

European Union Reference Laboratory for Rabies

TECHNICAL REPORT FOR 2013

February 2014

The ANSES Nancy Laboratory for Rabies and Wildlife has been nominated as Union Reference Laboratory (EURL) for rabies since 1 July 2008. The functions and duties of the European Union Reference laboratory (EURL) for rabies are described in the Commission Regulation (EU) No 415/2013 of 6 May 2013 laying down additional responsibilities and tasks for the EU reference laboratories for rabies, bovine tuberculosis and bee health and amending the Regulation (EC) No 737/2008 designating the EURL for crustacean diseases, rabies and bovine tuberculosis. The Commission Regulation (EU) No 737/2008 also amends Annex VII to Regulation (EC) No 882/2004 of the European Parliament and of the Council.

I. Plan of the Work Programme for 2013

Activity 1:

- 1.1 **To provide technical support to National References Laboratories (NRLs)** by producing, storing and supplying biological materials and enlarging the rabies virus collection.
- 1.2. **To provide technical support to NRLs** by performing confirmatory diagnosis tests and typing at request.

Activity 2:

- 2.1. **To provide training to laboratories** according to the needs highlighted by the inter-laboratory test results.

Activity 3:

- 3.1. **To organize Inter-laboratory test on the reference rabies diagnosis tests: the fluorescent antibody test (FAT) and the rabies tissue culture inoculation test (RTCIT).**
- 3.2. **To organize Inter-laboratory test on the molecular biology techniques: the RT-PCR and the Real Time PCR techniques.**
- 3.3. **To collect data and information on methods of rabies diagnosis used through European Union and to standardize them.**
- 3.4. **To collect data and results of tests carried out in the EC.**

Activity 4:

- 4.1. **To organize an annual meeting for NRLs.**
- 4.2. **To keep abreast of development in surveillance, epidemiology and prevention of rabies throughout the world** by participating in meeting, workshop and conferences.

Activity 5:

- 5.1. **To update the EURL Internet website.**
- 5.2. **To maintain and enlarge the rabies strain database.**

Activity 6:

- 6.1. **To conduct research programmes on molecular epidemiology of rabies in Baltic countries.**
- 6.2. **To conduct comparison of different Real Time PCR methods.**

II. Technical report for 2013

A. Projects funded by the UE under the EURL for Rabies mandate

(Responsibilities and tasks set out in Commission Regulation (EC) No. 737/2008 of 28 July 2008 and amended in the Commission Regulation (EU) No 415/2013 of 6 May 2013)

Activity 1: Technical support

Sub Activity 1.1.:

Technical support: producing, storing and supplying biological materials and virus collection

Work plan: The EURL will undertake the production of new virus batches and supply rabies viruses and biological materials to the National Reference Laboratories (NRLs) at request.

Work completed: New batches of viruses (lyophilised or frozen) were produced to cover laboratory requests, to enlarge the rabies virus collection but also for the purpose of the inter-laboratory tests. Controls (FAT, RTCIT, Real Time, RT-PCR, Sequencing, virus titration) were performed to ensure the validity of each new batch produced.

In 2013, four new strains have been implemented in the EURL rabies virus collection: Roe deer strain from Ex-Yugoslavia dated from 1999, Red fox strain from Germany dated from 1990, FYROM red fox strain from 2011 and Greek red fox strain from 2012. The objective is to enlarge the geographical and species origin of the EURL rabies virus collection. These new strains have been produced and will be supplied to the NRLs upon request and in compliance with national shipping regulations.

Batch of viruses produced by the rabies EURL for diagnosis techniques in 2013:

Batch name	Storage condition	Species	Strain name	Original species affected	Country of origin
CVS27 01-13	Lyophilised, +4°C		CVS27	<i>Fixed strain</i>	/
FYROM 37-12	Liquid nitrogen	RABV	/	<i>Vulpes Vulpes</i>	FYROM
Greece 36-12	Liquid nitrogen	RABV	/	<i>Vulpes vulpes</i>	Greece
Yougoslavie 05-13	Liquid nitrogen	RABV	/	<i>Capreolus capreolus</i>	Yugoslavia
RABV Rd allemand 14-13	Liquid nitrogen	RABV	/	<i>Vulpes vulpes</i>	Germany
GS7 16-13	Liquid nitrogen	RABV		<i>Vulpes vulpes</i>	France
FYROM 10-13	Lyophilised, +4°C	RABV	/	<i>Vulpes Vulpes</i>	FYROM

Cn Viv Estonie 15-13	Lyophilised, +4°C	RABV	/	<i>Nyctereutes procyonoides</i>	Estonia
EBLV1b Lurcy Lewis 08-13	Lyophilised, +4°C	EBLV-1	EBLV-1b	<i>Eptesicus serotinus</i>	France
EBLV2 13-13	Lyophilised, +4°C	EBLV-2	EBLV-2	<i>Myotis daubentonii</i>	UK
ABLV 06-13	Lyophilised, +4°C	ABLV		<i>Pteropus alecto</i>	Australia

- The set of viruses presented above can be used as positive controls in rabies diagnosis tests (FAT, RTCIT, MIT and molecular techniques). Homogenates of healthy red fox brains were also produced and are available to NRLs as negative controls in rabies diagnosis tests and for the purpose of inter-laboratory tests (Batch "Negatif 17-13").
- Cross-sections of red fox teeth were prepared at request to provide NRLs with positive or negative controls for tetracycline detection in the teeth (bait uptake control).
- Until recently, cell cultures were provided. However since early 2010, the EURL is no longer able to provide cell lines (BSR, BHK, N2a, etc.). The ATCC, which is the official producer of cell lines, has set up a mutual transfer agreement that makes it impossible to laboratories to sell or supply cells without contracting an agreement. Drawing up an agreement with ATCC incurs financial costs.

Sub Activity 1.2:

Technical support: confirmatory tests (rabies diagnosis, typing)

Work plan: The EURL will receive, examine and report on samples submitted by EU Member States and type strains from NRLs upon request. FTA® papers will be offered to NRLs to simplify and reduce the cost of shipping samples.

Work completed:

In 2013, five samples from EU member states have been submitted for rabies diagnosis confirmation to the EURL for Rabies. Sample submitted for rabies diagnosis and found positive with reference techniques have been analysed for typing (see table below). A report was edited for each submitted sample.

