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Nancy Laboratory
for rabies and wildlife

European Union Reference Laboratory for Rabies

European Union Reference Institute for Rabies Serology

WHO Collaborating Centre for Research and Management in Zoonoses Control

OIE Reference Laboratory for Rabies

EUROPEAN UNION REFERENCE LABORATORY
RABIES

European Union Reference Laboratory for Rabies

TECHNICAL REPORT FOR 2016-2017

March 2018

ANSES - French Agency for Food, Environmental and Occupational Health & Safety
Technopôle agricole et vétérinaire - BP 40 009 - 54 220 Malzéville Cedex - France

The ANSES Nancy Laboratory for Rabies and Wildlife has been nominated as European 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. This regulation amends the Regulation (EC) No 737/2008 designating the EURL for crustacean diseases, rabies and bovine tuberculosis as well as the Commission Regulation (EU) No 737/2008 Annex VII to Regulation (EC) No 882/2004 of the European Parliament and of the Council.

I. Work Programme planned for 2016-2017

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

Activity 3:

3.1. **To organise Inter-laboratory test on the reference rabies diagnosis tests: the fluorescent antibody test (FAT), the rabies tissue culture inoculation test (RTCIT) and molecular biology techniques (Real Time and RT-PCR).**

3.2. **To collect data and information on methods of rabies diagnosis used through European Union and to standardise them.**

3.3. **To organise Inter-laboratory test to evaluate serological tests used by NRLs for the follow-up of ORV campaigns.**

3.4. **To organise Inter-laboratory tests to evaluate tetracycline and age determination techniques.**

3.5. **To collect data and results of tests carried out in the EC.**

Activity 4:

4.1. **To organise an annual workshop for NRLs.**

4.2. **To keep abreast of development in surveillance, epidemiology and prevention of rabies throughout the world** by participating in conferences.

4.3. **To manage and update the EURL website.**

Activity 5: **To compare and evaluate the different Real Time PCR methods.**

II. Technical report for 2016-2017

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

The EURL will provide full assistance to the NRLs concerning their requests as regards laboratory techniques related to rabies diagnosis, typing and follow-up of oral vaccination campaigns.

Sub Activity 1.1:

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

Work plan: The EURL rabies virus collection will be maintained (storage in liquid nitrogen). Depending on outbreaks and opportunities, new rabies virus strains will be produced and stored to enlarge the rabies virus collection.

The biological materials that will be available for rabies diagnosis to the NRLs are:

- Positive controls infected with RABV, EBLV-1, EBLV-2, ABLV, DUVV, BBLV species (strains available in the laboratory and subject to the consent of the owner of the strain) and negative controls for rabies diagnosis and for typing;
- Lyophilised preparations of fixed reference viruses (CVS 11 for *in vitro* tests and CVS 27 for *in vivo* tests).

The biological materials and facilities that will be available for follow-up of oral vaccination campaigns are:

- CD-ROM describing the operating procedure for determining tetracycline presence;
- Fox teeth samples (positive and negative controls for determining tetracycline presence).

Other technical support available to the NRLs:

- Experimental station capacities with mice, cats, dogs, foxes and raccoon dogs: support to laboratories willing to obtain strains of certain rabies viruses produced on animals.

Work carried out in 2016: 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.

In 2016, two field strains have enlarged the EURL batch rabies virus collection: A EBLV-1 bat strain from Luxembourg isolated in 2013 and a rabies vaccine induced strain, isolated in Slovenia in 2012 (Table 1). These new strains have been imported in the laboratory, produced and stored to enlarge the geographical and species origin of the EURL rabies virus collection and will be supplied to the NRLs upon request and in compliance with national shipping regulations. Since the EURL designation, an average of 3 new strains per year are produced on mice, validated by FAT, RTCIT, RT-PCR, qPCR and typing (the analysis are repeated 3 times to ensure homogeneity of the production). Each new batch is composed of 10-40 vials of 800µl of virus suspension and is stored in liquid nitrogen.

