Editorial

Opening issue of the Journal of Reference

Reference activities are one of the Agency's operational missions and a pivotal part of its sophisticated health monitoring system. The Afssa business plan establishes several strategic goals regarding reference activities: participation in both national and EU-level (NRL and CRL) reference discussions, heightened exchange and collaboration with the French Institute for Public Health Surveillance (InVS) and the National Centres of Reference (CNR), assistance in organising and coordinating both certified and recognised laboratory networks, and acknowledgment of Afssa's benchmark role in both the EU and worldwide, thanks to its reference laboratory and collaborating centre mandates. Afssa also aims to actively promote the dissemination of scientific knowledge and health safety awareness and their acceptance by all concerned parties. Our role is also to consolidate and enhance the effectiveness of the scientific and technical support provided to our governing bodies – especially in the programming of health inspection and monitoring plans – and to pursue our commitment to coordination and participation in standardisation commissions and working groups in technical areas in relation to our activities.

This is the first issue of Afssa's Journal of Reference. In it, we will discuss the scientific and technical aspects of methods of analysis and their role in the regulatory system, the reinforcement of collaborative efforts, the detection of emerging risks and many other topics. Its subscription-based online format makes it easy to use and we will be publishing it on a regular basis to exchange information on our reference activities and in so doing enable you take full advantage of them. Promoting an even flow of information among members of the lab network and all those involved is one of the journal's main goals.

Whether you work in a control laboratory, a reference laboratory in another member country, a national centre of reference or a ministry, or whether you are a decision-maker or veterinary practitioner, this journal has been created especially for you.

As we are all well aware of the vital role played by reference activities in surveillance, I am confident that everyone working in this rigorous and formative sector will take full advantage of this new tool.

The Journal of Reference also exists in French.

P. Briand, Director General, Afssa

Contributions may be solicited or submitted spontaneously (Please send all contributions to ds@afssa.fr).
Focus on a laboratory

In epidemic circumstances

When an episode of clustered cases – of a bacterial infection, for example – occurs in a human population, it is all the more urgent to pinpoint its exact source in cases where morbidity and/or lethality rates are high. Whether transmission is direct or through food, knowing the origin of the epidemic outbreak makes it possible to take the necessary measures to halt its transmission. Health authorities can rely on two types of support: (a) interventional epidemiology, with descriptive and analytical methods (essentially patient questionnaires, circumstantial studies and case-control studies) and statistical tools that usually make it possible to pinpoint the epidemic’s origin, with differing degrees of precision, from moderate to very high, and the calculation of relative risk which is often crucial to coordinating microbiological investigations; and (b) phenotype and genotype characterisation of bacterial or viral strains taken from human case clusters (by the NCR) and isolated from possible/probable food sources or the environment (by the NRL). The characterisation of strains of human origin generally makes it possible to confirm their clonality, demonstrating high probability that the epidemic comes from a single source, while characterisation of strains isolated from a suspicious food or environmental source makes it possible to identify the source and to provide proof of its origin.

In endemic circumstances

By comparing phenotypes and/or genotypes of strains isolated from the environment or food with human isolates, the origin of an emergent phenomenon (multiple antibiotic resistance, a new serotype, a particularly virulent strain, etc.) can be deduced and sometimes even rapidly ascertained. Thus, focused studies comparing isolate populations from human and food sources can help to determine the role played by various food supply chains in a given endemic situation and ascertaint the origin of isolates within the chain (transport, slaughterhouse, etc.).

In either situation, it is the characterisation and comparison of isolates that makes it possible to support or confirm the analytical epidemiology data. This approach will be all the more solid if characterisation is precise and able to confirm the molecular identity of the strains – or their very close relationship. This is known as molecular epidemiology. The methods of phenotype, and especially genotype, characterisation for bacterial and viral isolates are many and varied. However the same methods must be used by both laboratory entities in order to enable the comparison of isolates from the NRLs and those from the NCRs. For example, they can be compared in terms of their profiles of antimicrobial resistance / susceptibility, their serotypes and using methods such as MLST (Multi Locus Sequence Typing) for molecular genotype characterisation. In addition, the antibiogramme techniques used in both laboratories must be sufficiently similar and robust so that the MICs (Minimum Inhibitory Concentrations) may be strictly comparable, the serotyping techniques reproducible, the MLST technique applicable to the same genes and, for each gene, to the same sequences, etc. If a characterisation technique such as bacterial DNA macrorestriction analysis followed by PFGE (Pulsed Field Gel Electrophoresis) is used, the technique used by both laboratories must be strictly identical so that the genotype profiles obtained can be compared, which may require inter-laboratory testing and validation stages. Ultimately, strain comparisons are often needed to confirm the identity of two isolates.

A close, permanent and ongoing collaboration between the two types of reference laboratories is therefore not simply a regulatory requirement, it is also a veritable public health obligation. What is the logic behind it?

Point of view

NRLs – NCRs: Why work together?
P. Martin, Afssa, Scientific Department, Maisons-Alfort (France)

Many pathogens have both a National Reference Laboratory (NRL) and a National Centre of Reference (NCR). Such is the case each time a disease caused by a transmissible agent – a bacteria, virus or parasite – is found to be zoonotic and presents a problem or major threat to human public health. In the NCR designation decision (Order dated November 29, 2004, establishing the designation process and missions of the National Centres of Reference in transmissible disease control), it is requested that the NCRs collaborate with their NRL counterparts whenever necessary. It is likely that in the NRL designation decision, the same request will be made. Collaboration between the two types of laboratories is therefore not simply a regulatory requirement, it is also a veritable public health obligation. What is the logic behind it?
**Lab news**

**Equine diseases CRL designation**
**Afssa, Maisons-Alfort and Dozulé laboratories (France)**

The newly-created “Equine Disease” Community Reference Laboratory (CRL) mandate was granted to Afssa for a five-year period as of July 1, 2008 (EC regulation no. 108/2008). Its activities will cover all the major and emerging equine diseases.

