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Editorial

The Focus section of this new issue of *EuroReference* includes a presentation of the European Union Reference Laboratory for Parasites. Based in Rome (Italy) since 2006, the EURL works on zoonotic helminths and protozoa. It carries out inter-laboratory proficiency trials, training, diagnostic activities and standardization of analytical methods on a routine basis. Here, we learn about its epidemiological work on the prevalence of four species of nematodes from the genus *Trichinella* circulating in Europe.

Also in this issue, two articles present some major developments in European legislation: the first, published in the Focus section, expands on the application of the new European Regulation on the protection of animals and the constraints it imposes on animal experimentation in research and reference laboratories; the second article outlines the revision of the European Regulation (EC) No 882/2004 on official controls. It will be described in further detail in the next issue of *EuroReference*.

The Point of View gives an example of genome sequencing, a promising method for the future of microbiology diagnostic and reference laboratories, illustrated here by its application to contagious equine metritis.

This issue, rich in diversity, also looks back over two years of inter-laboratory trials of different techniques for rabies diagnosis. It also reports on the discussion meetings of the French reference community in the fields of animal health, food safety and plant health.

A little over a year after the start of a new phase in the life of *EuroReference*, we are on track to achieve our objectives. Three European members (Italy, Belgium and EPPO – the European & Mediterranean Plant Protection Organization) have joined the editorial committee and will soon be joined by a fourth (Poland). We are happy to welcome them to the life and activity of the journal. Our previous two thematic issues, which were substantial and authoritative, strengthened our approach and broadened the journal's readership. The next thematic issue, in 2014, will most likely be devoted to the surveillance networks for pathogens and contaminants in Europe.

We hope you will enjoy reading this issue of *EuroReference* and that it will be a valuable source of information for your professional work.

The editorial team

Summary

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Focus

The European Union Reference Laboratory for Parasites

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The Istituto Superiore di Sanità, Rome, Italy, was appointed European Union Reference Laboratory for Parasites (EURLP) in July 2006. The EURLP has been accredited according to the ISO/IEC 17025:2005 Standard, and has applied to be accredited as Proficiency Testing provider in accordance with the ISO/IEC 17043:2010 Standard. The main target parasites are zoonotic helminths of the genera *Trichinella*, *Anisakis*, *Pseudoterranova*, *Echinococcus*, *Taenia*, *Diphyllobothrium*, *Opisthorchis*, *Fasciola*, and zoonotic protozoa of the genera *Cryptosporidium*, *Giardia*, *Sarcocystis*, and *Toxoplasma*. The main tasks of the EURLP are to support and coordinate the network of National Reference Laboratories (NRL) for Parasites by supplying reference materials, identifying foodborne parasites, training NRL personnel, and organising proficiency testing and workshops. The EURLP provides scientific support in the field of foodborne parasites to DG SANCO, EFSA and ECDC of the European Commission, and to international organisations such as the FAO, OIE and WHO.

Introduction

Parasites are a group of human and animal pathogens including both unicellular (protozoa) and multicellular (helminths and arthropods) organisms. Some of these organisms are transmitted to humans through the consumption of food originating from animals infected or contaminated with these pathogens, i.e. the foodborne parasites.

In 2005, the Directorate General for Health and Consumers of the European Commission launched a call to select potential candidates for designation as Community Reference Laboratories (CRLs, now known as European Union Reference Laboratories, EURLs) operating in the areas of feed and food safety and animal health. The networks of EURLs and National Reference Laboratories (NRLs) are essential tools in the framework of official feed and food control. Their role has been recognised by Regulation (EC) No 882/2004 of the European Parliament and of the Council of 29 April 2004 on official controls performed to ensure the verification of compliance with feed and food law.

The creation of the EURL-NRL network for foodborne parasites has been an excellent opportunity to harmonize methods and epidemiological surveillance among European countries, to exchange reference materials, and to organise proficiency testing. The Istituto Superiore di Sanità, Rome, Italy was appointed EURL for parasites (EURLP) in July 2006 (this was renewed in 2011).

The EURLP tasks cover a broad spectrum of parasites, both protozoa and helminths (**Table 1**), circulating in Europe and those at risk of being imported into the EU by the animal and food trade. Unlike foodborne bacteria, most of which contaminate food and feed, many foodborne parasites (e.g., *Anisakis* spp., *Pseudoterranova* spp., *Trichinella* spp., *Echinococcus* spp., *Taenia* spp., *Diphyllobothrium* spp., *Opisthorchis felineus*, *Toxoplasma gondii*, and *Sarcocystis* spp.) grow in the host tissues or organs and consequently all measures to prevent their transmission should be taken primarily at the farm level, not at the slaughterhouse nor in the food processing industries. The diseases caused in humans by these parasites are generally characterized by a long incubation period (from weeks to years), a large number of asymptomatic or mild infections, and often by a chronic course (e.g., alveolar and cystic echinococcosis). In the EU, mortality rates due to foodborne parasites are quite low. In the field of detection of foodborne pathogens, parasites are unusual since there are no standard methods (ISO and/or CEN standards) and a lack of certified reference materials. Therefore, most available detection methods are in-house ones developed by public research and health institutions, which in some cases are able to provide reference materials from their own repositories.

The EURLP

The Unit for Gastroenteric and Tissue Parasitic Diseases of the Department of Infectious, Parasitic and Immune-mediated Diseases was appointed EURLP (http://www.iss.it/crlp/index.php?lang=2). The EURLP has been accredited according to the ISO/IEC 17025:2005 Standard, and has applied to be accredited as Proficiency Testing provider in accordance with the ISO/IEC 17043:2010 Standard. Eighteen people: nine scientists and nine technicians, are working on foodborne parasites for the diagnosis (parasitological, molecular, serological) in animals, foodstuff and humans, epidemiological surveillance of foodborne parasitic zoonoses, organisation of proficiency testing (PT) and ring trials, training of personnel from Member States (MSs) and non-EU countries, development of new diagnostic tools, production of reference materials (e.g., parasite strains, antigens, nucleic acids, reference sera), development of ISO standards, applied and basic research, and technical and scientific support to the European Commission, European Centre for Disease Prevention and Control (ECDC), European Food Safety Authority (EFSA), Food and Agricultural Organization (FAO), World Organization for Animal Health (OIE), World Health Organization (WHO) and International Commission on Trichinellosis (ICT).

The most important target foodborne parasites circulating in Europe (**Table 1**) are zoonotic helminths of the genera *Trichinella*, *Anisakis*, *Pseudoterranova*, *Echinococcus*, *Taenia*, *Diphyllobothrium*, *Opisthorchis*, *Fasciola*, and zoonotic protozoa of the genera *Cryptosporidium*, *Giardia*, *Sarcocystis*, and *Toxoplasma* (Dakkak, 2010; Pozio, 2008; Pozio *et al.,* 2013). Among the broad spectrum of foodborne zoonotic parasites, most of the efforts, time and resources of the EURLP are devoted to parasites of the genus *Trichinella*. This arises from

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Table 1. Foodborne parasites circulating in Europe and those at risk of being introduced in Europe by animal and food trade.

Protozoa	Main hosts	Route/s of transmission to humans	Distribution in Europe	Severity of the infection in humans
Cryptosporidium spp.	livestock, wildlife, humans	ingestion of oocysts contaminating vegetables, water or fomites, contact with infected animals and humans	cosmopolitan	from asymptomatic to severe; death in immunosuppressed people
Giardia duodenalis	humans	ingestion of cysts contaminating vegetables, water or fomites, contact with infected animals and humans	cosmopolitan	from asymptomatic to mild, more severe in children
Sarcocystis spp.	cattle, pigs (intermediate hosts), humans (definitive hosts)	ingestion of raw beef and raw pork	cosmopolitan	from asymptomatic to moderate
Toxoplasma gondii	cats (definitive hosts), all warm-blooded animals (intermediate hosts)	ingestion of tissue cysts in raw meat, ingestion of oocysts shed by cat faeces which contaminate vegetables, water or fomites	cosmopolitan	from asymptomatic to severe; death of foetus and immunosuppressed people
Helminths	**Main hosts**	**Route/s of transmission to humans**	**Distribution in Europe**	**Severity of the infection in humans**
Diphyllobothrium spp.	freshwater fish (intermediate hosts), fish-eating mammals (definitive hosts)	ingestion of raw freshwater fish	Foci in Estonia, Finland, France, Greece, Italy, Lithuania, Poland, Sweden	mild infection
Taenia saginata	cattle (intermediate hosts), humans (definitive hosts)	ingestion of raw beef	cosmopolitan	mild infection
Taenia solium	pigs (intermediate hosts), humans (definitive hosts)	ingestion of raw pork ingestion of eggs shed by infected people	could be present in small foci in eastern Europe	from asymptomatic forms (taeniasis) to severe forms (neurocysticercosis)
Taenia multiceps	sheep (intermediate hosts), dogs, wolves (definitive hosts),	ingestion of eggs shed by dogs	in areas used for sheep grazing	from asymptomatic to severe forms (coenurosis)
Echinococcus granulosus	sheep, goats, cattle, pigs (intermediate hosts), dogs (definitive hosts)	ingestion of eggs shed by dogs	prevalent in southern Europe	from asymptomatic to severe forms; death occurs rarely
Echinococcus multilocularis	wild rodents (intermediate hosts), foxes, raccoon dogs, dogs (definitive hosts)	ingestion of eggs shed by foxes, raccoon dogs, dogs	prevalent in central Europe	from asymptomatic to severe forms; death occurs rarely
Fasciola hepatica	cattle, sheep	ingestion of raw water plants contaminated with immature parasite larvae or by drinking water	cosmopolitan	from asymptomatic to mild forms
Opisthorchis felineus	freshwater fish (intermediate hosts), fish-eating mammals (definitive hosts)	ingestion of raw freshwater fish of the family Cyprinidae	13 EU countries	from asymptomatic to severe forms
Anisakis spp.	marine crustaceans, cephalopods, fish (intermediate hosts), sea mammals (definitive hosts)	ingestion of raw sea fish and cephalopods	cosmopolitan	from asymptomatic to severe forms; allergies
Trichinella spp.	domestic and sylvatic swine, carnivores	ingestion of raw meat	cosmopolitan	from asymptomatic to severe forms; death occurs rarely

