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EuroReference

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Winter 2012 Issue No. 8

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Editorial

We are pleased to bring you Number 8 of *EuroReference*. After three years, we thought it important to take stock of this publication's existence. *EuroReference* has no less than 3000 subscribers and each issue is viewed by nearly 4000 unique readers. These numbers have risen steadily since the journal was launched.

With eight issues published since the first one appeared in summer 2009, the biannual rhythm that we established at the outset has been maintained. From 2013, we will be publishing three issues a year, to enable us to keep you even better informed about news or key events. It is interesting to analyse the number of visits to the journal website. These are influenced by the time of publication, but for issues published outside the summer period, there are typically between 1200 and 1400 visits in the following month. Each visitor views several pages at each visit, reading an average of 2.6 pages. The number of monthly visits has doubled (figures recorded outside the "peaks" coinciding with the release of each issue), rising from 400 a month in 2009 to about 800 a month in the first half of 2012. If this trend continues, it would suggest that some readers are now paying regular visits to the site.

The journal, which appears in both French and English, has mostly received contributions from French teams (excluding the special issue on bioterrorism). In the first few months we focused on setting up and developing the publication. Our ambition now is to give it a more European reach. To achieve this, several new non-French members have agreed to join a renewed Editorial Board in 2013. We will be introducing them in the next issue's editorial.

Lastly, we are pleased to announce a special issue for next year, devoted to plants. This is currently being prepared, and is due to appear in spring 2013.

In the meantime, this issue contains a special one-off article: a mini-glossary of terminology used in the reference field: fidelity, reproducibility—all will be explained. In the Focus section, we examine the impact of two health crises—bluetongue and Schmallenberg—on a research and reference laboratory, and how they are managed. We report on the start of an original approach to building a dialogue between accredited laboratories and NRLs. We will return to this in the next issue of *EuroReference*. In the Surveillance Networks and Research sections, you can find out about Salmonella surveillance, and a sampling protocol enabling MRLs to be proposed for veterinary drugs in honey. Finally, there are articles on methods for serodiagnosis of contagious bovine pleuropneumonia by immunoblotting, and quantitative PCR for monitoring abortions due to Q fever in ruminant livestock.

We hope you enjoy reading this issue.

The Editorial Committee

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Glossary

Glossary of terms taken from the XP U 47-600-1, NF ISO 5725-1 standards and the Pr NF ISO 99999 (NF X 07-001) *International Vocabulary of Metrology (VIM)*

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M. Laurentie (2012). Glossary of technical terms for the validation of laboratory analytical methods in the framework of a quality process, EuroReference, N°8, ER08-12GL01 http://www.anses.fr/euroreference/numero8/

Glossary of technical terms for the validation of laboratory analytical methods in the framework of a quality process.

Method adoption

Prior to the 'routine' implementation of a duly characterised and validated molecular diagnostic method, method adoption is when the laboratory demonstrates its ability to undertake the analysis in question by showing that it has achieved the required performance level and/or the performance level announced in the characterisation and validation file for the method.

Bias (NF ISO 5725-1 standard)

Difference between the expectation of the test result and an accepted reference value.
NOTE: bias is the total systematic error as contrasted to random error. There may be one or more systematic error components contributing to the bias. A larger systematic difference from the accepted reference value is reflected by a larger bias value.

Repeatability condition (VIM 2.21)

Condition of measurement in a set of conditions that includes the same measurement procedure, same operators, same measuring system, same operating conditions and same location, and replicate measurements on the same or similar objects over a short period of time.

Reproducibility conditions

Conditions where test results are obtained with the same method on identical test items in different laboratories with different operators using different equipment over a sufficiently long period of time to consider that they are different test series.

Intermediate precision condition (VIM 2.23)

Condition of measurement in a set of conditions that includes the same measurement procedure, same location, and replicate measurements on the same or similar objects over an extended period of time, but may include other conditions involving changes.

Accuracy

Closeness of agreement between a test result and the accepted reference value.

Precision (of a measurement)

Closeness of agreement between test results obtained by replicate measurements under the same conditions
NOTE: precision depends only on the distribution of random errors and is not related to the true value or accepted reference value.

Precision (of an analytical method)

Closeness of agreement between test results obtained through a set of measurements under stipulated conditions.
NOTE 1: precision depends only on the distribution of random errors and is not related to the true value or accepted reference value.
NOTE 2: precision includes repeatability and reproducibility. An analytical method is precise when it produces very similar results both for the same operator taking multiple measurements and operators using it in different locations.

Trueness

Closeness of agreement between the average value obtained from a large series of test results and an accepted reference value.

Limit of detection (VIM 4.18)

Measured value obtained by a given measurement procedure for which the probability of falsely claiming the absence of a component in a material is , given a probability of falsely claiming its presence. This definition poses practical problems.

Limit of quantification

Lowest and/or highest concentration of an analyte that can be quantified under the described experimental conditions for the method. It is the lowest and/or highest concentration in the range of validity.

Mesurand (VIM 4.3)

Quantity intended to be measured.

Measurement (VIM 2.1)

Process of experimentally obtaining one or more values that can reasonably be attributed to a quantity.

Repeatability

Closeness of agreement between the results of successive measurements of the same measurand carried out under the same conditions of measurement.
NOTE: these conditions are called repeatability conditions. Repeatability conditions include: the same procedure, the same observer, the same measuring instrument used under the same conditions, the same location, repetition over a short period of time. Repeatability may be expressed quantitatively in terms of the dispersion characteristics of the results.

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Reproducibility

Closeness of agreement between the results of measurements of the same measurand carried out under changed conditions of measurement.

NOTE: a valid statement of reproducibility requires specification of the conditions changed. The changed conditions may include: method of measurement, observer, measuring instrument, reference standard, location, conditions of use, time. Reproducibility may be expressed quantitatively in terms of the dispersion characteristics of the results.

Analytical specificity

Ability to uniquely distinguish a target agent in the presence of other agents that are genetically similar to the target of interest and/or occupy the same ecological niche.

Diagnostic specificity

Proportion of known uninfected reference animals that test negative in an assay; uninfected reference animals that test positive are considered 'false positives'.

Validation of an analytical method

Confirmation through tangible evidence that requirements for a specific use or intended application have been fulfilled.

Verification stage consisting in comparing defined performance criteria values obtained when characterising a method with those that are expected or have been assigned beforehand (acceptability limits, targets to be reached) and declaring the analytical method as valid or invalid.

Verification

Provision of tangible evidence that a given entity fulfils specified requirements.

Point of view

Example of response to epidemics: the impact of two health emergencies (the emergence of the Bluetongue and Schmallenberg viruses) on a research and reference laboratory

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S. Zientara (2012). Example of response to epidemics: the impact of two health emergencies (the emergence of the Bluetongue and Schmallenberg viruses) on a research and reference laboratory, EuroReference, N°8, ER08-12PV01 http://www.anses.fr/euroreference/numero8/

Two viruses vectored by midges (the bluetongue virus and the Schmallenberg virus) emerged in northern Europe in 2006 and 2011 respectively. The Joint research unit (JRU) for Virology at ANSES's Maisons Alfort laboratory was confronted with the emergence of these two viruses in France. In just a few weeks, it was necessary to develop and validate serological and molecular diagnostic tools and to set up and coordinate a network of laboratories capable of processing thousands of samples while researching into the physiopathogenic mechanisms by which these infections operate. The structure of a JRU, where research teams work alongside the National Research Laboratory, enables it to meet these various requirements in a very short time.

Presentation of the Maisons Alfort UMR for Virology

The ANSES, INRA and ENVA Joint Research Unit (UMR) for Virology No.1161 (on the campus of the French National Veterinary School of Alfort - ENVA) focuses its activity on animal viral diseases representing a zoonotic and/or emerging risk. The UMR has a staff of about 40 people, working on: i) developing the most appropriate diagnostic tools for bio-monitoring and phylogenetics; ii) studying the physiopathology of some of these diseases, concentrating on the risks of inter-species transmission, especially from animals to humans; and iii) pursuing new approaches to vaccination, favouring those that can be administered orally. Apart from this applied and fundamental research, the UMR also houses reference laboratories at global level (OIE Reference Laboratory for epizootic haemorrhagic disease in deer), European level (EU Reference Laboratory for equine diseases) or national level (National Reference Laboratories – NLRs – for Bluetongue, foot-&-mouth disease, vesicular stomatitis, swine vesicular disease, African horse sickness and West Nile fever). This unusual association of ANSES-controlled reference activities and research activities helps the UMR confront emerging threats such as those that occurred in 2006 (with the emergence of bluetongue) or in 2011 (with the emergence of the Schmallenberg virus).

Brief history of the emergence of two vectored diseases in France and Europe

In August 2006, the European Commission gave official notification of the presence of bluetongue virus (BTV), a major pathogen for domestic and wild ruminants, in the Netherlands, Belgium and Germany. Although BTV had been circulating in the Mediterranean basin for several years, this was the first epizootic episode of the disease documented in northern Europe and the first time serotype 8 had been identified on the continent. At the end of 2006, six outbreaks were reported in the Grand-Duchy of Luxembourg and in France. Unexpectedly, the virus, which is vectored by midges of the *Culicoides* genus, survived over the winter period and spread rapidly over a large part of northern Europe in 2007 and 2008. Cattle were soon found to be heavily affected, while mortality in sheep reached 30% [3], in regions where the usual vector, *Culicoides imicola* (known to be responsible for the transmission of BTV in the Mediterranean basin), has never been found. In France, more than 50,000 outbreaks were reported between 2007 and 2008. However, the prophylactic measures that were rapidly taken (a massive vaccination programme) successfully controlled the epidemic and most of the countries concerned have now recovered their BTV-free status [5].

The sudden and unexpected emergence of serotype 8 of BTV (BTV-8) was a major animal health event for Europe and just a few years later history seemed to be repeating itself with the emergence of a new arbovirus affecting ruminants in northern Europe.

During the summer of 2011, several cases of febrile diarrhoea together with loss of appetite and a significant drop in milk production were reported in adult cattle in Germany (North Rhine-Westphalia), sometimes with clinical symptoms similar to those for BTV, giving rise to fears of a return of bluetongue. These symptoms were transitory and generally disappeared in a few days. The search for numerous pathogens in samples taken from affected cattle proved negative, despite the use of innovative techniques such as the Epizone Biochip 5.1, which contains more than 2000 virus primers. After many investigations had been carried out, in November 2011 the Friedrich-Loëffler-Institut (FLI) in Germany used high-speed sequencing without prior knowledge on blood samples from diseased cattle to identify nucleotide sequences belonging to a new virus that was given the name of the town the samples came from – the Schmallenberg virus (SBV) [1]. The implication of SBV in the clinical symptoms observed was later confirmed by the experimental infection of 9-month-old cattle, which showed that the viremia caused by the SBV seemed to be transitory (4 days) [1]. Analysis of the virus's gene sequence showed similarities with the Akabane, Aino and Shamonda viruses, which belong to the *Orthobunyavirus* genus in the *Bunyaviridae* family.