Table 1: Batch of viruses produced by the rabies EURL for the rabies virus collection in 2016:

Batch name	Storage condition	Species	Strain name	Original species affected	Country of origin	n vials (800µl of virus suspension)
Luxembourg 01-16	Liquid nitrogen	EBLV-1	DR0707	*Eptesicus serotinus*	Luxembourg	26
Slovenia 04-16	Liquid nitrogen	Vaccine Strain	DR0579	*Vulpes vulpes*	Slovenia	18

- The set of viruses presented above can be used as positive controls in rabies diagnosis tests (FAT, RTCIT, MIT and molecular techniques). Lyophilised batch viruses produced in the context of inter-laboratory test (section 3.1) can also be supplied as rabies diagnosis positive control to NRL at request.

Work carried out in 2017: New batches of viruses (lyophilised or frozen) were produced to cover laboratory requests and to enlarge the rabies virus collection.
In 2017, two field strains have enlarged the EURL batch rabies virus collection: An Italian RABV strain isolated in a red fox in 2010 and a Lithuanian RABV strain isolated in a red fox in 2015 (Table 2). These new strains have been imported, produced and stored in the laboratory to enlarge the geographical and species origin of the EURL rabies virus collection and will be supplied to the NRLs upon request and in compliance with national shipping regulations. Since the EURL designation, an average of 2/3 new strains per year are produced on mice, validated by FAT, RTCIT, RT-PCR, qPCR and typing (the analysis are repeated 3 times to ensure homogeneity of the production). Each new batch is composed of 10-40 vials of 800µl of virus suspension and is stored in liquid nitrogen.

Table 2: Batch of viruses produced by the rabies EURL for the rabies virus collection in 2017:

Batch name	Storage condition	Species	Strain name	Original species affected	Country of origin	n vials (800µl of virus suspension)
RABV Italie 03-17	Liquid nitrogen	RABV	Italy/red fox/V140/2010	*Vulpes vulpes*	Italy	20
Lituanie Renard 01-18	Liquid nitrogen	RABV	RABV2015 red fox	*Vulpes vulpes*	Lithuania	20

- The set of viruses presented above can be used as positive controls in rabies diagnosis tests (FAT, RTCIT, MIT and molecular techniques). Lyophilised batch viruses produced in the context of inter-laboratory test (section 3.1) can also be supplied as rabies diagnosis positive control to NRL at request.

- Cross-sections of red fox teeth and half jaws with identified status in age and tetracycline content were prepared at request to provide NRLs with positive or negative controls for tetracycline detection in the teeth (bait uptake control).

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 carried out in 2016: Three samples have been submitted for rabies diagnosis confirmation to the EURL for Rabies (Table 3). The first sample was a sample from Luxembourg identified as negative. Two Bulgarian positive samples were submitted for rabies diagnosis confirmation and tested negative for rabies by the EURL. The two samples harboured negative results for the two reference techniques (FAT, RTCIT). Moreover, RNA rabies virus was not detected with Conventional RT-PCR while detected at the limit of detection with the Real-time PCR technique (Taqman and SYBR Green). This has been potentially attributed to cross-contamination of samples. A report was edited for each submitted sample. The number of submitted samples received for typing only since 2012 is indicated in Table 3.

Table 3: Support of the EURL in rabies diagnosis confirmation and typing (NA: not applicable):

Anses ID	Reception date	Country	Species	FAT result	RTCIT result	RT-PCR result	Real time result	Report sent on
DR-1071	04/01/2016	Luxembourg	*Vulpes vulpes*	-	NA	NA	-	05/01/2016
DR-1203a	19/10/2016	Bulgaria	*Vulpes vulpes*	-	-	-	+ (at limit of detection)	09/11/2016
DR-1203b	19/10/2016	Bulgaria	*Vulpes vulpes*	-	-	-	+ (at limit of detection)	09/11/2016