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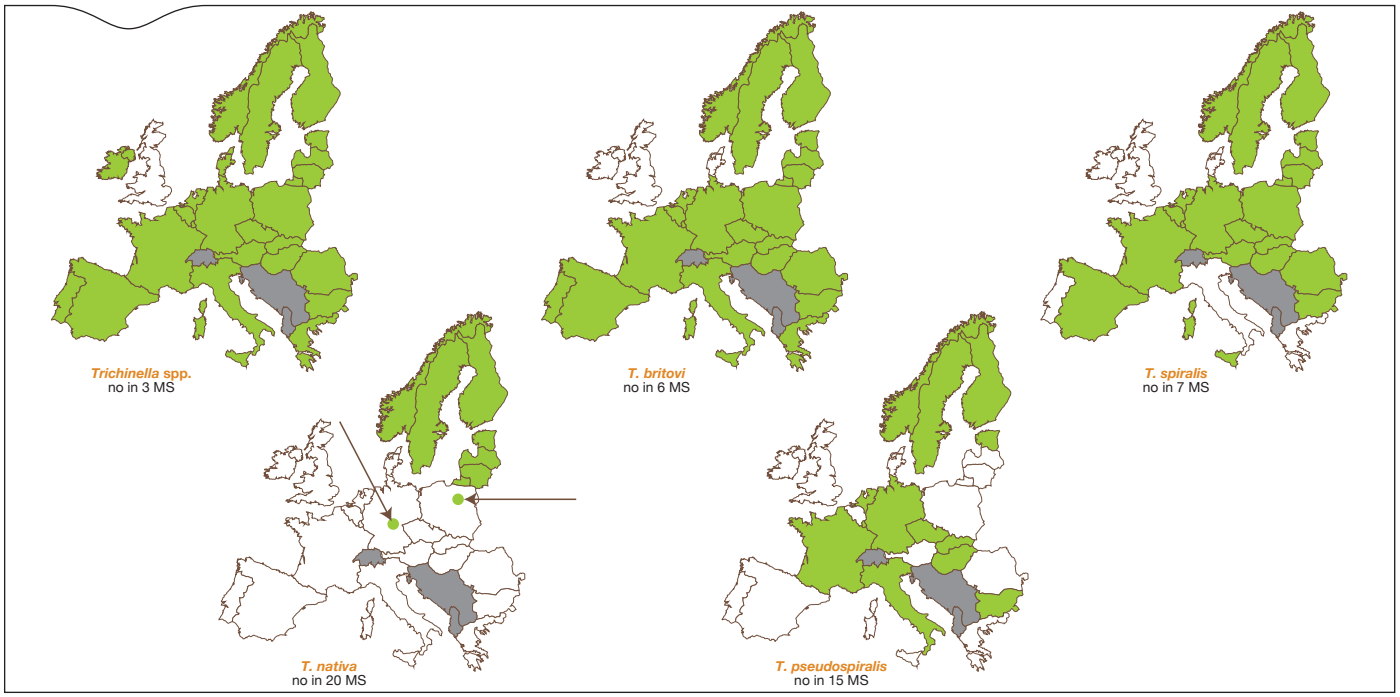

Figure 1. Distribution areas of *Trichinella* species circulating in countries of the European Union (EU). MS, EU member state; yellow MS, circulation of *Trichinella* sp.; white MS, absence of *Trichinella* sp.; brown MS, not an EU MS.

EU legislation, which has established the requirement to test for the presence of these parasites in all susceptible animals intended for human consumption (Commission Regulation, 2005), even if they do not represent the main problem for public health in the field of zoonotic parasites circulating in Europe.

Epidemiology of *Trichinella* spp. in the EU

Nematodes of the genus *Trichinella* infect a broad spectrum of animals, both wild and domestic, and humans, showing a cosmopolitan distribution (Pozio, 2007). In the European Union, *Trichinella* spp. are transmitted by a sylvatic cycle in most MSs with the exception of Cyprus, Great Britain, Luxembourg, and Malta, and by a domestic cycle in some foci in Bulgaria, Hungary, Lithuania, Poland, Romania, and Spain.

Four species of the genus *Trichinella* are circulating in Europe (**Figure 1**). *Trichinella spiralis,* which commonly infects domestic pigs and wild boars and, with a lower prevalence, carnivores such as red foxes, raccoon dogs and mustelids. The distribution of this species is characterized by spot foci in 19 MSs, but it has never been documented in Denmark, Greece, Italy or Portugal (**Figure 1**). *Trichinella britovi,* the most widespread species in the EU, has not been detected in Denmark, Ireland or Northern Ireland (UK). This species is prevalent in carnivores (red foxes, raccoon dogs, wolves, mustelids) and has a lower prevalence in wild boars (**Figure 1**). *Trichinella pseudospiralis* has been found in 12 MSs (Bulgaria, Czech Republic, Denmark, Estonia, Finland, France, Germany, Hungary, Italy, Netherlands, Slovakia and Sweden). This species is most prevalent in wild boars, whereas it has rarely been detected in other carnivores (raccoon dogs, red foxes, mink) (**Figure 1**). The distribution of the fourth species circulating in Europe, *T. nativa*, is restricted to Estonia, Finland, Latvia, Lithuania, and Sweden. Recently, it has been detected in single animals in Germany and Poland (**Figure 1**).

In Europe, the source of human trichinellosis varies by country. In the last decade, there has been a sharp reduction in human infections due to the consumption of pork from domestic pigs, whereas there has been an increase in trichinellosis in humans due to the consumption of meat from wild boars (Murrell and Pozio, 2011). Foci of trichinellosis due to the consumption of pork from backyard and free-ranging pigs still occur in disadvantaged areas of Bulgaria, Greece, Italy (Sardinia), Poland, Romania, and Spain. In France and Italy, the high number of human infections due to the consumption of horse meat has completely disappeared, thanks to controls at the slaughterhouse. Single infections or small foci have also occurred due to the illegal importation from eastern to western MSs of pork or pork-derived products not submitted to veterinary controls.

In the EU, about 400 million fattening pigs are slaughtered per year and the majority of them are reared in high containment level farms where *Trichinella* parasites have never been detected and will never be detected due to the mode of *Trichinella* transmission. These animals are thoroughly inspected at the slaughterhouse as a result of the substantial investment of manpower and resources. In the last 50 years, there have been no reports of *Trichinella* infections in pigs reared in high containment level farms, i.e. in more than 15 billion pigs (Alban *et al.,* 2011). However, only a small percentage of free-ranging and backyard pigs, which are at risk from *Trichinella* but are not intended for the large-scale retail trade, are tested. It follows that trichinellosis outbreaks still occur in Europe, mainly in poor and disadvantaged areas (**Figure 2**).

Focus

Today, according to current EU legislation, the European Commission cannot control the domestic markets of the MSs. However, it would be much more useful to shift all efforts and resources used today to investigate *Trichinella* in pigs from high containment level farms to the control of free-ranging and backyard pigs which, even if they account for only a low percentage of the total amount of pigs slaughtered every year in Europe, are at higher risk from these pathogens.
At the industrial level, most of the cost of *Trichinella* control is due to the delay in sending carcasses to the meat industry or to retail, pending the results of *Trichinella* testing, and is only partially linked to the examination itself. Alternatively, carcasses may be cut up at a cutting plant attached to or separate from the slaughterhouse, provided that the carcass, or the parts thereof, will not have more than one cutting plant as its destination, and the cutting plant is situated within the territory of the MS (Commission Regulation, 2005).

Figure 2. Encapsulated larva of *Trichinella spiralis* in the diaphragm pillar of a pig. Scale bar 100 µm.

Trichinella spp. activities carried out at the EURLP

Proficiency testing

Digestion method. One of the core duties of the EURLP is to organise PTs, as stated in Regulation (EC) No 882/2004 of the European Parliament and of the Council (Commission Regulation No. 882/2004). The purpose of this exercise is to test the ability of the NRLs to detect *Trichinella* larvae in pork and/or horse meat samples according to one of the approved methods reported in Annex 1 of Commission Regulation (EC) No 2075/2005 (European Commission, 2005). These PTs, whose management is fully computerized, have been held annually since 2007.
The test material forwarded to each laboratory consists of minced meat balls made with pork or horse meat, spiked or not (negative samples) with live *Trichinella* larvae. Up to now, the magnetic stirrer method for pooled sample digestion has been the technique most commonly used by participating labs; the mechanically assisted pooled sample digestion method/ sedimentation technique was used by 17.2% of the labs and the automatic digestion method for pooled samples up to 35g was used by 10.3% of the labs. A comparison of the results of the PT rounds performed up to now shows an improvement in the performance of labs applying the digestion method from 2007 to 2008, while fluctuating results were obtained from 2008 to 2013 (**Figure 3**).
Molecular identification of Trichinella larvae at the species level using PCR. According to Commission Regulation (EC) No 2075/2005 (Annex 1, Chapter I), when *Trichinella* spp. larvae are detected by digestion of animal muscles, the parasite samples are to be kept in 90% ethyl alcohol for conservation and identification at species level at the EURLP or NRLs. The purpose of this PT, organised since 2011, is to test the ability of NRLs to correctly identify the species circulating in Europe by means of molecular methods applied to single or pooled larvae.

Training

In the period 2006-2012, 38 people from 11 EU MSs, two non-EU countries in Europe, and from Australia, Brazil, Philippines, and Vietnam visited the EURLP to learn diagnostic tests to detect *Trichinella* larvae in meat samples, to identify these parasites at the species level by PCR, and to detect anti-*Trichinella* antibodies in animal and human sera.

Other activities

Diagnoses

The diagnostic activities of the EURLP are performed in accordance with the ISO/IEC 17025 Standard. At present, only the international standard ISO 15553:2006 Water quality - Isolation and identification of *Cryptosporidium* oocysts and *Giardia* cysts from water - has been published, but this method cannot be used to identify the pathogen at the species level, determine the host of origin or characterize the viability and/or infectivity of *Cryptosporidium* oocysts or *Giardia* cysts which may be present. The lack of international reference standard methods for the diagnosis of foodborne parasitic diseases is a major constraint in implementing a quality assurance system. To date, diagnostic methods have not been standardized at the European or international level, therefore there is a need to validate both commercial kits and tests developed in-house. The EURLP has developed, validated and accredited nine diagnostic methods (available as part of the services that

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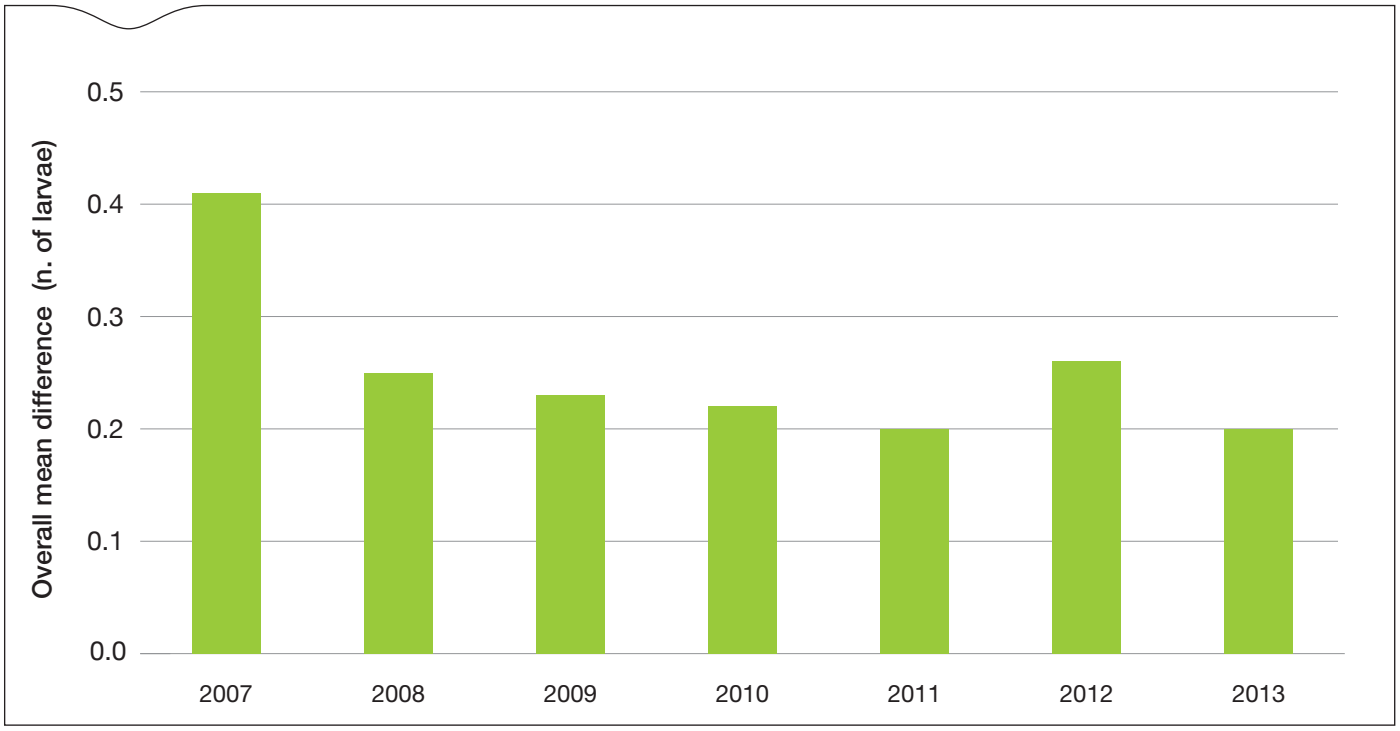

Figure 3. Results of seven rounds, from 2007 to 2013, of the proficiency test (PT) to detect *Trichinella* sp. larvae in muscle tissues according to the Commission Regulation (EC) No 2075/2005, organised by the EURLP for the National Reference Laboratories for Parasites. For each year, the overall mean difference (i.e., the mean of the relative difference between expected and observed values considering all samples and all laboratories), expressed as number of larvae, is shown; the lower the value of the overall mean difference, the better the result.

the EURLP provides to the NRLs) (**Figure 4**). Furthermore, the EURLP produces reagents and reference materials, and contributes to the development of international guidelines.