The FLI rapidly developed a test to detect the genome of the SBV by RT-PCR in real time, and the protocol was shared with several European partners. At the same time, a bio-surveillance scheme was set up across Europe.

In December 2011, the Netherlands for the first time reported SBV having a teratogenic effect on sheep, with similar characteristics to the effects observed with the Akabane and Aino viruses [1]. Female sheep, goats and cattle infected at the start of gestation were capable of transmitting the virus to their foetuses which then developed atypical malformations,

Point of view

most often leading to stillbirths or death of the offspring shortly after birth.

On 25 January 2012, the virus's genome was detected for the first time in France by our laboratory, in the brains of stillborn lambs from two farms in Moselle and Meurthe-et-Moselle (north-east France).

On 1 July 2012, 5234 outbreaks of SBV had been reported in Europe, 2865 in cattle, 2491 in sheep and 78 in goats (source: www.survepi.org).

Initial diagnosis of BTV and SBV viruses

In France, both in August 2006 and in January 2012, the NRL would have come under considerable strain if it had received all the biological samples taken from suspected cases of BTV or SBV. For example, for BTV in 2007, the Italian veterinary authorities insisted that some 100,000 cattle (grass-fed calves) exported from the centre of France to the Po Valley be tested individually by RT-PCR which the three people staffing the NRL could never have handled alone. The structure of the UMR, which combines research with reference activities, enabled us at least in the first few weeks of the two crises, to redeploy certain personnel (technicians from other teams interrupted their research projects and switched to helping their colleagues). We also worked in collaboration with various companies specialising in veterinary diagnosis (AES-ADIAGENE, LSI, IDvet, IDEXX and others) and asked them to develop molecular virology diagnosis kits that had to be sensitive, specific, inexpensive and automatable. For BTV, LSI and AES-ADIAGENE first used our in-house PCR test [2, 4] before developing their own methods. The same approach was later applied for the SBV crisis.

The same procedure was followed for the development of serology kits. We worked with IdVet to validate an ELISA diagnosis kit at the end of February 2012 [6]. It was in fact the first ELISA test developed anywhere in the world for detecting SBV antibodies.

In parallel with this, and with the support of the Directorate General for Food of the French Ministry of Agriculture, we set up, trained and organised a network of 66 departmental veterinary laboratories which were able to process several thousand biological samples a day (for BTV and SBV). The network first used the real-time RT-PCR kits created by LSI and AES ADIAGENE, developed and validated with help from our laboratory. (As a result, all samples found to be positive by RT-PCR were sent to the UMR for viral isolation, for both BTV and SBV). For SBV, the network was able to use the indirect ELISA serology test developed by IDvET for detecting antibodies to the nucleoprotein of the Schmallenberg virus.

We can therefore see that in each of these two health crises we were able to set up in only six weeks a large-scale diagnosis system consisting of an NRL and a network of over 60 laboratories capable of using real-time RT-PCR for the molecular diagnosis of infection by BTV or SBV, plus serodiagnosis for SBV.

Conclusion

The mutual respect and confidence that has been established between the different National Reference Laboratories on the subject of BTV since 2000 (when the virus first emerged in the Mediterranean basin) enabled us to exchange protocols and reagents rapidly and efficiently in order to start screening for BTV and SBV in the different countries of Europe. During the BTV crisis, the RT-PCR method that we had developed and validated [2] was transferred rapidly to our counterparts in the other European NRLs. Equally, for the case of SBV in France, it was during a meeting in Brussels in November 2011 that our German opposite numbers told us they had identified this new virus. By mid-December 2011, the specific RT-PCR for detecting SBV had been made available to the French NRL at Maisons-Alfort. From this time on, numerous scientific and technical exchanges have taken place between the national laboratories.

It can therefore be said that the BTV crisis had the effect of fostering scientific and professional relationships between the various national laboratories of the Member States of the European Union, which now share the information they hold, almost in real time. This is a considerable weapon in the event of emerging threats.

The Schmallenberg virus was detected in Germany by FLI using metagenomics. Considering the cost and highly specialised nature of this type of technology, cooperation clearly allows different laboratories to benefit from this type of method without investing the considerable sums that would otherwise be necessary, for what may prove to be only occasional use.

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Lab news

10th International meeting on microbial epidemiological markers, October 2-5, 2013

Sylvain Brisse, Institut Pasteur, Paris, France

The ability of microbes - bacteria, viruses, fungi and parasites - to mutate rapidly, disseminate and adapt to new hosts and environments, forces us to increase our capabilities for the early recognition of novel strains of pathogens, and to understand the factors that contribute to their diversity, evolution and dissemination. IMMEM-10 will address a variety of topics related to pathogen emergence, population-level diversity, evolution of virulence and antibiotic resistance, strain tracking, typing networks, public health and surveillance, novel typing approaches, high-throughput sequencing, genomics, and molecular epidemiology of infectious diseases. The meeting will take place at Institut Pasteur, in the heart of Paris.
For more information: http://www.immem-x.org

I3S - International Symposium "*Salmonella* and Salmonellosis" 27-29 May 2013, Saint-Malo, France

G. Clément, ISPAIA - ZOOPOLE développement, Ploufragan, France

The next International Symposium «*Salmonella* and Salmonellosis» will be held on 27th to 29th May 2013 at Saint-Malo France. This symposium will be arranged by ANSES, InVS, INRA, Institut Pasteur and ISPAIA - ZOOPOLE developpement. More than 400 participants will attend the symposium I3S 2013.
You can find all information at www.i3s2013.com .
Contact: i3s2013@zoopole.asso.fr

Lab news

An International Workshop in Paris: "*New Trends on Analytical Methods for Pesticides and Drug Residues*"

E. Verdon, ANSES-Fougères Laboratory, Fougères, France

As a follow-up of a previous meeting held in Paris in November 2009, a new scientific workshop is being jointly organised in April 2013 by the European section of the "AOAC International" (*Association of Official Analytical Chemists International*) and by ASFILAB (the French Association of Managers for Analytical Auality and Reliability). It will take place on 18 and 19 April at the Espace Saint Martin located in the heart of Paris close to the Centre Beaubourg.

This workshop, entitled *"New Trends on Analytical Methods for Pesticides and Drug Residues"*, will aim at discussing some of the latest advances in the analysis of chemicals in food. One of the sessions will be dedicated to veterinary drug residues and the other one to pesticide contaminants. It will include presentations of European projects based on new technologies such as hybrid high-resolution mass spectrometry, multi-class or multi-contaminant analysis methodologies, and new non-targeted approaches (metabolomics, etc.).

On this occasion, the ANSES Laboratory based in Fougères will participate in the organisation and chairing of the session dedicated to veterinary drug residues.

A preliminary programme can be obtained from the AOAC-Europe website: http://www.aoaceurope.com as well as the Asfilab website: http://www.asfilab.fr.

XVIIIth WVPAC Congress in Nantes, from 19 to 23 August 2013

G. Clément, ISPAIA - ZOOPOLE développement, Ploufragan, France

In August 2013, poultry veterinarians and scientists specialising in poultry health will hold their world conference in Nantes. This WVPAC2013 congress is being organised jointly by the French branch of the World Veterinary Poultry Association (WVPA) and ZOOPOLE Développement – ISPAIA. The WVPA has more than 2000 members and national branches in around 40 countries.

In Nantes, more than 1000 professionals are expected to take stock of research advances and development of new practices. This event will give them an opportunity to enhance their knowledge and networks. Experts from around the world will be attending WVPAC2013 and the extensive conference programme will cover important topical issues such as viral diseases, avian influenza, food safety, digestive health and mycotoxins. It will provide an update on the latest research, a roundup of new techniques and experiences in the field.

According to Nicolas Eterradossi, Chairman of the organising committee: "The French organisers have taken great care to put together a rich scientific programme at a high level, combined of course with an attractive social programme, to ensure the success of WVPAC2013, a major event for our poultry sectors. We look forward to welcoming the entire profession here to Nantes in August 2013!"

The call for papers is open until 20 December 2012.

For further information on WVPAC2013, please contact Geneviève Clément - wvpac2013@zoopole.asso.fr while the call for papers and all the latest news on the conference can be found at www.wvpac2013.org.

Lab news

Guide from the EURL for *Listeria monocytogenes*

B. Carpentier, L. Barre, ANSES - Laboratory for Food Safety, Maisons-Alfort, France

Publication of a guide on sampling in processing areas and equipment used in the production of foodstuffs with a view to detecting the presence of *Listeria monocytogenes*.

As part of the activities of the EURL for *L. monocytogenes*, a guide entitled "Guidelines on sampling the food processing area and equipment for the detection of *L. monocytogenes*" has just been published. It is available online at http://www.ansespro.fr/eurl-listeria/

This guide is intended to address shortcomings in the international standard on surface sampling techniques (ISO 18593), by giving specific recommendations on detecting *L. monocytogenes* in order to increase the probability of detecting this pathogenic bacteria when it is persistent in a production unit. In particular, the guide explains when and on which surfaces these samples should be taken.

This guide provides guidelines for operators in the ready-to-eat food sector who, in accordance with Regulation (EC) 2073/2005 on microbiological criteria for foodstuffs, have an obligation to take such samples.

Building a dialogue between NRLs and French accredited laboratories ('peripheral laboratories')

B. Gouget, ANSES – Scientific Affairs Department for Laboratories, Maisons-Alfort, France

In order to better identify the expectations of accredited laboratories in relation to NRLs, ANSES and the Joint Laboratories Service Unit (a scientific unit that fulfils the missions of the Directorate General for Competition Policy, Consumer Affairs and Fraud Control and the Directorate General for Customs and Indirect Taxes) invited all of France's accredited laboratories to complete an online satisfaction questionnaire. Each accredited laboratory was asked to fill out a separate questionnaire for each area of activity in which it undertakes official analyses. These areas of activity cover several NRLs and are defined in the five broad categories listed in the Ministerial Order for the appointment of NRLs (Ministerial Order of 19/10/2011 published in the Official Journal of 28/10/2011; http://www.legifrance.gouv.fr/affichTexte.do?cidTexte=JORFTEXT000024720808&dateTexte=&categorieLien=id): Biological contaminants found in foodstuffs – Chemical contaminants, residues and additives – Animal diseases – Plant health – GMOs.

The questionnaire was divided into 24 questions addressing the following points:

- scientific and technical support, methods and reference materials ;
- general organisation of ILPTs ;
- monitoring and alerts ;
- general perceptions of relations between the accredited laboratory and the NRLs.

This broad approach opened up some interesting lines of thought for all of the NRLs. On 13 November 2012, representatives from all of the NRLs worked together to analyse key factors to achieving successful relations between the NRLs and peripheral accredited laboratories as well as points for which an action plan (strengthening or easing) could be proposed together with the French certifying body and supervisory authorities.

A selection of points identified in this session will be presented and discussed with the accredited laboratories in a general meeting that will be held on 26 March 2013 (contact: ds@anses.fr).