Support of the EURL in rabies diagnosis confirmation:

Anses ID	Reception date	Country	Species	FAT result	RTCIT result	Real time result	Typing	Report sent on
DR-678	15/03/2013	Malta	<i>Canis lupus familiaris</i>	-	-	-	/	22/03/2013
DR-707	14/05/2013	Luxembourg	<i>Eptesicus serotinus</i>	+	+	+	EBLV-1	27/05/2013

DR-716	07/06/2013	Luxembourg	<i>Canis lupus familiaris</i>	-	-	-	/	13/06/2013
DR-750	27/11/2013	The Netherlands	<i>Canis lupus familiaris</i>	-	-	-	/	29/11/2013
DR-751	27/11/2013	The Netherlands	<i>Canis lupus familiaris</i>	-	-	-	/	29/11/2013

In May 2013, bat rabies was evidenced for the first time in Luxembourg (southern city of Differdange). The rabies virus, an EBLV-1b strain, was diagnosed in a serotine bat that bit a 29-year-old male person while he was asleep. The sample was submitted to the EURL by the Administration de la Nature et des Forêts of the Luxembourg. This first case evidence has been published in the manuscript “**First Isolation of a rabid bat Infected with European Bat Lyssavirus in Luxembourg. Servat A ; Herr J ; Picard-Meyer E ; Schley L ; Harbusch C ; Michaux C ; Pir J ; Robardet E ; Engel E ; Cliquet F. Zoonoses Publ. Health, 2014, doi: 10.1111/zph.12095**”.

Greece was a rabies free country since 1987. In 2012, nine rabies cases were detected in Northern Greece. Rabies diagnosis confirmation and sequencing of these first cases was performed in the EURL. All Greek isolates belong to the EE group, with a high degree of nucleotide identity with those from FYROM and Serbia, suggesting the movement of rabies-infected hosts in Western Balkan countries. The results of the phylogenetic analysis are included in the manuscript “**Reoccurrence of Animal Rabies in Greece. K.E. Tasioudi, P. Iliadou, E. Agianniotaki, E. Robardet, E. Liandris, S. Doudounakis, M. Tzani, P. Tsaroucha, E. Picard-Meyer, F. Cliquet, O. Mangana-Vougiouka. Emerg. Infect. Dis., 2014, 20, pp 327-329.**”

Activity 2: Training activities

Sub Activity 2.1:

Providing training to laboratories and possibly visiting them

Work plan: The Lyssavirus Unit of the laboratory is headed by Dr Florence Cliquet. The Unit is composed of 4 teams represented by 16 agents. Each team is headed by an experienced scientist who can provide expertise, scientific and technical support under the rabies EURL mandate in accordance with their competence area (diagnosis, molecular biology, virology and bait titration and epidemiology)

Upon NRL requests, the EURL will organise on-site training sessions on

- rabies diagnosis,
- typing, virus isolates
- virus titration and
- biomarker determination.

Work completed:

One training session was organized in the Bulgarian NRL in the frame of the EURL mandate (column mission of the budget report). The expertise in rabies diagnosis has been conducted from 03 to 05 April 2013 in the National Reference Laboratory for rabies, Sofia, Bulgaria. The objective of this visit was to meet Dr Darinka Ilieva, manager of the rabies diagnosis, and to review the procedure used to conduct the Fluorescent Antibody Test (FAT). This expertise was a pre-requisite for the Bulgarian NRL to participate successfully in the Interlaboratory Test for rabies organized annually by the EURL for rabies.

Country	N participants	Laboratory	Training title	Training place	Date
Bulgaria	1	NRL for Rabies National Veterinary Research Institute, Sofia	Rabies diagnosis by FAT	NRL for Rabies National Veterinary Research Institute, Sofia	03 April 2013 - 04 April 2013

In 2013, no training was provided in the Anses Nancy laboratory using the EURL for rabies EU budget (column training of the budget report).

Activity 3: Inter-laboratory tests and data collection

Sub Activity 3.1: Inter-laboratory tests to evaluate the recommended rabies diagnostic tests (FAT and RTCIT) (annual)

Work plan: An inter-laboratory test on the fluorescent antibody test (FAT) and rabies tissue culture inoculation test (RTCIT) will be conducted in 2013.

The different steps of the trials are the followings:

- Contacting all European laboratories (and possibly some from third countries after consultation and agreement with the EC) to establish a list of interested laboratories;
- Producing positive and negative reference materials (ten new batches will be produced for the need of the trial. A minimum of one month is necessary to produce and validate a new batch of virus *in vivo*);
- Testing the constituted panel;
- Distributing a panel of characterised samples for inter-laboratory comparison and validation;
- Interpreting all results of participating laboratories, then writing and dispatching a synthesis report.

Work completed:

The fifth inter-laboratory test for rabies diagnosis started on May 21th 2013. This test was based on the NRL analysis performed on 8 submitted samples. Each batch of virus produced for the trial was validated and tested for homogeneity and stability prior to sending. The panel test was sent to NRLs on the same day and NRLs were asked to perform rabies diagnosis on these samples using FAT and/or RTCIT.

For the first year, the EURL for rabies tried an online version of the technical questionnaires. The survey and statistics software “Sphinx iQ” was used. Four questionnaires were developed during spring 2013 with a total of 548 questions (FAT: 98, RTCIT: 220, conventional PCR: 109, and Real Time PCR: 121). In each questionnaire, several types of questions were submitted to the laboratories: open and multiple choice questions, tables, possibility for the laboratories to express themselves etc.. in order to collect the most accurate data. The questionnaires were online during the month of trial and answers, collected in an Excel sheet for each technique, were analysed at the EURL during the summer.

Twenty four NRLs from the EU participated in this trial. Report and analysis of the laboratory network results entitled “**Inter-laboratory test 2013 for rabies diagnosis: Fluorescent antibody test (FAT), Rapid tissue culture infection test (RTCIT), Reverse transcriptase polymerase chain reaction (RT-PCR) and Real time RT-PCR**”, including analysis of technical questionnaires and new recommendations were sent to NRLs by email in September 2013.