Other PTs

The EURLP also organises proficiency testing for the detection and/or identification of foodborne parasites other than those of the genus *Trichinella*, in accordance with the ISO/IEC 17043:2010 Standard.

Detection of Echinococcus spp. adult worms in the intestinal mucosa of the definitive host. The aim of this PT, organised in 2010, 2011, 2012, and 2013, is to correctly identify the presence of adult worms of *Echinococcus* spp. in a matrix of intestinal mucosa. A panel of three samples is forwarded to each participating NRL, each sample consisting of homogenized intestinal mucosa fixed in 70% ethanol, spiked or not (negative control) with adult worms of *Echinococcus* spp. Samples have to be analysed by the sedimentation and counting technique (SCT).

Detection of Anisakis spp. L3 larvae in fish fillets. The purpose of this PT, organised in 2009 and 2013, is to detect Anisakidae larvae in fish fillets by means of one of the following methods: i) candling; ii) compressorium; iii) UV examination after freezing; iv) digestion. A panel of three samples is delivered to participating NRLs, each sample consisting of 100g fish fillet sandwich spiked or not with *Anisakis* spp. L3 larvae free of their capsule.

Research activities on foodborne parasites and related animal infections

Many research activities on foodborne parasites were and/or are taking place at the EURLP since its appointment, namely:

- *Trichinella* spp.: the identification of intraspecific genetic variability of *Trichinella spiralis* and *T. britovi*, and the evaluation of the use of serology to monitor *Trichinella* infections in wild boars and/or red foxes;
- *Toxoplasma gondii*: the identification of oocyst stage-specific proteins;
- Anisakidae: the identification and development of analytical methods for the speciation of parasites belonging to this family;
- *Echinococcus* spp: the identification and development of analytical methods for the speciation of parasites belonging to this genus;
- the barcoding of zoonotic and non-zoonotic helminths and protozoa parasitizing domestic animals, foodstuffs and humans;
- *Cryptosporidium* spp: the development of analytical methods for the speciation of parasites belonging to this genus;
- *Alaria alata*: the collection of epidemiological data on the prevalence of this trematode worm in the wild boar populations of MSs.

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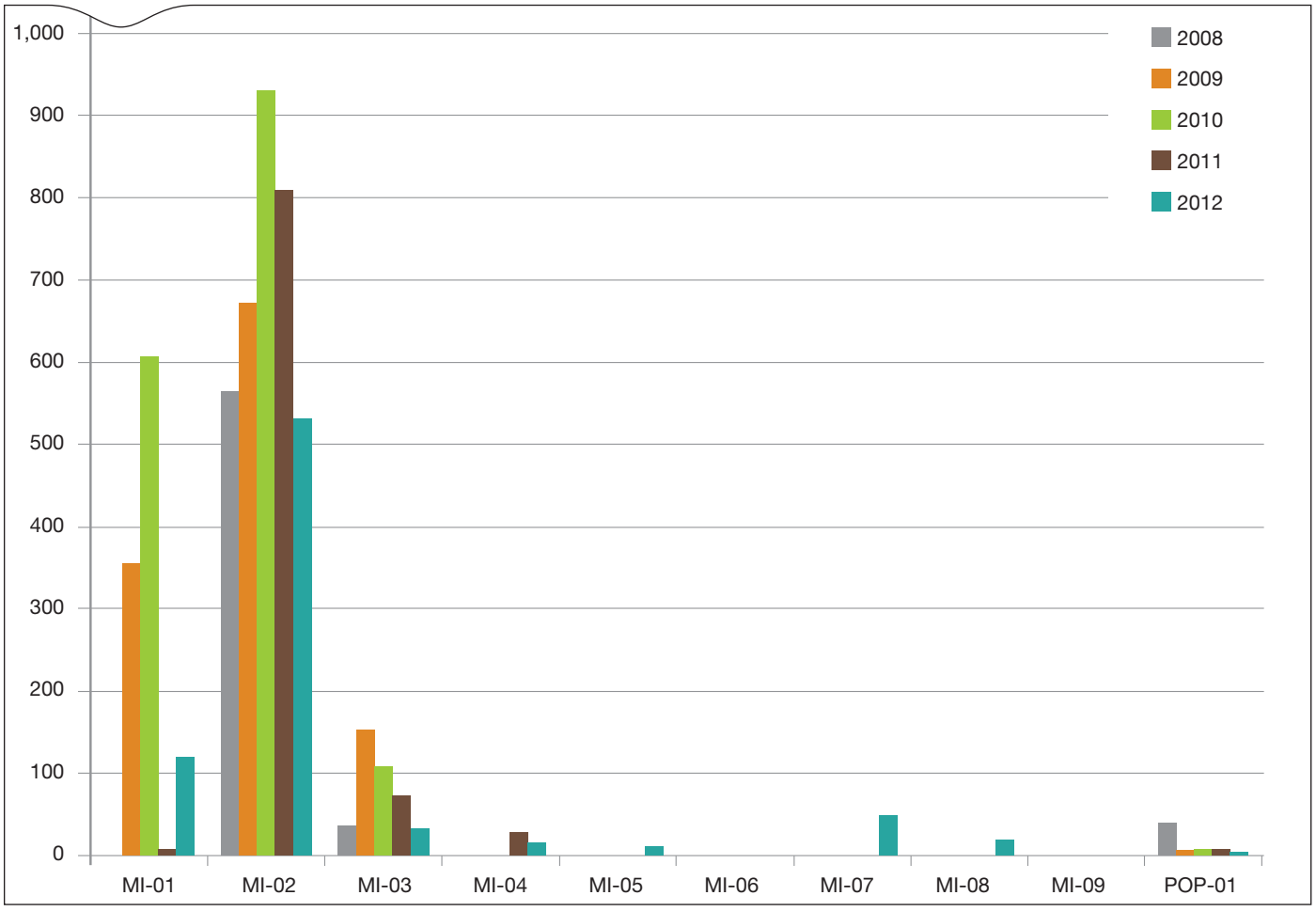

Figure 4. Samples analysed by accredited methods at the EURLP in the period 2008-2012.

MI-01: Detection of anti-*Trichinella* antibodies in swine serum by an indirect ELISA;
MI-02: Identification of *Trichinella* muscle larvae at the species level by multiplex PCR analysis;
MI-03: Detection of anti-*Trichinella* antibodies in human sera by an indirect ELISA;
MI-04: Identification of parasites of the family Anisakidae at the species level by PCR/RFLP;
MI-05: Identification of *Echinococcus granulosus* complex from hydatid cysts at the genotype/species level by PCR and sequencing;
MI-06: Identification of *Cryptosporidium* oocysts at the species level by PCR/RFLP;
MI-07: Detection of anti-*Opisthorchis* antibodies in human sera;
MI-08: Identification of *Opisthorchis* spp. eggs at the species level by PCR;
MI-09: Identification of *Giardia duodenalis* cysts at the assemblage level by PCR/RFLP;
POP-01: Magnetic stirrer method for pooled sample digestion for the detection of *Trichinella* in fresh meat (Commission Regulation (EC) No 2075/2005).

Standardization of analytical methods

Harmonization and standardization of diagnostic methods is one of the tasks of the EURLs. In this context, the EURLP participates in the work of the ISO/TC34/SC9 (International Organization for Standardization/Technical Committee 34 - Food products/Sub-Committee 9 - Microbiology) and the CEN/TC275/WG6 (European Committee for Standardization/ Technical Committee 275 - Food analysis - Horizontal methods/ Working Group 6 - Microbiology of the food chain). These two sub-committees, on the basis of the Vienna agreement, work together in order to speed up the standardization process and avoid duplication of work. The draft standard *ISO/DIS 18743: Microbiology of food and animal feed — Detection of Trichinella Larvae in meat — Physical method by digestion* was developed under the EURLP chair, and the Enquiry (DIS) ballot was issued in April 2013, to submit the draft to the ISO and CEN MSs for voting.

Workshops

Every year, the EURLP organises a two-day workshop for the personnel of the NRLs. Internationally recognized experts are invited to give a lecture in the field of foodborne parasitic zoonoses. On the first day of the meeting, each NRL presents the most important epidemiological data from the previous year and any possible problem related to diagnosis and control.

Focus

Conclusions

According to the European Commission regulations, every year the authorities of each MS provide epidemiological information on foodborne zoonoses to the ECDC and to EFSA for the human and animal side, respectively. These two European agencies process the data and publish an annual Community Summary Report. Unfortunately, the flow of data provided by the MSs is sometimes incomplete, and has not always been controlled and critically revised by national scientific institutions. As a consequence, the quality of published information is frequently low because of errors in data collection, processing and interpretation. One example is the reporting of *Trichinella* detection in pigs from high containment holdings. This showed the lack of a clear concept of what a 'high containment holding' is, frequently being confused with 'indoor farm'. In fact, by definition, *Trichinella* cannot circulate in a high containment holding where pigs are not fed with pork scraps (this parasite can be transmitted only by ingestion of *Trichinella*-infected muscle).

Moreover, the mandatory notification of human parasitic diseases and parasite control in animals, foodstuffs or drinking water, differs among MSs, making it difficult to compare the reported data. One of the most striking examples of this situation is the official data on human cystic echinococcosis (CE). In some EU countries, where cases of CE have been described in the scientific literature, there are often no reported cases in the official data, either because notification is not mandatory or due to reporting delays of several years or more.

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Focus

Application in French law of the new European regulations on the protection of animals used for scientific purposes: What progress has been achieved for animals? What constraints does this impose on research?

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Following European Directive 2010/63/EU, the French implementing legislation on the protection of animals used for scientific purposes was published in February 2013. These new regulations impose a number of limitations and constraints on scientific research and make the issue of animal suffering a primary concern.

Introduction

After two years of deliberation and collaboration between government authorities (the French Ministries of Agriculture and Research), professionals and animal welfare associations, the implementation of Directive 2010/63/EU (Directive, 2010) on the protection of animals used for scientific purposes was confirmed on 7 February 2013 with the publication of French texts in the Official Journal of the French Republic. This legislation takes the form of:

- a decree (Decree, 2013) on the protection of animals used for scientific purposes that *"establishes the conditions relating to the species concerned, the origin of animals, housing and care conditions, as well as the experimental procedures that must be complied with by user, breeder and supplier establishments if they are to be authorised to perform experimental procedures on animals or to raise or supply animals for this purpose. It lays down the conditions for accreditation and control of breeder, supplier and user establishments for animals used or intended to be used for scientific purposes.";*
- five inter-ministerial orders (Order, 2013a; Order, 2013b; Order, 2013c; Order, 2013d; Order, 2013e).