EuroReference
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Journal No. 8
Winter 2012

Networks

The *Salmonella* Network, a tool for monitoring *Salmonella* "from farm to fork"

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The ANSES Maisons-Alfort Laboratory for Food Safety is associated with the National Reference Laboratory for *Salmonella* (NRL-*Salmonella*) for the serotyping of *Salmonella* (Associate NRL). It coordinates the network for epidemiological surveillance of *Salmonella* in the food chain in France. This *Salmonella* Network is made up of about 140 French laboratories that send their serotyping results or strains for confirmation to the Associate NRL on a voluntary basis. By centralising all these results, it has been possible for more than 10 years to monitor trends in the isolation of *Salmonella* serovars in the food chain and detect the emergence of particular serovars or strains with characteristics critical to human health.

Background

Salmonella is one of the main microbiological contaminants responsible for foodborne illnesses in Europe. In 2010, EFSA reported 99,020 cases of human salmonellosis in Europe, although the decline in the number of annual cases observed for several years seems to be continuing (EFSA, 2012). In France, the number of foodborne illness outbreaks due to *Salmonella*, which has steadily declined since 2002, remained stable between 2009 and 2010 (InVS, 2012). In 2010, this bacterium was (or was suspected of being) responsible for 141 outbreaks of foodborne illness (20% of outbreaks with a confirmed or suspected agent), corresponding to 1357 human foodborne cases. Food items mainly involved are eggs and egg products, as well as meat.
Identification and characterisation of *Salmonella* remain essential for the epidemiological surveillance of contamination throughout the food chain and for the control of this pathogen.

The system for monitoring *Salmonella* and salmonellosis in France

Several organisations are involved:

- the National Reference Centre (NRC) for *Salmonella* at the Institut Pasteur performs serotyping of strains of human origin, sent by medical biology testing laboratories and hospital laboratories, and collects information on strains whose serovar has already been determined. These data are analysed in order to monitor changes in the number of *Salmonella* strains isolated from humans and detect outbreaks. The antimicrobial resistance of *Salmonella* is also studied;
- the French Institute for Public Health Surveillance (InVS), whose main task is to monitor the population's health status, analyses the signals sent by the NRC (clustered cases, outbreaks, etc.) and where necessary initiates investigations to identify any common source for the human cases. The aim is to take measures to limit the number of human cases (withdrawal and recall of a product, for example). The InVS also centralises and analyses data from the mandatory reporting of any foodborne illnesses notified to the Departmental Directorates for the Protection of Populations and Regional Health Agencies;
- the ANSES National Reference Laboratory (NRL) for *Salmonella* and its Associate NRL, the Maisons-Alfort Laboratory for Food Safety, deal with *Salmonella* strains of non-human origin. The Maisons-Alfort Laboratory for Food Safety characterises strains and coordinates a network of 140 food and veterinary testing laboratories, both public and private, known as the *Salmonella* Network, which collects strains from a variety of isolation contexts (self-inspections conducted by food-processing industries, official monitoring and control plans, investigations, food scares) and the epidemiological information associated with these isolates (David *et al.,* 2011).

Each year, in addition to the serotyping performed systematically, some strains are tested for their sensitivity to antimicrobials. Resistance mechanisms associated with phenotypes of interest to public health are studied. As a result of this, in 2009 the *Salmonella* Network identified a bacteria for the first time in food (*Salmonella* serovar *S.*I 4,12:i:- isolated from chicken meat) that carried the *armA* gene conferring high-level resistance to aminoglycosides of clinical interest (Granier *et al.,* 2011).
Centralising data on the phenotypic and genotypic characterisation of *Salmonella* collected by the *Salmonella* Network enables emerging clones to be detected and reveals epidemiologically related strains during investigations of episodes of clustered human cases.
Between 2005 and 2010, the *Salmonella* Network was called on 47 times by the Directorate General for Food and the InVS to identify potential sources of contamination and assist with epidemiological investigations.
The regular collection of serotyping information and results combined with a statistical time-series analysis of isolation of

Networks

Salmonella enables the detection of signals corresponding to a new or emerging situation of concern. The *Salmonella* Network has already shown its value to risk managers through its former alert function (Danan *et al.,* 2011).

Salmonella Network operation

The network has two objectives: (1) To provide food and veterinary testing laboratories with technical support for serotyping of *Salmonella* isolates, (2) To develop vigilance with respect to monitoring *Salmonella* isolated from the food chain ("from farm to fork") and detect signs indicating any unusual increase in a serovar.
Each year since 1997, a subscription charter has been signed by each partner laboratory (approximately 140 per year). Information in three areas is collected: (i) animal health and production (sick animals, healthy carriers or the farming environment); (ii) food hygiene (intended for human or animal consumption, slaughterhouse environment, cutting and processing units); (iii) the natural ecosystem.
Salmonella are isolated from samples taken throughout the food chain by numerous laboratories that currently provide good national coverage of first-line analyses. Almost all (97%) of France's public departmental laboratories are network members. The *Salmonella* serotyping method (Danan *et al.,* 2009) used by the Associate NRL on strains received for confirmation is implemented according to the NF EN ISO/IEC 17025 standard, under COFRAC accreditation (www.cofrac.fr , accreditation no. 1-2246).
The *Salmonella* Network partner laboratories send pure strain cultures or summary tables of serotyping results. For each result, information is collected about the sample's context, type and origin (Figure 1).
The data collected cannot be treated as prevalence data because the *Salmonella* Network receives no indication about the total number of tests performed. European regulations on zoonoses, which target certain farming sectors and serovars, impose a selective pressure that may have an impact on the feedback of information.
However, the relative stability of the network data and the similarities observed in the past regarding trends in certain serovars isolated in both humans (NRC) and food (NRL) underline the network's importance in the national *Salmonella* monitoring system. Its annual reports are available from http://www.ansespro.fr/reseausalmonella.

Main trends observed in recent years

The *Salmonella* Network collects about 15,000 *Salmonella* serotyping results each year (Table 1). Between 2005 and 2010, depending on the year, 55% to 65% of these serotyping results were obtained by the laboratories and sent to the *Salmonella* Network. In the remaining cases (35% to 45%), the strains were serotyped by the Associate NRL, either because the originator laboratory does not perform complete serotyping, the serotyping was more complex, or confirmation was needed in the context of official controls.
Each year, two thirds of the serotyping results ultimately obtained come from the "animal health and production" sector (of which 80% are obtained from partner laboratories and 20% by the Associate NRL) and one third from the "food hygiene" sector (40% and 60% respectively).
Figure 2 shows the overall decline in the relative annual share of the serovars *S.* Enteritidis and *S.* Typhimurium observed by the *Salmonella* Network. A similar observation was reported by the NRC for strains of human origin isolated between 2002 and 2010 (Jourdan-Da Silva and Le Hello, 2012). This decrease is probably due to the impact of control and management measures applied in the poultry sector in recent years.
The frequency of isolation of strains *S.*I 1,4,[5],12:i:-, known as

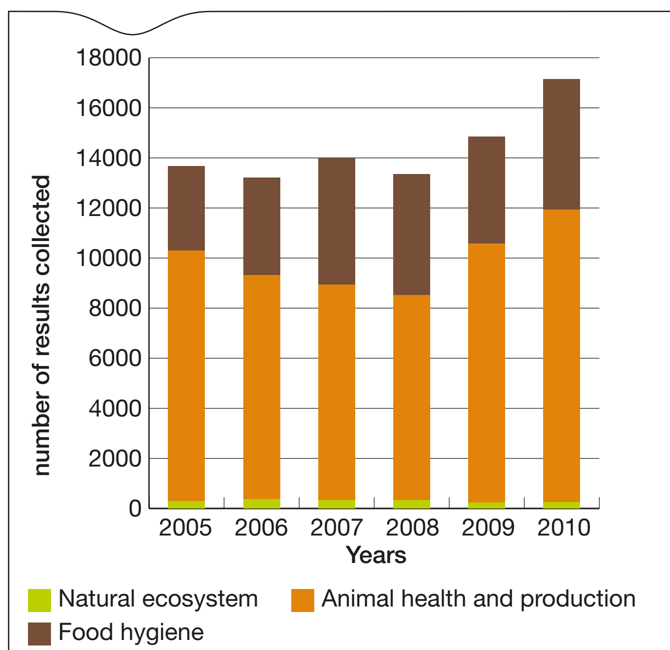

Figure 2. Number of *Salmonella* serotyping results recorded within the ANSES *Salmonella* Network between 2005 and 2010.

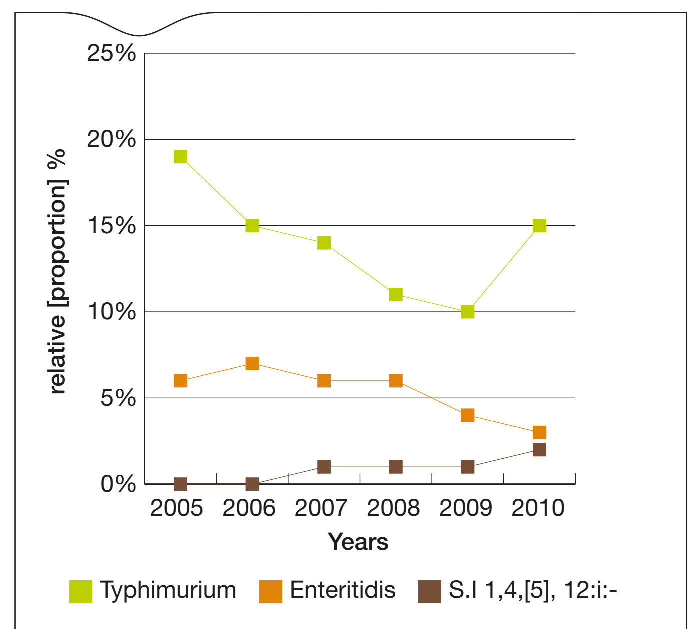

Figure 3. Trends between 2005 and 2010 in the relative proportions of serovars Typhimurium and Enteritidis in the *Salmonella* Network, emergence of the monophasic variant *S.*I 1,4,[5],12:i:-.

Networks

"monophasic variants" of *S*. Typhimurium, has been increasing for several years in humans (NRC data) and since 2008 in all the animal and production sectors monitored by the *Salmonella* Network (Figure 2; Table 2). These trends are consistent with the increase observed since 2007 in the number of outbreaks of clustered cases involving these strains in France (Danan *et al.,* 2012; Gossner *et al.,* 2012) and Europe (Bone *et al.,* 2010; Hopkins *et al.,* 2010).
The annual monitoring data presented in the inventories of *Salmonella* of non-human origin (2005 to 2010) available on the network's website, highlight a specific association of certain serovars with certain animal sectors or food types (Table 2), such as Dublin in dairy products, Indiana in poultry or Enteritidis in egg products.