The Laboratory for studies and research into animal diseases and zoonoses in Maisons-Alfort and the Laboratory for studies and research on equine diseases in Dozulé have united their skills and expertise to effectively carry out the mission of this new CRL. Its reference activities are shared by the two laboratories, with the Dozulé laboratory in charge of equine infectious anaemia, equine viral arteritis, equine herpes viruses, dourine and equine contagious metritis and the Maisons-Alfort laboratory responsible for West Nile fever, vesicular stomatitis and glanders.

**Crustacean diseases CRL designation**
**CEFAS, Weymouth Laboratory (United Kingdom)**

The “Centre for Environment, Fisheries & Aquaculture Science” (CEFAS), Weymouth Laboratory, United Kingdom, has been designated as the Community Reference Laboratory for crustacean diseases for the period of July 1, 2008 through June 30, 2013 (EC regulation no. 737/2008). This reference covers all the crustacean diseases, with a concentration on surveillance of exotic and endemic diseases with potential for emergence in EU aquaculture.

**Bovine tuberculosis CRL designation**
**VISAVET Universidad Complutense de Madrid (Spain)**

The Laboratorio de Vigilancia Veterinaria (VISAVET), Facultad de Veterinaria, Universidad Complutense de Madrid (Spain), has been designated as the Community Reference Laboratory for bovine tuberculosis for the period of July 1, 2008 through June 30, 2013 (EC Regulation no. 737/2008). In addition to its reference missions involving *Mycobacterium* sp., responsible for tuberculosis in animals, the CRL is also in charge of preparing, monitoring and distributing reference reagents to the National Reference Laboratories in order to standardise the tests and reagents used in the Member States and validate reference reagents, including antigens and tuberculins, submitted by the National Reference Laboratories for bovine tuberculosis.

**Designation of approved laboratories for the detection and quantification of phytosanitary product residues in plants and plant products**

The Massy Service Commun des Laboratoires (Joint Laboratory Service) is the National Reference Laboratory for research into pesticide residues in grains and foods. The Montpellier Service Commun des Laboratoires (Joint Laboratory Service) is the National Reference Laboratory for research into pesticide residues in fruits and vegetables. The National Reference Laboratory for single-residue method pesticide research is currently in the process of being designated. Department memo DGAL/SDPPST/N2009-8046 dated February 2, 2009 provides the list of approved laboratories for single-residue (excluding kepone) and multi-residue pesticide residue research in products of plant origin and for chromatography-based research with mass spectrometry detection of pesticides, impurities and phytosanitary product formulants.
Focus on a laboratory

Avian Influenza National Reference Laboratory (NRL)
V. Jestin, Afssa, Ploufragan (France)

Afssa’s Laboratory for studies and research on poultry, pig and fish farming in Ploufragan has been France’s NRL for avian influenza (AI) and Newcastle’s disease (ND) since 1992, the year the first European directives on the monitoring of these diseases were issued. This national reference activity, along with an OIE (World Organization for Animal Health) reference activity in infectious bursal disease and avian metapneumoviruses, is currently carried out, in conjunction with the NRL’s laboratory Avian pathology animal experimentation and breeding unit personnel can also provide the necessary support. When necessary, from other ministries. This article also situates the AI NRL in the global, and more specifically EU-level, network of AI surveillance and Newcastle’s disease (ND) since 1992, the year the first European directives on the monitoring of these diseases were issued. This national reference activity, along with an OIE (World Organization for Animal Health) reference activity in infectious bursal disease and avian metapneumoviruses, is currently carried out, in conjunction with the NRL’s laboratory Avian pathology animal experimentation and breeding unit personnel can also provide the necessary support. The AI NRL was also set up to provide the necessary responsiveness and to adjust the human resources deployed to the epidemic risk. In normal periods, to date, six full-time equivalent positions have been devoted to the AI/ND field of expertise. These individuals are mobilised in alternation according to the variable needs over the year and depending on the epidemic risk. Technical stand-by duty for weekends and holidays can be implemented when necessary. In crisis periods, such as in the winter of 2006, all the unit’s virology personnel were redistributed and mobilised into three teams ensuring a seven-days-a-week relay with extended hours. In addition to this workforce, the laboratory’s Avian pathology animal experimentation and breeding unit personnel can also provide the necessary support for the implementation of intravenous pathogenicity index (IVPI) tests in specific pathogen-free (SPF) chickens.

This article illustrates the way in which the AI NRL fulfils its NRL missions as set down in the decree dated January 5, 2005, namely the development and methodological validation, including standardisation, accredited laboratory network coordination, performance of official analyses (confirmation in particular), scientific monitoring and technology watch, and answering to all scientific and technical expert assessment requests from the Ministry of Agriculture and Fisheries and, if necessary, from other ministries. This article also situates the AI NRL in the global, and more specifically EU-level, network of AI reference laboratories and attempts to highlight the respective contributions of each to both research and expert assessment work on this topic.

Methodological development, validation, standardisation and accreditation

The AI/ND NRL has been accredited since 1997 for the COFRAC’s programmes 109 (animal serology) and 112 (animal virology) with minor extensions in 2002 and 2003, which cover the following official techniques: ND and AI H5, H6, H7 haemagglutination inhibition (HI) assay, AI agar gel immunodiffusion (AGID) tests, ovoculture isolation and haemagglutinin typing of haemagglutinating AI and ND viruses with the aid of reference sera and monoclonal antibodies (some of which were developed in the NRL in the 1980s), and haemagglutinating AI and ND viruses. An NRL researcher who had participated in their EU-level standardisation wrote the French reference texts and the corresponding standards thanks to his regular participation in AFNOR working groups on animal immunoserosology and virology. The reverse transcription polymerase chain reaction (RT-PCR)/sequencing procedures developed ten years earlier in the pigeon ND/paramyxovirus laboratory were applied to the AI diagnosis in the late 1990s. They were then brought up to date with the arrival of automatic sequencing and real-time RT-PCR techniques, then validated in collaboration with the CRAL and several other European pilot NRLs. This brought about the creation of the European Manual on AI Diagnosis which Council Directive 2005/94/EC draws on and which represents the regulatory foundation for EU measures in the fight against AI.