Participating NRLs in rabies diagnosis technique inter-laboratory trial (y: yes; n: no):

Continent	Country	Laboratory	Contact Name	Participation in			
				FAT	RTCIT	Con. RT-PCR	Real Time RT-PCR
Europe	Austria	AGES, Institute for Veterinary Disease Control	Dr. Elisabeth Vanek	y	y	y	y
(NRLs)	Belgium	Rabies Laboratory Communicable and Infectious Diseases	Dr. Bernard Brochier	y	n	n	y
	Bulgaria	National Diagnostic and Veterinary Research Institute	Dr. Darinka Ilieva	y	n	n	n
	Cyprus	Animal Health Laboratories	Dr. Vasiliki Christodoulou	y	n	n	y
	Czech Republic	State Veterinary Institute Prague	Dr. Miroslav Tomči	y	y	y	n
	Denmark	DTU National Veterinary Institute	Dr. Thomas Bruun Rasmussen	y	y	n	y
	Estonia	Estonian Veterinary and Food Laboratory	Dr. Katrin Mähar	y	y	n	y
	Finland	Finnish Food Safety Authority Evira	Dr. Tiina Nokireki	y	y	y	y
	France	ANSES Nancy Laboratory for rabies and wildlife	Dr. Florence Cliquet	y	y	y	y
	Germany	Federal Research Institute for Animal Health	Dr. Thomas Müller	y	y	y	y
	Greece	Institute of Infectious & Parasitic Diseases. Athens Centre of Veterinary Institutes	Dr. Konstantia Tasioudi	y	n	n	y
	Hungary	CAO VDD	Dr. Hakos Hornyak	y	y	y	y

	Italy	Istituto Zooprofilattico Sperimentale delle Venezie	Dr. Franco Mutinelli	y	y	y	y
	Latvia	Institute of Food Safety	Dr. Zita Muizniece	y	y	y	n
	Lithuania	National Food and Veterinary Risk Assessment Institute	Dr. Ingrida Jaceviciene	y	y	y	n
	Poland	National Veterinary Research Institute	Dr. Marcin Smreczak	y	y	y	y
	Portugal	Laboratório Nacional de Investigação Veterinária (LNIV)	Dr. Miguel Fevereiro; Dr. Isabel Almeida	y	y	y	n
	Romania	Institute for Diagnosis and Animal Health	Dr. Dragos Boncea	y	y	y	n
	Slovakia	State Veterinary Institute Zvolen	Dr. Slavomir Jerg	y	y	y	y
	Slovenia	VF / National Veterinary Institute	Dr. Peter Hostnik	y	y	y	y
	Spain	Centro Nacional de Microbiología, Instituto de Salud Carlos III	Dr. Juan E. Echevarría	y	y	y	n
	Sweden	Statens Veterinärmedecinska	Dr. Louise Treiberg Berndtsson	y	y	n	y
	The Netherlands	Central Veterinary Institute – WUR, Lelystad	Dr. Bart Kooi	y	n	n	y
	United Kingdom	Animal Health and Veterinary Laboratories Agency	Dr. Trudy Goddard	y	y	y	y
Total NRLs				24	19	16	17

Sub Activity 3.2: Inter-laboratory tests to evaluate molecular biology techniques (RT-PCR and Real Time) (annual)

Work plan: An inter-laboratory test on the molecular biology techniques (RT-PCR, real time PCR) will be conducted in 2013.

The different steps of the trials are the followings:

- Contacting all European laboratories (and possibly some from third countries after consultation and agreement with the EC) to establish a list of interested laboratories;
- Producing positive and negative reference materials (new batches will be produced for the need of the trial. A minimum of one month is necessary to produce and validate a new batch of virus *in vivo*);
- Testing the constituted panel;
- Distributing a panel of characterised samples for inter-laboratory comparison and validation;
- Interpreting all results of participating laboratories, then writing and dispatching a synthesis report.

Work completed:

The inter-laboratory test for molecular biology started in same time and with the same panel than the rabies diagnosis inter-laboratory test ones. All samples were sent to NRLs on the same day and NRLs were asked to perform rabies diagnosis on these samples using RT-PCR and/or Real Time PCR techniques. Sixteen NRLs from the EU participated in the RT-PCR test while seventeen NRLs participated in the Real Time RT-PCR test. Report and analysis of the laboratory network results entitled "**Inter-**

laboratory test 2013 for rabies diagnosis: Fluorescent antibody test (FAT), Rapid tissue culture infection test (RTCIT), Reverse transcriptase polymerase chain reaction (RT-PCR) and Real time RT-PCR”, including analysis of technical questionnaires and new recommendations were sent to NRLs by email in September 2013.

Sub Activity 3.3: Collecting data and information on the methods of rabies diagnosis used by laboratories for molecular biology techniques (annual)

Work plan: The procedures used by Member States for molecular biology techniques (FAT, RTCIT, RT-PCR, Real Time RT-PCR) will be collected via questionnaires on the techniques employed. Each step of the protocols will be analysed for all laboratories and compared to the OIE or/and WHO reference tests. A report will be written up with a synthesis for all procedures; special attention will be given to technical points that are different or adapted from the existing and standardised reference tests.

On the basis of the inter-laboratory test results and the synthesis of procedures used, a guide describing the main important points to consider in each step of the RT-PCR and Real Time procedures will be included in the inter-laboratory report. The objective of this guide is to obtain, as far as necessary, the standardisation of these methods within Europe.

Work done: Technical questionnaires on FAT, RTCIT, RT-PCR and Real-Time RT-PCR have been elaborated in the laboratory using Sphinx software and made available online. An average of 100 questions per questionnaire was asked to each laboratory. Analysis of these questionnaires provide precious information to highlight mismatch in laboratory procedures and can help to explain discrepancy results that occurred during the inter-laboratory test. Their analysis and potential impact on results as well as the subsequent recommendations on the techniques have been included in the inter-laboratory report “*Inter-laboratory test 2013 for rabies diagnosis: Fluorescent antibody test (FAT), Rapid tissue culture infection test (RTCIT), Reverse transcriptase polymerase chain reaction (RT-PCR) and Real time RT-PCR*”.

Sub Activity 3.4: Collecting data on tests carried out in the EC (annual)

Work plan: The EURL will request an annual report from each NRL. This will help to evaluate the number of tests performed in EU Member States for diagnosis, typing, virus titration, tetracycline detection, age determination and serology.

