. Some beekeepers participating in the two years of EPILOBEE may have benefited from the successive visits leading to an improvement of management practices and health conditions in the apiaries. The data collected during the two consecutive years for these beekeepers are under study.

Harmonisation of the training of bee inspectors set up in each Member States on sampling, observation and interpretation of clinical signs and detection of exotic arthropods in Europe were key factors to EPILOBEE success. The programme was a good opportunity to increase awareness among beekeepers taking part in EPILOBEE concerning the detection of clinical signs associated with the main parasitic and infectious diseases affecting honeybees.

Perspectives of the EPILOBEE programme

Representative and comparative data on honeybee health were collected over these two years, showing that the methodology implemented in EPILOBEE was feasible and repeatable. However, the methodology was adapted in each Member State taking into account their specificities. The specific diversity in data collection has been included in the statistical analyses. Further harmonisation of national procedures could be implemented at the European level by taking into account the specific characteristics of each Member State highlighted during EPILOBEE. EPILOBEE has shown that harmonisation of sampling protocols and field training is fundamental to collect comparable and robust data. During this programme, a large set of data was collected, requiring significant data management, edition and data mining. Since the programme was originally designed for fewer Member States than finally involved, it might have been necessary to reduce the extent of data collected to better adapt to the size of the project and thus ease overall data management. EPILOBEE was the essential first step for the recording of honeybee mortality and health status at a European scale through a descriptive surveillance programme. However, these two years should be prolonged in order to obtain a significant collection of data on colony mortality that could then be considered a baseline for future studies. For instance, during EPILOBEE, winter 2013-2014 was relatively warmer and shorter than winter 2012-2013, which was particularly long and cold throughout Europe. These two winters were opposite in terms of weather, showing the importance of long-term follow-up.

This descriptive programme, EPILOBEE, was a successful first step that will facilitate future implementation of projects (*e.g.* explanatory studies) examining other risk factors affecting colony health. For example, the study of potential causes such as pesticides, pathological agents, and food intake either on their own or in combination, could be integrated into future explanatory studies, such as case-control studies, in order to explore their role in honeybee colony mortality. These epidemiological projects require the joint commitment of all stakeholders and planned action strategies.

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This project was funded by the European Commission, ANSES through the EURL for bee health, and each Member State taking part in EPILOBEE. This programme involved thousands of different stakeholders over the two years of the project: beekeepers, field inspectors, scientists, laboratories and administrations. The EURL for bee health wishes to thank all participants for their substantial involvement in the successful implementation of EPILOBEE.

Mike Brown, Per Kryger, Franco Mutinelli, Marc Schäfer and Sophie Roelandt provided useful remarks and expertise during EPILOBEE through the EpiTeam.

The list of people who took part in this project under the name EPILOBEE Consortium is provided in Table 6.

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TABLE 6 / The EPILOBEE Consortium

Country	Name	Institutional affiliations
Belgium	De Graaf D.	Ghent University, Department of Physiology, Laboratory of Zoophysiology
	Méroc E.	NRL for honeybee diseases CODA-CERVA-VAR
	Nguyen B.K.	Ulg, Faculté Gembloux Agro-Bio Tech
	Roelandt S.	NRL for honeybee diseases CODA-CERVA-VAR
	Roels S.	NRL for honeybee diseases CODA-CERVA-VAR
	Van der Stede Y.	NRL for honeybee diseases CODA-CERVA-VAR
Denmark	Tonnersen T. Kryger P.	(NRL) Aarhus University
Estonia	Jaarma K. Kuus M. Raie A.	Estonian Veterinary and Food Board
Finland	Heinikainen S. Pelkonen S. Vähänikkilä N.	EVIRA, Veterinary Bacteriology Research Unit, Kuopio
France	Andrieux C.	DDPP du Cantal
	Ballis A.	Chambre d'Agriculture du Haut-Rhin
	Barrieu G.	DDPP des Bouches du Rhône
	Bendali F.	Direction Générale de l'Alimentation
	Brugoux C.	Groupement de Défense Sanitaire du Cantal
	Franco S.	LNR Abeilles Anses Sophia Antipolis
	Fuentes A.M.	Groupement de Défense Sanitaire de la Drôme
	Joel A.	DDPP Finistère
	Layec Y.	Groupement de Défense Sanitaire Apicole du Finistère
	Lopez J.	DDPP Indre et Loire
	Lozach A.	Groupement de Défense Sanitaire Apicole du Finistère
	Malherbe-Duluc L.	Groupement de Défense Sanitaire Indre et Loire
	Mariau V.	DDPP Indre et Loire
	Meziani F.	Direction Générale de l'Alimentation
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	Mutel S.	DDCSPP Haut-Rhin
	Oesterle E.	Groupement de Défense Sanitaire Indre et Loire
	Orlowski M.	DDPP de la Drôme
	Petit M.	DDPP Finistère
	Pillu P.	DDPP du Cantal
	Poret F.	Groupement de Défense Sanitaire du Cantal
	Viry A.	Laboratoire d'Analyses du Jura
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	de Craigher D.	University of Hohenheim, Apicultural State Institute, Stuttgart
	Genersch E.	Institute for Bee Research, Hohen Neuendorf
	Kaatz H.H.	University of Halle-Wittenberg, Zoology Dept., Halle
	Meixner M.D.	LLH Bieneninstitut Kirchhain
	von der Ohe W.	LAVES Institut für Bienenkunde, Celle
	Otten C.	Dienstleistungszentrum Ländlicher Raum, Fachzentrum Bienen und Imkerei Mayen
	Rosenkranz P.	University of Hohenheim, Apicultural State Institute, Stuttgart
	Schäfer M.O.	Institute of Infectiology, Friedrich-Loeffler-Institut, Greifswald - Insel Riems
	Schroeder A.	University of Hohenheim, Apicultural State Institute, Stuttgart

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Country	Name	Institutional affiliations
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	Arfara S.	Centre of Veterinary Institutes of Athens
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Quality assessment of biobank biological materials and reference specimens

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Abstract

Biobanks play a pivotal role in scientific progress and public health development as they can make available high-quality biological samples and provide access to associated data that are otherwise difficult to find for scientists in both human and veterinary medicine. The majority of relevant studies on epidemiology, pathogenesis, diagnosis and prevention of infectious diseases are based on obtaining biological specimens collected over long-term sampling. Moreover, the storage of specimens in biorepositories also offers the possibility of further evaluating samples with “next-generation” technologies that may not be available when the samples were originally collected. Furthermore, recent advances in molecular biology and genetics have increased the demand for properly preserved specimens and all relevant associated data on a large scale. Nevertheless, it is difficult to obtain samples with well-known features, except for those that are received from certified centres. The quality control assessment procedures used to evaluate the samples stored in the Biobank of Veterinary Resources (BVR) at the Lombardy and Emilia-Romagna Experimental Zooprophylactic Institute (IZSLER), have made it possible to ensure standard features and have improved the information related to stored biological materials.

Keywords

- Biobank
- Biological material
- Quality control
- One Health
- Veterinary medicine

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Introduction

About 75% of emerging and re-emerging human disease outbreaks throughout the world over the past two decades have been caused by pathogens of zoonotic origin (Hinchliffe, 2015). A wide range of animal species, both wild and domestic, could be reservoirs for these pathogens, which may be viruses, bacteria or parasites. Furthermore, increasing globalisation, livestock, pets and wildlife, as well as international trade and travel contribute to the spread of pathogens, and global warming is favouring the transmission of vector-borne diseases (Balogun *et al.*, 2016). Within this context, a transnational approach to zoonosis prevention and control programmes is required. In developing countries, contact between humans and animal populations in the surrounding environment is particularly close. Human health and animal health are inextricably linked; nevertheless, there is still separation between the human and animal health sectors (Rabinowitz and Conti, 2013). In order to overcome this gap, a global integrative concept, often referred to as "One Health", has been developed and strongly endorsed in the last few decades, reflecting the need for collaboration in the field of surveillance (Capps and Lederman, 2015; Scotch *et al.*, 2009).

Biobanking is an essential tool for ensuring easy availability of high-quality biomaterial collections that include essential samples and their associated data that are otherwise difficult for researchers to access (Zielhuis, 2012). In fact, supply of biological resources with poorly described features and stored in inadequate conditions is a recurring problem because samples may lack the required quality for research purposes (Carter and Betsou, 2011). Furthermore, an increasing number of test methods rely on the use of certified, stable and validated biological materials. In order to overcome these problems and meet modern requirements for biological materials, the Organisation for Economic Co-operation and Development (OECD, 2001) introduced a new concept of repositories of high-quality samples and information. Subsequently, to address these issues, the OECD (2007) published the *Best Practice Guidelines for Biological Resource Centres* (BRCs) and developed the document *Best Practice Guidelines for the Microorganism Domain*, with the purpose of ensuring that microorganisms held and supplied by BRCs meet high standards and are authentic. Furthermore, the World Health Organization (WHO, 2010) published the *Recommendations for the evaluation of animal cell cultures as substrates for the manufacture of biological medicinal products and for the characterization of cell banks*, to provide guidance to National Regulatory Authorities and National Control Laboratories and manufacturers on the basic principles and procedures for the characterisation of animal cells that are to be used in the manufacture of biological products. More recently, the World Organisation for Animal Health (OIE) provided guidelines on the preparation, validation and distribution of antibodies as International Reference Standards for antibody assays for infectious diseases of animals. Such standard preparations are designated by the OIE as primary reference standards for tests described in the *OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals*.

The field of veterinary research is rapidly evolving with new technologies and new standards. The European Technology Platform for Global Animal Health (ETPGAH) has identified the lack of biological material as one of the main gaps in the development of new effective tools for the control and prevention of animal diseases. Biobanks play a pivotal role in improving epidemiological research, which relies on the availability and quality of samples and the associated data. In particular, for retrospective studies and longitudinal designs for evaluating the course of diseases, the requirements for obtaining time-specific data are even stronger. Furthermore, biological materials are an essential resource for genomic research. Significant research is being carried out in genomics to improve efficiency of selection for healthier animals with disease-resistance properties.

As the need to access high-quality materials has increased at the global level, within the Biobank of Veterinary Resources (BVR) of the IZSLER, we have established a panel of quality tests to evaluate the features of the stored samples. These assays are specific for the

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different biological resources that are stored to be used as reference materials for research and other purposes. All these tests are performed in compliance with the UNI CEI EN ISO/IEC 17025 Standard and the biobank infrastructure is certified ISO 9001:2008. The migration to ISO 9001:2015 is currently in progress. The purpose of this paper is to briefly describe the quality controls performed on the major requested resources at the BVR, including cell cultures, bacteria, mycoplasmas and viruses, and to present various practical considerations for proper storage of biological materials.

1. Cell cultures

Cell lines are critical components of experiments and should be considered as standard reagents for research like other commercial laboratory products (Hughes *et al.*, 2007). In fact, cell line misidentification and contamination with microorganisms (such as bacteria, mycoplasmas, fungi and viruses), together with both genetic and phenotypic instability, are among the recurrent problems that can arise in cell culture laboratories. Contamination with microorganisms is quite simple to detect and is well regulated from the normative point of view. On the contrary, in the past, cell line authentication was not considered a real concern by the scientific community and it was not routinely performed.

As reported by Nardone (2007), misidentification and inter- and intra-specific cross-contamination of cell cultures represent a frequent and widespread problem with an estimated incidence of 18–30% cross-contaminated continuous cell lines, that makes scientific results unreliable and jeopardises the validity of data in literature (Hughes *et al.*, 2007; Parodi *et al.*, 2002). Frequently, the invading cells are better adapted to the culture conditions and grow faster than the original cells. Because of the morphological similarities of different cell lines, it is impossible to rely only on microscopic observations to screen for cross-contamination. In fact, with the progress made in karyotyping methods, it became apparent that about one third of all cell lines used in research were misidentified (Reid and Mintzer, 2012). Human cell lines are most frequently contaminated by HeLa cells, but also by a number of other rapidly-growing cell lines (Kniss and Summerfield, 2014). Nowadays, authentication testing should be considered an essential part of good cell culture practice to assure researchers that the cell line used is a valid experimental model (Capes-Davis *et al.*, 2010). In addition to authentication, there are other issues that should be considered when assessing the quality of a cell line (Almeida *et al.*, 2016). For these reasons, all cell types in the BVR undergo quality testing in order to evaluate the suitability of their features for their purpose. These controls are carried out using different techniques, including cell characterisation, authentication and microbiological testing.