The AI NRL then improved on its previous achievements and obtained the first COFRAC Animal Health PCR accreditation for four real-time RT-PCR techniques, it continues to improve on these techniques with the construction of an armoured RNA internal control that makes it possible to validate all the stages of the process.

Using real-time RT-PCR techniques and/or RT-PCR/sequencing, the AI NRL is able to perform the detection and full characterisation of all eight genes of all the influenza virus subtypes. The laboratory’s analytical capacity is also expressed...
in its full mastery of bioinformatics tools which enable it to identify specific mutations and monitor the genetic evolution of viruses, LP (low pathogenic) AI H5 in particular, as well as to study their antigenic evolution, within the limits of access to the relevant samples.

The NRL has also developed and/or validated ELISA influenza NP, N1, NS and M2 tests and within the framework of the EU programmes, is pursuing its work on the development of DIVA NP, N1, NS and M2 tests and within the framework of the EU programmes, is pursuing its work on the development of DIVA NP, N1, NS and M2 tests and within the framework of the EU programmes.

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Certified laboratory network coordination and adherence to the European and global laboratory network

Previously, the NRL offered regular training sessions for diagnostic laboratories specialising in poultry breeding, essentially in basic AI AGID (and Newcastle HI) techniques, and upon request, more specialised workshops (ovoculture in particular). In this way, a network of public and private laboratories specialising in poultry breeding was established. Now that the laboratories are well versed in these techniques, training sessions are only provided upon request (except for the specialised training sessions set up for the transfer of new techniques - NP-ELISA, H5/H7 HI, M and H5 gene real-time RT-PCR).

The NRL has organised yearly inter-laboratory proficiency tests (ILPTs) in accordance with programme 109 (in alternation with influenza AGID or HI - only Newcastle HI prior to 2002, then Newcastle HI and screening for H5/H7) since 1993 and since 2005 on the real-time AI M and H5 RT-PCR tests. Approximately fifteen laboratories participate. The NRL is also preparing for ILPT organiser accreditation.

The NRL participates in all the yearly European ILPTs organised by the CRL (VLA Weybridge, UK) in the domains of activity involving the detection and identification of viruses by RT-PCR / sequencing techniques and HI as well as the detection, quantification and typing of antibodies using AGID and HI techniques respectively.

Every year, in accordance with its obligations, the NRL provides a statement of identified viruses and observed serologically positive cases and also regularly presents scientific papers. In addition to its participation, with the CRL, in four European research programmes, the NRL also transmits strains of particular interest or that pose a problem in terms of identification to the CRL. The NRL is also a member of the FLU.LAB.NET network, that includes all the European NRLs and the major international reference laboratories and facilitates information exchanges. It also participates in working groups for data, expertise and biological material pooling within the framework of the EPIZONE network of excellence.

The AI NRL also contributes to the training of laboratory technicians and managers from other countries.

Official analysis performance (confirmation)

By implementing the above-cited techniques, the AI NRL performs all the confirmation analyses requested by the Directorate General for Food (DGAl) in contexts such as clinical suspicion, surveillance and export control. It also performs all the self-monitoring tasks necessary for surveillance of the Laboratory for studies and research on poultry, pig and fish farming’s SPF and conventional livestock. Moreover, in the early 2000s, during roll-out of the AI surveillance surveys, the AI NRL performed first-line analyses in minor poultry species sectors for which serological test validation was poorly documented; the NRL then progressively transferred these analyses to the accredited laboratories.

The NRL processed nearly 10,000 tests in 2008, versus close to 18,000 in 2006 (the year of the crisis). The AI NRL therefore performed the identification and virulence characterisation of all the HP (highly pathogenic) H5N1 viruses detected in the winter of 2006 and the summer of 2007 and demonstrated that the second outbreak was a new introduction of the virus, in addition to performing systematic monitoring of the genetic and antigenic evolution of the identified AI viruses (LP H5 virus in particular) and the emerging subtypes, in addition to regular requests to confirm positive H5 serological results.

A diagramme summarising the relationships between the certified laboratories and the AI NRL is shown in Figure 1. The certified laboratories perform first-line real-time RT-PCR tests: 1- to detect the presence of any subtype of influenza virus, and 2- if found to be positive, to detect the presence or absence of the H5 haemagglutinin subtype of the virus (green box, upper left-hand side). In the event of a positive result, the NRL takes over (all other areas of Figure 1): • either (if an H5 virus is detected) to immediately confirm or rule out the presence of highly pathogenic H5N1 clade 2.2 viruses (present in Europe in 2006-2008) and in all cases to confirm or determine the virulence characteristics of the H5 virus through sequencing of the haemagglutinin cleavage motif (red box, lower left-hand side); • or, if the H5 virus has not been detected, to immediately rule out or confirm the presence of H7 haemagglutinin virus subtypes, and if a positive result is found, determine the virulence characteristics of the H7 virus through sequencing of the haemagglutinin cleavage motif (box in the middle left-hand area).

The typing of previously identified H5/H7 viruses continues: the neuraminidase subtype is determined using RT-PCR methods; the strain is isolated in order to push investigations further in the event of a successful outcome: determination of antigenic characteristics, and if relevant, partial or complete genome
Focus on a laboratory

sequencing, possible in vivo testing, including an intravenous pathogenicity index (IVPI) test.

If H5/H7 viruses are absent, the most common (H6, H1, H3) and/or emergent (H9) subtypes are sought using RT-PCR methods, and if results are positive, the investigations continue as before.

If the aforementioned subtypes are absent, isolation is attempted in order to identify the virus using reference antisera and then confirmation and possible characterisation is performed using RT-PCR sequencing methods since the NRL is capable of identifying the H1-H15 and N1-N9 subtypes of avian influenza virus.