Work done: An annual activity questionnaire was sent to all National Reference laboratories (NRLs) on last February 2013 to collect and collate data on methods used and results of tests carried out in the Community in the frame of rabies control programmes (rabies cases, number of diagnosis and techniques performed within the year for passive and active surveillance, tetracycline detection tests on teeth, serological tests performed in the frame of monitoring of oral vaccination campaign, typing, etc.). Data

were collected and analysed. A review entitled “**Review of the analysis related to rabies diagnosis and follow-up of oral vaccination performed in NRLs in 2012**” representing the analysis performed in 2012 was produced. The document has been sent by e-mail to the network in December 2013.

Activity 4: Meetings and workshop

Sub-activity 4.1: Organising an annual meeting for NRLs (annual)

Work plan: On an annual basis, the EURL for rabies organised a workshop for gathering all National Reference Laboratories for rabies. The meeting is the opportunity to share information on rabies actualities and on the work that has been carried out during the year. Participants can be invited to deliver a presentation especially for Participants from countries where rabies still occurs. In 2013, the workshop will focus on the reference technique for rabies diagnosis using cell culture and on the harmonisation of the molecular biology methods for rabies diagnosis.

Work completed:

The sixth workshop for rabies was held on 13th and 14th November 2013 in Athens, Greece. The choice of this Member State as host country was decided following the re-emergence of rabies in the North of the country in October 2012 and was validated by the European Commission then by the Greek authorities.

Fifty-six participants from thirty-nine NRLs from European Union and third countries attended the meeting. An American expert, two representatives from the European Commission and EFSA and a delegation from the Hellenic Ministry of Rural Development and Food also participated to the workshop.

Presentations and discussions dealt with the rabies diagnosis tools, including a presentation on a comparison study on various real time RT-PCR kits and on a new test using direct rapid immunohistochemistry. Other presentations broached the control and the epidemio-surveillance of rabies issues. The recent cases of imported rabid animals in the EU were also discussed. The report of this workshop is under progress.

List of the countries with travel and accommodation supported by the European Commission:

1/ NRLs EU Member States

Country	Laboratory name and address	Names of representatives
AUSTRIA	AGES Institute for Veterinary Disease Control Moedling A-2340 Moedling, Robert Kochgasse 17	VANEK Elisabeth
BULGARIA	NRL of Rabies and monitoring the effectiveness of the vaccination 15 P. Slavejkov, Blvd. 1606, Sofia	ILIEVA Darinka

CROATIA	Laboratory for Rabies Savska cesta 143, Zagreb	BEDEKOVIC Tomislav
CYPRUS	Animal Health Laboratory Virology Section Veterinary Services 1417 Nicosia	CHRISTODOULOU Vasiliki
CZECH REPUBLIC	State Veterinary Institut Prague Statni veterinarni ustav Praha Sidlistni 136/24 Praha 6 - Lysolaje 16s 03	TOMCI Miroslav
DENMARK	DTU National Veterinary Institute Lindholm Kalvehave havnevej 51 DK-4771 Kalvehave	RASMUSSEN Thomas Bruun
ESTONIA	Estonian Veterinary and Food Laboratory Kreutzwaldi 30 Tartu 51006	MÄHAR Katrin
FINLAND	Finnish Food Safety Authority Evira Mustialankatu 3 00790 Helsinki	NOKIREKI Tiina
FRANCE	ANSES-Nancy Laboratory for rabies and wildlife Technopôle Agricole et Vétérinaire BP 40 009 54220 Malzéville	CLIQUET Florence ROBARDET Emmanuelle TOURDIAT Sylvie
GERMANY	Friedrich-Loeffler-Institut, Federal Research Institute for Animal Health Südufer 10 17493 Greifswald-Insel Riems	MÜLLER Thomas
HUNGARY	National Food Chain Safety Office Veterinary Diagnostic Directorate NRL for Rabies Budapest Tábornok u. 2 1149	HORNYAK Akos
IRELAND	Central Veterinary Research Laboratory, Department of Agriculture, Food and the Marine Laboratories, Backweston Campus, Stacumny Lane, Celbridge, Co. Kildare.	RALEIGH Patrick
ITALY	Istituto Zooprofilattico Sperimentale delle Venezie Viale dell'Università 10 35020 Legnaro (Padova)	MUTINELLI Franco
LATVIA	Institute of Food Safety, Animal Health and Environment BIOR Lejupes iela 3 RIGA, LV-1076	MUIZNIECE Zita
LITHUANIA	National Food and Veterinary Risk Assessment Institute Kairiukscio str.10 LT-08409 Vilnius	JACEVICIENE Ingrida
LUXEMBOURG	Laboratoire de Medecine Veterinaire de l'Etat 54, avenue Gaston Diderich Boite postale: 2081 1020 Luxembourg	SCHON Joseph
NETHERLANDS	Central Veterinairy Institute Houtribweg 39 8221RA Lelystad	KOOI Bart
POLAND	National Veterinary Research Institute Al. Partyzantów 57 24-100 Puławy	SMRECZAK Marcin

ROMANIA	Institute for Diagnosis and Animal Health Bucharest 63 Dr Staicovici Str, Sect 5, 050557, Bucharest	VUTA Vlad
SLOVAKIA	State Veterinary Institute Zvolen Pod Drahami 918 960 86 Zvolen	JERG Slavomir
SLOVENIA	Institute of Microbiology and Parasitology, Virology unit Gerbičeva 60, 1000 Ljubljana	RIHTARIC Danijela
SPAIN	Instituto de Salud Carlos III. Centro Nacional de Microbiología Ctra. Majadahonda-Pozuelo s/n 28220 Majadahonda, Madrid	ECHEVARRIA Juan Emilio
SWEDEN	SVA, National Veterinary Institute Travvågen 20 SE-751 89 Uppsala	BERNDTSSON TREIBERG Louise
UNITED KINGDOM	Animal Health and veterinary laboratory agency Woodham Lane New Haw Addlestone Surrey	FOOKS Anthony

2/ Invited Expert

USA	Global Alliance for Rabies Control 529 Humboldt St. Suite 1 Manhattan Kansas	RUPRECHT Charles
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Sub-activity 4.2: Keeping abreast of development in surveillance, epidemiology and prevention of rabies throughout the world and scientific assistance (multi-annual)

Work plan: The EURL will attend and participate in meetings, workshops and conferences in epidemiology and virology in regards to rabies (with prior Commission' agreement) and will also provide the European Commission with scientific advice and technical assistance at his request. The EURL will also continuously monitor new information available on rabies throughout the world.