Since animal welfare is a value of the Union upheld by Article 13 of the Treaty on the Functioning of the European Union, the new French regulations resulting from European Directive 2010/63/EU are based on the rule of the three Rs (Russell and Burch, 1959): "Replacement" of animals whenever possible, "Reduction" in their numbers in the procedures performed and "Refinement", i.e., limiting the harm caused to animals. These new regulations are resolutely focused on animal welfare and introduce significant changes in the use of animals for experimental purposes.

The provisions of the new French regulations apply when animals are used or intended to be used in experimental procedures, or when they are bred so that their organs or tissues may be used for scientific purposes. They concern vertebrate animals, and for the first time, larval forms able to feed themselves, foetal forms of mammals as from the final third of their normal development, and cephalopods.

On a practical level, a number of measures are to be introduced within user establishments (formerly known as animal experimentation establishments), as well as in supplier and breeder establishments, another new feature of these regulations.

Responsibilities and animal welfare

The provisions of this new regulatory framework include (1) the appointment of an animal welfare manager and (2) the setting up of a body responsible for animal welfare within each user, supplier and breeder establishment. Many tasks are delegated to these new functions. The manager is in charge of monitoring animal welfare in the establishment, supervising and following up authorised projects and monitoring the competence of staff designing or conducting experimental procedures, applying these procedures to animals, providing care for animals and killing them. The new body is responsible, among other things, for:

- advising staff on issues relating to animal welfare, care and housing,
- advising staff, in particular staff designing experimental procedures, on the implementation of the 3Rs,
- informing staff of technical and scientific developments relating to the application of this rule.

This body must also monitor the progress and results of projects using animals conducted within the establishment, and provide advice on animal rehoming programmes (that is the possibility that private individuals adopt animals (for domestic species) or the possibility to place animals in appropriate structures (for livestock and wildlife), when their health allows it at the end of the study. Although both functions are important and make a major contribution to animal welfare, the fact remains that this entails a considerable amount of work, which can be difficult to deal with in the current economic and budgetary climate in institutions - especially those in the public sector - required to use animals for experimental purposes.

Staff competence

Another important point concerns the competence of staff working in animal experimentation that results from their initial training, participation in a specialised animal experiment training programme (carried out no later than the year after they start work) and continuing education. The latter involves a minimum of three days of training every six years. Although at first sight this does not seem very much, this new provision is an important step that will enable all staff (not only scientists, but also technicians and animal attendants) to maintain their knowledge and keep abreast of new technologies and advances in the fields that concern them. All staff skills and training will be recorded in a skills booklet specific to each person, proving that their competence effectively matches their functions.

Authorisation of projects

One of the major changes to the previous system is the requirement to obtain authorisation prior to carrying out any project involving the implementation of one or more experimental

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procedures using animals. The project authorisation file includes a non-technical summary providing information on the project's objectives and the number and type of animals used. As this summary is intended to be published on the Ministry of Research's website and will therefore be accessible to the general public, scientists need to be vigilant to avoid disclosing confidential information.
Projects are authorised by the Ministry of Research for a maximum of five years. In another new development, when issuing authorisations this Ministry now relies on an ethics evaluation carried out by the ethics committee for animal experimentation affiliated to the establishment where the project will take place. In practical terms, all establishments conducting projects involving the use of animals for experimental purposes should therefore appoint an ethics committee, which must of course be operational and registered with the Ministry of Research. Most public and private French research organisations already have ethics committees, which play a vital role in ensuring that projects are undertaken while respecting ethics and animal welfare. The new regulation therefore formalises the work of these bodies that were already operating in compliance with the National Charter on the ethics of animal experimentation, whose philosophy is very close to the European (Directive, 2010) and French regulations (Decree, 2013). Currently, projects are submitted to the Ministry of Research by post, whereas online completion of applications and direct electronic submission are expected to be set up in the future, which should facilitate the process.

Care and housing

With regard to animal care and housing conditions, the new regulations entail a review of the requirements with the aim of improving the welfare of animals both in experiments and in breeding (Order, 2013a, Annex II). Establishments have until 1 January 2017 to acquire housing materials that meet the required standards. In addition, establishments are now required to implement appropriate enrichment techniques allowing the animals to express a wide range of normal behaviour. The establishment's *enrichment programme* should be regularly reviewed and updated. Individual housing may be allowed in accordance with the authorised project but should be limited as much as possible, and visual, auditory, olfactory and/or tactile contact should be maintained with other animals. Last but not least, the animals must be *checked daily* by a competent person. In fact, daily checks are already in place in organisations experimenting on dogs, cats, ruminants and non-human primates, and during projects potentially causing suffering in test animals (projects classified as 'severe'). However, these checks were not previously performed in most animal facilities housing rodents, species that are untroubled by the automatic distribution of food and can do very well without the presence of humans. This new requirement may cause problems in the organisation of work, particularly in smaller establishments, while the benefits are questionable for rodents, which are known to prefer being left alone to excessive handling and inspections.

Non-human primates (NHPs)

Finally, Non-human primates (NHPs) have a *special status* and are subject to specific measures. After much debate and as requested in the report on Directive 86/609/EEC (Evans, 2002; Directive, 1986), Europe, and by extension France, decided to consider NHPs as a special group of species governed by specific provisions. The first measure concerns the purpose of procedures involving NHPs that concern the avoidance, prevention, diagnosis or treatment of debilitating or potentially life-threatening human diseases. The second measure is the requirement for a scientific demonstration explaining that the purpose of the experimental procedure cannot be achieved by the use of species other than those belonging to the order of primates. Furthermore, the use of great apes (gorillas, chimpanzees and orang-utans) is prohibited except in the event of a specific waiver subject to approval from the European Commission. While the special protection of NHPs is a good thing because of their phylogenetic similarity to humans, it should however be considered that NHPs are not the only animals capable of experiencing stress, pain and suffering, and of emotions more generally. Does the special protection afforded to NHPs result in less protection for other animal species? Although this was not the intention of Directive 2010/63/EU and the ensuing French regulations, it appears that this is an indirect consequence.

Conclusion

In recognising animal suffering, these new regulations appear to be a step forward for the protection of animals used for scientific purposes, in terms of both improved standards of care and housing and the mandatory ethics evaluation for projects carried out by French scientists. It is worth noting, however, that when faced with increased costs and administrative burdens, questions should be asked about the future position of Europe and France in a context of increasing globalisation of research. Indeed, some countries are positioning themselves as possible alternatives to carrying out research projects in Europe, in particular research projects involving NHPs, which could not only lead to the "offshoring" of research, but also to less protection for animals in countries where the regulations in this area are not as advanced as in Europe. In addition, while animals continue to be essential models for fundamental and applied research, the fact remains that in recent years there has been great progress in the development of alternative methods to animal testing. One example of this is the establishment in 2007 of the national platform for the development of alternatives to animal testing (FRANCOPA, http://www.francopa.fr/web/francopa?page=home&out=txt&languageIhm=fre), which itself is a member of the European Platform, ECOPA (http://www.ecopa.eu/).

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Décret, 2013. Décret n°2013-118 du 1er février 2013 relatif à la protection des animaux utilisés à des fins scientifiques. NOR: AGRG1231951D

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Point of view

Whole genome sequencing, an efficient investment for developing diagnostic and epidemiology tools: the case of contagious equine metritis

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The number of available whole genomes has been regularly increasing while costs and sequencing times continue to decrease. Many laboratories — both public and private — participate in whole genome sequencing and this investment has radically transformed basic research and all fields that deal with biological processes. In the near future, whole genome sequencing will likely become a routine tool for microbiology diagnostic and reference laboratories.

Introduction

Genomics has seen great technological progress since the development of the first DNA sequencing methods and the publication of the first whole genome — that of the bacteriophage ΦX174 — in 1977. Today, sequencing an individual's genome only takes a few hours and costs little more than $1000, but the first whole human genome was obtained in 2003 after 13 years of work and cost roughly $2.7 billion. In bacteria alone, and only 20 years after the sequencing of the first genome of a living organism (*Haemophilus influenzae*, with a genome size of 1.83 Mb), nearly 4000 whole genomes are now available and four times this number are incomplete or being completed. Rapid progress in whole genome sequencing has been driven by the quest to understand, treat and predict diseases; to innovate in the fields of biotechnology, environment, agronomy, etc.; and also to better comprehend, on a basic science level, life and biological evolution.

A brief description of contagious equine metritis (CEM)

CEM is an economic threat for the horse industry (breeding, export, sales). This sexually transmitted and contagious bacterial infection appeared in the late 1970s in several regions around the world where horse dealing is particularly active. Today, the disease is still found worldwide, but mandatory testing has limited outbreaks to only a few cases. The clinical signs include inflammation of the endometrium in mares, which is typically accompanied by temporary infertility. Without treatment, horses (males and females) may become carriers of the disease for several years. Treatment failure seems to be frequent although none of the strains appear to show resistance to the administered antimicrobials. First classified in genus *Haemophilus*, the causative agent of CEM was reclassified in a new bacterial genus in 1985 named *Taylorella*, now made up of two species: *Taylorella equigenitalis*, which leads to an outbreak of a case of CEM, and *Taylorella asinigenitalis*, considered as non-pathogenic despite clinical signs of metritis following experimental intra-uterine infections in several mares. Despite

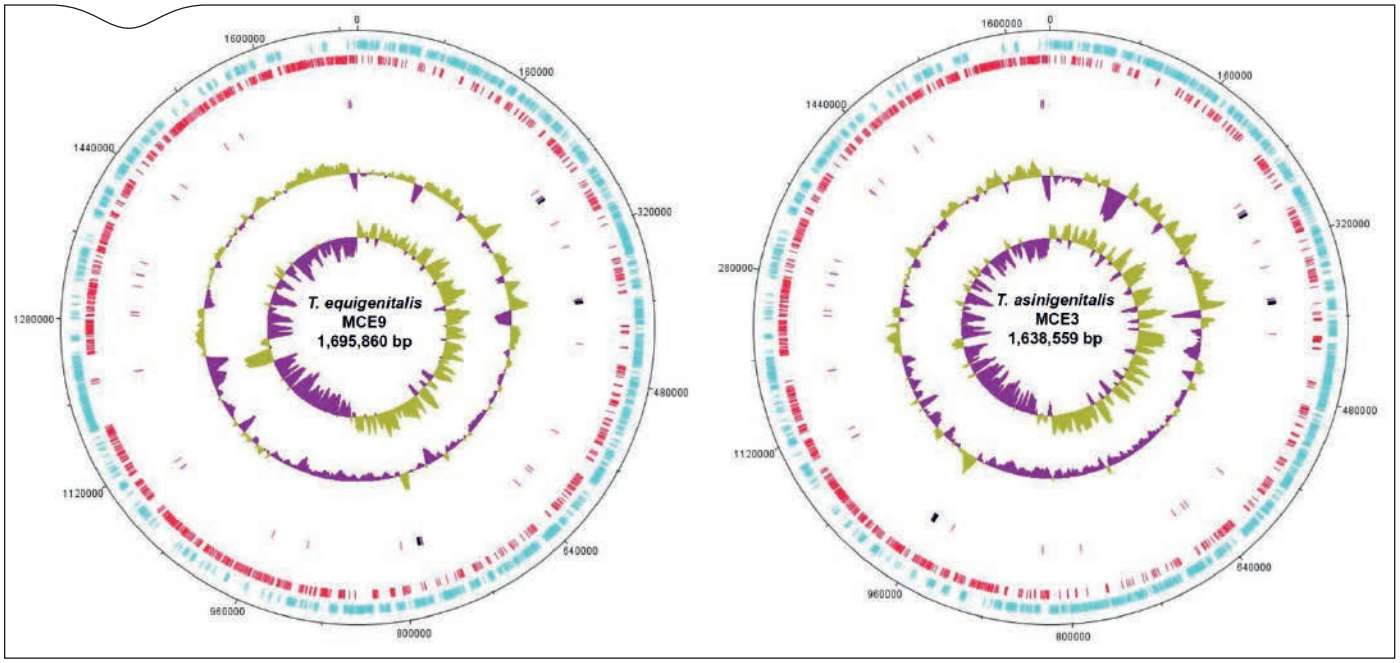

Figure 1. Genetic maps of the *T. equigenitalis* MCE9 and *T. asinigenitalis* MCE3 genomes.