Work carried out in 2017: Four samples have been submitted for rabies diagnosis confirmation to the EURL for Rabies (Table 4). The two first samples were two samples from Serbia finally identified as negative. Two samples from Luxembourg were also submitted for rabies diagnosis confirmation and tested negative for rabies by the EURL. All the samples harboured negative results for the two reference techniques (FAT, RTCIT). RNA rabies virus was not detected with Conventional RT-PCR and Real-Time PCR techniques in the Serbian samples and in the sample DR1462 from Luxembourg. Sample DR1552 from Luxembourg had RNA rabies virus detected at the limit of detection with the Real-time PCR techniques (Taqman and SYBR Green). This has been attributed to potential cross-contamination of samples. A report was edited for each submitted sample.

Table 4: Support of the EURL in rabies diagnosis confirmation and typing:

Anses ID	Reception date	Country	Species	FAT result	RTCIT result	RT-PCR result	Real time result	Report sent on
DR-1341	24/02/2017	Serbia	*Mus musculus*	-	-	-	-	31/03/2017
DR-1342	24/02/2017	Serbia	*Vulpes vulpes*	-	-	-	-	31/03/2017
DR-1462	31/05/2017	Luxembourg	*Bos taurus*	-	-	-	-	19:06/2017
DR-1552	05/10/2017	Luxembourg	*Felis silvestris catis*	-	-	-	+ (at limit of detection)	10/10/2017

Activity 2: Training activities

Work plan: The Lyssavirus Unit of the laboratory is headed by Dr Florence Cliquet. The Unit is composed of 4 teams represented by 15 agents. Each team is headed by an experienced scientist who can provide expertise, scientific and technical support under the rabies EURL mandate. The areas of expertise are diagnosis, molecular biology, virology, virus titration, biomarker determination and epidemiology.

Upon NRL requests, the EURL will organise training sessions on

- rabies diagnosis,
- molecular biology, typing,
- rabies virus production, rabies virus titration,
- biomarker determination.

The training will take place in the EURL (column “training”) or will take place in the facilities of the trained laboratories (column “mission” for the EURL staff) according to the needs outlined by the inter-laboratory test results.

Work carried out in 2016: One training session was organised for the Luxemburgish NRL in the frame of the EURL mandate (column training of the budget report). The expertise in rabies diagnosis FAT has been conducted from 21 to 23 September 2016 in the EURL, Nancy, France (Table 5). The objective of the training was to support the Luxemburgish NRL in updating and improving its capacity following the reorganisation of the laboratory and the reinstatement of the rabies diagnosis activity.

Table 5: Training organised by the EURL in 2016

Country	Number of participants	Laboratory	Training title	Training place	Starting date	Ending date
Luxembourg	2	Laboratoire de Médecine Vétérinaire de l’Etat	Training course on rabies diagnosis (FAT)	ANSES Nancy	21/11/2016	23/11/2016

Work carried out in 2017: One training session was organised for the Irish NRL in the frame of the EURL mandate (column training of the budget report). The expertise in rabies diagnosis FAT has been conducted from 26 to 28 June 2017 in the EURL, Nancy, France (Table 6). The objective of the training was to support the Irish NRL in updating and improving its capacity following the reorganization of the laboratory and the implementation of the BSL3 laboratory.

Table 6: Training organised by the EURL in 2017

Country	Number of participants	Laboratory	Training title	Training place	Starting date	Ending date
Ireland	2	Virology Division Central Veterinary Research Laboratory	Training course on rabies diagnosis (FAT)	ANSES Nancy	26/06/2017	28/06/2017

In 2016 and 2017, no mission was performed by the Anses Nancy laboratory using the EURL for rabies EU budget (column mission of the budget report).

Activity 3: Inter-laboratory tests, data collection and technique evaluation

Sub Activity 3.1: Inter-laboratory test to evaluate rabies diagnosis tests (FAT, RTCIT, Real Time, RT-PCR)

Work plan: To follow-up the performance of NRLs on rabies diagnosis, an inter-laboratory test on the fluorescent antibody test (FAT), rabies tissue culture inoculation test (RTCIT) and on the molecular biology techniques (RT-PCR, real time PCR) will be conducted in 2016.