Work completed:

One expert of the EURL for rabies participated in the "Rabies In The Americas (RITA)" congress that held in Toronto, Canada from 27th to 31st October 2013 (<http://www.rita2013.com/en/index.asp>).

The RITA meeting is an annual event that has been held since 1990 hosted in many countries across the Americas. The meeting provides an opportunity for researchers, health professionals, international, national and local managers of rabies programs, wildlife biologists, laboratory personnel and other people interested in advancing knowledge of rabies surveillance, prevention and control, to meet each other, to share their experiences and also to discuss the challenges to be met.

Following abstract submission, the EURL was invited to give an oral presentation entitled **Comparative assay of fluorescent antibody test results among twelve European National Reference Laboratories using various anti-rabies conjugates. Robardet E ; Andrieu S ; Cliquet F. Rabies in the Americas - RITA XXIV, 2013.** This study that has allowed highlighting variations in conjugates performances used within Europe was originally initiated in 2011 and then published in 2012 in the Journal of Virological methods.

Activity 5: Website and strain database management

Sub-activity 5.1: Website management

Work plan: The EURL website presenting the EURL's aptitudes and activities, a list of the NRLs, news of the laboratory network and agenda of EURL activities will be updated regularly.

Work completed:

An Internet website on the EURL's activities was launched in 2010 and is regularly updated since this date. The website is hosted at <http://www.ansespro.fr/eurl-rabies> and allows consultation of EURL reports, including the work programmes and technical reports. Each member of the NRL network has received a login and password. The website has been updated by including the news, activities and reports produced in 2013. In 2013-2014, a major redesign of all Anses EURL websites is scheduled. The new version of the EURL for rabies website should be available in 2014.

Sub-activity 5.2: Rabies Strain database deployment

Work plan: The EURL collects data from NRLs to be implemented in the genomic database of rabies strains isolated within the European Union. The implementation of a strain database was discussed in workshop in 2009 and the strain identification form has been proposed and adopted during workshops in 2011. This form is dedicated to be used by all NRLs to register rabies European strains in the database. The EURL will maintain and enlarge the genomic database of rabies strains isolated within the European Union. The objective is to overcome the lack of epidemiological information regarding rabies strains referenced in the main public sequence databases.

Work completed: The EURL is collecting data from the NRLs to be implemented in the genomic database of rabies strains isolated within the European Union. The submission of European rabies strains must be done by returning back the strain identification form to the EURL. The EURL staff is in charge of validating the data and implementing them in the strain database hosted on the EURL for rabies website. In 2013, 18 laboratories have been contacted several times to implement their data in the website.

Activity 6: Research Programmes

Sub-activity 6.1: Molecular epidemiology of rabies in Baltic countries:

Work plan: The EURL will carry out the collaboration with NRLs from Baltic countries to study the phylogeny of rabies in Baltic countries.

Work completed:

In 2012, only 7 rabies cases were detected on the total territory of the Baltic countries (no case in Estonia, 2 cases in Latvia and 5 cases in Lithuania). This Northeast European region could be potentially considered in few years as a “free of rabies” area. The low rate of cases detected nowadays follows the trend of a constant diminution of the number of rabies cases since the regular implementation in 2006 of large scale oral vaccination campaigns in these countries. This implementation has been facilitated by joining the European Union in 2004 and consequently taking part in the EU eradication and monitoring programmes of the EU Animal Health Strategy. The objective of this study is to provide a view of the phylogenetic and spatio-temporal evolution of rabies since the entry of these countries in the European Union. Rabies data from the three Baltic countries, Latvia, Lithuania and Estonia, have been combined. Surveillance data allow to assess the incidence of the disease and to appreciate the effectiveness of the oral vaccination programmes in parallel to the tetracycline and serological results obtained through the monitoring of the targeted population. The particularity of the Baltic countries is to host not only the principal rabies vector usually observed in other European countries (i.e. the red fox *Vulpes vulpes*) but also the raccoon dog (*Nyctereutes procyonoides*) which could have been a risk of failure in the fight against the disease. The comparison of the surveillance and the oral rabies vaccination monitoring data, as well as the comparison of the phylogenetic sequences are under analysis. The results of this study will be submitted to an international journal

Sub-activity 6.2: Comparison of different Real Time PCR methods

Work plan: The EURL will perform the comparison of the Real Time RT-qPCR using one step and two steps RT-PCR kits on several species of rabies virus held by the EURL. The “in-house” methods will be finally compared to commercially available kits for the determination of the analytical sensitivity (LOD) and performance of tests.

Work completed:

In 2013, two types of real-time SYBR-Green RT-PCR (one-step and two-step method) were compared using 10 commercial kits that were selected following the analysis of technical questionnaires sent by the NRLs. The comparison of the 10 kits was performed in triplicate on serial dilutions of EBLV-1 RNA

standard and pan-Lyssavirus primers shown to amplify all recognized rabies virus. For the study, three synthetic RNAs were generated from plasmid DNAs issued from the amplification of three species of rabies virus (RABV, EBLV-1 and EBLV-2). The comparison of the three RNA standards was performed by real time SYBR-Green RT-PCR (two-step method) showing that the PCR efficiency was significantly identical for the three generated synthetic RNAs. The two types of SYBR-Green real-time RT-PCR in one-step and in two-step showed a PCR efficiency less or more equivalent. However, the “optimized” one-step method showed an improved limit of detection compared to the “optimized” two-step method with respectively a limit of detection at 95% (LOD95%) of 24 copies/μl for the Quantitect master mix kit from Qiagen (one-step kit) and 41 copies/μl for the SuperScript III master mix kit from Invitrogen (two-step kit). The PCR efficacy was shown more or less equivalent for the 5 two-step RT-PCR kits tested with a detection up to 100 copies of RNA/μl, excepted for the master mix kit from Invitrogen which provided the best limit of detection (10 RNA copies/μl). Using the one-step method, The the best PCR efficacy (98 and 100%) was shown for two kits of the five one-step RT-PCR kits tested: the one-step Quantitect SG master mix kit from Qiagen (24 copies/μl) and the one-step Verso SG master mix kit from Thermo-Fischer with a detection up to 10 copies/μl for the two kits.