■ 1.1 Characterisation and authentication

Cell characterisation includes viability tests that are performed on all cell cultures before freezing and after thawing, in order to evaluate cryopreservation efficiency. Furthermore, trypan blue staining is usually performed along with cell culture proliferation. Several methods for the authentication of cell lines have been developed for the detection of inter- and intra-species cross-contamination (Reid, 2011). Isoenzyme profiling is the method suggested by the European Pharmacopoeia to detect interspecies cross-contamination; this analysis uses polymorphic enzymes that can be visualised as electrophoretic variants, giving rise to a specific pattern for each species (Nims *et al.*, 1998). This assay, if applied routinely in cell culture management, can greatly improve the detection of cellular cross-contamination. Furthermore, karyotyping can be used alone or to complement isoenzyme analysis, in order to reveal interspecies genetic differences to distinguish between cell lines with characteristic karyotypes (Ono *et al.*, 2007). Additionally, profiling of short tandem repeat (STR, also called genomic microsatellite) polymorphisms has been adopted for forensic work and by major repositories for the detection of intra-species differences in human cell lines and tissues (O'Brian, 2001; Reid and Mintzer, 2012).

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1.2 Microbiological quality controls

The most significant risk in cell culture laboratories is contamination by several microorganisms that include bacteria, fungi, yeasts, mycoplasmas, and endogenous and exogenous viruses. Contaminating microorganisms can be present in the sample tissue or organ from which the cells were derived. They can also be transferred with animal reagents or unintentionally introduced into the manufacturing process by inadequate laboratory practices.

Bacteria, yeasts and fungi contamination

Currently, specific tests for the detection of bacteria, yeasts and fungi are used as part of routine and regular quality control screening procedures for biological samples. These tests are usually performed in aseptic conditions to avoid interfering contaminations. Bacterial contamination in cell cultures is frequently evident to the naked eye, showing as sudden increasing turbidity and colour change of the culture medium, due to pH variation. Daily observation of cultures ensures early detection of contaminants and helps to prevent contamination of other cultures. Nevertheless, to detect low levels of contamination, samples from the cell cultures or supernatants should be inoculated either in liquid Tryptic Soy Broth (TSB) for the detection of aerobes, facultative anaerobes and fungi, Fluid Thioglycollate Medium (FTM) for the detection of aerobes and anaerobes, or onto solid growth media (Trypticase-Soya agar, Blood agar, Sabouraud's Dextrose agar and Malt Extract agar). These inoculated media are incubated for different amounts of time and at different temperatures (generally 25°C or 37°C), depending on the optimal conditions required for pathogen growth and depending on the testing standards used.

Although these conventional microbiological techniques are in routine, widespread use as standard sterility tests, they are based on inoculation of broth cultures that may not support the growth of all contaminating microorganisms. Alternative molecular methods, such as identification by PCR and DNA sequencing of ribosomal RNA may be used.

Mycoplasma contamination

Mycoplasmas have long been recognised as common contaminants of cells in continuous culture, but their presence could go unnoticed for months and even years. In fact, even though many *Mycoplasma* species produce severe cytopathic effects, others may cause very little evident morphological modification of the cultured cells (Drexler and Uphoff, 2002). As mycoplasma competes with the cells for the nutrients in the culture medium, typical signs of contamination consist of a reduction in the cell proliferation rate and changes in cellular physiology including gene expression, metabolism and phenotype (Nübling *et al.*, 2015). Even though these multiple effects do not affect the various cells in the same manner and to the same extent, mycoplasma contamination significantly impacts all cell cultures in terms of quality and safety, and may affect the scientific results of cell culture-based research, as well as the quality of biologics manufactured by cell culture in the biopharmaceutical industry (Armstrong *et al.*, 2010; Knezevic *et al.*, 2010; Laborde *et al.*, 2010). *Mycoplasma*-infected cell lines are themselves the most important source for further spreading of the contamination. Consequently, it is essential for all cell stocks and all new cultures entering a laboratory to be routinely tested for the presence of mycoplasmas. The most frequent contaminants of the bovine group of mycoplasmas are *Mycoplasma arginini* and *Acholeplasma laidlawii*: these species have a relatively wide host range as they have been isolated from cattle, sheep, and goats, and from a variety of other mammals, as well as from birds and insects (Drexler and Uphoff, 2002). In the past, these cell culture contaminants derived from bovine sera that were not routinely screened for mycoplasmas. *Mycoplasma orale* is the most frequent mycoplasma of human origin and it can be transmitted by the personnel if good laboratory practices are not followed. *Mycoplasma hyorhinis*, a common inhabitant of the nasal cavity of swine, also accounts for a high proportion of the contaminations (Drexler and Uphoff, 2002). Nowadays, several methods are available for the detection of mycoplasmas, including isolation on selective

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microbiological growth media, direct or indirect fluorescent staining, ELISA, immunostaining, and PCR-based techniques (Pisal *et al.*, 2016). In the past, culture in agar was considered the gold standard assay, but some "difficult" species of *Mycoplasma*, which require specialised culture conditions, can be missed.

The tests established for these organisms include: broth/agar culture, assays for mycoplasma-characteristic enzyme activities, and DNA staining. Currently, mycoplasmas are tested for in all cell cultures stored in the BVR by indirect staining with a fluorescent dye such as Hoechst 33258 which binds DNA. Among the wide variety of techniques that have been developed to detect *Mycoplasma* contamination of cell cultures, Uphoff and Drexler (2013) recommended PCR analysis, as it is considered the most reliable and useful detection method. Most primers use highly conserved sequences, attempting to detect a broad range of *Mycoplasma* species. On the contrary, direct staining of cultures is not recommended, as it often yields unclear results and will only reliably detect heavily contaminated cultures (Young *et al.*, 2010). Furthermore, the presence of *Mycoplasma* infection could be evaluated by several biochemical tests that detect mycoplasmal toxicity or enzymes.

Many of these methods are used in several commercial kits and are specifically able to detect viable organisms. By measuring the level of ATP in a sample, both before and after the addition of the substrate, a ratio can be obtained that is indicative of the presence or absence of mycoplasmas. This is measured indirectly with a luminometer, recording biolumination catalysed by the reaction of the ATP and luciferase.

Viral contamination

The risk of viral contamination is a common feature to all biologicals, whose production involves the use of reagents of animal or human origin. Viral contamination of cell cultures may arise from the source material (cell banks of animal origin, human or animal tissues that may contain endogenous viruses) or as adventitious (exogenous) agents introduced by laboratory handling or during the production process (Merten, 2002). The animal-derived materials used for the growth of the cells, such as animal sera, or for detaching cells, such as porcine trypsin, are of particular concern as many different animal viruses can potentially be present (Chen *et al.*, 2008). Their presence could influence the biology of cells in a significant way, as amongst other effects they may modify the transcription factor networks and change the susceptibility of these cells to infection by other viruses. Viral contamination can be evaluated by a panel of tests to detect pathogens, other endogenous viruses (such as retroviruses) and adventitious viruses. Usually, this panel of tests includes: electronic microscopy investigation for the observation of endogenous viruses, reverse transcriptase (RT) detection (as a general test for retroviruses), and indirect techniques such as immunofluorescence. Further tests can also be performed to find specific suspected agents, depending on the animal species of the sample and on the origin of the biological products used in the cultures.

In addition, the presence of suspected infectious adventitious viruses is investigated by *in vitro* direct methods: cell samples are co-cultured with susceptible cell lines (indicator cells) capable of detecting a wide range of viruses. As reported by Schiff (2005), a minimum of three cell lines that include a human diploid cell line (MRC-5), a monkey kidney cell line (Vero), and a cell type of the same species and tissue of origin are usually selected. After inoculation, cultures are incubated at 37°C in 5% CO2 for 5-7 days and observed daily for the potential cytopathic effect of several viruses. Virus detection by testing the inoculated cell culture for haemadsorption and/or haemagglutination at the end of the examination period is necessary for viruses with no observable cytopathic effect. At present, cell cultures can also be tested for a panel of potential adventitious viruses by PCR or real-time PCR analysis.

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2. Bacteria

The majority of bacterial strains stored in the Biobank of IZSLER are isolated during laboratory diagnostic activities on field-biological samples originating from farm animals, pets, and wildlife or isolated from feed and food of animal origin. Microorganism identification, including bacterial classification and pathogen detection, is essential for the correct diagnosis of diseases, the possible treatment of the infection, and the epidemiological investigation of outbreaks associated with microbial infections. Bacterial strains are identified either by phenotypic or genotypic tests. Phenotypic testing consists of a preliminary analysis to check the taxonomic identity of the isolated strain that often involves one or more phenotypic techniques, including the study of the biochemical profiles and metabolic properties of a microorganism by testing its growth requirements and enzymatic activities. Phenotypic identification methods are suitable for microorganisms with well-established growth parameters, and physiological and biochemical profiles. The biochemical tests are performed in specific growth media, compounds or growth conditions to stimulate an observable or measurable biochemical response of the microorganism, thereby enabling its identification and characterisation. Several commercial kits for biochemical tests like the API or the Vitek systems are currently available for rapid identification of microorganisms. Furthermore, the analysis of morphological traits can be performed to obtain an initial identification of a microorganism by routine techniques such as culture tests in specific culture media, and subsequent microscopic observation. Morphological properties include: shape, size, surface characteristics and pigmentation, cell wall characteristics (Gram-staining), sporulation characteristics, mechanisms of motility, and other cellular characteristics. Genotypic tests, mainly based on PCR, are carried out to detect genes of virulence. Moreover, 16S rRNA gene sequence analysis can be used to confirm the bacterial species (Iraola *et al.*, 2016). The development of molecular methods has greatly improved the ability to rapidly detect, identify and classify bacteria and also establish the taxonomic relationship among closely related genera and species. Identification, using molecular methods, relies on the comparison of the nucleic acid sequences (DNA, RNA) of a microorganism with documented data on known organisms. These methods are considered sensitive enough to allow detection of low concentrations of viable or non-viable microorganisms in both pure cultures and complex samples. Real-time PCR has proven useful for distinguishing specific sequences from a complex mixture of DNA. More recently, genome studies have been performed to characterise organisms. In the future, further information gained from complete profiling investigations of the transcriptome, proteome and metabolome may be available. Several modern technologies such as microarray analysis are increasingly used for the characterisation of microorganisms, with particular reference to some genotypic characteristics, including virulence determinants and antimicrobial resistance patterns (El-Adawy *et al.*, 2016). Finally, each isolated and propagated strain is subjected to quality controls for viability and purity by culture and microscopic investigation before biobanking. Each batch is tested again for viability after preservation and then, at regular intervals, for viability and compliance with the expected features

3. Mycoplasmas

The *Mycoplasma* genus includes several human and animal pathogens. Mycoplasmas are the smallest (0.2-0.8 µm in diameter) and simplest prokaryotes that lack a cell wall; the flexibility of their cell membrane allows them to pass through commonly used anti-bacteriological filters with diameters of 0.45 µm. These small bacteria depend on their hosts for many nutrients, due to their limited biosynthetic capabilities. The majority of the strains stored in the biobank of IZSLER are isolated from different animal species and selected among the pathogens of veterinary interest. Molecular assays are performed directly on the tissue or diagnostic sample. If the culture fails and *Mycoplasma* infection is suspected, the identification of the strain can be done by sequencing the PCR product. Once isolated from the field samples, these strains are classified for their morphology and growth behaviour, and purified by cloning. They are iso-

lated in liquid and/or on solid media and identified by biochemical and genetic methods. Colony cloning of the isolates is performed in order to evaluate their viability before the storage and to obtain the necessary amount of culture, to prepare the necessary number of lyophilised vials. Biochemical and/or molecular tests, including sequencing, are adopted as standardised methods. Furthermore, all strains provided for biobanking deposit are tested for viability and purity by sub-culturing, after time intervals planned for the different strains.