The different steps of the trials are the followings:

- Contacting all European laboratories (and possibly some from third countries after consultation and agreement of 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 validity, stability, homogeneity of 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 carried out in 2016: The eighth consecutive proficiency test for rabies diagnosis started on May 30th 2016. This test was based on the NRL analysis performed on 10 submitted samples. Each batch of virus used for inter-laboratory test purposes is produced on mice (Table 7) and was validated by testing the panel ten times in duplicate for homogeneity and four times for stability (by FAT, RTCIT, RT-PCR, qPCR and sequencing) 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 and/or Real Time and/or RT-PCR.

Table 7: Batch of viruses produced by the rabies EURL in 2016 for inter-laboratory test purposes:

Batch name	Storage condition	Species	Strain name	Original species affected	Country of origin	n vials
EBLV-2 05-16	Lyophilised, +4°C	EBLV-2	EBLV-2 03-12	*Myotis daubentonii*	United Kingdom	160
CVS 27 06-16	Lyophilised, +4°C	RABV	CSV 27 11-14	*Fixed strain*	/	140
BBLV 07-16	Lyophilised, +4°C	BBLV	DR 127900 Hemilly	*Vulpes Vulpes*	France	146

Slovakia 10-16	Lyophilised, +4°C	RABV	VB 1071, 2015	*Vulpes vulpes*	France	55
GS7 11-16	Lyophilised, +4°C	RABV	GS7 11-14	*Vulpes vulpes*	France	150

As for previous years, online technical questionnaires were used to collect information on the procedure used by participants. The survey and statistics software "Sphinx iQ" was used. A questionnaire was proposed for each evaluated technique. 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 six NRLs from the EU and 28 laboratories from third countries participated in this trial. Third countries participation was out of the scope of the EURL and performed without EURL budget. They included 10 laboratories from Europe, 4 laboratories from America, 8 laboratories from Africa, 5 laboratories from Asia, and one from Oceania. The number of participating EU laboratories remained stable compared to 2015.

The report entitled "***Inter-laboratory test for rabies diagnosis: Session 06-2016*** ", including an analysis of the laboratory network results as well as an analysis of technical questionnaires and recommendations was sent to NRLs by email in September 2016 and is available in the part under restrictive access of the EURL website (https://eurl-rabies.anses.fr/en/minisite/rabies/inter-laboratory-trials)

The lowest proportion of laboratories producing discordant results was found in the Real Time PCR test (3.7%), closely followed by RT-PCR (8.3%) (Table 8). The gold standard technique, the FAT, harboured 14% of laboratory with discrepant results. For the second consecutive year, the Real Time PCR test was the trial with the best laboratory performance level. In contrast to previous years, false negative results were more frequent than false positive results in both FAT and molecular biology techniques (RT-PCR and Real Time PCR). GS7 diluted samples presented higher percentages of discordant results in FAT. BBLV samples presented higher percentage of discordant results in Real Time PCR. The potential explanations of the discordant results based on the technical questionnaire analysis were included in the discussion section of the report.

In 2015, an Anses working group designed a questionnaire of satisfaction survey dedicated to participants of inter-laboratory test organised by the Anses laboratories. The EURL used this survey annually, at the end of the inter-laboratory campaign to assess participant satisfaction. 2016 survey indicated a global 100% satisfactory level (7 quite satisfactory, 34 satisfactory, 41/54 recipients of the survey participated) (Figure 1).