The results of the comparison of the two types of methods (one-step *versus* two-step) of the Real Time Sybr Green® RT-PCR were presented in 2013 at the 6th workshop for Rabies NRLs in Athens in Greece. The results of this study are under preparation to be published in an international journal.

Others:

In 2013, the EURL has also been asked to write an article for “Euroreference”, an Anses online magazine launched in June 2009, published three times a year, in English and French. Its mission is to facilitate the dissemination and sharing of information among all referencing stakeholders at European level. The review discussed a retrospective two year study of inter-laboratory trials results on rabies diagnosis and its key role in the harmonization of the methods (“***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.*** Robardet E ; Picard-Meyer E ; Servat A ; Cliquet F. *Euro Ref.*, 2013. - 18-26”) <http://www.ansespro.fr/euroreference/Documents/ER10-ReseauRage.pdf>.

Scientific papers and literature relating to rabies are continuously monitored all along the year.

B. Projects considered of interest for the EURL responsibilities and not funded by EURL funding

1. Research programmes and cooperation projects

- Cooperation with Greece (Support for Oral Vaccination):

In 2013, the laboratory has been contacted many times by the national authorities of Greece to provide some support for preparing the first oral vaccination campaign (performed in autumn 2013). One expert from the laboratory attended the launching meeting day to inaugurate the first flight of vaccine bait distribution (09 – 11 October 2013) and to provide advices to the experts of the ministry. Another expert from ELIZ (Entente interdépartementale de lutte contre les zoonoses) provided support for organizing the aerial distribution and dropping of vaccine baits from aircrafts (10 – 22 October 2013).

- Cooperation with Latvia:

From August 19th to August 23th 2013, one expert from the laboratory visited the Institute of Food Safety, Animal Health and Environment BIOR in RIGA, LATVIA in the framework of a collaborating project: Ensuring the rabies laboratory capabilities for the long-time disease prevention and control within the region. We provided support for the establishment of bat rabies surveillance (passive and pro-active) and advices on testing of bat samples for rabies. For the pro-active surveillance, some bat places were selected based on the bat species distribution in Latvia and training was given during the mission on bat sampling methods (saliva and blood samples).

An second expertise has also been conducted from 08 to 12 July 2013 at the Institute of Food Safety, Animal Health and Environment BIOR, Riga, Latvia. The objective of this visit was to assess the compliance of the rabies diagnostic laboratory and the animal facilities according to the biosafety requirements, and to prepare technical recommendations for the improvement of the current rabies testing premises and for the reconstruction of the animal facilities.

Twining France – Italy – Tunisia “Strengthening the capacities of the CNVZ”

The objective of the twining (TU11/ENP-AP/HE33 funded by the EC) was to improve the expertise of the Centre National de Veille Zoosanitaire regarding surveillance of animal diseases, training activities and communication tools. In the frame of the twining, a rabies expertise was organised from 03 to 07 June 2013 and one expert from Anses was invited to contribute to epidemiological data, to propose surveillance and control measures and to provide protocols for conducting dog population studies.

Coordination of Animal Disease Control and Eradication Programme in Bosnia and Herzegovina - Phase II

The terms of reference of this project funded by the EC are to provide technical assistance to the BiH authorities on wildlife rabies control and eradication. One expert from Anses was invited in Bosnia and Herzegovina (17 – 20 June 2013) to assess the laboratory support (two rabies laboratories were visited, one in Sarejevo, the other in Banja Luca) and to provide advices on several aspects of the present project.

- FP7-PREDEMICS (Preparedness, Prediction and Prevention of Emerging Zoonotic Viruses with Pandemic Potential using Multidisciplinary Approach (2011-2016)):

ANSES is a partner of the project and is involved in two workpackages in close collaboration with the Pasteur Institute of Paris.

The first workpackage consists to genetically characterize the bat rabies isolates recorded in France since 1989. Despite the fact that European bat lyssavirus type 1 (EBLV-1) is widespread in Europe, little is known about its evolutionary history. Most of these isolates originated from the serotine bats (*Eptesicus serotinus*), which suggest this animal species as the principal host. The aim is to undertake a comprehensive sequence analysis of the virus isolate to infer selection pressures, rates of nucleotide substitution, age of genetic diversity, geographical spread and population, growth rates of EBLV-1a and EBLV-1b in France, and in parallel, to study the population genetic of the serotine bats. 79 EBLV isolates have been selected, RNA extracted. The nucleotide sequence is currently being obtained using new generation sequencing (NGS).

The second workpackage consists to study the specificity evolution of strains passaged on foxes and dogs in experimental trials performed on caged animals. The dog remains the main reservoirs and rabies vector for infections in humans, and, likely too, for the inter-species RABV transmission with generation of viral lineages that spread to other taxa. However, the basic molecular mechanisms involved in the process of host adaptation in lyssavirus are poorly understood. The canine virus which genetic variant spread into the red fox and raccoon dog population in Europe in the 1930s provides a natural and experimentally reproducible model of adaptation. Homologous and heterologous experimental passages will be performed in dog and fox using various wild lyssavirus isolates specifically adapted. The Anses is presently determining the pathogenesis and efficiency of infection and replication of these different strains (fox strain to foxes; fox strain to dogs; dog strain to dogs; dog strain to foxes). Those trials have been initiated in September 2012 in our experimental facilities. In 2013, 5 foxes and 5 dogs have been inoculated with Moroccan strains.

- FP7-ICONZ (Integrated control of neglected Zoonoses : Improving human health and animal production through scientific innovation and public engagement (2009-2014)):

Anses is a partner of the project and is involved in two workpackages. The ultimate objective is to improve the knowledge on rabies and echinococcosis epidemiology in Morocco and in Mali.

In 2013, in close collaboration with the Swiss Tropical and Public Health Institute (Basel, Switzerland), we have participated to the analysis of the serological results obtained after blood sampling of dogs in

Bamako (paper in preparation). We have also received a rabies expert from the NRL of Bamako for a training in molecular biology and sequencing tools applied for testing 100 infected brain sampled in Mali (18 November – 06 December 2013). The phylogenic analyses of results are in progress.