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the slow growth of *Taylorella* bacteria on current culture media compared to the commensal flora of the genital tract, the official diagnosis of CEM is based on the isolation and biochemical identification of the pathogen. Moreover, the species must be confirmed by PCR because *T. equigenitalis* and *T. asinigenitalis* are phenotypically very similar.
The national reference laboratory (NRL) and European reference activities (carried out as part of the European Union Reference Laboratory for equine diseases) for CEM are based at the Bacteriology and Parasitology Unit of the ANSES Dozulé Laboratory for Equine Diseases.

Whole genome sequencing for reference activities, the case of CEM

For a reference laboratory whose role is to develop reliable tools for detecting and characterising pathogens as well as new typing methods, the lack of genetic information can drastically hamper progress. For example, in CEM, the theoretically close genetic relationship between genus *Taylorella* and other bacterial genera (e.g. *Bordetella* and *Haemophilus*) and the absence of sequence data other than ribosomal operons has limited the development of efficient diagnostic tools. To address this limitation, we undertook the *de novo* sequencing of the genomes of both *Taylorella* species in collaboration with the UMR1319 Micalis at INRA (Jouy-en-Josas, France); two high-throughput sequencing technologies were combined: 454 technology (Margulies *et al.,* 2005) coupled with "paired-end" reads (Fullwood *et al.,* 2013) to facilitate genomic assembly of both genomes, and Illumina (Solexa) technology (Bentley *et al.,* 2008). Both genomes were then annotated and compared (Hébert *et al.,* 2011; Hébert *et al.,* 2012; **Figure 1**).
The availability of genetic sequences has greatly aided the development of molecular diagnostic tools. To improve the reliability of CEM testing, we are currently collaborating to optimise a real-time multiplex PCR protocol that will improve upon the 16S ribosomal DNA sequences and therefore increase specificity. Knowledge of genome sequences also benefits the development of bacteriological or serological diagnostic methods. For example, the *in silico* reconstruction of the metabolic pathways of *Taylorella* species helps to better understand the bacteria's nutritional needs and develop a more appropriate culture medium to increase the sensitivity of the current official diagnosis method for CEM. The optimisation of this culture medium began in January 2013 in collaboration with the AES CHEMUNEX company (bioMérieux Industry, France).
There are several available molecular epidemiology tools, depending on the pathogen in question and the intended application (Sabat *et al.,* 2013). For genus *Taylorella*, we chose multilocus sequence typing (MLST), which is not only a robust, easy to perform and portable method, but also a standard method for global epidemiological surveillance of a disease and the study of the evolution of bacterial populations. Based on the annotated genomes of *T. equigenitalis* and *T. asinigenitalis*, we selected several genes that are shared between the two species and whose products are essential for cell survival. Seven of these genes were validated in 113 *T. equigenitalis* and 50 *T. asinigenitalis* strains collected in six countries over 35 years. This molecular epidemiology tool for CEM will eventually be shared with other NRLs in the European Union to determine the status of the *Taylorella* strains that circulate in Europe and to conduct retrospective studies on the available strain collections. MLST data (epidemiology of strains and DNA sequences from seven MLST markers) will be shared and made accessible in a *Taylorella*-specific database (http://pubmlst.org/taylorella/) hosted at the Department of Zoology, University of Oxford, UK (Jolley and Maiden, 2010).
Along with the development of diagnostic and molecular epidemiology tools, reference laboratories conduct research to enhance basic knowledge on pathogen biology and their infectivity in interaction with their host and the environment. The whole genome sequence will greatly help take this research forward over the long term. For example, from the comparison of the annotated genomes of *T. equigenitalis* and *T. asinigenitalis*, we have listed the potential colonisation and virulence factors that are common to both species and those that are specific to one of the two species. To shed further light on the genomic diversity of *Taylorella* species, we plan to sequence the genomes of some 10 strains in collaboration with ANSES' genomic/transcriptomic facility (at the Viral Genetics and Biosafety Unit of the ANSES Ploufragan-Plouzané Laboratory).

Routine use of whole genome sequencing

The routine use of whole genome sequencing has been addressed in several papers and review articles with, among others, a pilot study on *Staphylococcus aureus* and *Clostridium difficile* (Eyre *et al.,* 2012). All have demonstrated that the ever-decreasing costs and sequencing time have resulted in a dramatic change in the capability of microbiology diagnostic and reference laboratories. The most obvious applications are the identification of microorganisms (particularly those that are non-culturable, difficult-to-culture or highly pathogenic) and genotyping strains with optimal resolution in "real-time". The enterohemorrhagic *Escherichia coli* O104:H4 outbreak in Germany in 2011 (Mellmann *et al.,* 2011) and the recent identification of the causative agent of Theiler's disease (Chandriani *et al.,* 2013), which conventional identification techniques had failed to identify for more than a century, are good examples of these applications. Data from genome sequencing and new sequencing technologies can also be used to analyse specific target genes alone (e.g. antibiotic resistance genes) and thus reduce the cost of preparation, analysis and data storage.
It is however clear that, even though the technical constraints and costs incurred by generating sequence data are no longer limiting factors, as shown by the advent of 'push-button' sequencing, it seems difficult to envisage the routine use of whole genome sequencing as a method of diagnosis and epidemiology in the near future. Indeed, several technological advances have yet to be made to facilitate the analysis of data using software that can automatically interpret results and can be run by microbiologists who are not bioinformatics specialists, and to share data over common interfaces that are user-friendly and efficient enough to allow data incrementation and comparison with existing data. This last point implies the standardization of the formats and software from different sequencing platforms, and the suitability of infrastructures for the storage and transport of the large amounts of data generated.
In conclusion, although it is unlikely that whole genome sequencing will completely replace current diagnostic and epidemiology methods, it nevertheless represents a promising method for reference laboratories and offers greater possibilities than at present.

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Lab news

One-day event reference laboratory event: more than 100 participants

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ANSES recently organised a one-day forum bringing together heads of laboratories involved in the surveillance and control plans implemented by the health authorities in the fields of animal and plant health, and food safety. The event was co-organised with the Joint Laboratories Service Unit of the Ministries of the Economy and the Budget. The aim was to better address the needs of accredited laboratories and thereby to consolidate the surveillance networks.

Our food supply is often the result of a complex chain of activities, including crop and livestock production, as well as processing, storage and distribution processes. As a result, various types of microorganisms and chemical substances may be found in food and must therefore be monitored.

Food safety is based on the accountability of all the players involved in the food chain, from "farm to fork". Each player must implement a risk management plan aimed at preventing microbiological and chemical contamination. This plan includes self-monitoring, which involves regular analyses carried out by manufacturers and distributors themselves on samples taken from raw materials and finished products, enabling early warnings to be given in the event of contamination.

Recent incidents have highlighted the need for this regulatory system to include surveillance and controls performed by the public authorities themselves, in a completely independent manner. Moreover, the systems in place constantly need to be updated to take into account new knowledge and to tackle new emerging contaminants, with the help of the latest technologies such as high throughput sequencing, genomic and proteomic techniques, and mass spectrometry.

A targeted surveillance system

For some pathogens, like viruses, bacteria and parasites, or major chemical contaminants, the health authorities have set up a surveillance system, supported by a network of reliable laboratories that can perform official analyses. For each of these regulated pathogens or contaminants, the health authorities designate approved laboratories that are authorised to perform analytical testing.

The surveillance programmes primarily concern the main foodborne microorganisms such as *Salmonella*, *Listeria*, or *Campylobacter* bacteria, and certain toxic substances that can be found in food, including marine biotoxins, mycotoxins and heavy metals. Control and surveillance plans are also implemented to monitor agents that cause the most common animal diseases (rabies, foot and mouth disease, and avian influenza) and agents that affect plant health (GMOs prohibited in Europe, invasive plant species, etc.).

A "reference" laboratory is appointed for each regulated target hazard. This laboratory guarantees the reliability of analyses carried out by all the accredited laboratories. In this area, ANSES has 68 national reference mandates, nine European mandates, and about 20 international mandates.

How do reference laboratories work?

Reference laboratories may have a national mandate, as National Reference Laboratory (NRL), coordinating a network of departmental laboratories in France, a European mandate, as European Reference Laboratory (EURL), coordinating a network of NRLs, or an international reference mandate as a WHO, OIE or FAO[1] Collaborating Centre. Depending on the specific pathogen or contaminant and its level of circulation, the number of accredited laboratories to coordinate can vary from just a few to nearly a hundred. To guarantee the reliability of analyses performed by the network of laboratories, the reference laboratory proposes training sessions on the new analytical methods it develops, and organises inter-laboratory proficiency tests (ILPTs) to verify the accuracy of official analyses.

Valuable discussions

Along with the Joint Laboratories Service Unit, a scientific department within the Directorates General for Competition, Consumer Affairs and Fraud Control (DGCCRF) and Customs, ANSES recently organised a one-day forum bringing together heads of French National Reference Laboratories (NRLs) working in the Agency's areas of expertise, and the representatives of the accredited laboratories designated by them.

The purpose of the event was to identify ways in which reference laboratories can better address the needs of accredited laboratories. More than 100 participants attended the meeting in Maisons-Alfort on 27 March. The presentations and discussions focused on four areas:

- From development to transfer of methods;
- Reference materials, control of reagents;
- Inter-laboratory proficiency testing;
- Scientific and technical discussions between accredited laboratories and National Reference Laboratories.

These exchanges helped to develop a consensus on the challenges in this area, and to better define the issues facing accredited laboratories and their expectations. This will enable optimisation of working procedures, thereby consolidating surveillance networks.

The Agency is planning to make this a recurrent event, held every two years.

This meeting was part of the Agency's priority objective of reinforcing the control and detection resources made available to the public authorities by the reference laboratories. It follows on from the creation of a Reference Board in 2011, bringing together heads of the Agency's NRLs, a body that was extended to all French NRLs in 2012.

1. Respectively World Health Organization, World Organization for Animal Health, and Food and Agriculture Organization of the United Nations

Lab news

2013 Annual scientific meeting of MedVetNet

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The last annual scientific meeting of MedVetNet – "One health, one medicine: sharing challenges for combating zoonoses" - held on 24 to 25 June 2013, in Copenhagen, Denmark, hosted by the Technology University of Denmark, Lyngby, Denmark. MedVetNet 2013 targets human and animal health researchers working on the examination, implications and eradication of foodborne zoonoses using the "One Health" approach. Much of the work done through this approach has been cross-disciplinary - including a variety of sectors - and its implications reach beyond the study and eradication of zoonoses. The meeting aimed to present the broad range of disciplines relevant to the One Health approach, as well as keynote presentations dedicated to combining these disciplines to faciliate economic development in zoonotically-plagued regions. http://www.medvetnet2013.eu/fileadmin/filer/David/MedVetNet2013/MVN2013Flyer02.pdf

Short summary of the 2nd EPPO Workshop for heads of plant pest diagnostic laboratories

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EPPO Secretariat, Paris, France

EPPO is intergovernmental organisation responsible for European cooperation in plant protection in the European and Mediterranean region (more information on EPPO was recently published in EUROREFERENCE and is available in http://www.ansespro.fr/euroreference/). Since 1998, EPPO has established a work programme in the area of diagnostics to harmonize procedures across the region. Among the different activities conducted in the framework of this programme, EPPO organises Workshops and Conferences.