Phylogenetic studies and reassortment research are then performed. The most interesting strains are added to the collection to be used in pathogenesis research work and vaccinology as well as in methodological development to update existing tests, validate new ones, etc.

Figure 1. Avian influenza virus detection and identification process

Scientific and technical monitoring and support

Through the contribution of these same researchers to international-level reference and research activities, the NRL fulfils its scientific and technical monitoring mission on a regular basis. As for expert opinion reporting, thanks to its senior researchers, the NRL has always been there to provide responses to the solicitations of its governing bodies – and the Ministry of Agriculture and Fisheries in particular. In fact, these requests were quite intense during the establishment of the initial AI monitoring plans and then during their updating and extension due to the increased risk of HP H5N1 virus introduction in France, as well as during the development of an associated vaccination and monitoring plan and the discovery of the health situation on domesticated water fowl farms. In addition, two senior NRL researchers have been participating in the collective expert evaluation process at Afssa (Chairmanship of the emergency collective expert working group, avian influenza ECEAG), Afset (French Agency for Environmental and Occupational Health Safety) and EFSA (European Food Safety Authority). One of them has also participated in expert reporting missions for the FAO (Food and Agriculture Organization) and the OIE (World Organization for Animal Health).

Conclusion

Thanks to its efficient utilization of powerful and reliable tools, its constant investment in methodological development, its structure and responsiveness and the coordinated involvement of its personnel in research and/or expert reporting activities, the AI NRL effectively fulfils all its official obligations. In doing so it is able to provide all the scientific and technical support needed for monitoring avian influenza in France and to share its expertise with laboratories in requesting third-party countries.

References


Figure 1. Avian influenza virus detection and identification process
Avian chlamydiosis: working towards the identification of new strains
K. Laroucau, Afssa, Maisons-Alfort (France)

The genus Chlamydia, along with the genus Chlamydia, make up the family Chlamydiaceae which currently is comprised of six species: Chlamydophila (C.) abortus, C. caviae, C. felis, C. pecorum, C. pneumoniae and C. psittaci (Everett et al., 1999). Avian chlamydiosis (also known as ornithosis-psittacosis) is a zoonosis caused by C. psittaci. The serovars that comprise this species are host-specific: the A serovar is most often found in psittacines, the B serovar in pigeons, the C in ducks and geese, the D in turkeys, the E in pigeons and ratites and the F in psittacines. This classification is based on the use of a panel of monoclonal antibodies. The new molecular biology tools provide heightened precision and detail for this classification. Thus the alternative genotyping tools Micro-Array (Sachse et al. 2008a and 2008b) and MLVA (Laroucau et al., 2008a), very recently described, reveal a wider diversity within this species.

Since avian chlamydiosis affects many wild and domesticated birds throughout the world, the infection has been described in 467 species belonging to 30 different orders. Symptomatology of the disease in animals depends on the virulence of the strain and the sensitivity of the bird, although C. psittaci infections most often remain latent or inapparent.

The infection is transmitted to humans through aerosol inhalation or by direct contact with droppings or infected respiratory secretions. Psittacosis is difficult to diagnose, it's sometimes asymptomatic. The incubation period is most often from 5 to 14 days. It clinically presents as a flu syndrome with fever, myalgia and headache or as atypical pneumonia. Due to late administration of appropriate treatment, complications such as acute respiratory syndromes may arise, which in rare cases can lead to patient death. However, with early appropriate treatment, the disease remains benign and recovery is rapid.

In 2005, 2006 and 2007, 17, 15 and 28 human cases, respectively, were reported to the French Institute for Public Health Surveillance (InVS) by the National Centre of Reference (NCR) which provides passive surveillance of human psittacosis (Laroucau et al., 2008b). Over this period, several human outbreaks related to the mule duck have been the subject of investigations linking the animal to humans (Laroucau et al., 2008c).

However, faced with the meagre data currently available regarding both humans and animals, the potential severity of the human version of the disease and the persistence of epidemic outbreaks in different professional contexts, a two-year descriptive study of psittacosis in humans, coordinated by the InVS, was initiated in January. Its goal is to determine the incidence of hospitalised human cases in the 15 highest poultry producing French departments and the frequency of cluster cases as well as to describe patient exposure, the strains involved in both humans and animals and the characteristics of the farms involved in order to improve understanding of the factors favouring animal-to-human transmission and provide guidance regarding prevention and control measures (http://www.invs.sante.fr/surveillance/psittacose/default.htm).

As part of this study, serological and/or bacteriological analyses of human samples are performed by the NCR (University of Bordeaux 2), while Afssa handles samples collected from birds present in the patients’ environment. When possible, the human and bird samples are genotyped and compared. The initial results of this study can be found on the Internet site provided above.

The molecular tool developed by Afssa is capable of detecting and quantifying all the bacteria in the Chlamydiaceae family, including C. psittaci.

Using this tool, investigations were conducted last year on chickens, ducks and guinea fowl following several cases of unexplained atypical pneumonia in a small fowl slaughterhouse in Charente.

Chlamydiaceae bacteria were detected by PCR in the various species of birds examined. The cloacal excretion levels were very high in certain animal batches. The genotyping of samples using specific C. psittaci tools was only successful for one of the positively diagnosed samples (sample collected on a batch of ducks).

The preliminary DNA microarray analysis of one of the atypical samples (Sachse et al., 2006) showed that it was a strain belonging to the Chlamydiophila genus, but that it was completely different from all the species described up to now.

The samples were cultured and six strains have already been isolated. Thanks to the partial sequencing of the RNA16S and ompA genes, these strains have been found to possess virtually identical RNA16S sequences, while the ompA sequences were all different. Phylogenetic analysis has confirmed the preliminary data delivered by the DNA microarray.

In Europe, three other sequences similar to those obtained in this study have recently been described from samples taken from hens in Germany (Gaede et al., 2008). The use of tools broadly targeting the Chlamydiaceae family now makes it possible to detect new strains as well as new species.