4. Viruses

Viruses are obligate intracellular parasites that require living systems for their replication. Viral culturing is an amplification method that increases the amount of the pathogen, facilitating detection and characterisation. Culture methods allow the detection of many different viruses, including some that are not suspected when the culture is established, and can provide an isolate of viable virus that can be further characterised and stored for future studies. Both reference and field viruses isolated from several animal species are stored in the biobank of IZSLER. Several methods can be used to detect and identify viruses for diagnostic purposes and are mainly based on serology tests and end-point or real-time PCR. These assays amplify specific viral genome sequences known to be characteristic of a virus with a nucleotide sequence available in database collections. In addition, two types of electron microscopy methods are available for viral particle detection: direct or immuno-electron microscopy. With direct methods, negative staining is most often used and the specimens may be used directly or the virus particles may be concentrated before negative staining, to increase the sensitivity of the detection level. Immuno-electron microscopy methods may be particularly useful for viral identification and classification if the number of viral particles present is small. Furthermore, cell-associated viruses can be isolated from several types of samples and grown in adherent or suspension cell cultures or chorio-allantoic membranes of embryonated hen's eggs. The main principle for isolating viruses is to choose the most suitable cell line and subsequently carry out several amplification passages to increase the virus titre, in order to produce the "master samples" and the "working samples". These batches are tested for potential microbiological contamination by microbiology, virology, serology and molecular biology methods. Contamination may take place at various steps of the manufacturing process, including the starting sample, the amplification procedure itself (through biological reagents and media), or during inadequate laboratory handling. Currently, contamination by extraneous viruses is verified through several assays based on molecular biology techniques. Bacterial contamination is verified through the inoculation of non-selective culture media, and the absence of *Mycoplasma spp.* in the final stock is assessed by real-time PCR, as also reported for cell cultures.

5. Storage of biological storage

Proper storage of the biobank resources includes the use of cryovials and labelling systems that will withstand the storage conditions: vessels and labels are selected for extended storage periods. Depending on specimen features, the intended use, and the estimated length of storage, bio-specimens are usually stored at −80°C, at −196°C (liquid nitrogen) or at –189°C (vapour phase nitrogen). Otherwise, microorganisms and viruses can undergo a lyophilisation process that ensures the viability of freeze-dried samples for extended periods at 4°C or at –20°C. Temperature represents a potential risk in the storage process and is one of the main points that must be controlled to maintain sample integrity. In fact, in addition to safety and security of the buildings to protect against fire, unauthorised access, and other usual hazards, monitoring temperature within mechanical freezers and refrigerators is an essential quality assurance measure. For these reasons, all freezers of the infrastructure are fitted with real-time temperature monitoring and alarm systems. The corresponding temperature logging information is automatically transferred through an electronic interface to the biorepository management system and is linked to the samples stored in the corresponding freezer. If a measured value is no longer between the upper or lower limits, an alarm is sent to the staff

members. Any outage of electrical power is compensated by an independent system such as a generator with locally controlled production. To preserve biological resources in the case of a disaster or unexpected events, part of the samples stored at the BVR are intended as backup samples and are located in a separate infrastructure for safety and security reasons. This additional “mirror banking” ensures that if samples are compromised for whatever reason, replicate aliquots of good integrity will still be available.

CONCLUSION

Biobanking is an emerging multidisciplinary and dynamic activity that involves the collection and preservation of several types of samples, but the potential value of a biobank depends on the quality of samples and on the maintenance of their integrity. Biobanks with well characterised specimens will be essential for future research and development efforts for retrospective studies, epidemiological investigations, and for providing reference materials used in assay standardisation, validation, and proficiency testing programmes. Although there has been significant progress in this field, several issues remain to be addressed at the global level concerning the whole process of biobanking, such as the lack of a harmonised approach to standard procedures for processing samples and saving the related data. Currently, there is significant variability regarding the collection, processing and storage of the majority of biological materials available for research and diagnosis, and regarding their associated data. To overcome this problem, the existing procedures provided in the guidelines published by the international organisations mentioned above could be developed for the evaluation of quality assessment parameters. In this regard, one of the main projects that will be launched in the near future by the BVR and all the OIE Reference and Collaborating Centres is to standardise the quality controls of the stored reference materials.

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Development of detection methods within the framework of a flexible scope accreditation in accordance with the ISO/IEC 17025 Standard: experience at the French Plant Health Laboratory

Véronique Wilson*[1], Renaud Ioos[1].

Abstract

In its role as National Reference Laboratory, the Mycology Unit of the ANSES Plant Health Laboratory is tasked to develop and validate detection and identification methods for phytopathogenic fungi.

In 2014, the laboratory obtained an extension of its accreditation in accordance with the ISO/IEC 17025 Standard to use methods that are developed and validated in-house, within a flexible scope framework. Most of these methods are based on molecular biology techniques.

This article presents the various actions implemented to develop and validate new detection methods under accreditation, and the adjustments that the laboratory made to its quality management system to integrate this methodological activity.

Keywords

- Flexible scope framework
- ISO/IEC 17025:2005 Standard
- Method validation
- Plant pests
- Quality management system

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Introduction

The French Agency for Food, Environmental and Occupational Health & Safety (ANSES) has 11 reference and research laboratories, including the Plant Health Laboratory. This laboratory is made up of six technical units specialised in the detection and identification of organisms harmful to plants. The Mycology Unit located in Malzéville is the National Reference Laboratory (NRL) for the detection and identification of phytopathogenic fungi.

The Mycology Unit's main mission is to develop and validate specific detection tools for phytopathogenic fungi or oomycetes of interest potentially posing a risk to the national territory, that are officially regulated or included in quarantine lists, and that are emerging in France or other countries. Requests for development may come from external sources, relate to the unit's NRL mandate, or be initiated directly by the laboratory itself. Most of the tools developed are based on molecular biology techniques. The methods developed by ANSES can then be transferred to accredited laboratories to carry out routine analyses. As a result, the unit tries to develop detection tools making use of real-time PCR techniques that have higher performance and are far easier to standardise.

The Mycology Unit of the Plant Health Laboratory is accredited by the French Accreditation Committee (COFRAC) in accordance with the ISO/IEC 17025:2005 Standard for detection and identification analyses of phytopathogenic fungi and oomycetes. In 2014, the Mycology Unit decided to apply for an extension to its accreditation to use methods that it had developed and validated in-house, within a flexible scope framework. To do this, the laboratory made use of the quality management system it developed to carry out analyses, to which it added a chapter specifically on the implementation and traceability of new method development and validation.

Developing new detection methods under accreditation

■ Ensuring the admissibility of the request

The requester sends the specifications presenting the parasite of interest, the plant matrix (fruit, twig, seed, etc.) and the intended objectives on the basis of the implementation context, i.e. rapid method for border controls, low-cost method for serial analyses, or higher sensitivity than the reference method.

The laboratory examines the admissibility of the project on receipt of the request. This study takes into account a variety of criteria such as the conditions of implementation depending on the availability of the biological materials (*e.g.* pests not present in the European Union), limiting factors (*e.g.* obligate biotrophic species), the actions required (training, equipment purchases, etc.), and also constraints in terms of personnel availability. The laboratory also evaluates how demanding the request is concerning various criteria (*e.g.* complete absence of false positives).

The head of the unit then decides on the admissibility of the project. The project may be admissible, provisionally admissible (need for funding, establishment of a partnership, etc.) or not admissible.

■ Describing the project precisely

The development of new methods begins with establishing the current state of knowledge. This step involves documenting the various scientific techniques or approaches related to

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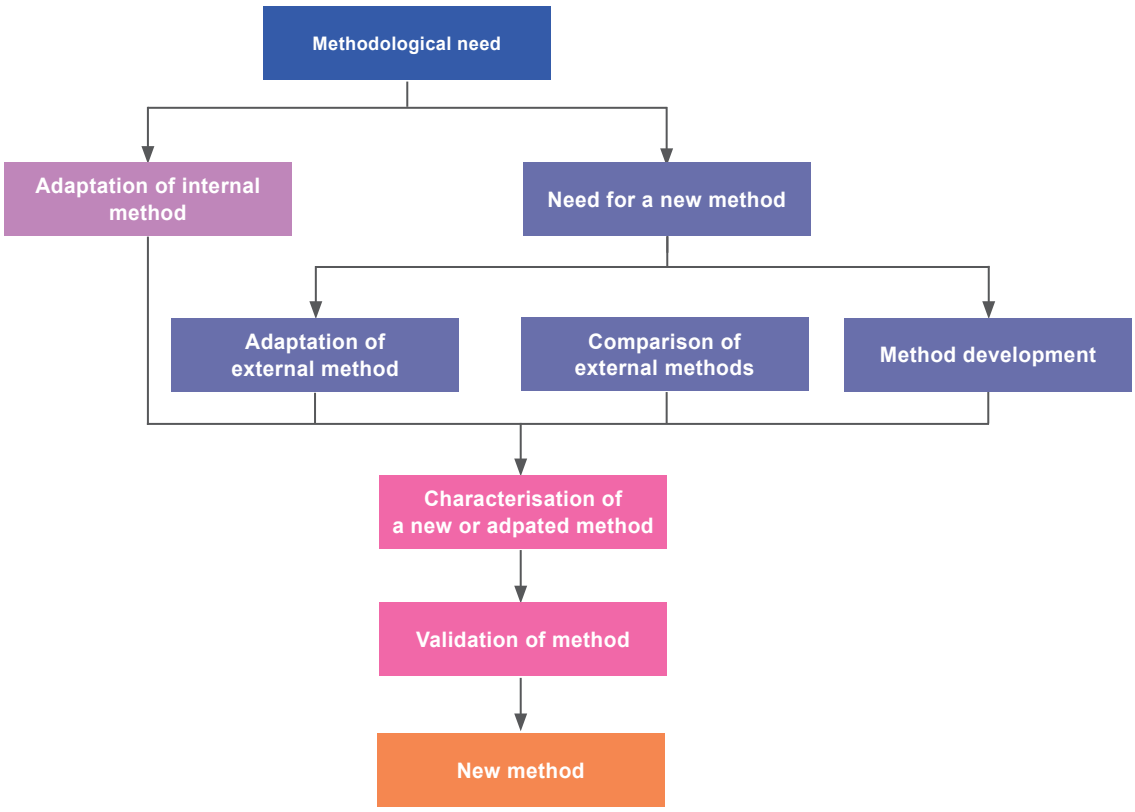
the project. For each of the publications, the advantages and disadvantages are described in detail. Analysis of these data makes it possible to define the best possible approach or approaches to meet the project objectives. The four main approaches retained by the laboratory are: adaptation of an In-house method, adaptation of an external method, comparison of external methods, and lastly development and optimisation of a new method. The analysis completed on the basis of the current state of knowledge enables the project leader to describe the project precisely and to select the various steps required to develop a new method (Figure 1).

FIGURE 1/ Summary diagram of the various steps required to develop a method on the basis of the selected approach.



Adaptation of an in-house method

Adaptation of an in-house method involves changing a method that has already been developed and validated by the Mycology Unit to adjust it to the needs of the laboratory or client. Method adaptation is required when changing a reagent, consumables, experimental parameters, or critical equipment. It includes a simplified characterisation step and a validation step.

For each change that can be made to the method, performance criteria to be re-characterised and practical aspects to be followed are defined by the laboratory.

Adaptation of an external method

Adaptation of an external method occurs when, following assessment of the current state of knowledge, it appears that only one method corresponding to the request is available publicly (scientific literature, international protocol, etc.), and that it can be used to develop the new

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method. Performance criteria of this adapted method are characterised and validated.

Comparison of external methods

Comparisons of methods are carried out when various protocols that fulfil the requirements of the request are already available in the scientific literature. Following an assessment of the current state of knowledge, the project leader selects at least two methods available in the scientific literature that in principle respond to the request. Comparisons of methods involve evaluating and comparing several performance criteria, as a first step, in order to retain only the protocol that is most suitable, in view of its complete characterisation and validation.

Method development

A new method is developed when no satisfactory method in terms of the request is available in the scientific literature or in international protocols. Method development is the process of designing and optimising the various steps in the method in which the most important physical, chemical, and biological parameters are evaluated and adjusted to suit the intended application of the method (adaptation to the matrix, to the analyte, or to the practical conditions in which the method will be used).

■ Characterising the method's performance criteria

In most cases, the methods already described in the literature use equipment, reagents, and consumables that are completely or partially different to those commonly used in the laboratory. Except in exceptional cases, such as a reagent or equipment indicated in publications as mandatory, adaptation of an external method will be carried out using reagents, consumables, and equipment similar to those in the original protocol, but available and commonly used in the laboratory.