Since 2011, Workshops for Heads of plant pest diagnostic laboratories are organised to promote exchanges of views on strategic issues for diagnostics. The second Workshop took place in Hammamet, Tunisia on 2013, 5-7 september. The main theme of the Workshop, "Plant pest diagnostic laboratories: towards national and regional reference laboratories", was chosen based on suggestions made by heads of laboratories.

The Workshop started with presentations from different laboratories of their experience with reference laboratories. A presentation of the European Union Reference Laboratory (EURL) for bees health was made as well as a presentation of the revision of the Regulation (EC) No 882/2004 by the European Commission Directorate General Safety of the food Chain (SANCO). The scope of this regulation will extend to the plant health and plant reproductive material sectors (seeds included) so that a network of National Reference Laboratories and EURLs can also be established in the plant health sector.

In addition to the plenary presentations, three subgroups were formed to discuss the following topics:
- Accreditation what are the needs of laboratories?
- Organisation of proficiency testing (PT) and test performance studies (TPS): what are the challenges?
- Roles of National Reference Laboratories (NRL)
- Reference material
- Possible scope of regional reference laboratories

Each group reported back to the plenary. Discussions in subgroups were very active and several recommendations have been identified as a result of these discussions.
The summary of the main conclusions and recommendations of this Workshop can be downloaded at http://archives.eppo.int/MEETINGS/2013_conferences/labs_tunisia.htm

Lab news

Preparation of a European body of legislation on official controls: "Healthier animals and plants and a safer agri-food chain"

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On 6 May 2013, the Commission adopted a set of four proposed regulations that seek to modernise, simplify and strengthen the regulatory framework relating to official controls covering not only the food chain, but also animal health, and the health of plants and plant reproductive material (seeds and plants). These four regulations will replace around 70 pieces of legislation.

The rules on organisation and quality of controls in the current Regulation (EC) No 882/2004, known as "Official Controls", will be adapted to these three sectors that were not previously covered by the current regulations, and will undergo improvements. Regulation (EC) No 882/2004 had been adopted in the narrower framework of Regulation (EC) 178/2004 (the "Food Law") and of the "Hygiene Package".

The legislative work of the European Parliament and the Council begins at the end of May but, according to the Commission, the new provisions are unlikely to come into effect before 2016. The provisions on the analytical methods and official laboratories appear in Chapter IV, Articles 33 to 41. The conditions for designation as an official laboratory still involve accreditation, but include exceptions and the possibility of temporary designation when there has been a change in methods or in a crisis situation.

The articles on reference laboratories and centres are grouped under Title III, in Articles 91 to 99 of the "Official Controls" proposal. The principle of creating a reference network including both the European Union reference laboratories and the national reference laboratories is maintained, and has been extended to plant health.

http://europa.eu/rapid/press-release_IP-13-400_en.htm
or
http://ec.europa.eu/dgs/health_consumer/animal-plant-health-package

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anses
French agency for food, environmental and occupational health & safety

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Networks

Two year study of interlaboratory trial results on rabies diagnosis (Fluorescent Antibody Test, Rabies Tissue Culture Infection Test, Mouse Inoculation Test, PCR techniques): a starting point towards the harmonization of the methods

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Two interlaboratory trials on rabies diagnosis techniques were organised over the period 2009-2010 by the European Union Reference Laboratory (EURL) for Rabies. Results revealed that laboratories produced the highest proportion of concordant results in PCR techniques (90.5%), FAT (87.1%), followed by RTCIT (70.0%) and MIT (35.0%) in 2009 and in FAT (85.0%) and PCR techniques (80.6%) followed by RTCIT (77.3%) in 2010. Independently of the year, the molecular techniques were the techniques presenting the lowest rate of false negative results, while RTCIT and MIT (performed in 2009 only for the latter) were the techniques with the lowest proportion of false positive results. Considering the participant laboratories results, the FAT gold standard technique presented a better specificity than RT-PCR with 1.6% false positive in 2009 and 5.8% in 2010 and a better sensibility than RTCIT with 1.6% false negative results in both 2009 and 2010. In both molecular biology techniques and FAT trial, false negative results were notified on bat strains only, highlighting the need to improve result quality more specifically on such strains. The analysis of technical questionnaires and procedures provided by participating laboratories revealed variations of methods that could cause inconsistencies between the results. In 2009, the impact of the number of readers for slide examination during the FAT was underlined as a significant factor affecting the results of laboratories, confirming the necessity of two independent readers for routine rabies diagnosis. Such findings highlight the need for all rabies diagnosis laboratories to improve harmonization of procedures. To facilitate this work, and as a first step, recommendations on the most commonly used reference techniques, FAT and RTCIT have been stated. Theses recommendations were establish based on the OIE and WHO international recommendations and on an update of the knowledge of critical factors known to affect the results.

Introduction

Rabies is a neurotropic and lethal infectious disease caused by a rhabdovirus of the *Lyssavirus* genus. This disease remains a significant public health concern in many parts of the world as it is still responsible for an estimated 55,000 human deaths annually, mainly in Asian and African children (WHO, 2005). Because the clinical diagnosis of animal rabies is not reliable, the rabies diagnosis is obtained by laboratory post-mortem investigations on brain tissues. This is generally undertaken for examination of animals that have bitten a person or have potentially caused human exposure, but also in the context of rabies surveillance in wildlife to assess the epidemiological situation in infected countries. As an example, in Europe, 63,218 animals including 4 893 positives ones, were tested in 2012 (Source: Rabies Bulletin Europe data http://www.who-rabies-bulletin.org/).

Although various post-mortem diagnostic methods have been published, three reference techniques are commonly used and are currently recommended by both the World Health Organization (WHO, 1996; WHO, 2005) and the World Organization for Animal Health (OIE, 2011). The first technique is the Fluorescent Antibody Test (FAT), which detects viral antigens using specific fluorescent anti-rabies antibodies (Dean *et al.,* 1996). This technique is considered the gold standard and allows rapid direct identification of the virus at low cost. The two other techniques involve virus isolation for the detection of infectivity of particles: the Rabies Tissue Culture Infection Test (RTCIT) is an *in vitro* technique using cell culture (Webster and Casey, 1996) while the Mouse Inoculation Test (MIT) is an *in vivo* technique using intra-cerebral virus inoculation in susceptible mice (Koprowski, 1996). However, for ethical, financial and rapidity reasons, it is preferable to use the *in vitro* technique (OIE, 2011). In the last three decades, molecular tools have been widely developed and used (Fooks *et al.,* 2009; Dacheux *et al.,* 2010). As a result, there are a large number of molecular tests that can be used to complement conventional rabies diagnosis. A condition precedent to the systematic ongoing collection, analysis, comparison and interpretation of rabies data and the dissemination of information is a reliable rabies diagnosis (Cliquet *et al.,* 2010). The latter is also an indispensable condition in human medicine as regards the administration of adequate and timely post-exposure prophylaxis (WHO, 2005; WHO 2010). To reach this goal, an appropriate harmoniz ation scheme is necessary. This can be established through the comparison of laboratory results and their method used. For this purpose, the EURL for rabies has implemented an annual inter-laboratory trial on rabies diagnosis techniques under EU directive ((EC, 2008) Council Directive 737/2008). This article reports the data from the two first extensive annual interlaboratory trials (FAT, RTCIT, MIT and PCR) and the consequent recommendations performed to increase the harmonization of the reference techniques.

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Materials and Methods

Two interlaboratory trials were organised over the period 2009-2010. Two different sample panels were used in 2009 while a single panel was prepared in 2010. In 2009, one panel was dedicated to the reference diagnosis techniques (FAT, RTCIT and MIT) while the other one was exclusively dedicated to the molecular biology techniques (RT-PCR, Real-time PCR). The two panels comprised the same batches of viruses and were sent the same day to all participating laboratories. In 2010, a single sample panel was dedicated to the reference techniques (FAT and RTCIT) and also to the molecular biology techniques (the ring trial on MIT was organised in 2009 only). For each trial, laboratories were requested to analyse the panel using their own current procedures.

1. Participating laboratories

National Reference Laboratories (NRLs) from Member States of the European Union and from third countries were invited to take part in the interlaboratory trials (**Figure 1**). In 2009, thirty-two laboratories were involved in the rabies diagnosis interlaboratory trial for reference techniques, including 21 European NRLs and 11 laboratories from third countries. Thirty-two laboratories performed the FAT, 20 performed the RTCIT and 8 performed the MIT. For the interlaboratory trial dedicated to the PCR techniques, twenty-one participating laboratories were involved. Participants included 17 European NRLs and four laboratories from third countries. In 2010, forty-two laboratories participated in the interlaboratory trial. Participant laboratories for 2010 included 24 European NRLs and 18 laboratories from third countries. Forty laboratories performed the FAT, 23 performed the RTCIT and 31 performed a PCR technique.

2. Constitution and composition of the panels to be tested

Each batch of virus was produced by intra-cerebral inoculation of animals (mice, red foxes, raccoon dogs or dogs, depending on the strain) according to animal experimentation instructions provided by the French Ethical Committee. For each virus batch, collected brains were mixed together to ensure homogeneity, divided up into 1ml tubes and then freeze-dried. Rabies (RABV) strains used in the interlaboratory trials were GS7 (strain from a naturally infected fox in France), raccoon dog (raccoon dog strain from Poland), Ariana (dog strain from Tunisia), EBLV-1b (European Bat *Lyssavirus* type 1, subtype b, strain from France) and EBLV-2 (European BAT *Lyssavirus* type 2, strain from the United Kingdom), ABLV (Australian Bat *Lyssavirus* strain) and negative samples (negative red fox brain).
The panel for the interlaboratory trial for reference diagnosis techniques was composed of 8 randomly blindly coded samples (GS7, EBLV-1b, EBLV-2, Ariana, Ariana weak (Ariana diluted sample providing weak fluorescent signal in FAT), Raccoon dog, 2 negatives) while the panel for the interlaboratory trial for PCR techniques was composed of 7 blindly coded samples (GS7, EBLV-1b, EBLV-2, Ariana, Raccoon dog, 2 negatives). The panel used in the interlaboratory trial of 2010 consisted of 7 blindly coded samples (GS7, EBLV-1a (European Bat *Lyssavirus* type 1, subtype a, strain from France), EBLV-2, ABLV, 3 negatives).