In food hygiene

Among *Salmonella* isolated from pork meat, the relative proportion of serovar Typhimurium has been stable since 2005 (30 to 35% of the sample panel), while that of serovars Derby and *S*.I 4,[5],12:i:- increased from 20% to about 40%, and from 0 to 5.5% respectively. For delicatessen meats, Typhimurium and Derby remain the most frequently identified serovars, but the growing relative importance of serovar *S*.I 4,[5],12:i:- between 2008 (3.4%) and 2010 (10%) is noteworthy.
The relative proportion of *S*. Typhimurium in dairy products is decreasing (12% in 2005 compared with 6% in 2010). Concerning egg products, the few isolates identified in this food category only emphasise the relative stability of serovars Typhimurium and Enteritidis between 2005 and 2010.
With regard to the hygiene of duck carcasses, meat and offal, the serovars Indiana, Typhimurium and Kottbus are the most frequently isolated and have been relatively stable since 2005. The distribution of serovars is much more variable for the "turkey" and "*Gallus gallus*" sectors, although since 2005 the main serovars have remained Agona, Bredeney, Derby, Hadar, Indiana and Typhimurium in turkeys, and Enteritidis, Indiana, Typhimurium and most recently Paratyphi B in *Gallus gallus*. The serotype *S*.I 4,[5],12:i:- has also emerged in the "turkey" and "*Gallus gallus*" sectors since 2009.

In animal health and production

Since 2005, Senftenberg has been the serovar most frequently isolated from the *Gallus gallus* and turkey farming environments whereas in the duck sector it is the serovar Indiana. In the cattle sector, Typhimurium, Montevideo and Dublin are predominant, with relative stability since 2005, isolated both from farming environment samples and in the context of animal disease. In the pork sector, each year since 2005, the two main serovars (Typhimurium and Derby) have accounted for between 60% and 80% of all *Salmonella* isolates.

Conclusion

Despite not providing consolidated data on prevalence, the *Salmonella* Network provides an appreciation of the diversity and spatiotemporal evolution of isolated serovars, for the entire food chain. In particular, it is a source of information on rare serovars or those not covered by the regulations, and can act as an alert mechanism for the health authorities.
The voluntary mobilisation of the *Salmonella* Network's partner laboratories and the close collaboration between the reference laboratories (NRC and NRL) are essential prerequisites to the efficient running of the national *Salmonella* monitoring system. Coordination and regular assessment of the *Salmonella* Network's operation, harmonisation of analytical methods and data repositories to be shared, and the resources and communication tools implemented are critical to achieving monitoring objectives.

Table 1. Relative frequency (%) of the main serovars detected within the *Salmonella* Network, by food category, in 2010 (N = total number of isolates)

SEROVARS	Poultry (N = 629)	Egg products (N = 35)	Pork (N = 1155)	Delicatessen meats (N = 523)	Beef (N = 154)	Dairy products (N = 815)	Animal feed (N = 1113)
TYPHIMURIUM	14.2	5.7	30.6	32.5	37.7	5.8	2.9
DERBY	3.2	0	37.4	17.9	5.8	5.4	1
HADAR	1.9	0	0	0.2	0	0	0.1
MONTEVIDEO	1.4	0	0	0.2	2.6	2.9	19.9
INDIANA	25.6	0	0	1.3	0.6	0	0.4
AGONA	2.7	0	0.5	3	1.3	4	0.9
DUBLIN	0	0	0	0.2	10.4	57.6	0
ENTERITIDIS	4.7	22.9	0.3	0	0.7	0.3	0.3
MBANDAKA	1.7	22.9	0.3	0.2	13	2.5	6.3
RISSEN	0.5	0	2.2	9.8	0	0.4	3
S.I 1,4,[5],12:i:-	3.5	0	5.5	10	11	1.3	0.5
S.IIIb 61:[k]:1,5,7	0	0	0	0.2	0.7	5.6	0
TOTAL %	**59.4**	**51.5**	**76.8**	**75.5**	**83.8**	**85.8**	**35.3**
Total number of serovars identified (100%/category)	**51**	**11**	**44**	**50**	**30**	**37**	**109**

Networks

We would like to thank all the partner laboratories that regularly send strains and epidemiological information to the Salmonella Network.

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Networks

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Livraison colis / souches : Pôle HQSA - Unité CEB - 22, rue Pierre Curie - 94700 Maisons-Alfort

anses

FICHE DE RENSEIGNEMENTS *SALMONELLA*

Ne rien inscrire dans ce cadre
N° Colis HQSA : .... *Souche n°:* ....

HYGIENE DES ALIMENTS / ECOSYSTEME NATUREL

(remplir une fiche par souche envoyée)

☐ **Sérotypage** ☐ **Typage moléculaire**

Votre réf de souche : ....
Autres réf : n° alerte DGAl : .... n° alerte DDPP : ....

Date du prélèvement : ...../........../..........

Code postal (ou département) du prélèvement : ....

Adresse du laboratoire :

Abonné ☐	Non Abonné ☐

Code laboratoire :

▶ **Contexte du prélèvement**

☐ Contrôle "Exploitants" (Autocontrôle) ☐ Contrôle "Autorités" (Contrôle officiel)
☐ Plan de Surveillance/Plan de contrôle DGAL/DGCCRF* : Note de service N°
☐ Enquête, étude ☐ Autre :

Le prélèvement a t-il été réalisé dans un contexte de toxi-infection alimentaire? ☐ Oui ☐ Non
Si oui, nombre de malades : ....

▶ **Caractéristiques du prélèvement** — Merci d'être précis en remplissant le cadre ci-dessous

ALIMENTS DESTINES A L'HOMME
(de l'abattoir à la consommation)

Type du prélèvement
☐ Produit alimentaire ☐ Environnement

Préciser le site du prélèvement :
☐ Abattoir ☐ Atelier de fabrication ☐ Distribution

Catégorie d'aliment
☐ Viande ☐ Produit de charcuterie ☐ Lait et produit laitier
☐ Œuf et ovoproduit ☐ Produit de la pêche ☐ Eau
☐ Produit végétal ☐ Autre produit

Nature du prélèvement :

Le produit est-il ☐ cuit ou ☐ cru ?

Filière :
☐ Bovine ☐ Porcine ☐ Caprine
☐ Equine ☐ Ovine
☐ Aviaire* : poulet de chair, poulet sous label, dinde, canard, pintade,
Autre (préciser) : ....
☐ Autre filière : ....

ECOSYSTEME NATUREL

Nature : (pour les eaux, préciser : eau de mer, de rivière, ...)

▶ **Caractères sérologiques de la souche**

☐ OMA ☐ OMB ☐ O: 4,5 ☐ O: 3,10,15 ☐ O: 9 ☐ O: 6,7,8
☐ H: i ☐ H: E ☐ H: h ☐ H: 1 ☐ H: 2 ☐ Autres : ....

Sérotype présumé : ....

* Entourer la mention qui convient.

Figure 1. Example of the form used by the *Salmonella* Network to collect information associated with an isolate from a food intended for humans or from the ecosystem.

Journal No. 8 Winter 2012

Research

Towards the development of robust protocols for the establishment of MRLs for veterinary drugs in honey

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Despite widespread concern about the decline in the health of bee colonies in Europe and the rest of the world, beekeepers have access to relatively few veterinary medicines to treat bee diseases. This situation is unlikely to improve in the absence of an internationally agreed protocol for the establishment of Maximum Residue Limits (MRLs) for these medicines in honey. Therefore, a research project has been initiated to develop a statistically valid and harmonised sampling protocol to provide the robust scientific data needed to assist regulators in proposing MRLs for veterinary drugs in honey.

Introduction

Whilst rigorous guidelines exist for calculating the withdrawal time for veterinary medicines in most food producing species, these are not well defined for bees/honey. There is currently no robust protocol for conducting studies which would provide the data necessary to support an application for approval of a treatment and to establish a Maximum Residue Limit. The situation is not helped by the fact that bees are considered a minor species so there is little financial incentive for pharmaceutical companies to invest in the development of new treatments.

The European Commission "*Notice to applicants and Guideline - Veterinary medicinal products - Establishment of maximum residue limits (MRLs) for residues of veterinary medicinal products in foodstuffs of animal origin (Volume 8)*" states that for honey, a depletion study (to determine a withdrawal period) should comprise 5 samples from each of 5 hives, at time points defined according to the period of treatment and the production of the honey. Although helpful, these guidelines could be subject to interpretation.

Outside of the European Union there are no international agreements on setting MRLs in honey. This was highlighted by the fact that the Joint FAO/WHO Expert Committee on Food Additives (JECFA) recently informed the Codex Committee on Residues of Veterinary Drugs in Food (CCRVDF) that there were no recommendations and/or procedures that JECFA could follow for setting MRLs in honey (FAO 2011). In response to this the Codex Committee on Residues of Veterinary Drugs in Food (CCRVDF) set up a working group to:

- collate data on treatments that have been authorised throughout the world ;
- identify common or related parameters used when authorising treatments ;
- propose a risk assessment policy for setting appropriate limits in honey (Codex 2011).

The working group reported that substances to be considered as possible bee treatments should be categorised on the basis of known toxicity, fate and behaviour. For substances considered to be safe it was proposed that a residue study could be waived (e.g. thymol). For substances (e.g. tetracycline) with an established Acceptable Daily Intake (ADI) and/or MRL in a food producing animal or food commodity extrapolation to bees may be possible subject to a depletion study which would also determine an appropriate marker residue. Substances

which are not approved for use in food animals, or which are new drug entities, would require a full residue study, which may not be financially viable for a minor species such as bees.

Such residue studies will set a challenging task because of the need to take into account many variables. Previous studies have identified a large number of factors, including the variability of residue concentrations within and between hives, the effects of timing and application of the treatment, the properties of the substance used for treatment as well as seasonal and climatic factors. A recent study (Fussell et al 2012) concluded that the size of the sample collected and the number of hives in the study should be sufficiently large to minimise the variability in the

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measurement of residue concentrations, including sampling, to acceptable values.

The CCRVDF working group considered the evidence available from a recent study (Fussell 2012), together with previous studies, and has proposed a draft protocol for conducting residue studies for honey. The CCRVDF recommendation is to collect all of the honey in supers available from a minimum of five treated hives at each of a minimum of eight different time points. The intervals between the time points should be set to characterise the typical depletion profiles observed in previous studies (see Figure 1). A minimum of five 'control hives' should not be treated while being maintained at a separate location at a distance sufficient to avoid cross contamination by drifting of bees from treated hives.

The Veterinary Medicines Directorate, an agency of the UK Government, is funding a new study to evaluate this CCRVDF draft protocol. The project, being conducted by the Food and Environment Research Agency in the UK, will focus on the development of statistically based methods for collecting samples of honey. It will take place over a minimum of three years in order to take into account seasonal and climatic variations. This is important because the ways in which bees store and move honey within and between hives could influence the distribution of the medicines. The first phase of the project, a field study involving five control hives and 45 treated hives started in May 2012. The hives were treated in late Spring with a 'model compound' (ciprofloxacin), a fluoroquinolone antimicrobial, which was dissolved in syrup solution and applied onto the bees between the frames. Ciprofloxacin was selected because it is stable and yields residues that are measurable by Liquid Chromatography tandem quadrupole Mass Spectrometry.