The project leader selects the performance criteria to characterise, following the assessment of current knowledge. He or she defines the way they are characterised: expected performance values, statistical tests required, and types of samples to test, etc. In the framework of laboratory activities, the method is characterised by evaluating the various performance criteria presented in Table 1 (non-exhaustive list). According to our procedure, robustness is always characterised; the other optional criteria are selected on the basis of the specifications.

For each of these criteria, the laboratory has described how they will be evaluated (*e.g.* use of DNA extracts at standardised concentrations to determine analytical specificity) and defined the expected limit values (*e.g.* the reproducibility of the method must be greater than or equal to 80%).

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TABLE 1 / Non-exhaustive list of the performance criteria to characterise.

Mandatory criteria *	Evaluation of the efficacy of a PCR reaction	
	Analytical sensitivity:	Determination of the smallest detectable quantity of the target that it is possible to measure with a defined certainty.
	Inclusivity:	Ability of the method to detect the target taxon regardless of geographical origin and host, etc.
	Analytical specificity:	Ability of the test to provide a negative result for a non-target organism.
	Repeatability:	Consistency between successive and independent results obtained with the same method and using an identical test sample in identical conditions.
	Reproducibility:	Consistency between results of individual tests performed on an identical test sample and using the same method obtained by operators using different equipment.
	Diagnostic sensitivity:	Proportion of infested or infected samples yielding a positive result with the test of interest.
	Diagnostic specificity:	Ability of the test to provide a negative result for a healthy sample.
Optional criteria *	Robustness:	Ability of the method to remain unaffected by small deliberate variations in the experimental parameters described in the method.
	Evaluation of the quality of DNA extraction by an external (monoplex) or internal (multiplex) real-time PCR test targeting the 18S gene.	
	Ability of the test to be used in multiplex, i.e. to be used in parallel with other PCR tests in real time in the same reaction tube (*e.g.* test for another target, internal control of DNA extraction, etc.).	
	Evaluation of the minimum number of test samples to be used.	
	Ease of use and transfer.	
	Estimate of all the costs generated to produce the results: personnel, infrastructure, liquids, consumables, reagents, etc.	

*According to ANSES Generic guidelines for method validation.

■ Validating the new method

The ISO/IEC 17025 Standard indicates that “validation is the confirmation by examination and the provision of objective evidence that the particular requirements for a specific intended use are fulfilled”.

Validation corresponds to recognition of the ability of the method to meet the intended use. It involves comparing the values for the performance criteria with the expected values for the method. The validation phase must also confirm through tangible evidence that the level of method performance complies with the requester’s specifications (e.g. cost and duration of analysis) (Table 2).

The new method is considered suitable for the intended use, and validated, if the values for the performance criteria as described in the description of the projects are achieved.

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TABLE 2 / Example of validation of performance criteria. Extract from the validation report of the method for detection of *Plasmopara halstedii* by real-time PCR (Ioos *et al.*, 2012).

<table>
<tr><th>Performance critera</th><th>Results obtained</th><th>Expected results</th><th>Validation decision</th></tr>
<tr><td>Characteristics of the real- time PCR reaction</td><td>The effectiveness of the reaction is evaluated at 1.20 for monoplex reactions, and at 0.87 for duplex reactions. The detection threshold of the target is not affected by the duplex reaction from a qualitative point of view when calibrated plasmid solutions are tested because the detection limit remains the same. However, from a quantitative point of view, a mean lag of about 3 cycles is obtained at the detection limit. Ultimately, it was demonstrated that the mean Ct value obtained from DNA extracts of contaminated sunflower seeds (N=27) was not significantly different between a qPHAL monoplex test and a qPHAL + 18S uni duplex test (F=1.02; p=0.320). The R2 calculated for a monoplex reaction in a diluent of ultrapure water is 0.99</td><td>Duplex reaction as effective as monoplex reaction 0.80<E<1.20
R2 ≥0.98</td><td>Use in duplex format possible

OK</td></tr>
<tr><td>Repeatability and reproducibility</td><td>
<table>
<tr><th>Target</th><th>Target concentration[a] (number of plasmid copies in the PCR tube)</th><th colspan="2">CV (%)</th></tr>
<tr><th></th><th></th><th>intra-assay</th><th>inter-assay</th></tr>
<tr><td rowspan="3">P. halstedii qPHAL-F/-R PCR product</td><td>2.26 10^4</td><td>0.45</td><td>2.21</td></tr>
<tr><td>2.26 10^3</td><td>0.52</td><td>1.52</td></tr>
<tr><td>2.26 10^2 [b]</td><td>1.98</td><td>1.69</td></tr>
<tr><td>P. halstedii DNA</td><td>n.d.[c]</td><td>1.74</td><td>4.04</td></tr>
</table>
[a] Plasmids in which was inserted the qPHAL-F/-R region, diluted in a background of H.annuus DNA.
[b] This concentration was determined as 10 times the limit of detection of the test.
[c] Total DNA extract from a naturally infected H. annuus seed sample (02 FU)

The qualitative repeatability and the quantitative reproducibility are both 100%</td><td>Reproducibility and repeatability >80%
Coefficients of variation<10%</td><td>OK</td></tr>
<tr><td>Other criteria
Duration of analysis</td><td>Estimated duration of new method: 1 day
Duration of former method MH/07/24: 2 days</td><td>At least as short as the reference method</td><td>OK</td></tr>
</table>

A development and validation dossier is prepared for each new validated method. This dossier contains the following information:

- the requester's specifications,
- analysis of the current state of knowledge,
- a description of the methodological project,
- records of characterisation and, if necessary, optimisation,
- documents related to thought processes, all planned protocols, tests and raw data,
- handling sheets,
- validation report,
- etc.

Adapting the management system to method development

The laboratory decided to make use of the existing quality management system to perform PCR or real-time PCR detection analyses. The tests used for method development most often require the same resources (facilities, equipment, etc.). However, when necessary, specific new provisions for method development were implemented to meet the requirements of the ISO/IEC 17025 Standard.

■ Personnel

The laboratory personnel are in charge of method development, and if needed, trainees are recruited specifically for this project. Qualification and maintenance of operator competence

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is ensured by regularly conducting detection analyses that rely on molecular biology techniques. These analyses performed under accreditation are subject to regular audits, during which the competence of operators is assessed.

Integration of this new activity in the quality management system required the creation of a new key function called “project leader”. Qualification criteria based on initial training and professional experiences have been defined. This competence is evaluated annually.

The project leader’s role is to formalise the project, manage and follow up its implementation, and ensure the validation of the method.

Equipment and consumables

The material used in methodological development projects is the same as that used in analyses under accreditation. All the critical equipment is assessed in terms of metrology.

Plastic consumables, including tubes, microtubes, PCR tube strips or real-time PCR tube strips, pipette microtips, etc., are, as far as possible, the same as those used in the context of analyses under accreditation.

Reagents

The reagents used (enzymes, master-mix, buffers, etc.) are, as far as possible, the same as those used in analyses under accreditation. However, the project may aim to test and evaluate new reagents that are not yet used by the laboratory. In this case, like for reagents already used by the laboratory, batches are tracked and used in accordance with the manufacturer’s recommendations in terms of preparation and storage. Procedures of the quality management system for the purchase, reception and suppliers evaluation apply for these new reagents.

Project review

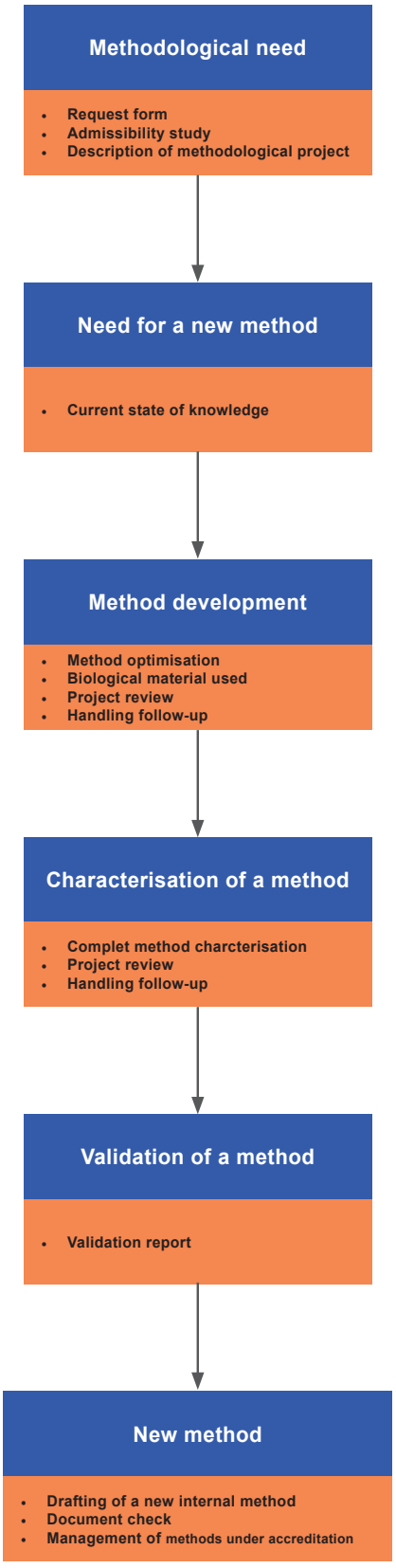
The project leader and operators carry out one or more reviews during the project. The reviews are aimed at evaluating the conduct of the project with regard to the initial project plan or its revisions. At each method development phase, the project leader can decide to revise the initial choices if necessary. Review is also required when results are not those that were expected. Project review is also a regular milestone helping to ensure traceability of activities and to check that no documents are missing. Lastly, it is a chance for the members of the team to exchange opinions. Each project review gives rise to a summary report used to track conclusions and the decisions made.

Traceability and data management

Traceability must ensure that required information is available to reproduce all or part of the results obtained during method optimisation and characterisation of the performance criteria. Records must also provide proof that resources used (reagents, equipment, operators, etc.) are suited to the task.

A unique feature of the project is the traceability method chosen by the laboratory. New forms are not only used for the traceability but they also provide a checklist for operators and project leaders. All the forms to fill are

FIGURE 2 / New specific forms to fill for each step of method development.



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ready to use with specific empty fields. The objective is to ensure that all the project leaders work in the same way and more importantly, that no criterion is omitted during method optimisation or characterisation of the performance criteria. As such, any failure to perform a step (or failure to characterise a criterion) must systematically be justified.

Detailed forms have been developed for each step. To guide the project leader, a summary diagram of all the specific forms for method development has been included in the project description sheet (Figure 2).

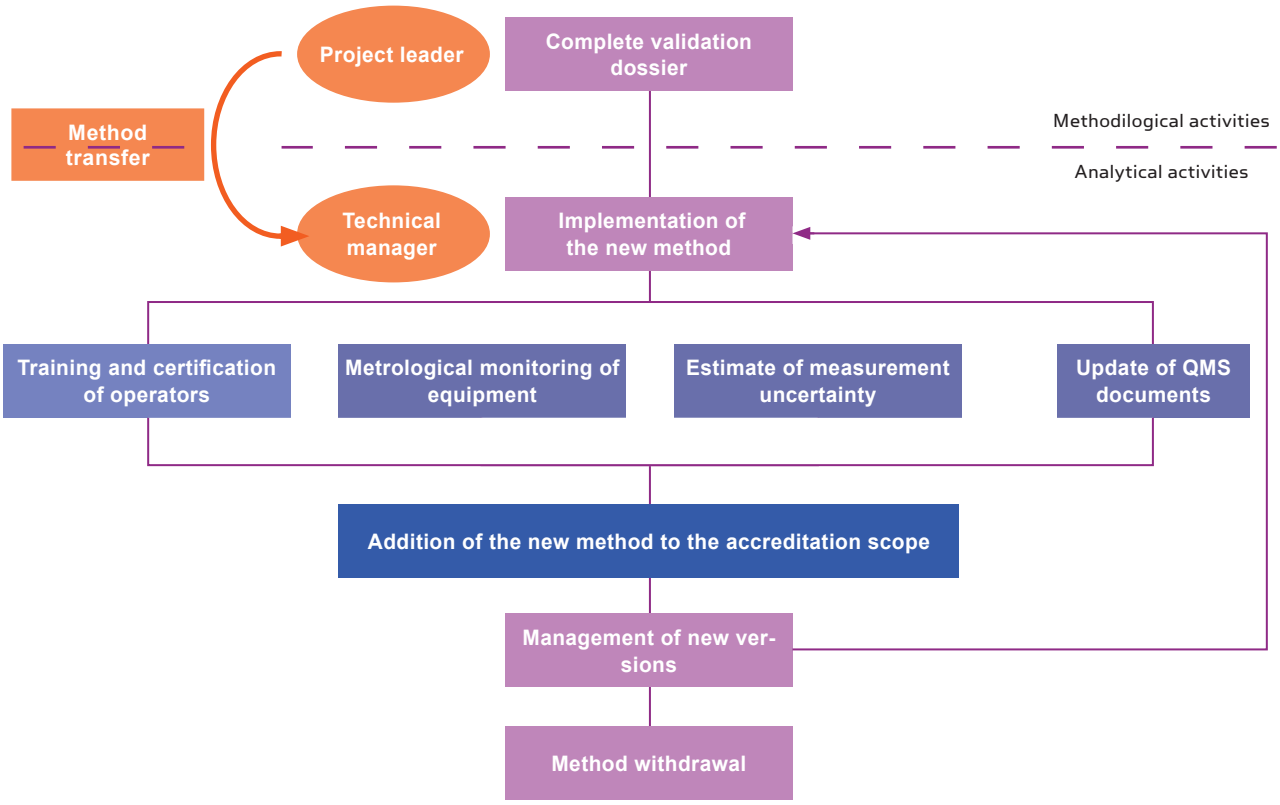
■ Integrating the method in the flexible scope accreditation

Once the validation report is complete, quality manager carries out a document check on the entire dossier of the new method or on the items specific to adaptation of an in-house method. Any technical or organisational deviation observed, and the corresponding corrective measures taken, are governed by procedures for non-compliance with provisions and non-compliant work.