Table 8: Rabies diagnosis laboratory discrepancies in 2016 session (percentage are given in brackets).

discordant results n/N (%)	**FAT**	**RTCIT**	**RT-PCR**	**Real Time PCR**
Number of laboratories	7/50 (14.0)	5/31 (16.1)	3/36 (8.3)	1/27 (3.7)
Negative samples (false positives)	**0/50 (0)**	1/31 (3.2)	**0/36 (0)**	**0/27 (0)**
Positive samples (false negatives)	9/397 (2.2)	6/248 (2.4)	3/282 (1.1)	1/205 (0.005)
CVS 27	1/50 (2.0)	1/31 (3.2)	**0/36 (0)**	**0/27 (0)**
RABV GS7	**0/50 (0)**	**0/31 (0)**	**0/36 (0)**	**0/27 (0)**
RABV GREECE	**0/50 (0)**	2/31 (6.5)	**0/36 (0)**	**0/27 (0)**
RABV MOROCCO	**0/50 (0)**	**0/31 (0)**	**0/36 (0)**	**0/27 (0)**
Total RABV	1/200 (0.005)	3/124 (2.4)	**1/144 (0.007)**	**0/108 (0)**
RABV GS7 diluted	7/50 (14)	1/31 (3.2)	1/36 (2.8)	**0/27 (0)**
EBLV-1	**0/49 (0)**	1/31 (3.2)	1/34 (2.9)	**0/25 (0)**
EBLV-2	**0/49 (0)**	**0/31 (0)**	1/34 (2.9)	**0/24 (0)**
BBLV	1/49 (2.0)	1/31 (3.2)	**0/34 (0)**	1/21 (4.8)