2. Participation in international congress and meeting

- TAIEX meeting:

The laboratory has been invited as speaker in the AGR 53855 Workshop “Regional policies to control rabies in the Southern Mediterranean” in Paris, France, the 15-16 October 2013. The ANSES rabies expert has been requested to present a talk entitled “The European Union Reference Laboratory (EURL) in the frame of rabies surveillance and control in Europe and in third countries”.

Rabies Regional Crisis Meeting – Tirana, Albania, 12 April 2013
The event was organised within the framework of the European Commission-funded project (IPA Multibeneficiary Project - Western Balkans) to support the control and eradication of animal diseases within the seven countries of the Western Balkans.. The laboratory has been invited as speaker (“Strategies, plans and coordination - lessons learned in France”) The meeting was intended to enable countries in the southernmost parts of the Balkan Peninsula to:

- 1) Share experience in rabies surveillance and the implementation of vaccination programmes;
- 2) Establish an operational network between the relevant authorities;
- 3) Agree on harmonised strategies for surveillance, vaccination, monitoring and public awareness.

- BTSF training (2013 - 2014)

A BTSF training entitled Training on movements of dogs and cats has been initiated at the end of 2013 and will continue in 2014. The laboratory is involved in one training course as speaker with two presentations (“Scientific background to justify the legislative measures regarding the control of rabies and *Echinococcus multilocularis* in the context of the movements of dogs and cats” and EU-approved rabies serology laboratories”). The technical content of the training mainly covers EU Legislation on import, trade-in and non-commercial movements of dogs, both from third countries and within the EU.

- RITA congress: Toronto, Canada from 27-31 October 2013.

The Rabies in the Americas (RITA) meeting is a worldwide annual rabies event. This international meeting provides an opportunity for researchers, managers of rabies programs interested in advancing knowledge of rabies surveillance, prevention and control, to meet each other, to share their successes and also to discuss the challenges to be met.

Two oral presentations were performed by the Lyssavirus Unit of the laboratory:

- *Validation of the FAVN test as a serological potency assay to replace the in-vivo potency tests of inactivated rabies vaccine for veterinary use / Servat A ; Cliquet F. Rabies in the Americas - RITA XXIV, 2013*
- *Comparative assay of fluorescent antibody test results among twelve European National Reference Laboratories using various anti-rabies conjugates / Robardet E ; Andrieu S ; Cliquet F. Rabies in the Americas - RITA XXIV, 2013*

- Taiwan:

Our laboratory was invited to take part in two conferences at the end of 2013 following a rabies outbreak in ferret badgers in a country considered until now as rabies-free.

- 1) International Expert Meeting – Challenges and Opportunities in the Prevention and Control of Rabies” (Taipei, 30 – 31 August, 2013). The objective of the conference and also to close meeting with representative of the ministry of agriculture was to provide scientific information and recommendations to the relevant authorities regarding rabies control, prevention and surveillance in human, wildlife and domestic animals. Discussions were also dedicated on the pathogenesis of ferret badger rabies strain and the different methods/protocols to be put in place for experimental studies on caged ferret badgers but also in caged dogs. A certain number of experts from China, USA, Japan, France, Philippines and Taiwan were invited to give a talk. The invited Anses expert gave one lecture: “Evaluation and Practice on Oral Immunization of Wild Animals in Europe”, F. Cliquet.
- 2) International Conference on Prevention and Control of Rabies in Taiwan (Taipei, 30 September 5 October 2013)
The aims of this conference was to create a discussion forum where experts from France, Thailand, USA, Australia, China, Japan, Philippines, Vietnam and Taiwan discussed rabies related issues nowadays with a view to provide a better understanding of rabies in animals and human, stimulate scientific discussion and develop policy-relevant approaches and analysis for decision-makers. The Anses Nancy laboratory participated to the meeting by giving two special lectures during the conference:
 - *Means for rabies surveillance, control and monitoring of oral vaccination campaigns used in Europe / Robardet E ; Cliquet F. International Conference on Prevention and Control of Rabies, 2013*
 - *Different existing laboratory methods used for rabies surveillance and monitoring / Servat A; Cliquet F. International Conference on Prevention and Control of Rabies, 2013*

The last two days of the symposium were dedicated to workshop on rabies diagnosis also where the laboratory gave different lectures:

- *Inter-laboratory testing in the field of rabies / Robardet E ; Wasniewski M ; Cliquet F. International Conference on Prevention and Control of Rabies, 2013*
- *Rabies diagnosis techniques / Servat A; Robardet E; Cliquet F. International Conference on Prevention and Control of Rabies, 2013*
- *Rabies serological assays / Servat A; Wasniewski M; Cliquet F. International Conference on Prevention and Control of Rabies, 2013*

- China:

The laboratory was invited by the CDC (Beijing, China) the 24rd October to give a presentation on the activities of the ANSES Nancy laboratory especially on rabies field. During this visit, the laboratory was visited and discussed on rabies laboratory methods were initiated.

The laboratory was also invited by the Veterinary Research Institute Academy of Military Medical Sciences (Changchun) to give a technical and scientific assistance on the seroneutralisation method (FAVN test) for the rabies antibody titration in serum samples (28rd October to the 6th November 2013). This laboratory intends to take part in the next rabies serology proficiency test organized in April 2014.

3. Organisation of international meeting

The Anses-Nancy Laboratory for Rabies and Wildlife is also the EURL for rabies serology since 2000. Within this frame, it organises each year an inter laboratory trial (Decision 2010/436/EU of 09 August 2010). On 1st and 2nd October 2013, the EURL for rabies serology organised its 4th meeting for the laboratories of its network. The objectives of the meeting were to discuss about the procedure for the serology proficiency testing and associated legislation aspects (Commission Decision 2010/436/EU). Serological testing on domestic carnivores was largely discussed as well as the sero-monitoring of wildlife following oral vaccination campaigns, particularly new promising tools available for serological testing. The results of a collaborative study using a ELISA kit for testing wildlife samples were also presented.