The honey from all frames in the individual supers [(1)] from each hive will be extracted, combined and thoroughly mixed to produce a bulk sample to represent that particular super. The results of replicate analysis will be averaged to give the mean residue concentration in each of the individual supers. The results for the individual supers will then be averaged to give the mean residue concentration for each hive. Therefore, the smallest unit for the purposes of statistical analysis will be an individual super. If the results from 2012 demonstrate that satisfactory data can be obtained using fewer hives, then this will be reflected in the experimental design for second phase of the project due to start in 2013. It is envisaged that a reduction in hive numbers in subsequent phases in Years 2 and 3 will encourage collaborators from around the world to contribute. This would enable an assessment of the effects of a greater number of factors including the different genetic strains of bees, different bee husbandry practices, different treatment practices and geographic factors, leading to the generation of a much more comprehensive data set.

Anses-Fougères, as a Reference Laboratory for veterinary drug residues in food, expressed a specific interest in this study. Following the recent signing of a Memorandum of Understanding between Fera-York and Anses-Fougères for potential future scientific collaboration on veterinary drug residues, Anses-Fougères will contribute analytical expertise for the determination of antimicrobial residues in honey and be involved in the statistical assessment of the emerging data.

The objective of this research project is not to support the authorization of any specific product but to develop a protocol that can be used to generate robust residue depletion data.

Data from these experiments will provide statistical information that could be used to better define the experimental requirements for a suitable residue depletion study and hence assist with the calculation of fit-for-purpose withdrawal periods.

Conclusion

It is anticipated that this study will provide a statistically valid protocol that can be used by the UK and others to assist with the establishment of MRLs in honey.

The study will directly support international initiatives within the EU, the Codex Committee on Residues of Veterinary Drugs in Food and JECFA.

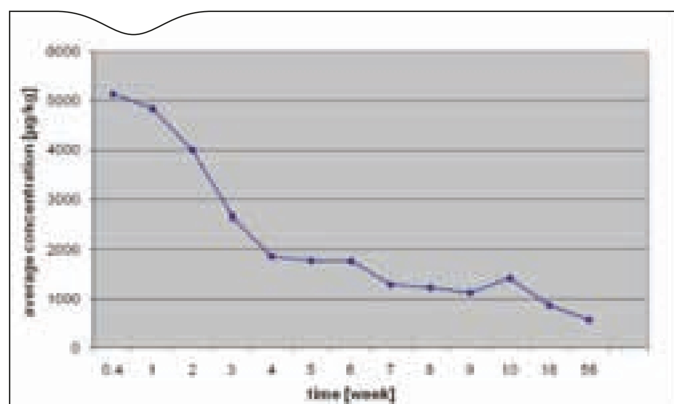

Figure 1. Depletion of ciprofloxacin in super honey collected up to 56 weeks after treatment (based on results averaged from nine individual hives

Spray application of medicine in syrup solution

(1) Supers are the individual boxes that are placed above the brood box, and in which honey is stored prior to collection for human consumption.

Research

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Acknowledgements

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Methods

Serodiagnosis of contagious bovine pleuropneumonia by immunoblotting

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Contagious bovine pleuropneumonia (CBPP) is a bacterial disease of cattle caused by *Mycoplasma mycoides* subsp. *mycoides* biotype Small Colony (MmmSC). CBPP is listed by the World Organisation for Animal Health (OIE) as notifiable and notification is also mandatory on a national level. This results in strict health control measures, including depopulation and restrictions on livestock movements and exports.

Once a worldwide livestock affliction, CBPP now only persists in Africa and some areas of Asia. Europe has been considered CBPP-free since 1900 inspite of a few local outbreaks. The last outbreak was widespread, affecting all of south-western Europe between 1980 and 1999. It resulted from the insidious spread of a new, less virulent variant of MmmSC (Nicholas *et al.*, 1996).

The attenuated and chronic forms of PPCB are difficult to detect. They are the main source of spread and can often only be detected by systematic serological testing. These subclinical forms of the disease are typical of infections with the contemporary European variant of PPCB. The complement fixation (CF) test and competitive ELISA are the OIE-approved serological tests. However, their lack of sensitivity makes them inefficient in cases of low prevalence and in infection with the less immunogenic European strain of CBPP. Only the routine use of the immunoblotting test (IBT) in Europe made it possible to eliminate the disease in the last affected areas in Portugal in 1999 when all other strategies had failed (Nicholas *et al.*, 2008). This highly sensitive and highly specific test (Schubert *et al.*, 2011) is recommended by the OIE as a confirmatory test. However, as it is currently described in the OIE manual (2008), IBT lacks reproducibility and robustness. The only international IBT inter-laboratory proficiency test (ILPT), carried out in 2009, showed that results vary widely among laboratories. Here, we propose some improvements to standardise reagent production as well as the serodiagnosis procedure to increase reproducibility and to set up quality controls.

Reagent production: selecting the strain, preparing the antigen and controlling quality

The choice of the strain to use is an extremely critical point. The use of the B103 strain, the reference strain used by Regalla *et al.* in 1999 to develop the IBT method, is highly recommended and should be used to ensure consistent results among laboratories. The antigen is then prepared from a culture grown in mycoplasma broth medium (Poumarat *et al.,* 1991) and concentrated to 10^{11} to 10^{12} CFU/mL (Table 1). It is absolutely essential to ensure that the cultured strain correctly expresses the five specific proteins targeted in the IBT, which are 110, 98, 95, 60-62 and 48 kDa in size, as defined by Gonçalves *et al.* (1998). The expression levels of these proteins can vary according to the MmmSC strain used and the selected clone. Thus, prior to use, each antigen batch must be assessed with positive and negative reference sera (Table 1).
The culture must be checked for contamination by other mycoplasmas. Trace levels of contamination can be detected using the colony blot method (Gaurivaud *et al.,* 2004) with a MmmSC-specific monoclonal antibody (Brocchi *et al.,* 1993). The mycoplasma protein concentrate can be aliquoted and stored at -20°C up to one year.

Preparation of antigen strips

The OIE manual recommends separating the extracted proteins on a 5-15% gradient-resolving polyacrylamide gel. This polyacrylamide gradient ensures optimal separation of all the MmmSC proteins and was used by Gonçalves *et al.* (1998) to identify the set of five consensus antigenic proteins specific to

MmmSC. However, protein separation is optimal only if proteins migrate to the gel concentration appropriate for their molecular mass. For this reason, in practical terms, reproducibility is often difficult to control, mainly for two proteins, the 95 and 98 kDa proteins. Use of a 7% acrylamide gel (Schubert *et al.,* 2011) offers a compromise, with good separation of the proteins and better reproducibility between batches of antigen strips. To reduce the preparation time, commercial, ready-to-use gels can be used, such as Invitrogen NUPAGE 7% Tris-Acetate gels

Methods

and Bio-Rad Mini-Protean TGX Any kD gels (Figure 1A). Mini-Protean TGX Any kD gels were chosen for their homogeneity in band intensity for the 95 and 98 kDa proteins in IBTs with positive sera. It should be noted that commercial gels do not necessarily guarantee the separation of proteins in the 95 - 98 kDa size range (e.g. 7.5% Mini-Protean TGX gels from Bio-Rad). After electrophoresis, the proteins are transferred to a nitrocellulose membrane using a semi-dry transfer protocol (Gravel, 2002), which has several advantages over the wet transfer protocol: it is less time-consuming, several gels can be transferred simultaneously and a standard power supply can be used because a lower voltage is required (Kurien and Scofield, 2006; MacPhee, 2010). The transfer of proteins ranging from 48 to 110 kDa occurs after 30 to 45 min at 25 V in Towbin

Table 1. Description of the steps involved in the serodiagnosis of CBPP using the immunoblotting test (IBT)

Steps	Methods	Technical notes	Quality control
Antigen preparation	Preculture, in mycoplasma broth medium (Poumarat *et al.*, 1991), of the B103 strain (isolated in Portugal in 1986 from bovine lung tissue, Gonçalves *et al.*, 1998)	Strain B103 is available from the LNIV (Laboratório Nacional de Investigação Veterinária, Lisbon, Portugal) and from ANSES, Lyon Laboratory (UMR Ruminant Mycoplasmoses, 31 avenue Tony Garnier, 69364 Lyon cedex 07)	
	Seeded at 1:100 in 150 mL of mycoplasma broth medium. Incubation at 37°C, 5% CO_2, 48 to 66 h		Absence of mycoplasma contamination checked by "colony blotting" with a MmmSC-specific antibody
	Centrifugation at 12,000 xg, 30 min at 4°C. Mycoplasma pellets are washed three times in PBS (10 mM sodium phosphate, 150 mM NaCl, pH 7.4)		
	The pellet is resuspended with 1 mL of PBS and homogenised by repeated pipetting with a thin tip needle. The mycoplasma concentrate is stored at -20°C up to one year in 50 µL aliquots		The presence of the 110, 98, 95, 60-62 and 48 kDa proteins is checked by IBT with positive and negative reference sera [(1)]
Production of antigen strips	1 volume of the antigen diluted to 1:2 in distilled water is mixed with 1 volume of Laemmli buffer (62.5 mM Tris-HCl pH 6.8, 25% glycerol, 2% SDS, 0.01% bromophenol blue, 5% β-mercaptoethanol) and boiled for 5 min	The denaturation buffer affects the quality of protein separation. Any buffer other than Laemmli buffer must be checked by IBT	
	Separation by electrophoresis on a Bio-Rad Mini-Protean TGX Any kD gel (Buffer: 2.5 mM Tris, 19.2 mM glycine, 0.01% SDS, pH 8.3) or on an Invitrogen NUPAGE 7% Tris-Acetate gel (NUPAGE buffer, Tris-acetate SDS), according to the manufacturer's recommendations	Electrophoresis is stopped when the 40 kDa protein of the Invitrogen NOVEX Sharp protein standard reaches the bottom of the gel	
	The gel, the nitrocellulose membrane (0.45 µm) and the filter papers (extra-thick filter papers, Bio-Rad, reference 170-3969) are allowed to equilibrate in Towbin buffer (25 mM Tris, 192 mM glycine) for 5 min at room temperature		
	Semi-dry transfer in Towbin buffer for 30 to 45 min at 25 V with the Trans-Blot SD or the Trans-Blot Turbo system from Bio-Rad		The quality of the transfer is checked by staining the membrane with R-RPOB from Sigma-Aldrich (according to the manufacturer's recommendations). The membrane is destained with 10 mM EDTA, pH 8.0 and rinsed twice with distilled water
	The membrane is blocked with 40 mL of blocking solution per membrane for 2 h at room temperature Blocking solution: 50 g/L dry skim milk, 75 g/L glycine, 10 g/L egg albumin, stored at -20°C up to 6 months		Batches of blocking buffer are tested by IBT with positive and negative reference sera
	The membrane is washed three times at room temperature for 15 min with 40 mL of TBS, 0.1% Tween 20 and once for 15 min with 40 mL TBS. TBS: 20 mM Tris, 500 mM NaCl pH 7.4	If the membrane is not washed, the intensity of the reaction will be reduced	
	The membrane is cut into antigen strips (one membrane makes one batch of strips). The strips are dried at room temperature and then stored in an air-tight tube at -20°C up to one year		Two strips per batch are tested by IBT with positive and negative reference sera