Once the dossier is considered complete, the method can be implemented to carry out analyses in the context of flexible scope accreditation.

The project leader forwards the method to the technical manager in charge of detection analyses using the method. This person trains and certifies operators, estimates measurement uncertainty, and drafts quality documents required for traceability of analyses (Figure 3). For the Mycology Unit at the Plant Health Laboratory, the qualification phase of the technical manager and the operators is facilitated by the fact that they have most often participated in method development.

FIGURE 3 / Addition of a new method to the accreditation scope presented (type B flexible scope)



ACCREDITATION

Conclusion

The extension of the quality management system to methodological activities made it possible to define precisely the various steps in method development for the detection of phytopathogenic fungi, and to implement effective traceability.

The main difficulty encountered in developing the procedure to describe this process was to establish a list of the various types of cases found in method development for plant diseases that was as exhaustive as possible. Applying this procedure then required the creation of many record forms. Since traceability is often considered a very time-consuming constraint, most of the forms were developed to assist in writing. As such, for each step, the form was designed to be as exhaustive as possible.

This procedure and the associated forms provide practical assistance to project leaders and operators but require caution in terms of regular review.

In addition, because of this traceability, the project leader has all the necessary information to draft a scientific publication. As the project moves forward, all the characterisation and validation data, and all the metrological guarantees, are recorded. A corresponding, peer-reviewed scientific publication helps to demonstrate the value of the project.

Flexible scope accreditation enables the Mycology Unit of the Plant Health Laboratory to integrate methods or withdraw them from its scope depending on its needs as a National Reference Laboratory, for instance in health crisis situations or for emerging parasites. In this way, the laboratory can quickly respond to requests, while maintaining a quality management system that fulfils the requirements of the ISO/IEC 17025 Standard and that is suitable for the unit's size.

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METHOD DEVELOPMENT

BactoScan FC: conversion system for results at the national level in Italy and reproducibility of total bacterial count testing four years after implementation

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Abstract

BactoScan and BactoCount are automated instruments for the determination of the total bacterial count (TBC) in milk through an alternative routine method. Results are given in impulses, but the TBC is officially expressed in colony-forming units per ml (CFU/ml), making a conversion system necessary in order to transfer results onto the official scale. In Italy, these instruments were introduced at the beginning of the 1980s, and today amount to more than 50 units. The initial huge number of conversion lines was gradually reduced over the years until 2012, when a single conversion relationship, developed by a joint NRCBMQ – NRLMMP project, was finally made available to Italian laboratories. In fact, it has been adopted by almost all the laboratories that routinely use these instruments. This article examines the results of about 50 proficiency tests (PTs) organised by the Italian Breeders Association (AIA) on a national scale in the period 2003-2016, for which laboratories were asked to provide results in impulses and in CFU, according to their own current conversion system. A retrospective statistical analysis of the results enabled us to assess the changes in the reproducibility of the results expressed in both units of measurement over time: that is, in impulses (mainly dependent on instrumental performance) and in CFU (also dependent on the conversion line used). In particular, we demonstrate the effect of applying the national conversion system developed via the 2008-2012 harmonisation project.

Keywords

- Milk
- Total bacterial count
- Reproducibility of findings

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METHOD DEVELOPMENT

Introduction

The availability of the fully automated flow cytometry instruments BactoScan (Foss) and BactoCount (Bentley) for the determination of the total bacterial count (TBC) in raw milk with an alternative method has led, in a few decades, to an impressive improvement in laboratory performances for this parameter. This improvement involves especially repeatability, standardisation, speed of response, and reduction of costs compared to the same indicators related to the reference method ISO 4833-1:2013, since the reference method is a pour plate colony count method characterised by a predominantly manual and subjective component.

In particular, the speed of response has made these instruments extremely valuable for milk control laboratories, reducing the analysis time from 3 days for the reference method to a few minutes, making this alternative method particularly suitable for the dynamic workflow of milk processing.

Unfortunately, the drawback of this instrumental method is that results are obtained in impulses (IBC), whereas the official limit for TBC reported in Regulation (EC) No 853/2004 is stated in colony-forming units/ml (CFU/ml). The need to express the results in CFU/ml requires us to "convert" the instrumental results. Over about thirty years, this apparently simple step has led to a considerable number of studies and investigations carried out by several researchers or, individually by laboratories equipped with these instruments.

The different conversions applied by laboratories have had a remarkable impact on the final reproducibility of the results obtained since we are dealing with high technology instruments, characterised fundamentally by excellent precision.

The number of Italian laboratories expert in raw milk control on large numbers of samples per day is traditionally high (from about 20 to over 40 in the last two decades) and this has increased the impact of using different conversions more than in other countries. These conversion methods included internal systems, approaches adopted from other laboratories, or acquired directly from instrument manufacturers.

In 2008, the Reference Centre for Bovine Milk Quality at the IZSLER launched a national project for the evaluation of a single conversion system with the collaboration of 15 Italian laboratories (Bolzoni and Marcolini, 2010a; 2010b). In the following years, the work was strengthened through the participation of the Italian NRL-MMP (ISS). In order to harmonise the control of TBC, 33 public and private laboratories throughout the country were involved, and in 2012, a single national conversion system for bovine milk controls specific to BactoScan FC was validated (Bolzoni *et al.*, 2015).

This conversion was gradually implemented throughout the country, and it can be estimated that almost all the laboratories that currently control TBC with BactoScan FC in Italy have adopted this conversion system.

This work aimed at assessing the improvement in reproducibility achieved over time through the gradual reduction in the number of different conversion systems in use, until the adoption of the single conversion line. The work examined the collection of results obtained during periodic proficiency testing for TBC in raw milk using flow cytometry instruments (4 rounds per year on average) organised and implemented by the Standard Milk Laboratory of the Italian Breeders Association (AIA) since 2003 across Italy. This comprehensive database provides a dynamic picture of deviations among laboratories, with reference both to the results expressed in impulses and those expressed in CFU, calculated after applying the current conversion system used by each of the laboratories at the time of the PTs.

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Thirty years of "conversion" in brief

Obviously, it is not a simple task to solve a 30-year technical-scientific issue. After all, if we were to ask the question of how each bacterium or mass of bacteria behaves in each milk sample to form a colony in the conditions of the pour plate method, we would have to conclude that we do not know the answer. Let us recall briefly the core of the problem: automated instruments are able to count – with very good precision – all kinds of viable bacteria present in each raw milk sample analysed. However, it is not possible to deduce *a priori*, and with the same accuracy, how many colonies the same bacteria would have developed if analysed with the reference method. Unfortunately, the variability of the relationship of the results obtained with these two count methods is very highly dependent on the type and amount of bacteria in the sample, as well as metabolic conditions and growth and multiplication requirements, but also non-biological factors such as the energy applied when mixing the sample before analysis.

Of course, it is possible to develop a mathematical function that represents the correlation between impulses and CFU obtained in the laboratory from a representative number of samples, so as to correctly extrapolate the same function to the entire milk population from which it is derived, by statistical inference. Nonetheless, we are well aware that from the practical point of view, this is merely a type of “compromise”.

Among the many factors that may affect the variability of this relation, it must be highlighted that part of the microorganisms of a milk sample cannot grow and form colonies in the specific conditions of time, temperature, medium and atmosphere of incubation of the reference method. In addition, it should not be overlooked that the "gold standard" – meaning the result obtained with the reference method – is characterised by performances for repeatability and reproducibility that are less favourable than those obtained with instrumental counting.

Thirty years of studies, hypotheses and discussions cannot be summarised here, but we can point out the significant milestones:

- Description of the characteristics of the opto-fluorimetric method (Grappin *et al.*, 1985);
- Log transformed values and conversion through multiple change points procedure (MCPP) (Kaereby, 1990);
- Criteria and conversion modes (Shuren *et al.*,1991);
- Appropriateness of linear conversion with respect to polynomial conversion (Dasen *et al.*, 1990);
- Production of conversion relationships by single laboratories on a geographical basis (various authors from the 1990s to date);
- Study of the impact of factors influencing the conversion relationship (season, time between milking and analysis, temperature, mixing mode of the sample, etc.) (various authors from the 1990s to date);
- Issue of relevant International Standards (the main ones include ISO 21187, 2004; ISO 16140, 2003 and ISO 16297, 2013).

Regarding Italy, the first studies date back to the beginning of the 1980s for BactoScan III and to the end of the 1980s for BactoScan 8000. Since then and until 2008, the laboratories

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involved worked independently and in very different ways. Starting in the early 2000s, the introduction on the market of BactoScan FC resulted in a progressive convergence to two basic conversions: Shuren *et al.*, 1998; Bolzoni *et al.*, 2000 and Bolzoni *et al.*, 2001. Starting in 2010, the conversion system developed in the first part of the above-mentioned Italian project was gradually adopted by the participating laboratories. At the end of 2012, the national conversion system, produced and validated in the second part of the project, finally became representative of the entire national territory and became a technical reference for Italian laboratories.

Materials and Methods

Since the end of the 1980s, the Standard Milk Laboratory of the Italian Breeders Association has been performing PTs concerning the main analytical parameters of milk. These activities, initially directed to the laboratories of the Regional Breeders Associations and aimed at managing instrument calibration, were gradually extended to the vast majority of public and private laboratories working in the field.

PTs dedicated to the total bacterial count with flow cytometric instruments were started around 2003 with approximately four rounds per year to date (each consisting of 4 samples with different levels of bacterial contamination). The number of laboratories has of course changed over the years, as well as the types of instruments used. However, on the whole, the available volume of results represents an invaluable source of information covering a 13-year period.