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The pathogenicity for humans of these strains has not yet been confirmed and still needs to be examined. The human sera collected from patients will be analysed soon.

A preliminary study conducted with the French National Institute for Agricultural Research (Inra) and using a murine model has shown no virulence in these new strains, as opposed to *C. psittaci* and *C. abortus*. The new strains seem closer to the *C. pecorum* strains. The species, which is also very heterogenous, is non-invasive in the same murine model. However, despite a number of misconceptions, certain strains of *C. pecorum* have been linked to cases of abortion in ruminants. The new molecular tools now make it possible to provide better strain characterization and as a result to more effectively identify the species involved. Certain strains of these new *Chlamydophila* species may present varying levels of virulence.

Another issue that needs to be addressed is that of the source of contamination for these new *Chlamydophila* species. Four infected batches came from the same batch of breeding hens. However, according to ompA gene sequences, the four strains isolated from these batches are all different. Environmental investigations are currently being carried out and could help improve general understanding of the origin of contamination and of Chlamydiacea pathogenesis.

### References

Staphylococcal enterotoxins are a family of 23 exoproteins with a mass of 22 to 30 kDa secreted in food matrices contaminated with enterotoxigenic strains of Staphylococcus aureus. Food will only become toxic if conditions favourable to both high bacterial growth and toxinogenesis are fulfilled.

Due to the emetic properties of some of these enterotoxins, their ingestion can cause gastro-intestinal symptoms including nausea and vomiting, sometimes followed by diarrhoea. In 2007 in France, enterotoxins were the second-ranking counted cause of bacterial food poisoning, with 16.2% of outbreaks and 15.8% of cases (Jourdan-Da Silva and Vaillant, 2008).

The diagnosis of a case of staphylococcal food poisoning can only be ascertained when at least one of the following factors has been confirmed:

• S. aureus count in the suspected food source exceeding $10^5$ colony-forming units per gramme;
• detection of staphylococcal enterotoxins in the food matrix.

Two major problems exist when attempting to confirm a diagnosis of toxic staphylococcal outbreaks. First, while these pathogens are sensitive to heat treatments applied to food, the enterotoxins are not. And secondly, currently available staphylococcal enterotoxin detection methods do not cover the entire range of 23 enterotoxins described to date.

Thus, in order to offer an innovative alternative to the immuno-enzymatic assays used for staphylococcal enterotoxin detection in food matrices, the Laboratory for the Study of Proteome Dynamics (EdyP) of the CEA (French Atomic Energy Commission) in Grenoble and Afssa’s Laboratory for studies and research on food quality and food processes have developed a method for measuring these contaminants using mass spectrometry.

In this method (PSAQ – Protein Standard Absolute Quantification), full-length recombinant proteins that perfectly match the biochemical properties of the natural target proteins are utilized as an internal standard. These PSAQ standards are isotope-labelled by incorporating $^{13}$C$_6$, $^{15}$N$_2$ L-lysine and $^{13}$C$_6$, $^{15}$N$_4$

The aim of the ProteomiqES project is to ascertain the origin of toxin-based staphylococcal outbreaks. To do this, we have developed a new analytical approach using molecular tools and physicochemical protein separation tools in addition to the microbiological and immunochemical methods traditionally used. The results provide an interesting initial application in cases of collective toxin-based food poisoning.
L-arginine during their cell-free biosynthesis (ProteoMaster system, Roche).

These internal PSAQ standards behave like the natural target proteins (Figure 2) and can be added before treatments are performed on the sample, thus avoiding biased results due to the analysis process itself (Figure 3). The PSAQ is therefore the only technique to take into account the yields of the prefractionation, purification and trypsic digestion stages.

This methodology was successfully applied to quantify the staphylococcal enterotoxin type A in various complex food matrices (Dupuis et al., 2008; Hennekinne et al., 2008). These initial results suggest that this method could be applied to the other staphylococcal enterotoxins and eventually used in the analytical process for etiological characterisation of food poisoning within the framework of our reference missions on both the national and EU level. A joint NRBC research project involving the EdyP Laboratory and Afssa's Laboratory for studies and research on food quality and food processes began in early 2009 and will pursue the development of quantitative mass spectrometry techniques for analysing staphylococcal enterotoxins in foods.

References

**Methods**

**Determination of heavy metal content of foodstuffs of animal origin: closed-system mineralisation by microwave digestion and measurement using inductively coupled plasma mass spectrometry (ICP-MS)**

T. Guérin, L. Noël, Afssa, Maisons-Alfort (France)

This article describes the method for determining the arsenic, cadmium, lead and mercury content of foodstuffs of animal origin using closed-system mineralisation by microwave digestion and measurement with inductively coupled plasma mass spectrometry. This technique is employed by the Heavy Metals National Reference Laboratory (group B3c as per annex I of Council Directive 96/23/EC of 29 April 1996 on measures to monitor certain substances and residues thereof in live animals and animal products) of Afssa’s Laboratory for studies and research on the quality of foods and food processes (Maisons-Alfort).

**The technique**

The inductively coupled plasma mass spectrometry (ICP-MS) technique for determining the arsenic, cadmium, lead and mercury content of foodstuffs following closed-system microwave mineralisation includes:

1. digestion of the sample with nitric acid in a hermetically sealed vessel placed in a pressurised container with application of high temperature and pressure in a closed-system microwave oven;
2. preparation of the test solution by taking up the mineral deposit with deionised water;
3. measurement of the arsenic, cadmium, lead and mercury content using inductively coupled plasma mass spectrometry: the test solution, obtained through closed-system microwave mineralisation, is nebulised and the aerosol is transferred into a high-frequency argon inductively coupled plasma (ICP). High temperatures are applied in order to desolvate the aerosol and atomise and ionise the elements. The ions are extracted from the plasma using a two-cone assembly and transferred to a mass spectrometer where they are separated according to their mass-to-charge ratio and then detected. This technique cannot be applied to oils, fats or matrices with high-lipid content.