(1) The positive reference sera used are the 511-49 and 511-56 sera from the Abdo et al. (1998) study. The negative reference sera come from French cattle that have tested negative for CBPP using competitive ELISA and IBT

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Table 1. Description of the steps involved in the serodiagnosis of CBPP using the immunoblotting test (IBT) (following)

Steps	Methods	Technical notes	Quality control
Serodiagnosis by IBT	For each tested serum, an antigen strip is immersed in 1 mL of dilution solution and 200 µL of serum and then incubated for 2 h at room temperature with gentle shaking. Dilution solution: 0.1% dry skim milk (Bio-Rad "blotting-grade blocker" reference 170-6404), 0.1% egg albumin, stored at 20°C up to 6 months		Batches of dilution solution are tested by IBT with positive and negative reference sera. Positive and negative reference sera are used as controls for each batch of strips
	The strips are washed three times at room temperature for 15 min in TBS, 0.1% Tween 20 and once for 15 min in TBS		
	The secondary antibody is diluted in dilution solution, 1 mL is added to each strip and incubated for 2 h at room temperature with gentle shaking. The secondary antibody: immunoaffinity-purified peroxidase-conjugated anti-bovine IgG (Sigma-Aldrich reference A5295)		The dilution of the secondary antibody is determined during the test by IBT with positive and negative reference sera when each new batch is received
	The strips are washed three times at room temperature for 15 min in TBS, 0.1% Tween 20 and once for 15 min in TBS		
	During the last wash, the substrate is prepared (30 mg of 4-chloro-1-naphtol dissolved in 10 mL methanol joined to 50 mL PBS and 30 µL hydrogen peroxide)		Batches of 4-chloro-1-napthol are tested on dot blots with dilutions of secondary antibodies to assess the reaction and check for the presence of any non-specific precipitation
	The strips are incubated with 2 mL of substrate until bands start to darken. The reaction is stopped by washing the strips in distilled water	The time it takes for the bands to darken is based on the positive and negative controls	
	The results are interpreted by comparing the lane containing the tested serum with those of the positive and negative controls		

buffer with the Trans-Blot SD or the Trans-Blot Turbo transfer system (Bio-Rad) (Table 1). When the filter papers, gels and membranes are assembled, bubbles or gel debris can hinder transfer resulting in the absence of one or more proteins from a lane in the membrane (this corresponds to an antigen strip). Since batches are tested on two randomly chosen strips, a transfer problem on one lane may go unnoticed and cause false negative or uninterpretable results, thereby extending analysis time. The homogeneity and the quality of the protein transfer must therefore be checked by using a reversible staining procedure that does not interfere with the IBT, such as the R-PROB reagent sold by Sigma-Aldrich (MacPhee, 2010). This step also allows the lanes to be marked for cutting the membrane into strips and for tracing the migration fronts to align and compare strips and therefore minimise edge effects. The membranes are then blocked (Table 1) and cut into antigenic strips.
By using ready-made gels and semi-dry electrotransfers, only 5 h are needed to prepare the antigen strips from the frozen protein extract. The number of strips produced ranges from 20 to 50, depending on the system used. The strips are dried (nitrocellulose membranes must be used, because they are easier to rehydrate compared to polyvinylidene fluoride membranes) and stored at -20°C for up to one year in an air-tight tube. Using reagents prepared this way, IBT serodiagnosis can be completed in 6 h.

Serodiagnosis by IBT

Each serum to be tested is diluted to 1:6 in a solution the composition of which (Table 1) influences the intensity of the reaction and the background noise. Store-bought dry skim milk, commonly used as a blocking agent in western blotting (MacPhee, 2010) is not recommended due to the great variability among batches and suppliers and also due to the high background noise that occurs with certain sera, hindering interpretation (Figure 1B). The skim milk sold by Bio-Rad keeps background noise down (Figure 1C). Any new batch of skim milk must be assessed beforehand by running the IBT with positive and negative reference sera.
The prescribed secondary antibodies are immunoaffinity-purified anti-bovine IgG (Sigma-Aldrich) to ensure detection specificity. Upon reception, each new batch of antibody must be tested to determine the appropriate dilution and ensure similar sensitivity among antibody batches.
To read the IBT results, it is essential that the position of the 110, 98, 95, 60-62 and 48 kDa bands always be identified against a positive control serum for which the IBT profile is well characterised (reference serum, Table 1). The identification of the bands of interest is also facilitated by using several size standards with different molecular masses (Sagedi *et al.*, 2003) and a negative control serum. Then, the interpretation of the results is done by comparing the profiles of the controls against those from the tested sera. The interpretation of IBTs can sometimes be problematic when other bands appear near the MmmSC specific bands, since an edge effect can cause a slight shift in band position. In extreme cases, the strip can be cut in two lengthwise and one strip can be revealed with the positive serum and the other with the ambiguous serum. Joining the two halves of the strip then allows for precise comparison of the test serum profile with that of the positive control.

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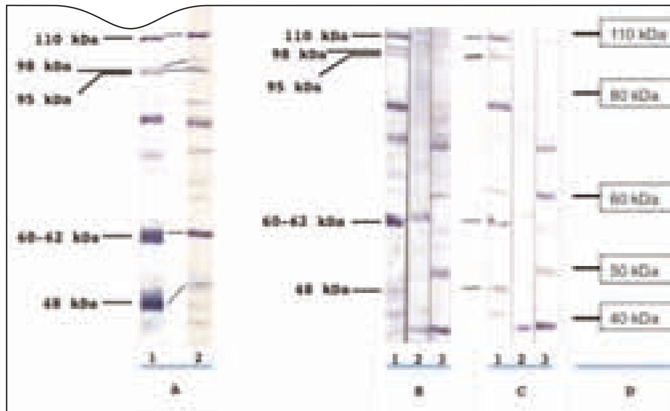

Figure 1. Immunoblotting test for CBPP serodiagnosis: influence of the type of gel and the serum dilution solution used on the obtained profiles.

A : profile of a positive serum on a Bio-Rad Mini-Protean TGX Any kD gel (lane 1) or an Invitrogen NUPAGE 7% Tris-Acetate gel (lane 2),

B and C: tested sera were diluted in a dilution solution made up with store-bought powdered skim milk (B) or Bio-Rad brand skim milk (C). Lane 1 contains the positive serum and lanes 2 and 3 the negative sera.

D: position of proteins in the molecular mass standard (Novex sharp protein standard from Invitrogen).

In conclusion, carefully choosing the consumables (i.e. reagents and materials) and identifying the critical steps in IBT can significantly improve repeatability and should enhance reproducibility, the main weakness of the IBT method as revealed in the last ILPT in 2009. IBT is regularly performed in our laboratory to test suspicious or positive sera, which occur frequently for the CF test and occasionally for the competitive ELISA.

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Adoption by a network's laboratories of a validated quantitative real-time PCR method for monitoring Q fever abortions in ruminant livestock

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In France, Q fever is a disease of concern for the state, both in terms of animal and public health. However, there is as yet little knowledge of epidemiological situations or the exposure risks. To ensure the relevance of both risk assessment and risk management, it therefore seemed essential to implement a surveillance scheme for serial abortions due to Q fever in ruminant livestock.

A key feature of this scheme is the production of reliable, comparable data by a network of participating veterinary analysis laboratories. To achieve this, the PCR methods to be used were first validated in accordance with the new French standards XP-U47-600-1 and XP-U47-600-2. Also with regard to standards, the National Reference Laboratory (NRL) for Q fever organised the adoption of the PCR methods by the laboratories. This adoption phase involved testing to verify that the laboratories obtained the expected performance, as determined during the validation step with regard to limits of detection and accuracy of quantification.

All of the laboratories successfully adopted the methods. The overall analysis of these adoption results is presented to demonstrate for the first time the consistency of the laboratories network's PCR results.

Introduction

Q fever, caused by the bacterium *Coxiella burnetii*, is widespread both geographically (found throughout the world) and in terms of affected animal species (Rousset *et al.,* OIE 2010). In ruminants, the disease is characterised by abortions and can cause significant economic losses (Touratier *et al.,* 2012). In addition, *C. burnetii* is a zoonotic agent, whose transmission to humans occurs primarily by air. Infected animals may shed the bacterium and contaminate the environment. Bacteria can persist in the environment as pseudospores and then be disseminated. The occurrence of cases or outbreaks in the population appears to depend on a combination of factors favouring its airborne diffusion, such as a site's topography or weather conditions (Forland *et al.,* 2012). Nevertheless, the greatest risk of environmental contamination appears to be associated with abortion episodes in livestock, combining both a large number of shedding animals and shedded high individual loads (De Bruin *et al.,* 2012; De Crémoux *et al.,* 2012). Surveillance of farms affected with clinical Q fever has been considered, in order to gain a better understanding of the situation with this disease and its development in France, with a view to assessing the means of control. This surveillance is to be coordinated by the National Platform for Epidemiological Surveillance in Animal Health, created recently in response to the guidelines adopted in 2010 at the national consultation on the health sector (*Etats généraux du sanitaire*) organised by the French Minister for Agriculture.

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The surveillance scheme will involve 10 *départements* for three years in order to apply the most rational optimisations with a view to standardising methods (sampling in farms, testing in laboratories and interpreting results). A protocol was therefore proposed for the detection of an episode of Q fever in livestock (Service Memo DGAL/SDSPA/N2012-8188 of 11 September 2012). This protocol is mainly inspired by the conclusions drawn in 2007 by a national working group on Q fever. It was also taken up at European level as a proposal for surveillance based on a passive system (Sidi-Boumedine *et al.,* 2010). PCR analysis performed in the laboratory is a crucial part of this diagnostic procedure. It enables the detection and quantification of bacteria in vaginal (sheep, goats), endocervical (cattle) or placental swabs (all ruminant species) from sampled females. The result is then interpreted in relation to a bacterial load threshold, determined according to expert opinion. The distributions of quantitative data from the surveillance protocol will be studied in order to adjust this clinical threshold, if necessary, depending on the animal species but also on the type of sample.
Therefore, based on the needs and objectives of the surveillance to be implemented, work was undertaken to harmonise and validate the quantitative real-time PCR (qRT-PCR) to be used for the molecular diagnosis of abortive Q fever. Two manufacturers (Adiagene and LSI) submitted their qRT-PCR kits for validation according to a standard procedure proposed by the NRL, and in keeping with the recommendations of the new French standard XP U47-600-2 relating to PCR in animal health, published by AFNOR in June 2011. The supplier reports were reviewed and validated by the NRL in December 2011 according to predetermined performance criteria. Meanwhile, a validation certificate was also issued to one of the network's laboratories that had developed its own method. These validated methods were then approved by the Ministry of Agriculture during accreditation of the laboratories.