For this work, data from about 50 PTs were examined, corresponding to a total of more than 200 milk samples analysed in the period between October 2003 and September 2016, by a number of laboratories ranging from a minimum of 15 in 2003 to a maximum of 46 in 2012 (accounting for the average number of 34 participating laboratories over the entire period

FIGURE 1 / Example of a synthetic result report of the elaboration provided by the organizer - PT of September 2015, unit of measure: Log Impulses/µl and Log CFU/µL

RING TEST TOTAL BACTERIAL COUNT – SEPTEMBER 2015

Log Impulses*1000/ml - **Repeatability – Reproducibility- Outliers**

Sample	Valid laboratories	Mean	r	R	Sr	SR	RSDr	RSDR	RSDL
1	41	2.381	0.053	0.259	0.019	0.092	0.784	3.850	3.769
2	42	2.716	0.032	0.090	0.011	0.032	0.412	1.176	1.102
3	42	3.350	0.024	0.203	0.009	0.072	0.256	2.146	2.130
4	39	3.928	0.014	0.204	0.005	0.072	0.129	1.838	1.834

General means

	Mean	r	R	Sr	SR	RSDr	RSDR	RSDL
	3.094	0.034	0.199	0.012	0.070	0.395	2.252	2.209

RING TEST TOTAL BACTERIAL COUNT – SEPTEMBER 2015

Log CFU*1000/ml - **Repeatability – Reproducibility- Outliers**

Sample	Valid laboratories	Mean	r	R	Sr	SR	RSDr	RSDR	RSDL
1	41	1.786	0.052	0.278	0.018	0.098	1.023	5.495	5.399
2	42	2.095	0.030	0.127	0.010	0.045	0.499	2.137	2.078
3	42	2.688	0.024	0.273	0.008	0.096	0.312	3.588	3.574
4	39	3.224	0.017	0.202	0.006	0.071	0.186	2.216	2.208

General means

	Mean	r	R	Sr	SR	RSDr	RSDR	RSDL
	2.448	0.033	0.228	0.011	0.080	0.505	3.359	3.315

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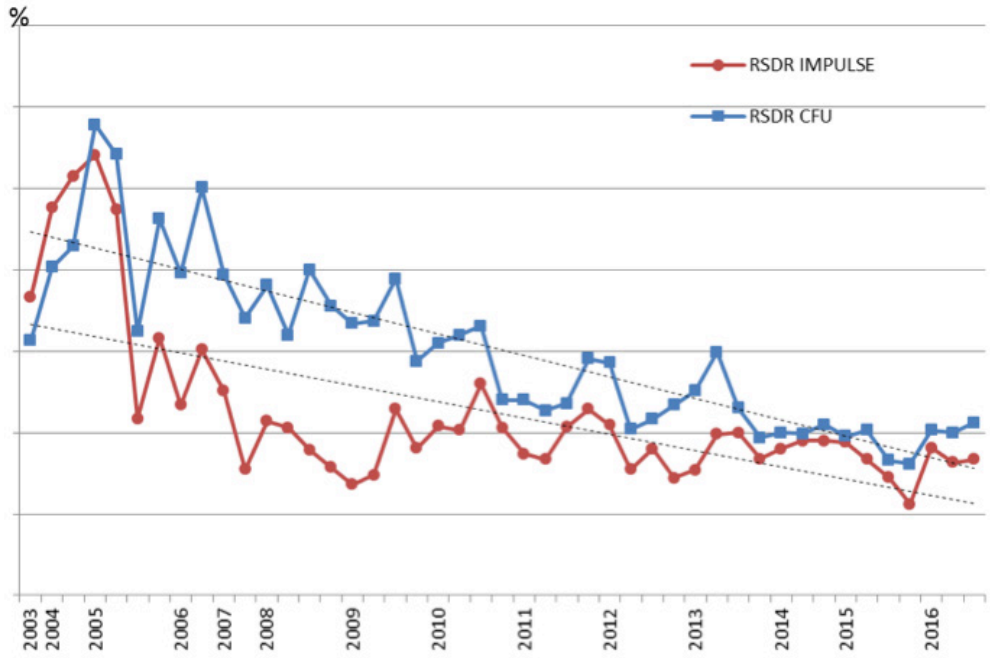
considered and 40 for the last six years). In the early years, the instruments available were BactoScan 8000 and BactoScan FC (initial ratio of 1:1), plus 1 BactoCount. The BactoScan 8000 model, which was used in 2006 in less than 10 laboratories, was gradually and completely replaced by the FC model by 2008. Moreover, as of 2008, two laboratories using BactoCount instruments also participated in the PTs.

The statistical analyses of the individual rounds have always been performed by AIA on the pooled data (with in-house software based on ISO 5725-2, 1994), without any distinction concerning the instruments used. Hence, no specific information is available for this study, but this is probably of little importance due to the disproportion in numbers consolidated in the last ten years.

The reports for each PT managed by the Standard Milk Laboratory of AIA provide, with reference to each sample and for each laboratory, the determination of the conventional indicators of dispersion and comparison with the reference values for the z-score evaluation for both units of measurement. The overall evaluation of each PT is also accompanied by graphics and tables for the two different units of measurement. An example of the summary page of a PT (September 2015) is provided in Figure 1, which shows the data in log impulses and log CFU for each sample with reference to: the number of valid laboratories (non-outlier labs), the average values, repeatability (r) and reproducibility (R) with standard deviations (Sr, SR) and finally, the estimated relative standard deviation of repeatability (RSDr), of reproducibility (RSDR) and of laboratory (RSDL); the same indicators are reported in the lower part as an overall estimate for the single PT.

The statistical analysis carried out in this study was performed using Excel software and taking into account the RSDR and RSDL values expressed in linear units and obtained from each PT or each sample, throughout the last 13 years. This unit of measurement was chosen for its consistency with the data available for the entire period, since the statistical evaluation on log transformed results was introduced by the PT organiser only recently.

FIGURE 2 / RSDR trend for impulses and CFU for PTs organized in the period 2003-2016



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Results and discussion

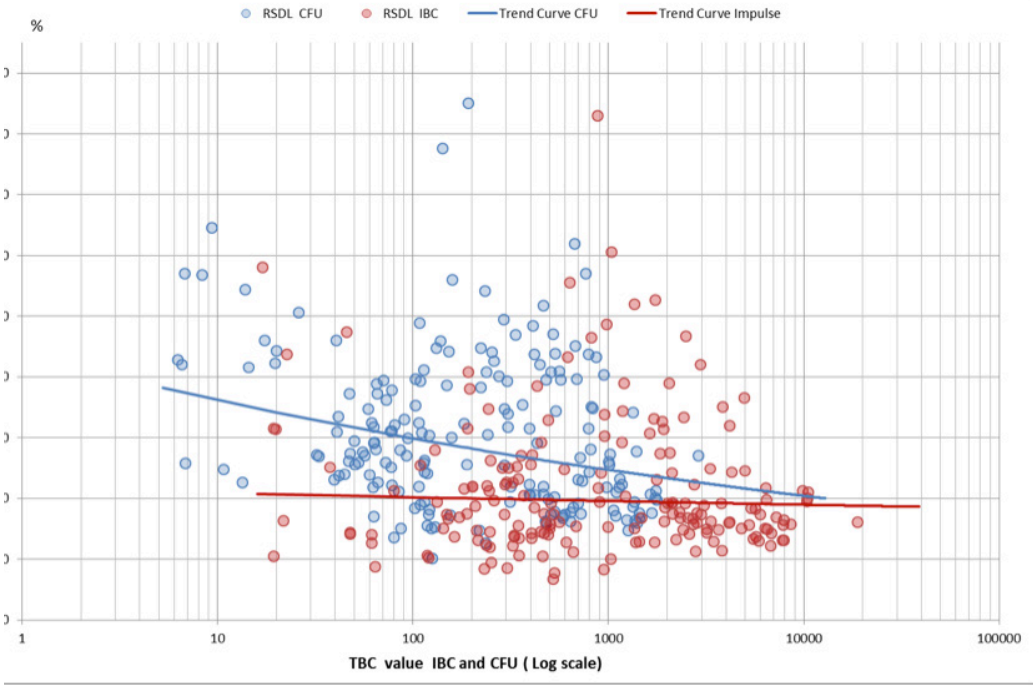
The first indicator chosen to estimate the trend of the "conversion effect" over time is the average RSDR value of each PT calculated on the valid results given both for the instrumental measurement unit (impulses) and after the conversion into CFU, as calculated by each participant. While the first results are instrument-dependent, the second ones are also dependent on the conversion mode in use in each laboratory, at the time of the PT.

Figure 2 shows the RSDR trend for the results given in both units of measurement. The values used for this graphic are those reported by the organiser as "general average of the PT" and show the linear trend estimated for the two series of values and their different slopes.

A more accurate evaluation can be obtained by replacing the indicator RSDR with RSDL, which represents the relative standard deviation among laboratories. For any given level, RSDR represents the overall reproducibility of the PT and incorporates both RSDr (independent of the different conversions applied by each laboratory during the PT) and RSDL (likely conditioned, among the other causes, by the variety of the conversion systems used).

Figure 3 shows the distribution of the RSDL values for the individual samples (given in impulses and in CFU). This specific distribution of values shows the influence of samples with very low bacterial contaminations on the overall evaluation, given the percent expression. This effect can be seen in the left part of the distribution, where the percentages for the individual samples appear to deviate significantly from the trend curve for CFU.

FIGURE 3 / Distribution and trend of RSDL values for individual samples (Pulses and CFU)



After removing 12 samples below 20 CFU/ml, a second analysis, similar to that plotted in Figure 2, was performed.

Figure 4 presents the RSDL values for the two units of measurement, calculated for each individual sample in the series of PTs considered. In the same figure, the results are reported both for the individual samples (shaded indicators) and for the averaged values for each sample group (solid indicators); the mobile media lines (solid lines), calculated as the rolling

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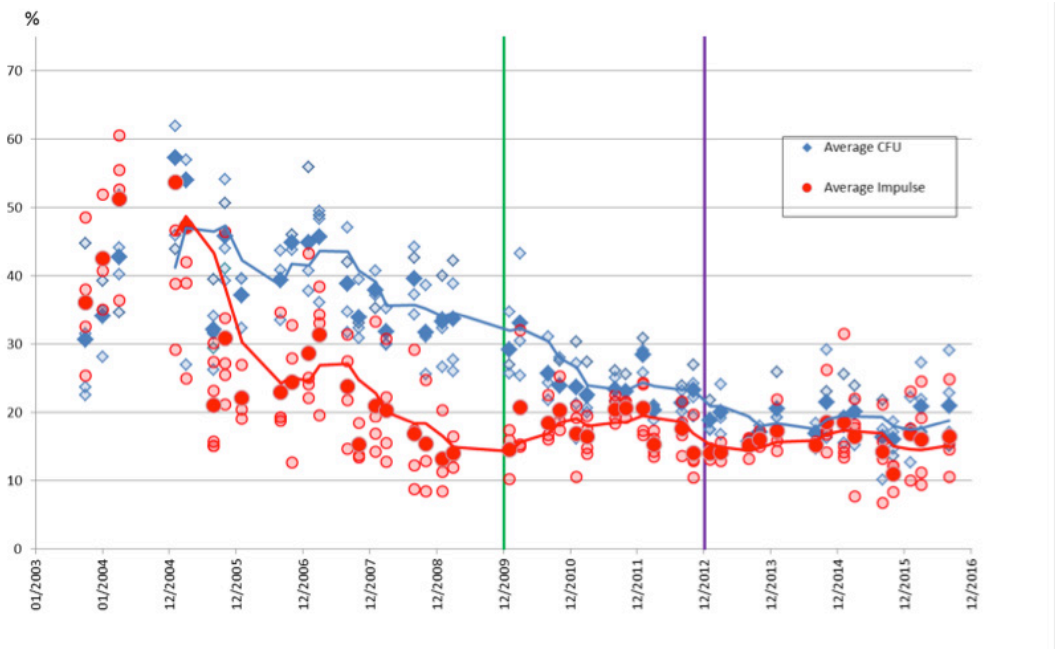
FIGURE 4/ RSDL values for impulses and CFU (for each sample and for averaged results); rolling averages and project phases



average of 4 consecutive values are also represented. Finally, the two vertical lines represent the temporal phases of the entire project.

The general trend shown in the graph is consistent with the previous results plotted in Figure 2. Moreover, it shows not only the gradual reduction of both the RSDL values over time, but also the clear tendency for the approachment of the two series of data. This enables us to draw the following conclusions:

Impulses - As an indicator of the performance of each instrument used by the participating laboratories, there is a clear improvement (reduction of the average RSDL from levels close to 50% for the first rounds to less than 20% for the most recent ones). This can be explained mainly by actions aimed at ongoing monitoring, maintenance or modification of instruments (for example autofocus, cleaning treatments of circuits, etc.) carried out after the rounds in the first period or implemented by outlier laboratories. In fact, the most significant improvement is concentrated in the rounds related to the first 2 or 3 years, and since 2009 substantial stabilisation to "physiological" values has been reached.

In addition, the gradual decommissioning of BactoScan 8000, completed in 2008 in favour of the new FC model, seems to support this observation.