**Equipment and reagents**

Specific equipment includes quartz or PTFE (Polytetrafluoroethylene) digestion vessels (Matras), preferably fitted with safety disks (Anton Paar or equivalent); a closed-system microwave oven (Anton Paar Microwave or equivalent); an inductively coupled plasma mass spectrometer (VG PQ ExCell or equivalent). Reagents (nitric acid – HNO₃ – in particular, in a concentration equal or superior to 65%) are of Suprapur grade or equivalent). Reagents (nitric acid – HNO₃ – in particular, in a concentration equal or superior to 65%) are of Suprapur grade or equivalent). Reagents (nitric acid – HNO₃ – in particular, in a concentration equal or superior to 65%) are of Suprapur grade or equivalent). Reagents (nitric acid – HNO₃ – in particular, in a concentration equal or superior to 65%) are of Suprapur grade or equivalent).

**Procedure**

1. **Preliminary treatment: preparation of test samples**
   
   Homogenise the sample in compliance with the recommendations set down in EN 13804:2002.

2. **Closed-system microwave mineralisation**

   **Test portion**

   Place a portion of the sample weighing between 0.2 g and 0.5 g (dry matter) or between 0.5 g and 1 g (wet matter) in the digestion vessel. Add 3 mL of deionised water, then 3 mL of nitric acid to the digestion vessel and close.

   To ensure that there has been no contamination, each series of analyses should include a blank test (a matrix-free test performed with the same amounts of reagents that undergoes digestion at the same time as the samples).

   To control the analysis method, each series of analyses should contain a reference material containing a known amount of the elements of quantify. This reference material should undergo digestion under the same conditions as the sample under examination.

3. **Digestion**

   Install the digestion flask on the rotor, then apply the appropriate digestion programme. Oven programming (power / time, for example) should be performed according to manufacturers’ recommendations. For microwave systems, normal digestion times are from 15 to 30 minutes. In general, oven programmes include a low-power phase that lasts a few minutes, followed by one or several high-power phases. A gradual increase between selected phases is recommended so as to avoid pressure spikes inside the vessel. To reduce the temperature and pressure inside the digestion vessel, a cooling phase is included at the end of the programme.

   The final state of digestion of the sample depends on the digestion temperature. In general, the higher the temperature, the less residual carbon is left in the solution and the better the quality of the mineral deposit. The digestion solution should be limpid, without any suspended particles, and its volume should be practically the same as before digestion.

   After digestion, open the vessels, then rinse the covers and walls with deionised water, take up in polypropylene flasks, add 0.25 mL of the internal standard solutions of indium, bismuth, rhenium and yttrium at 1 mg L⁻¹ in a 6% HNO₃ solution and dilute to 50 mL with deionised water. Repeat with the mineralisation blank.
The below programme is provided as an example:

<table>
<thead>
<tr>
<th>Steps</th>
<th>Initial intensity (W)</th>
<th>Time (min)</th>
<th>Final intensity (W)</th>
<th>*Cooling rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>500</td>
<td>20</td>
<td>800</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>800</td>
<td>10</td>
<td>1,000</td>
<td>1</td>
</tr>
<tr>
<td>3</td>
<td>0</td>
<td>20</td>
<td>0</td>
<td>3</td>
</tr>
</tbody>
</table>

*Ventilation rates can vary upwards from 0 to 3 (arbitrary unit).

3. Inductively coupled plasma mass spectrometer (ICP-MS)

**Conditions for operation**

Stabilise the ICP-MS for 20 to 30 minutes after start-up. The operating conditions listed below are provided as an example, using a VG PQ Excell.

**Nebuliser:**
Concentric type, flow rate: 0.9 mL/min
Nebulisation chamber with double coolant compartment

**Generator:**
Incident power: 1,350 W
Reflected power: < 5W

**Gas flow rate:**
Plasma: 15 L.min⁻¹
Vacuum: Expansion: 2.0 mbar
Nebuliser: 0.9 L.min⁻¹
Intermediate: 2.0 10⁻⁴ mbar
Auxiliary: 0.9 L.min⁻¹
Analyser: 7.6 10⁻⁷ mbar

**Sampler:**
Skimmer cone: nickel 1.0 mm orifice
Sampler cone: nickel 0.75 mm orifice

**Acquisition parameters:**
Number of channels: 500
Number of scans: 500
Dead time: 160 µs
Total acquisition time: 60 s

After having optimised and calibrated the machine (the calibration solutions contain 2, 5, 10 or 20 µg.mL⁻¹ of arsenic, lead, cadmium and mercury and a uniform 5 µg.mL⁻¹ of the internal standard solutions of indium, bismuth, rhenium and yttrium), the samples can be analysed. The role of optimisation is to increase the device's sensitivity while reducing interference. In order to ensure internal quality control, the set of calibration solutions is analysed regularly (every five samples); blanks, reference material, doped samples and doubles are also analysed.

**Interference**

Isotopic interference is the overlapping of two peaks representing two isotopes with the same atomic mass. The table below shows isotopic interferences for the different isotopes of arsenic, cadmium, lead and mercury.
Predominant atomic species present in the plasma, originating in the plasma itself (Ar, H, O, N, C), or in acids or solvents used, can form molecular ions through recombinations when leaving the interface. The table below shows the molecular ion interferences on arsenic, cadmium, lead and mercury:

<table>
<thead>
<tr>
<th>Analyte</th>
<th>m/z</th>
<th>Interference</th>
</tr>
</thead>
<tbody>
<tr>
<td>As</td>
<td>75</td>
<td>$^{40}\text{Ar}^{35}\text{Cl}$, $^{40}\text{Ca}^{35}\text{Cl}$</td>
</tr>
<tr>
<td>Cd</td>
<td>111</td>
<td>$\text{MoO}^+$, $\text{MoOH}^+$</td>
</tr>
<tr>
<td></td>
<td>114</td>
<td>$\text{MoO}^+$, $\text{MoOH}^+$</td>
</tr>
<tr>
<td>Hg</td>
<td>202</td>
<td>$\text{WO}^+$, $\text{RuRu}^+$, $\text{HgH}^+$</td>
</tr>
<tr>
<td>Pb</td>
<td>207</td>
<td>$\text{PbH}^+$</td>
</tr>
<tr>
<td></td>
<td>208</td>
<td>$\text{PbH}^+$</td>
</tr>
</tbody>
</table>

To reduce these interferences, the use of nitric acid, which does not provide any additional interfering species (unlike sulphuric acid), is recommended. Formation of these species depends on the power of the plasma, nebulisation flow rate and matrix.