Prior to the routine implementation of the method, the French standard recommends conducting adoption tests to verify that the user has been able to achieve the performance claimed by the supplier. Adopting a molecular diagnostic method involves confirming the performance of firstly the PCR step (recommendations in Chapter 11 of the Standard XP U47-600-1, pages 30-32) and secondly the complete analytical method (pages 32-33) in terms of limit of detection and accuracy of quantification. This adoption stage was not required for the one laboratory in the network that had validated its own in-house method. All the other laboratories in the network returned results that complied with the criteria required for the adoption testing.
The purpose here is to analyse all the results in order to obtain a preliminary assessment of the PCR method implemented within the network's laboratories in the context of the surveillance scheme. In addition, the description of this pioneering exercise, conducted under real conditions, will serve to facilitate assimilation of the standard's requirements by future adopters.

Table 1. Distribution of commercial reagents, matrices and devices based on the 11 data sets obtained

Set code	PCR kit manufacturer	Negative biological matrix	Real-time thermal cycler	DNA extraction kit
j	ADIAGENE	Bovine vaginal mucus	Stratagène MxPro 115108	Macherey Nagel Nucleospin Tissue
d	ADIAGENE	Caprine vaginal mucus	Applied Biosystems Abiprism 7500 SDS	Qiagen QIAamp DNA Mini kit
a	ADIAGENE	Bovine vaginal mucus	Applied Biosystems Abiprism 7500	Qiagen QIAamp DNA Mini kit
c	ADIAGENE	Bovine placental cotyledons	Applied Biosystems Abiprism (2*)	Qiagen QIAamp DNA Mini kit
b	LSI	Bovine vaginal mucus	Applied Biosystems Abiprism 7500	Macherey Nagel Nucleospin Tissue
e	LSI	Bovine vaginal mucus	Stratagène MxPro 115108	Macherey Nagel Nucleospin Tissue
k	LSI	Bovine vaginal mucus	Applied Biosystems Abiprism (2*)	Qiagen QIAamp DNA Mini kit
f	LSI	Bovine vaginal mucus	Biorad CFX96 and Biorad CHROMO4 (2*)	Macherey Nagel Nucleospin Tissue
h	LSI	Bovine vaginal mucus	Roche Light Cycler 480	Qiagen QIAamp DNA Mini kit
i	LSI	Bovine vaginal mucus	Applied Biosystems Abiprism 7500 (2*)	Qiagen QIAamp DNA Mini kit
g	LSI	Bovine vaginal mucus	Applied Biosystems Abiprism 7500 (2*)	Qiagen QIAamp DNA Mini kit

*2 different thermal cyclers tested

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Description of the quantitative real-time PCR (qRT-PCR) methods

Two commercial PCR methods were assessed in this interlaboratory adoption test: the ADIAVET® COX REALTIME kit, # ADI143 (Adiagene, France) and the TaqVet™ *Coxiella burnetii* - Absolute Quantification kit, # FQPAQ (LSI, France). The requirements of French standard XP U47-600-2, as well as the performance criteria predefined by the NRL, were applied to characterise and validate the methods required for the diagnosis of abortions. Each qRT-PCR kit was associated with two defined methods for extraction and purification of total DNAs using reagents on silica columns (Nucleospin Tissue, Macherey-Nagel, France, and QIAamp DNA mini kit, Qiagen, France). Validation focused on the endocervical, vaginal or placental matrices. As a minimum, one of the three biological matrices from one of the three animal species was to be assessed, since the inhibitory effect on the PCR is considered equivalent in these samples. The two kit manufacturers conducted validation experiments on the same thermal cycler model: ABI Prism 7500® from Applied Biosystems.

The two methods were validated to allow absolute quantification of *C. burnetii* between at least 1.10^3 and 1.10^6 genome equivalents (GEs) per mL of sample. The target is an IS*1111* insertion sequence specific to the genome of *C. burnetii* (Berri *et al.*, 2003). The plasmid standard for each kit, which enables a calibration curve to be constructed, was verified and linked to the genomic DNA standard of the NRL for Q fever. The result is given in GEs of the Nine Mile reference strain. The genome of this reference strain contains 20 copies of IS*1111*. Thus, the result is placed into perspective considering that each bacterium has an average of 20 copies of IS*1111*. Each of the two kits simultaneously detects a second target specific to the genome of cattle, sheep and goat cells in order to obtain information about the validity of the total DNA extraction and to check for possible inhibitory effects of the sample on the PCR. The manufacturer's instructions were drafted in consultation with the NRL to specify the detailed procedure and the validated performance characteristics of both the PCR and the complete method. They serve as the standard official protocol.

Number and distribution of "method adoption" data sets

In total, 11 data sets were obtained: seven with the LSI kit and four with the Adiagène kit (Table 1). Of the nine departmental laboratories in the network required to undergo adoption testing on the commercial methods, two laboratories performed the exercise on both the proposed kits. The laboratories used the qRT-PCR kits in combination with one or other of the two extraction methods (seven sets with the Qiagen kit and four sets with the Macherey Nagel kit).

In total, 16 different thermal cyclers were used (representing 5 models), with most laboratories being equipped with the Abiprism 7500® model. Five laboratories possessing more than one thermal cycler conducted checks on two different models with a view to their routine use.

PCR limit of detection (LD_{PCR})

The limits of detection for kits A and B respectively were defined as 1.5 and 1.0 GEs per PCR. These values correspond respectively to 300 and 200 GEs per mL that can be detected in 95% of cases.

A measured DNA reference material was provided by the NRL. Each laboratory performed two independent tests of three repetitions of a DNA level three times the LD_{PCR}. Detection of all six tests verified the expected LD_{PCR} performance (Table 2 A). The term fidelity (or intermediate fidelity), used hereafter, expresses repeatability and intra-laboratory reproducibility.

Operators obtained mean Ct values of 33.41 and 34.23, with kits A and B, respectively (Table 3). Standard deviations of fidelity did not exceed 0.50 Ct, and standard deviations of reproducibility were 1.32 Ct for the four laboratories that used kit A (24 measurements at the level 900 GE/mL) and 0.87 Ct for the seven tests performed with kit B (42 measurements at the

Table 2. Designs for the experiments conducted for the adoption of a validated PCR method for Q fever diagnosis in the context of abortion disease surveillance in ruminants

A. Limit of detection of PCR and the complete method (in the presence of measured reference material)

Adoption step	Levels tested	Number of independent tests*	Minimum number of operators	Number of replicas	Number of measurements	Acceptability
PCR	3 x LD_{PCR}	2	1	3	6	100% of results positive
Complete method	5 x LD_{Method}	2	1	2	4	100% of results positive

B. Scope and limit of quantification of PCR and the complete method (in the presence of measured reference material)

Adoption step	Levels tested	Number of independent tests*	Minimum number of operators	Number of replicas	Number of measurements	Acceptability
PCR	Range (5 points including LQ_{PCR})	1	1	5 range points and 5 replicas of LQ_{PCR}	10 (including 6 LQ_{PCR})	Bias below 0.5 log_{10} GEs/mL for each level
Complete method	5 x LQ_{Method}	2	1	2 (with two 5-point ranges)	14	100% of results positive and quantified with bias below 0.7 log_{10} GEs/mL

*1 for each thermal cycler
LD, limit of detection; LQ, limit of quantification

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level 600 GE/mL). The coefficients of variation (CV) of fidelity were below 1.47%. The method yielded CV of reproducibility of 3.96% with kit A and 2.54% with kit B.

Limit of detection for the complete method (LD_{Method})

The complete method involves two distinct phases: extracting DNA from the biological sample, and then amplification by PCR of the target sequence on this extracted DNA. To date, the method's performance has been validated for two common methods for extracting total DNA on silica columns (Nucleospin Tissue, Macherey-Nagel and QIAamp DNA Mini kit, Qiagen) according to the instructions in Standard XP U47-600-2 (Chapter 7.3). It is interesting to note that the limits of detection were similar for PCR and the complete method with either kit: 300 GEs per mL with kit A and 200 with kit B.

Testing was also performed using another reference material provided by the NRL, this time consisting of quantified bacteria. Representative samples were prepared by adding a known number of *C. burnetii* bacteria to one of the biological matrices under study (Table 1). For these, the laboratories were asked to obtain the quantity of negative matrices needed for adoption testing: cell suspensions prepared from vaginal swabs for small ruminants, endocervical swabs for cattle, or placental cotyledon regardless of the ruminant species (1 swab being resuspended in 1 ml of PBS pH7.4). As a minimum, one of the three biological matrices from one of the three species has to be submitted for adoption.

Following a principle similar to the LDPCR adoption step, the LDMethod was confirmed at a concentration level corresponding to 5 times the LDMethod analysed in duplicate under intermediate fidelity conditions over the entire process of DNA extraction and PCR amplification of the target (Table 2 A). The Ct values obtained by the laboratories were extended from 31.09 to 34.49 for the level 1500 GEs per mL (deviation of 3.4 Ct over 16 measurements) with kit A and from 31.71 to 36.20 (deviation of 4.49 Ct over 28 measurements) for the level 1000 GEs per mL with kit B (Table 3). The dispersion of detailed results for each laboratory reached a deviation of 2.23 Ct but was mostly between 0.80 and 1.50 Ct (results not shown). The overall means were 32.71 and 34.30 with kits A and B respectively. The CVs of both fidelity and reproducibility were relatively close to those calculated for the LDPCR for kit A and slightly higher for kit B. The overall results showed a CV of reproducibility of less than 4%.

Quantification and limits of quantification: LQ_{PCR} and LQ_{Method}

In PCR, the limits (LQ_{PCR}) were determined at 400 and 300 GEs per mL with kit A and kit B respectively. Regardless of the PCR kit used, the validated limit of quantification was 2.70 log_{10} GEs/mL for the complete method, or 500 GEs/mL, bearing in mind that the diagnostic threshold is currently set (by expert opinion) at 4 log_{10} GEs/mL (Service Memo DGAL/SDSPA/N2012-8188 of 11 September 2012). In addition, the methods were validated to ensure linear quantification over 5 orders of magnitude to at least 1.106 GEs per mL (or bacteria per swab).

Confirmation of quantification performance was assessed, using the measured reference materials, by calculating the bias between the expected quantified value and that obtained, whether for the gene amplification step alone or for the complete method. The maximum bias permitted was set at 0.5 log_{10} for PCR and 0.7 log_{10} for the complete method (Table 2 B). Calculations had to be performed on data expressed in logarithm form.

For PCR, the experimental design followed involved conducting a test for the 5 levels of ten-fold serial dilution constituting the calibration range, including six copies of the last level representing the LQ_{PCR}. The bias was to be calculated for each level and for the five replicates of LQ_{PCR}.

For the complete method, two tests were performed for the quantification of a sample in duplicate. In total, four positive quantified results were obtained for the level of 5 times the LQ_{Method}, i.e. 3.40 log_{10} GEs per mL regardless of the kit used (2500 bacteria/swab).