Colony-forming unit - As an indicator of the conversion mode used by the participating laboratories, the decreasing RSDL values show a similar trend, and this must clearly be associated with greater uniformity of the results in impulses. However, as expected, the decrease appears to be delayed in time and in particular is more consistent than that of impulses: we believe that these two aspects are those that indirectly confirm the gradual effect of the conversion change adopted by the participating laboratories in the period considered. Although the milestones of this gradual process date back to 2010 and 2013 (first and second phase of the project), it is difficult to identify a net change in the plotted trends. The new conversion mode was in fact progressively implemented after the dissemination of the results of the two phases of the project. Moreover, some laboratories intentionally transitioned to the new conversion in 1-2 years with the aim of reducing the possible impact on the classic levels of TBC results they had been producing in their geographic area.

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The residual difference, albeit minimal and apparently constant, seems to persist even in the last rounds and could be accidental, given the very low level: the average RSDL of PTs from 2013 to 2016 is calculated as 20.6 for CFU and 16.5 for impulses, *i.e.* a minimal difference that theoretically should be destined to disappear. At present, the possible cause of this permanent difference could be the persistence of conversion modes other than the national system among the participants in the PTs. This applies, for example, to the two BactoCount systems currently in use, but probably also to a few laboratories in the dairy industries and private laboratories which, although participating in national rounds of PTs, still maintain their own conversion lines. Another possible source of this difference could be that values in CFU and in impulses are evaluated independently by the organiser of the PT. In this way, the selection of outliers could lead to the occasional exclusion of different laboratories in the two series of results.

Conclusions

A retrospective analysis of the results of about 50 collaborative rounds organised by the AIA in Italy in the last 15 years has provided evidence to assess the changes in laboratory performances for the determination of TBC in raw milk over time.

Information from the periodic rounds on TBC with flow cytometry instruments carried out since 2003 has highlighted that the overall reproducibility level of the alternative method has been markedly improved over time, in particular after the initiative undertaken between CRQLB and NRL-MMP to evaluate, define and transfer a single conversion line at the national level.

It is important to highlight that this type of instrument is essential in order to perform an efficient and timely control of raw milk and to allow the food business operator to implement the appropriate measures in time so as to correct the situation in case milk fails to meet the criteria stated for TBC. The many laboratories in Italy equipped with these instruments allow highly accurate, hygienic, continuous control of the raw milk produced every day by thousands of farms throughout the country, with acceptable costs and an exceptionally short time of analysis. The performances of the laboratories can be considered established and, in addition to showing that the level of reproducibility of TBC results for milk produced in Italy is highly satisfactory, demonstrate the uniformity of evaluation of this parameter throughout the country.

The type of work carried out and the results obtained could be taken as a reference in national contexts characterised by similar operating conditions, such as a high number of laboratories and farms, and climate and environmental differences on farms.

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DISPATCHES

New regulation on official controls: what changes for official laboratories, and for national and EU reference laboratories?

Françoise KREMER*[1] , Loïc CARTAU[1]

Introduction

In May 2013, the European Commission put forward three new regulations concerning animal health, plant health, and controls and other official activities. The regulation on transmissible animal diseases, called the “Animal Health Law”, was published on 31 March 2016 (Regulation (EU) 2016/429) [EU, 2016a]. The regulation on organisms harmful to plants, called the “Plant Health Law”, was published on 23 November 2016 (Regulation (EU) 2016/2031) [EU, 2016b]. And the regulation on “official controls” intended to replace Regulation (EC) No 882/2004, was agreed on politically in June 2016 under the Dutch presidency of the Council. The final text has been adopted by the European Parliament and the Council in March 2017. This text will be applicable at the same time as the plant health regulation (“Plant Health Law”), *i.e.* as of 14 December 2019, will change the obligations of official laboratories, and will set up a framework for European Union Reference Laboratories (EURLs) and National Reference Laboratories (NRLs). Concerning the EURLs and NRLs, the provisions will be applicable one year after publication of the “official controls” regulation.

The main principles underpinning Regulation (EU) No 882/2004 are maintained but the new text will provide new information and clarifications.

Keywords

- European Union
- Official controls
- Laboratories
- Revision of regulation

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DISPATCHES

■ Official laboratories: clarity on the accreditation obligation

The competent authorities in each Member State in charge of official controls and other official activities will, as is currently the case, appoint official laboratories tasked with carrying out official analyses. These appointments will indicate the expected tasks and the framework of cooperation with the competent authority of the Member State. In addition to requirements concerning equipment and personnel, the official laboratory will need to guarantee its impartiality, and the absence of any conflicts of interest when carrying out official analyses. It will need to be accredited for the analytical methods implemented as part of official analyses [ISO/IEC 17025:2005]. However, exemptions to this obligation of accreditation are planned, specifically:

- for the detection of Trichinella in meat, for laboratories that perform only this analysis;

- for the cases that will be indicated subsequently by delegated act of the European Commission;

- temporarily, when replacing analytical methods, when the method itself is changed, or in emergency situations.

These exemptions are only possible if the laboratory provides guarantees, *i.e.* depending on the case: accreditation for similar methods, satisfactory results in inter-laboratory proficiency testing, or supervision by the competent authorities of the Member States.

Lastly, the new regulation will strengthen the requirements for the competent authorities of the Member States to check that the conditions for appointment as an official laboratory are still complied with. If necessary, the competent authorities are required to withdraw the appointment.

■ European reference activities are reinforced for reference missions in animal protection and food fraud. The EURL-NRL system is maintained and generalised for analytical activities

The regulation also provides for the possibility of appointing European reference centres in animal protection as well as European reference centres on the authenticity and integrity of the food chain.

Concerning analytical laboratories, the EURL and NRL system is maintained for the areas of animal health and food safety, and expanded to include plant health. The European Commission will firstly need to establish the need for a reference laboratory at the European level in a specific regulation (established by delegated act). The new text provides for appointment of European Union Reference Laboratories via a public selection process with regular review of mandates. However, a laboratory will need to be appointed as the EURL for a minimum period of 5 years. The new text is supplemented with appointment conditions and obligations, such as the absence of conflicts of interest or the availability of personnel and equipment. The work programme, including in particular the activities related to analytical methods, inter-laboratory proficiency testing, and reagents and reference materials, will need to be established on the basis of the scope of competence and the missions identified by the European Commission.

The obligation for the Member States to appoint a National Reference Laboratory (NRL) for each EURL is maintained. The same obligations as those of the EURLs, for instance in terms of conflicts of interest and availability of personnel and equipment, will apply.

The improvement and distribution of analytical methods, as well as consistency in their implementation, will continue to rely on a system bringing together European Union Reference Laboratories, National Reference Laboratories, and official analysis laboratories, specifically through the organisation of inter-laboratory proficiency testing.

The text will be published in the next few weeks, with early implementation, *i.e.* one year and 20 days after publication for the articles regarding EURLs and NRLs, while the remainder of the text will widely come into force at the same time as the Plant Health Law.

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http://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32016R2031&rid=1

ISO/IEC 17025:2005 - General requirements for the competence of testing and calibration laboratories, 28 pp. www.iso.org

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Go COMPARE!

"One serves all" next-generation sequencing framework

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COMPARE (Collaborative Management Platform for detection and Analyses of [Re-] emerging and foodborne outbreaks in Europe) is a multidisciplinary research network that is set up with the common vision of becoming the enabling analytical framework and globally linked data and information sharing platform system for the rapid identification, containment and mitigation of emerging infectious diseases and foodborne outbreaks.

The system sets out to integrate state-of-the-art strategies, tools, technologies and methods for collecting, processing and analysing sequence-based pathogen data in combination with associated (clinical, epidemiological and other) data, for the generation of actionable information to relevant authorities and other users in the human health, animal health and food safety areas.

Next-generation sequencing (NGS) used for whole genome sequencing (WGS) or whole community sequencing (WCS or metagenomics) enables generation of complete genomic information from the isolate or sample, independent of both the sector (public health, veterinary health or food safety), and the type of pathogen (viruses, bacteria or parasites). The outputs (sequence data) provide one common language that can be exchanged and compared between laboratories and over time, in combination with other associated data defined here as "metadata" including contextual data (*e.g.* data on sample type and process, clinical, microbiological, epidemiological and other data), primary data (raw sequence reads) and derived data (*e.g.* genomic alignments of reads, assemblies and functional annotation data

Keywords

- Data sharing platform
- Emerging infectious diseases
- Foodborne outbreak
- Laboratory proficiency testing
- Research network
- Sequence analysis

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sets). COMPARE aims to harness the rapid advances in these technologies to improve identification and mitigation of emerging infectious diseases and foodborne outbreaks.

To this end, COMPARE is establishing a “One serves all” analytical framework – as depicted in Figure 1 on the following page – presenting the different components of the COMPARE Analytical Framework.

COMPARE runs from 1 December 2014 to 30 November 2019. The consortium (Table 1) has been awarded EUR 20 million funding under the European Union’s Horizon 2020 research and innovation programme under grant agreement No 643476.

Table 1 / The COMPARE consortium consists of the following members:

1	Technical University of Denmark (DTU), Denmark	16	University of Cambridge, UK
2	Erasmus University Medical Center (Erasmus MC), The Netherlands	17	Tierärztliche Hochschule Hannover (TIHO) / The University of Veterinary Medicine Hannover (TiHo), Germany
3	Statens Serum Institute (SSI), Denmark	18	Universidad de Castilla- la Mancha (UCLM), Spain
4	Friedrich-Loeffler-Institut (FLI), Germany	19	Fondation Mérieux, France
5	Agence nationale de sécurité sanitaire de l’alimentation, de l’environnement et du travail / French Agency for Food, Environmental and Occupational Health & Safety (ANSES), France	20	Aristotelio Panepistimio Thessalonikis / Aristotle University of Thessaloniki (AUTH), Greece
6	Robert Koch-Institute (RKI), Germany	21	Institut Français de Recherche pour l’Exploitation de la Mer / French Public Institute for Marine Research (IFREMER), France
7	European Molecular Biology Laboratory (EMBL), UK	22	Erasmus University Rotterdam, The Netherlands
8	Istituto Superiore di Sanità (ISS), Italy	23	The Australian National University (ANU), Australia
9	Rijksinstituut voor Volksgezondheid en Milieu / National Institute for Public Health and the Environment (RIVM), The Netherlands	24	Magyar Tudomanyos Akademia Wigner Fizikai Kutatokozpont / Wigner Research Centre for Physics, Hungarian Academy of Sciences, Hungary
10	Animal and Plant Health Agency, UK	25	Civic Consulting GmbH (CIVIC), Germany
11	University of Edinburgh (UEDIN), UK	26	Responsible Technology (RT), France
12	University of Bonn Medical Centre (UK-Bonn), Germany	27	University of Bologna (UNIBO), Italy
13	Academisch Medisch Centrum Universiteit van Amsterdam / Academic Medical Center (AMC), University of Amsterdam, The Netherlands	28	Leibniz Institut Deutsche Sammlung von Mikroorganismen und Zellkulturen / German Collection of Microorganisms and Cell Cultures (DSMZ), Germany
14	Universiteit Antwerpen / University of Antwerp (UA), Belgium	29	Wellcome Trust Sanger Institute (WTSI), UK
15	Artemis One Health Research Institute (Artemis), The Netherlands		

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Within the project, the first two work packages are researching approaches to support how more efficient risk-based sampling can be carried out, and the identification and harmonisation of laboratory protocols for samples intended for next-generation sequencing. All work packages are listed in Table 2.

Risk-based sampling inventory

WP1 leader: Dr Amie Adkin (amie.adkin@apha.gsi.gov.uk)

Understanding the extent of existing sampling protocols for generating clinical and diagnostic data arising from food, human, livestock and wildlife populations helps to predict the characteristics of samples that are likely to be supplied or made available through existing surveillance systems. We have been developing an inventory of existing, and where possible harmonised, protocols in order to map the types of samples that are currently recommended at the EU or international level for known diseases of public and veterinary health importance. Work is underway to make this list openly available to other researchers. The work required was across the different disciplines of human clinical data and the equivalent animal information. This was accessed through the various websites of multinational organisations and EU-FP7 projects, and gave rise to some surprising comparisons. Firstly, accessing livestock information was, overall, easier and these datasets were structured more logically than those held for human health. However, for all areas, information was dispersed and sometimes incomplete, leading to a feeling of a treasure hunt! The accessibility and coverage of datasets made available from several organisations was commended including the World Organisation from Animal Health (OIE) and US Centres for Disease Control and Prevention (CDC). Whilst the inventories are still being completed, they have already proven useful for one of the contributors currently working at Food and Agriculture Organization of the United Nations (FAO).