**Example of correction:** $^{40}\text{Ar}^{35}\text{Cl}$

$I(75\text{As}) = I(75\text{mass}) - 3.127 \times [I(77\text{mass}) - 0.826 \times I(82\text{Se})]$

With 3.127 = Abundance $^{75}\text{ArCl}$ / Abundance $^{77}\text{ArCl}$ and 0.826 = Abundance $^{77}\text{Se}$ / Abundance $^{82}\text{Se}$

Non-spectral interferences (matrix effects) are due to the difference in behaviour of analytes in a simple environment (calibration) or in the presence of a more complex matrix (samples). In order to reduce these interferences, samples are highly diluted. The addition of internal standards can also offset changes in sensitivity due mainly to the presence of dissolved salts.

**Presentation of results**
The arsenic, cadmium, lead and mercury content (C) of the sample, expressed in mg.kg$^{-1}$, is equal to:

$$C = \frac{(C_e - C_b) \times \frac{50 \times f}{m}}{50}$$

50 = final solution volume (mL)
Ce = test solution concentration (mg.L$^{-1}$).
Cb = blank test solution concentration (mg.L$^{-1}$).
m = test portion mass (g).
f = possible dilution factor.
## 2008 ILPT results

<table>
<thead>
<tr>
<th>Organiser</th>
<th>ILPT</th>
<th>Method</th>
<th>Date (2008)</th>
<th>Participating laboratories</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>RAEMA (Network of analyses and exchanges in food microbiology)</strong></td>
<td>Enterococcus sp.</td>
<td>Flora count: mesophilic aerobic microorganisms, enterobacteria, total and thermotolerant coliforms, beta-glucuronidase-positive E. coli, sulphite-reducing anaerobes, Clostridium perfringens, coagulase-positive staphylococcus, L. monocytogenes, Salmonella</td>
<td>March</td>
<td>434</td>
</tr>
<tr>
<td></td>
<td>Citrobacter sp.</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>Escherichia coli</td>
<td></td>
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<tr>
<td></td>
<td>Clostridium perfringens</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Staphylococcus aureus</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>Salmonella Anatum</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Listeria monocytogenes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><strong>Afssa - Ploufragan - Brest</strong></td>
<td>Immunoserology - Avian influenza</td>
<td>Serum AGID</td>
<td>May and October</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Immunoserology - Newcastle's disease</td>
<td>Serum haemagglutination inhibition technique</td>
<td>May and October</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Immunoserology - CSF (classical swine fever)</td>
<td>Serum ELISA, commercial kits</td>
<td>September and November</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Serum virus neutralisation</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Virology - CSF (classical swine fever)</td>
<td>Real-time RT-PCR on organ samples</td>
<td></td>
<td>May and November-December</td>
</tr>
<tr>
<td></td>
<td>Virology - Fish (infectious hematopoietic necrosis, viral hemorrhagic septicaemia)</td>
<td>Isolation and identification using an immunological method (neutralisation or immunofluorescence or ELISA or PCR)</td>
<td></td>
<td>October-November</td>
</tr>
<tr>
<td></td>
<td><strong>Afssa - Niort</strong></td>
<td>Immunoserology - CAEV (caprine arthritis-encephalitis virus)</td>
<td>Serum ELISA, commercial kits</td>
<td>September and October</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Immunoserology - Visna-Maedi</td>
<td></td>
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<tr>
<td></td>
<td>Immunoserology - Brucellosis</td>
<td>Serum ELISA, buffered antigen test, complement fixation test or Wright serum-agglutination test</td>
<td>March (2nd session) and October</td>
<td>88</td>
</tr>
<tr>
<td></td>
<td>Immunoserology - Brucellosis</td>
<td>Bovine milk ELISA</td>
<td>October</td>
<td>18</td>
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<tr>
<td></td>
<td>Diagnosis of animal trichinellosis</td>
<td>Artificial digestion of muscle samples</td>
<td>Mars-April and October-November</td>
<td>75</td>
</tr>
<tr>
<td></td>
<td>Immunoserology - West Nile</td>
<td>Serum ELISA, commercial kits</td>
<td>September and October</td>
<td>5</td>
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<tr>
<td></td>
<td>Virology - Bluetongue</td>
<td>Real-time RT-PCR on EDTA blood samples</td>
<td>November-December</td>
<td>61</td>
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<tr>
<td></td>
<td><strong>Afssa - Maisons-Alfort</strong></td>
<td>Phycotoxins</td>
<td>Amnesic toxins in shellfish meat</td>
<td>October</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Heavy metals</td>
<td>Lead, cadmium and mercury contamination in fishery products</td>
<td>September</td>
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<tr>
<td></td>
<td></td>
<td>Listeria monocytogenes</td>
<td>Detection of Listeria monocytogenes in salmon</td>
<td>October</td>
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<tr>
<td></td>
<td></td>
<td>Somatic cells</td>
<td>Raw milk somatic cell count</td>
<td>December</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Staphylococcal enterotoxins</td>
<td>Detection of staphylococcal enterotoxins in lyophilised cheese</td>
<td>September</td>
</tr>
<tr>
<td>Organiser</td>
<td>ILPT</td>
<td>Method</td>
<td>Date</td>
<td>Participating laboratories</td>
</tr>
<tr>
<td>-----------------------</td>
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<tr>
<td>Afssa - Lyon</td>
<td>Escherichia coli</td>
<td>Agar diffusion antibiogramme</td>
<td>October</td>
<td>59</td>
</tr>
<tr>
<td></td>
<td>Salmonella</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Staphylococcus sp.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Streptococcus sp.</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>Pasteurella sp.</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>Identification of the pathogenic prion protein for the diagnosis of BSE (bovine spongiform encephalopathy)</td>
<td>LIA, WB, ImmunoStrip, ELISA or EIA, commercial kits</td>
<td>November</td>
<td>57</td>
</tr>
<tr>
<td></td>
<td>At different concentrations and in pure or mixed cultures: Mycoplasma (M.) agalactiae, M. bovis, M. mm Large Colony</td>
<td>Mycoplasma detection by isolation for diagnosis and screening</td>
<td>November</td>
<td>32</td>
</tr>
<tr>
<td>Afssa - Dozulé</td>
<td>Virology - AVE (equine viral arteritis)</td>
<td>Virus isolation in cell culture and sperm sample gene amplification</td>
<td>October</td>
<td>9</td>
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<tr>
<td></td>
<td>Immunoserology - AVE (equine viral arteritis)</td>
<td>Serum seroneutralisation</td>
<td>October</td>
<td>12</td>
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<tr>
<td></td>
<td>Immunoserology - EIA (equine infectious anaemia)</td>
<td>Serum agar gel immunodiffusion, commercial kits</td>
<td>October</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>Bacteriology - CEM (contagious equine metritis)</td>
<td>Isolation and identification of Taylorella equigenitalis (NF U47-108)</td>
<td>January-February</td>
<td>79</td>
</tr>
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<td></td>
<td>IF CEM (contagious equine metritis)</td>
<td>Immunofluorescence identification of Taylorella equigenitalis (DGAL/SDSPA/SDRRCC/N2007-8262)</td>
<td>January and March</td>
<td>42</td>
</tr>
<tr>
<td>Afssa - Sophia-Antipolis</td>
<td>Immunoserology - BVD (bovine viral diarrhoea)</td>
<td>Serum ELISA, commercial kits</td>
<td>April-May</td>
<td>43</td>
</tr>
<tr>
<td></td>
<td>Immunoserology - IBR (infectious bovine rhinotracheitis)</td>
<td>Individual serum and milk ELISA, commercial kits</td>
<td>April</td>
<td>98</td>
</tr>
<tr>
<td></td>
<td>Immunoserology - SAO (Salmonella Abortusovis)</td>
<td>Sero-agglutination</td>
<td>April</td>
<td>18</td>
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<td></td>
<td>Immunoserology - BO (Brucella ovis)</td>
<td>Complement fixation</td>
<td>April</td>
<td>14</td>
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<td></td>
<td>Immunoserology - Chlamydiosis</td>
<td>Serum ELISA, commercial kits</td>
<td>April</td>
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<td></td>
<td>Complement fixation</td>
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<td>14</td>
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<tr>
<td>Afssa - Nancy</td>
<td>Immunoserology - Rabies</td>
<td>Serum seroneutralisation</td>
<td>April (2nd session) and September</td>
<td>3 et 56</td>
</tr>
<tr>
<td>CIRAD</td>
<td>Immunoserology - Bluetongue</td>
<td>Serum ELISA, commercial kits</td>
<td>February and April</td>
<td>81</td>
</tr>
<tr>
<td>(Agricultural Research for Developing Countries) Montpellier</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LDGO</td>
<td>Immunoserology - Hypodermasis</td>
<td>Milk ELISA, commercial kit</td>
<td>November</td>
<td>16</td>
</tr>
<tr>
<td>(Côte-d’Or Departmental Laboratory) Dijon</td>
<td></td>
<td>Serum ELISA, commercial kits</td>
<td>February (2nd session)</td>
<td>3</td>
</tr>
</tbody>
</table>
Afssa training programmes