3. Control and stability of the panels

In 2009, the stability of the two test panels was assessed by analysing them after 10 days at room temperature. In 2010, stability of the panel was evaluated by testing the samples after 7 and 14 days at room temperature. Under these conditions all panels revealed that the positive samples remained positive

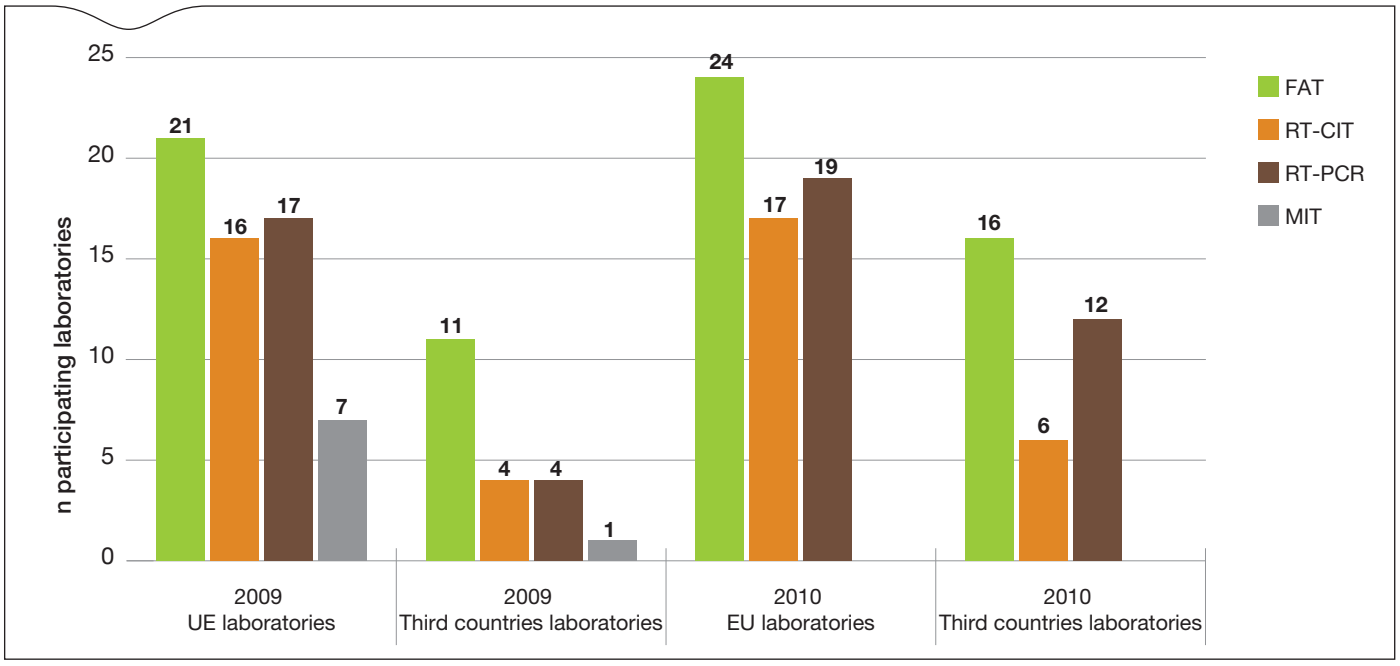

Figure 1: Number laboratories from EU Member States and from third countries participating in the 2009 and 2010 rabies diagnosis inter-laboratories trials.

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and negative samples remained negative for FAT, RTCIT and RT-PCR (data not shown). Every batch of virus used in the trials was verified by RT-PCR and typed before and after the interlaboratory trial.

4. Shipment conditions of the panels

The 2009 and 2010 panels were shipped at ambient temperature by an international certified carrier, under UN2814 conditions, in accordance with International Air Transport Association regulations (IATA, 2009) and the "European Agreement concerning the International Carriage of Dangerous Goods by Road" (ADR, 2009). All the panels except one were received during the time range where stability was ensured. Since all the results received were concordant, even the data for the panel received after the delay of guaranteed stability were included in this study. For both year laboratories were recommended to store the panel at 4°C as soon as received and until the analysis was performed.

5. Technical questionnaire

In 2009, a technical questionnaire, approaching each procedure step of the tested methods, was sent to the participating laboratories at the same time as the panels. In 2010, the original technical procedures of the participating laboratories were requested. The analysis of the technical questionnaires and the technical procedures of FAT and RTCIT were examined in order to highlight and discuss the small variations that could impact the results.

Results

The **Table 1** gives the overall results for the 2009 and 2010 trial according to the different techniques used.

1. Fluorescent antibody test (FAT) results

In 2009, four laboratories (12.9%) returned discordant results represented by one false positive (1.6% of negative samples) and three false negative (1.6% of positive samples) results. All laboratories produced satisfactory results for RABV strains (GS7, Ariana, Ariana weak, raccoon dog). Two false negatives were found for EBLV-1 (6.5% of EBLV-1 samples) and one for EBLV-2 (3.2% of EBLV-2 samples). In 2009, FAT false negative results were consequently found in bat strains only.

In 2010, six laboratories (15.0%) returned discordant results in FAT. Seven tests (5.8%) were identified as false positive results and 3 tests (1.9%) provided false negative results. False negatives were identified for the EBLV-1a strain (2.5%), EBLV-2 strain (2.5%) and ABLV strain (25.0%). No false negative was observed with the RABV strain. As in 2009, false negative results were found in bat strains only. Concerning the analysis of the procedures, one factor was identified in 2009 as affecting significantly the results (details of the analysis are presented in the report of rabies diagnosis interlaboratory trial 2009 (Robardet, 2010). Generally, two readers examine the slide (67% of laboratories) while 22% of laboratories have more than 3 readers and 11% have a single reader. The number of laboratories with discordant results (2/3 laboratory for 1 reader, 1/17 laboratory for 2 readers, 2/7 laboratory with discordant results for 3 reader) differed significantly according to the number of reader examining the slides (pc2 (Yates correction) = 0.03), the laboratories with two readers being those presenting the lowest proportion of discordant results.

2. Rabies tissue culture infection test (RTCIT) results

In 2009, four laboratories (30.0%) returned discordant results with six false negative results (6.3% of positive samples). Three of them were found for the EBLV-2 strain (representing 18.8% of tests on this strain), two were identified in the Ariana strain samples (12.5% of the Ariana strain samples) and the last was identified with the raccoon dog strain (6.3% of the raccoon dog strain). Considering these results, the proportion of false results in the interlaboratory trial differs depending on the strains, with the highest proportion of false results with the EBLV-2 strain followed by the Ariana strain and the raccoon dog strain.

In 2010, analysis of laboratory results revealed that five laboratories (22.7%) obtained discordant results. They returned three false positive results (4.6% of negative samples) and seven false negative results (8.0% of positive samples). Further

Table 1. Results of the 2009 and 2010 interlaboratory trials.

	2009		2010	
	n Discordant/Total	% Discordant and Confidence interval	n Discordant/Total	% Discordant and Confidence interval
FAT				
Number of laboratories	4/31	12.9 [4.2 - 30.8]	6/40	15 [6.2 - 30.5]
Negative samples	1/62	1.6 [0.1 - 9.8]	7/120	5.8 [2.6 - 12.1]
Positive samples	3/186	1.6 [0.5 - 5.1]	3/160	1.9 [0.5 - 5.8]
GS7	0/31	0 [0.0 - 13.7]	0/40	0 [0.0 - 10.9]
Ariana	0/31	0 [0.0 - 13.7]	_	_
Ariana (weak)	0/31	0 [0.0 - 13.7]	_	_
Raccoon dog	0/31	0 [0.0 - 13.7]	_	_
EBLV-1a	_	_	1/40	2.5 [1.3 - 14.7]
EBLV-1b	2/31	6.5 [1.1 - 22.8]	_	_
EBLV-2	1/31	3.2 [0.2 - 18.5]	1/40	2.5 [1.3 - 14.7]
ABLV	_	_	1/40	2.5 [1.3 - 14.7]

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Table 1. Results of the 2009 and 2010 interlaboratory trials. (continued)

	2009		2010	
	n Discordant/Total	% Discordant and Confidence interval	n Discordant/Total	% Discordant and Confidence interval
RTCIT				
Number of laboratories	4/16	30 [8.3 - 52.3]	5/22	22.7 [8.7 - 45.8]
Negative samples	0/40	0 [0.0 - 10.9]	3/66	4.6 [1.2 - 13.6]
Positive samples	6/96	6.3 [2.6 - 13.6]	7/88	8 [3.5 - 16.2]
GS7	0/16	0 [0.0 - 24.1]	0/22	0 [0.0 - 18.5]
Ariana	2/16	12.5 [2.2 - 39.6]	_	_ _
Ariana (weak)	0/16	0 [0.0 - 24.1]	_	_ _
Raccoon dog	1/16	6.3 [0.3 - 32.3]	_	_ _
EBLV-1a	_	_	3/22	13.6 [3.6 - 36.0]
EBLV-1b	0/16	0 [0.0 - 24.1]	_	_ _
EBLV-2	3/16	18.8 [5.0 - 46.3]	1/22	4.5 [0.2 - 24.9]
ABLV	_	_	3/22	13.6 [3.6 - 36.0]
mit				
Number of laboratories	6/8	75 [35.6 - 95.5]		
Negative samples	0/16	0 [0.0 - 24.1]		
Positive samples	11/48	22.9 [12.5 - 37.7]		
GS7	1/8	12.5 [0.7 - 53.32]		
Ariana	2/8	25 [4.45 - 64.4]		
Ariana (weak)	0/8	0 [0.0 - 40.3]		
Raccoon dog	1/8	12.5 [0.7 - 53.3]		
EBLV-1b	1/8	12.5 [0.7 - 53.3]		
EBLV-2	6/8	75 [35.6 - 95.5]		
RT-PCR				
Number of laboratories	2/21	9.5 [1.7 - 31.8]	6/31	19.4 [8.2 - 38.1]
Negative samples	3/42	7.1 [1.9 - 20.6]	8/93	8.6 [4.1 - 16.7]
Positive samples	0/101	0 [0.0 - 4.6]	1/120	0.8 [0.0 - 5.2]
GS7	0/21	0 [0.0 - 20.0]	0/31	0 [0.0 - 13.7]
Ariana	0/21	0 [0.0 - 20.0]	_	_ _
Raccoon dog	0/21	0 [0.0 - 20.0]	_	_ _
EBLV-1a	_	_	0/31	0 [0.0 - 13.7]
EBLV-1b	0/20	0 [0.0 - 20.0]	_	_ _
EBLV-2	0/18	0 [0.0 - 21.9]	1/31	3 [0.2 - 18.5]
ABLV	_	_	0/27	0 [0.0 - 15.5]

analysis confirmed that false negative results were restricted to bat strains EBLV-1a (13.6% of EBLV-1 samples), EBLV-2 (4.5% of EBLV-2 samples) and ABLV (13.6% of ABLV samples).

3. Mice inoculation test (MIT) results

This test was performed in 2009 only and included eight volunteer participants. Six laboratories (75.0%) presented discordant results with 11 false negative results (22.9% of tests on positive samples). One false negative was found for the EBLV-1b strain (12.5%) and six false negative results for the EBLV-2 strain representing 75.0% of tests on this strain. One false negative result was detected on the GS7 strain (12.5%), two false negative results were identified with Ariana samples (25.0% of tests on the Ariana strain) and one false negative result for the raccoon dog strain (25.0% of tests on the raccoon dog strain). In summary, the higher proportion of false results was obtained with the EBLV-2 strain followed by the Ariana strain and the raccoon dog, GS7 and EBLV-1b strains.

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4. PCR results

Discordant results were detected in two laboratories (9.5%) with three false positive results (7.1% of tests on negative samples). No false negative was found for the EBLV-1b, EBLV-2, GS7, Ariana and raccoon dog strains. In 2010, discordant results were identified in six laboratories (19.4%). The discordant results included eight false positive results (8.6% of negative samples) and one false negative result (0.8% of positive results). False positive and false negatives results were resulting from different participating laboratories. The single false negative result was observed for the EBLV-2 strain (3% of EBLV-2).

Discussion

1. Fluorescent antibody test (FAT)

Eighty seven and 85 percent of laboratories involved in the FAT trial produced satisfactory results in 2009 and 2010 respectively. Although terrestrial rabies involving the classical rabies virus RABV is the most commonly investigated disease, bat rabies is also diagnosed more and more frequently. This highlights the need to include such strains in the process of diagnostic quality estimation. The study shows that no errors were produced on the RABV strains detection while all bat strains (EBLV-1, EBLV-2 and ABLV) were involved in false negative results. Infection by RABV being responsible of the majority of human deaths, results of this trial revealed high performance of laboratories suggesting a high quality level for the public health management of the classical rabies. In contrast, results on bat strains highlight the need for laboratories to improve the sensitivity of their diagnostic methods on such strains. As these strains do not provide the same type of fluorescence than the conventional RABV strains, false negative results could be due to difficulties to identify the fluorescence obtained from these strains. Analysis of the technical questionnaires performed in the light of the inter-laboratory results indicated that the number of slide readers could have a significant impact on the reliability of the FAT results. Two persons should independently and systematically read all slides and then compare their results to avoid any error of interpretation. In the event that their results are the same, they should be validated and if they are discordant, a third person (with more experience) should decide. Even if

Table 2. Recommendations of the EURL for the FAT

"Strong recommendations" are considered inderogable because their modifications have already been demonstrated as affecting significantly the results. "Recommendations" are not compulsory but some variations could potentially affect the results, it is consequently recommended to follow them in order to maximize the European harmonization. "At laboratory decision" are acceptable variations not demonstrated as affecting significantly the results.