Optimising and harmonising handling protocols

WP2 leader: Prof Dr Martin Beer (martin.beer@fli.de)

Careful sample handling is a crucial step in gaining high-quality information from next-generation sequencing to ensure maximum benefit for clinical and public health. Samples have to be treated with great care to minimise significant shifts in the microbial community composition of samples during transportation. This is an important prerequisite in order to display the initial sample situation within the sequencing outcome, and to successfully detect causative agents via sequencing. Therefore, work package 2 (WP2) is addressing the harmonisation of standards for sample handling as a basis for other tasks in the COMPARE project. During the first year of the project, an inventory of commonly used protocols with respect to collection, handling, transport and storage of samples was conducted via a survey. Based on survey results, experiments were designed to investigate the influence of various treatments and handling procedures such as fixation, storage temperature and duration on different sample matrices such as tissue, body fluids, faeces, sewage samples, as well as ticks and insects containing pathogens.

In parallel, sample-processing pipelines including pathogen inactivation, nucleic acid extraction, and subsequent processing until sequencing were developed and are being intensively tested for different matrices (*e.g.* tissue, ticks, bacterial suspensions, and food samples). Protocols providing best results regarding quality and quantity of extracted nucleic acids as well as sequence reads have already been disseminated in the form of Laboratory Operating Procedures (LOPs) for their further review and application in the laboratories of COMPARE members.

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These sample-handling experiments will be tested and sample-processing pipelines further refined during the coming months. The ambitious aim of these optimisation steps is to develop and to provide one protocol for all samples for metagenomics.

Figure 1 / The COMPARE analytical framework

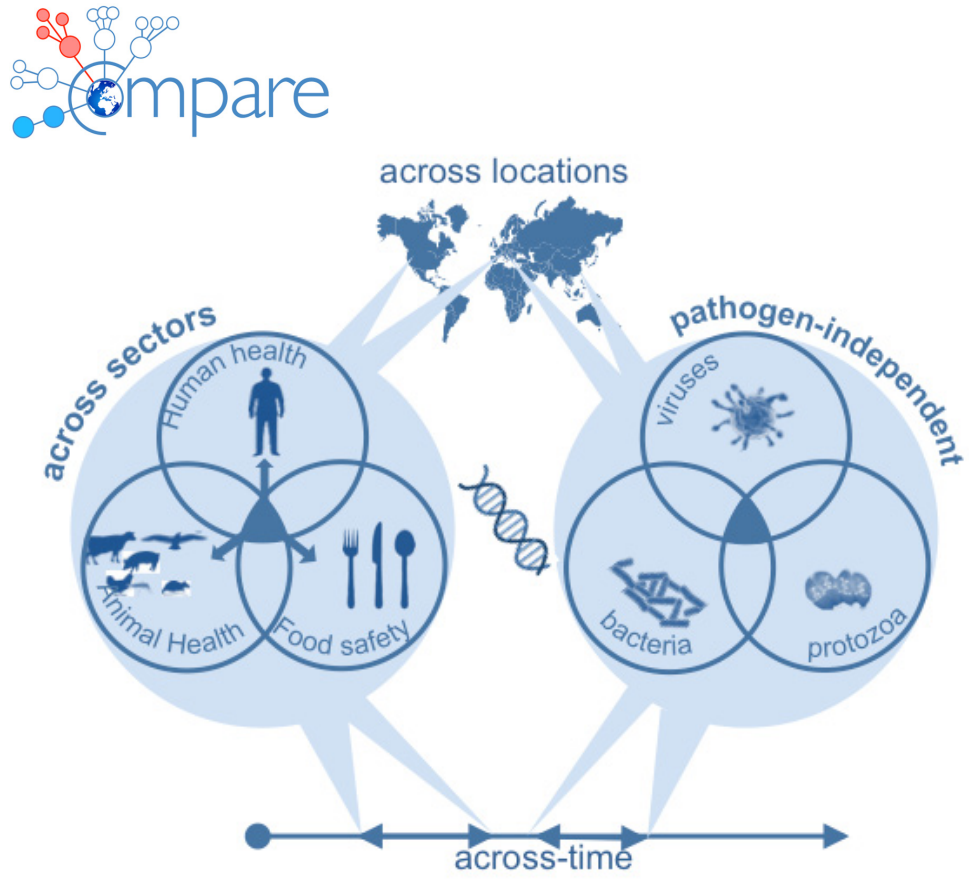

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Table 2/ COMPARE Work Packages, WP leaders and overall tasks

Work Package	Title	WP Leader and Co-leader	Overall task of WP
WP1	Risk assessment and risk-based strategies for sample and data collection	WP leader: Amie Adkin, DEFRA/APHA (10) amie.adkin@apha.gsi.gov.uk Co-leader: Christian Gortazar, UCLM (18) christian.gortazar@uclm.es	To develop risk assessment models and risk-based sampling and data collection strategies for NGS-based analyses of foodborne and (re-) emerging infections.
WP2	Harmonised standards for sample processing and sequencing	WP leader: Martin Beer, FLI (4) martin.beer@fli.bund.de Co-leaders: Simone Caccio, ISS (8) simone.caccio@iss.it	To develop harmonised analytical workflows for generation of high-quality NGS data in combination with relevant metadata for pathogen detection and typing across sample types, pathogens and domains
WP3/6	From comparable data to actionable information: Analytical workflows for frontline diagnostics	WP leader: Surbhi Malhotra, UA (14) surbhi.malhotra@uantwerpen.be Co-leaders: Constance Schultsz, AMC (13) c.schultsz@gmail.com, Anne Pohlmann, FLI (4) anne.pohlmann@fli.de	To develop an analytical workflow for the use of single isolate and metagenomic NGS in human and veterinary clinical microbiology. To assess the feasibility of NGS/WGS/WCS for clinical diagnostic use and hospital epidemiology
WP4/7	From comparable data to actionable information: Analytical workflows for foodborne pathogen surveillance, outbreak detection and epidemiological analysis	WP leader: Eva Møller Nielsen, SSI (3) emn@ssi.dk Co-leaders: Tine Hald, DTU (1) tiha@food.dtu.dk, Michel-Yves Mistou, ANSES (5) michel-yves.mistou@anses.fr	To develop a general analytical workflow for population-based disease surveillance, outbreak detection and epidemiological modelling of foodborne infections.
WP5/8	From comparable data to actionable information: Additional tools for detection of and response to (re-) emerging infections	WP leader: Ron Fouchier, EMC (2) r.fouchier@erasmusmc.nl Co-leader: Mark Woolhouse, UEDIN (11) mark.woolhouse@ed.ac.uk	To develop cross-sector and cross-pathogen methods for support of emerging pathogen identification and characterisation in support of outbreak investigations and epidemiological analysis.
WP9	COMPARE data and information platform	WP leader: Guy Cochrane, EMBL (7) cocharne@ebi.ac.uk Co-leaders: Ole Lund, DTU (1) lund@cbs.dtu.dk, Istvan Csabai, WIGNER (24) csabai.istvan@wigner.mta.hu	Support rapid sharing, integration and analysis of sequence-based pathogen data in combination with other contextual metadata; the system will be linked to existing and future complementary systems, networks and databases such as those used by ECDC, NCBI and EFSA.
WP10	COMPARE risk communication tools	WP leader: Emilio Mordini, RT (26) emilio.mordini@responsibletechnology.eu	To design and develop appropriate risk communication tools and strategies for stakeholders.
WP11	User consultations	WP leader: Marion Koopmans, EMC (2) m.koopmans@erasmusmc.nl Co-leader: Frank Aarestrup, DTU (1) fmaa@food.dtu.dk	To design the COMPARE systems' analytical workflow and its main components based on the expert inputs and associated information needs of its intended future users and other stakeholders.
WP12	Barriers to open data sharing	WP leader: George Haringhuizen, RIVM (9) george.haringhuizen@rivm.nl Co-leader: Jørgen Schlundt, DTU (1) jschlundt@ntu.edu.sg	To identify, clarify and, as far as feasible, develop practical solutions for Political, Ethical, Administrative, Regulatory and Legal (PEARL) barriers that hamper the timely and open sharing of data through COMPARE.
WP13	Dissemination and training	WP leader: Frank Aarestrup, DTU (1) fmaa@food.dtu.dk Co-leader: Marion Koopmans, EMC (2) m.koopmans@erasmusmc.nl	To ensure that relevant stakeholders of COMPARE are adequately informed about COMPARE's progress and results and have access to the training they need in order to apply the harmonised workflows, analytical tools and data resources developed and implemented by COMPARE in their pathogen detection and outbreak response activities.
WP14	Cost-effectiveness framework	WP leader: Pieter van Baal, EUR (22) vanbaal@bmg.eur.nl Co-leader: Frank Allewedlt, CIVIC (25) alleweldt@civic-consulting.de	To develop a standardised framework for estimating the cost-effectiveness of the COMPARE system and related methods and tools, including the value of safety.
WP15	Management	WP leader: Frank Aarestrup, DTU (1) fmaa@food.dtu.dk Co-leader: Marion Koopmans, EMC (2) m.koopmans@erasmusmc.nl	To implement the appropriate organisational structures and processes to ensure COMPARE's compliance with the EU Grant Agreement and the COMPARE Consortium Agreement (CA).

Contributing organisations for this issue:

ANSES	Agence nationale de sécurité sanitaire de l'alimentation, de l'environnement et du travail *French Agency for Food, Environmental and Occupational Health & Safety* www.anses.fr	Maisons-Alfort	France
AECOSAN	Agencia Española de Consumo, Seguridad Alimentaria y Nutrición / *Spanish Agency for Consumer Affairs, Food Safety and Nutrition* http://www.aecosan.msssi.gob.es	Madrid	Spain
AGES	Österreichische Agentur für Gesundheit und Ernährungssicherheit GmbH/ *Austrian Agency for Health and Food Safety Ltd* www.ages.at	Vienna	Austria
APHA	Animal and Plant Health Agency www.gov.uk/government/organisations/animal-and-plant-health-agency	Addlestone / Weybridge	United Kingdom
BfR	Bundesinstitut für Risikobewertung *Federal Institute for Risk Assessment* www.bfr.bund.de	Berlin	Germany
CODA-CERVA	Centrum voor Onderzoek in Diergeneeskunde en Agrochemie / Centre d'Etude et de Recherches Vétérinaires et Agrochimiques *Veterinary and Agrochemical Research Centre* www.coda-cerva.be	Brussels	Belgium
FERA Science Ltd		York	United Kingdom
FLI	Friedrich Loeffler Institut *Federal Research Institute for Animal Health* www.fli.de	Riems	Germany
ISS	Istituto superiore di Sanità *Italian National Institute of Health* www.iss.it	Rome	Italy
IZSAM	Istituto Zooprofilattico Sperimentale dell'Abruzzo e del Molise *Experimental Zooprophylactic Institute of Abruzzo and Molise* www.izs.it	Teramo	Italy
IZSLER	Istituto zooprofilattico della Lombardia e dell'Emilia Romagna *Experimental Zooprophylactic Institute of Lombardy and Emilia Romagna* www.izsler.it	Brescia	Italy
NVWA	Nederlandse Voedsel en Warenautoriteit (Ministerie van Economische Zaken) / *Netherlands Food and Consumer Product Safety Authority (Ministry of Economic Affairs)* www.nvwa.nl	Wageningen / Utrecht	Netherlands
EPPO/OEPP	European and Mediterranean Plant Protection Organization Organisation européenne et méditerranéenne pour la protection des plantes www.eppo.int	Paris	France
PIWET (NVRI)	Państwowy Instytut Weterynaryjny. Państwowy Instytut Badawczy *National Veterinary Research Institute* www.piwet.pulawy.pl	Pulawy	Poland
SCL	Service commun des laboratoires *French Ministries for Economy and Finances* www.douane.gouv.fr / www.economie.gouv.fr/dgccrf/Le-service-commun-des-laboratoires-DGDDI-et-DGCCRF	Paris	France
SVA	Statens Veterinärmedicinska Anstalt / *National Veterinary Institute* www.sva.se	Uppsala	Sweden
WBVR	Wageningen Bioveterinary Research http://www.wur.nl/en/Expertise-Services/Research-Institutes/Bioveterinary-Research.htm	Lelystad	Netherlands

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