1st half 2009

June 4 and 5, 2009, Afssa - Dozulé (France)
- Training in serological diagnosis of equine infectious anaemia (EIA) using agar gel immunodiffusion (AGID). Training programme for approved analytical laboratories or laboratories which have officially requested approval.
  Contact person: A. Hans (a.hans@afssa.fr)

June 10-12, 2009, Afssa - Dozulé (France)
- Training in serological diagnosis using seroneutralisation and in virological diagnosis using virus isolation in cell culture and PCR for equine viral arteritis (EVA). Training programme for accredited analytical laboratories or laboratories which have officially requested accreditation.
  Contact person: A. Hans (a.hans@afssa.fr)

June 17-19, 2009, Afssa - Dozulé (France)
- Training in bacteriological diagnosis and immunofluorescence methods for contagious equine metritis. Training programme for accredited analytical laboratories or laboratories which have officially requested accreditation.
  Contact person: S. Petry (s.petry@afssa.fr)

1st half 2009, Afssa - Sophia-Antipolis (France)
- Training in Coxiella cell culture isolation technique.
  Contact person: R. Thiery (r.thiery@afssa.fr)

1st half 2009, Afssa - Nancy (France)
- Training in parenteral vaccines. For European NRL researchers.
  Contact person: E. Robardet (e.robardet@afssa.fr)
- Training in rabies diagnosis. For European NRL researchers.
  Contact person: E. Robardet (e.robardet@afssa.fr)

Afssa workshops

1st half 2009

June 15 and 16, 2009, Afssa Maisons-Alfort (France)
- CRL staphylococcus workshop
  Contact person: B. Lombard (b.lombard@afssa.fr)

June 2009, Afssa - Fougères (France)
- Workshop on the assessment of transfer of the multi-antibiotic (post-)screening method using CL/SM-SM. For French departmental analytical laboratories (NRL activity)
  Contact person: B. Roudaut (b.roudaut@afssa.fr)
- Workshop on colorant residues in farmed fish. For the national reference laboratories of the 27 EU member states (CRL activity)
  Contact person: E. Verdon (e.verdon@afssa.fr)