Procedure step	Strong recommendation	Recommendation	At laboratory decision
1. Part of the brain	Brainstem and cerebellum or Ammon's horn (two replicates per sample)		
2. Preparation of slides	Impression/ smear method		
3. Drying before fixation		15-30 min at Room Temperature	
4. Fixation	Acetone; -20°C; 30min	Control and samples slides placed in separate rinse containers	
5. Drying before staining		15-30 min at RT	
6. Staining	Strictly follow manufacturers' recommendations		Addition of Evan's blue to conjugate
	37°C; 30 min; humid chamber		
7. Washing	Control and sample slides placed in separate rinse containers	Soaking 2 x 5min in PBS	
8. Mounting	Buffer mounting > or equal to pH 8.5 A strong concentration of glycerol may provide diminished staining		
9. Reading	Read slides within 2 hours after mounting	Final magnification from 200 to 400	
	2 independent trained readers		
10. Controls	Include positive and negative controls of the targeted virus species in each session		

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no significant influence of other factors has been highlighted, due to the weak number of discordant results, potential small variations in the procedure are already known to affect, even critically, the sensitivity and specificity of the FAT. The area of the brain examined (Bingham and van der Merwe, 2002), the duration and type of fixation (Upcott and Markson, 1971), the nature of the FITC labelled anti-rabies antibody conjugate (Robardet *et al.,* 2013), the alkalinity of the mounting medium (Durham *et al.,* 1986; Pital and Janowitz, 1963), the proportion of glycerol (Rudd *et al.,* 2005) and the use of appropriate microscopy filters (Lewis *et al.,* 1973) are part of these factors. Regarding these information, the EURL has established its recommendations (**Table 2**). These recommendations were established with the consultation of the NRLs during the 2010 EU Workshop for Rabies and are in agreement with other international recommendations (WHO, 1996; OIE, 2011).

2. Rabies Tissue Culture Infection Test (RTCIT)

Seventy percent of participating laboratories and 77.3% produced satisfactory results in 2009 and 2010 respectively. Unsatisfactory results were obtained on the negative, EBLV-2, EBLV-1a, Ariana and raccoon dog strains, suggesting that false results occurred independently of the type of species. Only the RABV strain issued from the red fox (GS7) displayed no discordant result either in 2009 or in 2010. Although not significant, the proportion of successful laboratories was greater in 2010 than in 2009, suggesting a slight increase in quality for the results using RTCIT.

Table 3. Recommendations of the EURL for the RTCIT

Procedure step	Strong recommendation	Recommendation	At laboratory decision
1. Material	Microplate or Labtek		
2. Cell culture	Cell line: Neuroblastoma		E-MEM; Dulbecco's Modified Eagle Medium (D-MEM); Glasgow Minimum Essential Medium (G-MEM)
	Medium: Eagle's minimal essential medium (EMEM) without essential amino acids + 10% FBS + Antibiotics		
	Trypsination must be performed when cell monolayer at 80% confluence		
3. Inoculum preparation	Preparation at 10%		Frosting/ defrosting step
	Grinding medium: cell culture + antibiotic		
	Centrifugation at low temperature		
4. Inoculation (microplate)	Monolayer 80% confluence		Volume of medium and inoculum
4. Inoculation (Labtek)	Monolayer 80% confluence	50µl of inoculum and 400µl of medium per well (105 cell /ml)	
5. Incubation	From 48h to 96h; 36°C ±2; 5% CO2		
	Change medium at 72h		
6. Washing	PBS	Soaking 2x	
7. Fixation	100% acetone (Labtek); 80% acetone (microplate)		
8. Drying		15-30 min at Room Temperature	
9. Staining	Follow strictly the manufacturer's recommendations		Use of Evan's blue
	37°C; 30min		
10. Washing	PBS	Soaking 2x for microplate, Soaking 2x 5min for Labtek	
11. Reading	2 independent trained readers	General magnification from 200 to 400	
12. Controls	Include positive and negative controls of the targeted virus species at each session		

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Analysis of the technical questionnaire showed that different strains of cells were commonly used. Murine neuroblastoma cells are known to be superior to all others tested for the isolation of field virus strains (Rudd and Trimarchi, 1989; Webster and Casey, 1996) and should be used preferentially for suitable diagnosis. Each laboratory must also ensure that the medium used in the laboratory is adapted to cell culture, considering that supplementing culture medium with serum (foetal calf serum, 10%) could enhance growth. Considering the cell density, the cell number per well, the volume of the inoculums and duration of the incubation, there is great diversity in the steps of cultivation of the virus in cells. Whatever the amounts used, it is necessary to ensure that the monolayer of cells reaches 80% confluence at the end of the incubation. It is also recommended to change the medium after the first 24h when incubation duration is up to 72h (OIE, 2011). Reference guides have established a fixation step in 70-80% acetone for 30 minutes at room temperature for tests on microplates (Webster and Casey, 1996) and in 100% acetone for 30 minutes at -20°C for tests on Lab-Tek glass chambers (Barrat *et al.,* 1988). Suitable conditions for the reading step are the same as for the FAT technique. The summary of EURL recommendations is proposed in **Table 3**. These recommendations were also established with the consultation of the NRLs during the 2010 EU Workshop for Rabies and are in agreement with other international recommendations (WHO, 1996; OIE, 2011).

3. Mouse Inoculation Test (MIT)

Twenty-five percent of participating laboratories produced satisfactory results. A percentage of 22.9% false negative tests was obtained. The interlaboratory trial for MIT thus recorded the highest proportion of false negative results among the techniques investigated. The MIT interlaboratory sensitivity was low mainly due to the high number of false negatives on the EBLV-2 strain. To avoid any loss of sensitivity and specificity on this technique, young Swiss albino strain mice should preferably be chosen, particularly baby mice less than three days old if possible, as they are more sensitive to the rabies virus (Koprowski, 1996). Mice transported to the laboratory should undergo an adaptation stage for a minimum of three days so that mice likely to die as a result of rough transport conditions may be discarded before the experiment (Koprowski, 1996). Antimicrobial agents must be added to the brain tissue preparation to avoid non-specific mortality (streptomycin at 1560UI/ml and penicillin at 500UI/ml) (Koprowski, 1996). To avoid interference phenomena it is also preferable to prepare the suspension tissue for inoculation at 10% (Koprowski, 1996). To avoid animal distress and suffering, as well as non-specific mortality, any intra-cerebral injection should be conducted under anaesthesia. It must be emphasized that half of the participating laboratories did not anaesthetize mice before the inoculation step. This contradicts ethical regulations and efforts made to avoid suffering of animals used for experimental and scientific purposes (European Council Directive 86/609/EEC, 1986). Wherever possible, virus isolation in cell culture should replace mouse inoculation test (WHO, 2005; OIE, 2011).

4. Molecular biology techniques (RT-PCR: Real-time PCR and conventional RT-PCR)

90.5% of participating laboratories produced satisfactory results in 2009 and 80.5% in 2010. No false negative samples were recorded during the trial of 2009, while 0.83% was detected in 2010, consisting of a false negative result on an EBLV-2 sample. Although statistically insignificant, the proportion of false positive results in 2009 (7.1%) was lower than in 2010 (8.6%). False positive results were found only in a laboratory using both two-step RT-PCR and Real Time PCR. Comparison of the technical questionnaires revealed a high number of techniques and protocol variations. Each laboratory used its own validated reagents, primers, commercial kit for RNA extraction, for generation of cDNA or for one-step RT-PCR. Although molecular biology tools are not currently recommended for routine post-mortem diagnosis, they are more and more widely used. However, if not performed by highly-trained staff in the field of molecular biology, this very sensitive technique involves a high risk of contamination and therefore may generate false positive results especially with nested PCR. The considerable development of these techniques in recent decades has resulted in a wide variation in molecular biology techniques (hnRT-PCR, one-step hnRT-PCR, Real-time PCR). Laboratories must therefore take particular care to verify the validity of these highly sensitive techniques with the help of international guidelines on quality assurance (OIE, 2011).

5. Techniques comparisons

Comparisons of the different techniques demonstrate that the RT-PCR techniques produced the lowest rate of false negative results, and were consequently the most sensitive, while RTCIT and MIT techniques produced the lowest proportion of false positive results and were the most specific. Conversely false positive rate in RT-PCR technique was the highest level among techniques used while false negative rate was higher in RTCIT and MIT techniques. The high sensitivity of PCR, which makes PCR a powerful research tool, means that extreme care must be taken to avoid generating false positive results. FAT was found to be a good compromise as only a few false positive and false negative results were obtained (in bat strains only). Each technique involves different components of the virus (viral antigen for FAT, viral infectivity for RTCIT and MIT and viral RNA for RT-PCR) and consequently leads to different results of specificity and sensitivity. While it was not possible to detect rabies by FAT or RTCIT due to antigen degradation and loss of virus viability, RT-PCR was shown to detect RNA in putrefied samples (David *et al.,* 2002) or in samples examined after long term storage (Lopes *et al.,* 2010). RT-PCR can therefore be used in a broader range of conditions, as example, on impregnated FAT® paper after 43 days of storage at room temperature (Picard-Meyer *et al.,* 2007) while it is not possible to perform FAT or RTCIT in such conditions.

6. Harmonization scheme

As a first step, this study underlines that many variations of procedure occur between laboratories and this even among reference techniques described in OIE (2011) and WHO manuals (1996 and 2005). As only a few modifications to a technique can lead to a drastic reduction the sensitivity and specificity of a test drasticall (Rudd *et al.* 2005), any change, even minor, must be accompanied by an adequate test validation estimating its impact on the results (McElhinney *et al.,* 2008). To use comparable and efficient methods, international institutions (WHO, OIE and the European Commission) increasingly recommend the use of standardized test methodologies. At the European Level, the European Commission has mandated European Union Reference Laboratories (EURL) to harmonize

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the diagnosis techniques used for animal diseases (Council Directive (EC) 737/2008). Such a scheme has been initiated by consensual discussion between NRLs and the EURL in the light of existing standardized techniques. First recommendations based on OIE, WHO international recommendations and on an update of the knowledge of critical factors that could affect the results have been proposed at European level to improve the standardization of the two most commonly used reference techniques, i.e. the FAT and the RTCIT.

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Agenda

2013 Annual scientific meeting of Epizone

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The 7th EPIZONE Annual Meeting - "Nothing permanent except change" - will be held in Brussels, Belgium, on 1-4 Oct 2013 and will be hosted by CODA-CERVA. "Nothing permanent except change" is particularly true when it comes to epizootic viral diseases and has become even more so due to the increase in international movements of animals, animal products and people, but also due to the possible deliberate introduction of pathogens. Moreover, the EU's non-vaccination and eradication policy is maintained in most circumstances to allow unrestricted trade of animals and their products worldwide. All these factors make surveillance the basis for recognition of disease status and the subsequent impact on trade. In this context, early detection of potentially epizootic diseases is of the utmost importance. We will continue to focus on the EPIZONE themes for improving disease control through the integration and collaboration of research in diagnosis, intervention strategies, risk assessment, surveillance and epidemiology.
http://www.epizone-eu.net/7th-annual-meeting.aspx

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