The EURL for serology experts gave four oral presentations:

- *Results of the collaborative study on a commercialised ELISA kit / Wasniewski M ; Kempff S ; Labadie A ; Rieder J ; Schereffer JL ; Tribout L ; Cliquet F. Rabies serology meeting, 2013*
- *Evaluation of an ELISA to detect rabies antibodies in wild (foxes and raccoon dogs) and domestic carnivores (dogs and cats) / Cliquet F ; Guiot AL ; Schereffer JL ; Tribout L ; Mähar K ; Wasniewski M. Rabies serology meeting, 2013*
- *Reference serum of dog origin. Batch 3 / Barrat J ; Cliquet F ; Monchatre-Leroy E ; Wasniewski M. Rabies serology meeting, 2013*
- *Procedure for rabies antibody proficiency testing / Cliquet F. Rabies serology meeting, 2013*

Organized in the magnificent frame of the Lucien Cuénot amphitheatre of the Museum Aquarium of Nancy, the meeting met great success, gathering 48 scientific experts from 34 laboratories and one representative from the European Commission.

4. Training of third or EU countries laboratories not under EURL mandate funding

From the beginning of 2013, 8 training courses counting a total of 12 participants were organized by ANSES Nancy in the frame of the rabies activities (Rabies diagnosis, typing, virus production and titration, cell culture maintenance, serology, tetracycline detection and epidemiology, etc.):

Country	N participants	Laboratory	Training title	Training place	Date
Greece	1	Virology department, institute of infectious and parasitic diseases Ministry of rural development and food athens centre of veterinary institutes, Athens	Rabies diagnosis with references and molecular biology techniques	ANSES Nancy, Malzéville	16 January 2013 - 20 January 2013
Indonesia	1	Disease Investigation Centre, Region VII Maros	Serology in the frame of a PhD study on the effect of 3 rabies vaccines in local Indonesian dogs with FAVN and ELISA test	ANSES Nancy, Malzéville	03 February 2013 - 15 February 2013
Kazakhstan	2	ANTIGEN Co. Ltd Almaty,	Titration and vaccine control	ANSES Nancy, Malzéville	11 February 2013 - 22 February 2013
Montenegro	3	Diagnostic Veterinary Laboratory, Podgorica	PCR diagnostic of rabies	Diagnostic Veterinary Laboratory, Podgorica	26 March 2013 - 28 March 2013
Greece	2	Virology department, institute of infectious and parasitic diseases Ministry of rural development and food athens centre of veterinary institutes, Athens	Tetracycline detection in teeth and phylogenetic analysis of rabies virus	ANSES Nancy, Malzéville	02 April 2013 - 04 April 2013
Greece	1	Virology department, institute of infectious and parasitic diseases Ministry of rural development and food athens centre of veterinary institutes, Athens	Typing and phylogenetic analysis of rabies virus	ANSES Nancy, Malzéville	02 April 2013 - 04 April 2013

United Kingdom / Mexico	1	School of Veterinary Science, University of Liverpool	End-of-studies internship	ANSES Nancy, Malzéville	03 June 2013 - 28 June 2013
Mali	1	Laboratoire Central Vétérinaire Bamako	Typing and phylogenetic analysis of rabies virus	ANSES Nancy, Malzéville	18 November 2013 - 06 December 2013

5. International publications related to rabies published in 2013

1. *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.* Robardet E ; Picard-Meyer E ; Servat A ; Cliquet F. *Euro Ref.*, 2013. - 18-26
2. *Isolation of Bokeloh bat lyssavirus in Myotis nattereri in France.* Picard-Meyer E ; Servat A ; Robardet E ; Moinet M ; Borel C ; Cliquet F. *Arch. Virol.*, 2013, vol. 158, n° 11. - 2333-2340
3. *Genetic strain modification of a live rabies virus vaccine widely used in Europe for wildlife oral vaccination.* Cliquet F ; Robardet E ; Picard-Meyer E. *Antiviral Res.*, 2013, vol. 100, n° 1. - 84-89
4. *Epidemiology and molecular diversity of rabies viruses in Bulgaria.* Robardet E ; Ilieva D ; Iliev E ; Gagnev E ; Picard-Meyer E ; Cliquet F. *Epidemiol. Infect.*, 2013. - 1-7
5. *Official batch control of rabies veterinary vaccines: current situation and perspectives in the European Union.* Servat A ; Cliquet F. *Altern. Lab. Anim.*, 2013, vol. 41, n° 1. - 10-11
6. *Comparative assay of fluorescent antibody test results among twelve European National Reference Laboratories using various anti-rabies conjugates.* Robardet E ; Andrieu S ; Bruun Rasmussen T ; Dobrostana M ; Horton DL ; Hostnik P ; Jaceviciene I ; Juhasz T ; Muller T ; Mutinelli F ; Servat A ; Smreczak M ; Vanek E ; Vazquez-Moron S ; Cliquet F. *J. Virol. Methods*, 2013, vol. 191. - 88-94
7. *Serosurvey of dogs for human, livestock, and wildlife pathogens, Uganda.* Millan J ; Chirife AD ; Kalema-Zikusoka G ; Cabezon O ; Muro J ; Marco I ; Cliquet F ; Leon-Vizcaino L ; Wasniewski M ; Almeria S ; Mugisha L. *Emerg. Infect. Dis.*, 2013, vol. 19, n° 4. - 680-682
8. *A step forward in molecular diagnostics of lyssaviruses - Results of a ring trial among European laboratories.* Fischer M ; Wernike K ; Freuling CM ; Muller T ; Aylan O ; Brochier B ; Cliquet F ; Vazquez-Moron S ; Hostnik P ; Huovilainen A ; Isaksson M ; Kooi EA ; Mooney J ; Turcitu M ; Rasmussen TB ;

Revilla-Fernandez S ; Smreczak M ; Fooks AR ; Marston DA ; Beer M ; Hoffmann B. *PlosOne*, 2013, vol. 8, n° 3. - 1-9

9. Evaluation of an ELISA to detect rabies antibodies in orally vaccinated foxes and raccoon dogs sampled in the field. Wasniewski M ; Guiot AL ; Schereffer JL ; Tribout L ; Mähar K ; Cliquet F. *J. Virol. Methods*, 2013, vol. 187. - 264-270

10. Molecular characterisation of rabies virus strains in the republic of Macedonia. Picard-Meyer E ; Mrenoshki S ; Milicevic V ; Ilieva D ; Cvetkovikj I ; Cvetkovikj A ; Krstevski K ; Dzadzhovski I ; Robardet E ; Gagnev E ; Iliev E ; Plavsic B ; Kirandjiski T ; Cliquet F. *Arch. Virol.*, 2013, vol. 158, n° 1. - 237-240