The calibration curves were assessed after each test by visually examining the alignment and uniform distribution of

Table 3. Ct values obtained during testing to verify limits of detection

	A				B			
	Limit of detection		Limit of quantification		Limit of detection		Limit of quantification	
Levels verified	$3LD_{PCR}$	$5LD_{Method}$	LQ_{PCR}	$5LQ_{Method}$	$3LD_{PCR}$	$5LD_{Method}$	LQ_{PCR}	$5LQ_{Method}$
Number of GEs in log_{10} / mL	2.95	3.18	2.60	3.40	2.78	3.00	2.48	3.40
Number of GEs / mL	900	1500	400	2500	600	1000	300	2500
Number of laboratories	4	4	4	4	7	7	7	7
Number of repetitions	6	4	8	4	6	4	8	4
Number of measurements	24	16	32	16	42	28	56	28
Mean value obtained	33.41	32.71	33.72	32.01	34.23	34.30	35.06	32.93
Minimum/maximum value	32.03 / 35.90	31.09 / 34.49	32.09 / 36.54	29.60 / 33.66	32.67 / 36.10	31.71 / 36.20	33.65 / 36.66	30.81 / 34.50
Standard deviation of fidelity	0.46	0.55	0.54	0.40	0.50	0.80	0.50	0.65
Standard deviation of reproducibility	1.32	1.00	1.37	1.44	0.87	1.34	0.85	1.15
CV fidelity (%)	1.38	1.67	1.61	1.25	1.47	2.34	1.43	1.98
CV reproducibility (%)	3.96	3.49	4.05	4.49	2.54	3.92	2.43	3.50

CV, coefficient of variation; LD, limit of detection; LQ, limit of quantification

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points, as well as the absence of outliers. In addition, two criteria documented in the kit instructions were verified: the value of the correlation coefficient R^2 greater than 0.90 and PCR efficiency between 85 and 115%. The calibration curves of 33 different PCR assays showed good reproducibility of the Ct values obtained as a function of concentration (Figure 1). Standard deviations of reproducibility varied from 0.42 to 1.31 Ct. The corresponding CVs were between 3.63 and 4.52% with kit A and between 1.91 and 3.00% with kit B. The overall means of 33 ranges (11 sets) found for the slope, y-intercept and efficiency were respectively -3.41, 43.33 and 97%. The network of laboratories involved in surveillance therefore seems capable of providing results with a good degree of consistency. The acceptance criteria to be complied with for the quantification curves, as well as the high number of points (5 points) in the range, help to ensure this reproducibility.

The standard deviations of both fidelity and reproducibility, from concentrations measured at the LQ_{PCR} and the LQ_{Method}, were entirely similar regardless of the kit (Table 4). The CVs of fidelity and reproducibility were higher for the concentration measurements obtained with the complete method than for PCR alone. As suspected, the DNA extraction step introduces factors that influence the result.

The laboratories provided measurements of concentrations that were within the predetermined bias limits. PCR alone clearly achieved greater precision than the complete method (Table 4). The maximum acceptability limit set at 0.7 log_{10} for the complete method means that variations up to a factor of 5 can be detected compared to the target level. In other words, a deviation of 2.33 Ct is permitted.

Taken together, the maximum bias for the nine laboratories was 0.67 (results not shown). Most of the measurements (96/132, or 73%) showed a bias of less than 0.25. However, with the complete method, fewer biases (18/44 or 41%) were less than 0.25. A bias of less than 0.25 log_{10} indicates that the measurements have a maximum variation of a factor of 1.78 compared to the expected concentration, or less than one Ct. A comparison between the laboratories of the measurements at the level of $5xLQ_{Method}$ (2500 bacteria/swab) showed that the minimum and maximum amounts measured ranged from 2.73 to 3.84 (seven sets) with kit B and from 2.72 to 3.93 (four sets) with kit A (Table 4). The precision of the measured concentrations shown in box plots appears similar between the two kits (Figure 2).

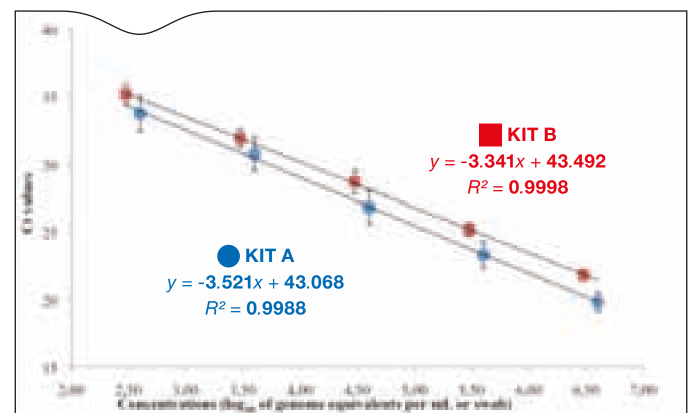

Figure 1. Calibration curves, between the Ct values and concentrations introduced, obtained with quantification standards of known DNA concentration, provided with each of the PCR kits.

The means and standard deviations of interlaboratory reproducibility were calculated from Ct values obtained from 12 ranges with the Adiagène kit (4 data sets) and 21 ranges with the LSI kit (7 data sets).

Table 4. Concentrations measured (in log_{10} / mL)

	A		B	
Levels expected Number of GEs in log_{10} / mL Number of GEs / mL	**LQ_{PCR}** 2,60 400	**$5LQ_{Méthode}$** 3,40 2500	**LQ_{PCR}** 2,48 300	**$5LQ_{Méthode}$** 3,40 2500
Number of laboratories	4	4	7	7
Number of repetitions	8	4	8	4
Number of measurements	32	16	56	28
Fidelity				
Mean concentration found	2.62	3.16	2.48	3.18
Minimum/maximum concentration	2.16 / 2.94	2.72 / 3.93	2.05 / 2.77	2.73 /3.84
Standard deviation of fidelity	0.15	0.24	0.15	0.21
Standard deviation of reproducibility	0.16	0.34	0.15	0.31
CV fidelity (%)	5.85	7.49	6.02	6.52
CV reproducibility (%)	6.23	10.79	6.17	9.63
Precision				
Mean bias in absolute value	0.13	0.35	0.12	0.31
Minimum bias in absolute value	0.01	0.03	0.00	0.02
Maximum bias in absolute value	0.44	0.67	0.42	0.66

CV, coefficient of variation; LD, limit of detection; LQ, limit of quantification

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Conclusions and outlook

Surveillance of abortion diseases in ruminants is currently a priority issue in animal health in France. However, a recent inventory highlighted the heterogeneity of the diagnostic procedures used for abortions (Touratier *et al.,* 2012). Homogeneous differential diagnosis protocols are currently being proposed in collaboration with professional organisations. For Q fever, surveillance has been designed in conjunction with that for brucellosis, a disease that has now been eradicated and that therefore requires surveillance that can ensure a rapid response. Because of its pilot scale, this scheme should be flexible enough for all the adjustments needed in terms of feasibility and cost. The developments implemented should help to improve the procedures for other abortion diseases.

Because the PCR tool for the diagnosis of Q fever is a fundamental part of surveillance, a standardised validated method was essential: to enable surveillance data to be analysed, it is important to ensure that the results obtained are comparable. The method's performance characteristics are key components, not only for the analysis laboratory but also for the statistician analysing the surveillance data (Laurentie and Delmas, 2011). Two French standards relating to PCR in animal health were recently developed to assist with the production of reliable data (Standards XP U47-600-1 and -2). In accordance with these standards, work was undertaken to provide a standard method and determine its performance characteristics. The next necessary step was to verify that the network's laboratories were capable of achieving the performance specified under operational conditions for routine analysis. To our knowledge, this is the first interlaboratory adoption test for a PCR method involving peripheral laboratories and kit manufacturers. The results of the PCR method adoption exercise provided a first glimpse of the level of performance within the laboratories and served to verify the consistency of the results.

In a previous study to evaluate quantitative real-time PCR targeting the gene IS*1111*, by an interlaboratory test, the overall agreement of results in Ct values was deemed acceptable. Deviations in Ct obtained by the seven participating laboratories ranged from 4.0 to 7.2 depending on the seven positive DNA

Figure 2. Box-plot representation of the precision of the measured concentrations (bias in $\log_{10}$ / mL)

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samples tested (Jones *et al.,* 2011). In the adoption exercise presented here, involving nine laboratories, the quantitative results provided by the validated and adopted PCR method are all far more comparable. The interlaboratory Ct deviations ranged from 2.57 to 4.49 at low and medium levels ranging from 300 to 2500 GEs/mL (Table 3). The maximum CV of reproducibility was 4.49%. Standard deviations of fidelity for Ct values were generally between 0.40 and 0.80 Ct (CV between 1.25 and 2.34%).

This adoption test showed that for the expected concentration measurements, a similar level of accuracy (fidelity combined with precision) was obtained regardless of the kit used. For example, for the precision of the complete method, the measurements found for the tested level of 2500 bacteria per mL ($5xLQ_{Method}$) varied from 2.72 log_{10} (525) to 3.93 log_{10} (8511) bacteria per mL (Table 4). The mean absolute bias was 0.3 log_{10}, i.e. a factor of 2 between the expected and measured concentration. In terms of dispersion of measurements, the laboratories achieved CVs of fidelity below 8% and CVs of reproducibility below 11%.

Moreover, the bias values, like the coefficients of variation, can serve as initial baseline data for this method that has been officially defined, validated and shared by a network of laboratories. More substantiated interlaboratory reproducibility should be estimated by participation in regular interlaboratory tests. It is especially important to correctly define the level of the diagnostic threshold. It is also essential for each laboratory to produce a control chart on the critical point formed by the threshold. This involves incorporating a control determined to 4 log_{10} bacteria per ml in each extraction series. This internal reference material thereby serves as a method control. It also enables monitoring in the form of an intra-laboratory control chart (limit of bias acceptability set at less than 0.7 log_{10}). The measurement uncertainty should be taken into account in interpreting the results. A measurement at 2000 bacteria/swab will be interpreted as a highly positive result because it corresponds to the lower limit of the threshold level at 10,000 bacteria/swab.

Finally, information on the accuracy capabilities of the results around the threshold should help progress towards a simplified and less expensive method in the future (i.e. use of a threshold standard instead of a range of five quantification standards).

Acknowledgements:

The team at the NRL for Q fever would like to thank Maëlle LEBORGNE and Béatrice BLANCHARD from Adiagène, as well as Sandrine MOINE-BOULEY and Eric SELLAL from LSI for their constructive cooperation in conducting validations and preparation for adoption. They also wish to express their gratitude to Claire PELLETIER from LDA Saône-et-Loire for the relevant discussions held during the validation phase for their in-house method.

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Publication and design
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Translation: Coup de Puce, D. Pottratz, B. Vallantin
Creation/Development: L. Lelyon, C. Leterq, Parimage
ISSN 2110-5294

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and Occupational Health & Safety
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