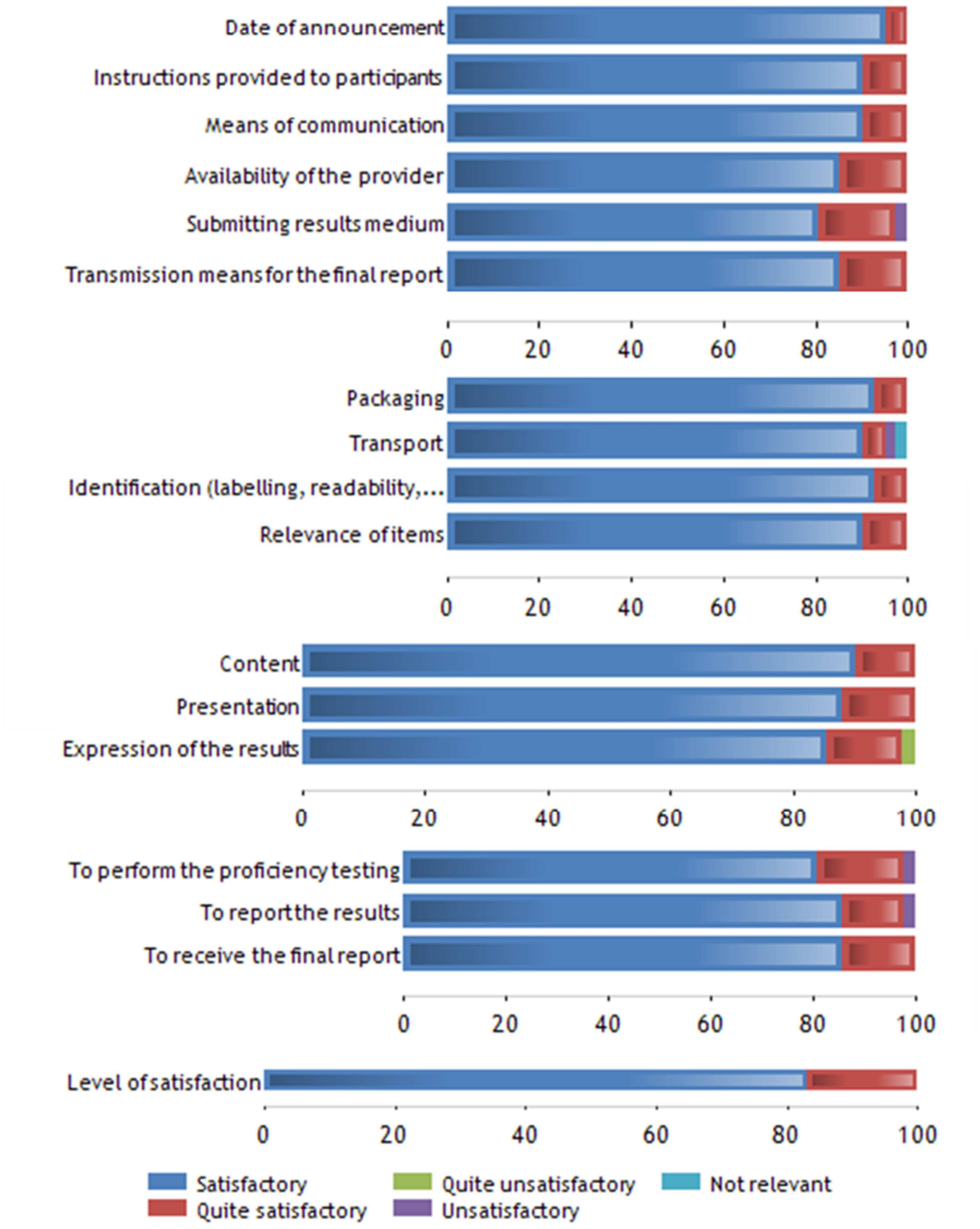

Figure 1: Results of the Anses satisfaction survey regarding the inter-laboratory test for Rabies diagnosis –session 2016

Work carried out in 2017: The ninth consecutive proficiency test for rabies diagnosis started on May 29th 2017. This test was based on the NRL analysis performed on 10 submitted samples. Each batch of virus used for inter-laboratory test purposes was produced *in vivo* (Table 9) and was validated by testing the panel ten times in duplicate for homogeneity and seven times under various conditions for stability (by FAT, RTCIT, RT-PCR, qPCR and sequencing) 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 and/or Real Time and/or RT-PCR.

Table 9: Batch of viruses produced by the EURL in 2017 with EURL budget for inter-laboratory test purposes:

Batch name	Strain name origin	Country of origin	Date of production	Produced on	Storage condition	n vials
NEG 01-17	/	France	24/02/2017	*Vulpes vulpes*	Lyophilised, +4°C	310
RABV Slovaquie 02-17	Slovaquie 13-16	Slovakia	19/04/2017	*Mus musculus*	Lyophilised, +4°C	150
CVS27 04-17	CSV 27 lot 11-14	Fixed strain	21/04/2017	*Mus musculus*	Lyophilised, +4°C	150
RABV Bulgarie 05-17	RABV Bulgarie lot 09-14	Bulgaria	31/05/2017	*Mus musculus*	Lyophilised, +4°C	151
ABLV 06-17	ABLV 12-09	Australia	27/06/2017	*Mus musculus*	Lyophilised, +4°C	150
EBLV-1a 09-17	EBLV1a 29-12	United Kingdom	09/08/2017	*Mus musculus*	Lyophilised, +4°C	150
EBLV-2 11-17	EBLV2 03-12	France	05/10/2017	*Mus musculus*	Lyophilised, +4°C	150
GS5 15-17	ATTON RA-0334	France	19/12/2017	*Vulpes vulpes*	Lyophilised, +4°C	154

Twenty five NRLs from the EU and 23 laboratories from third countries participated in this trial. Third countries participation was out of the scope of the EURL and performed without EURL budget. They included 8 laboratories from Europe, 5 laboratories from America, 6 laboratories from Africa, 3 laboratories from Asia, and one from Oceania. The number of participating EU laboratories remained stable compared to previous years (Figure 2).

The report entitled "***Inter-laboratory test for rabies diagnosis: Session 09-2017*** ", including an analysis of the laboratory network results as well as an analysis of technical questionnaires and recommendations was sent to NRLs by email in September 2017 and is available in the part under restrictive access of the EURL website (https://eurl-rabies.anses.fr/en/minisite/rabies/inter-laboratory-trials)

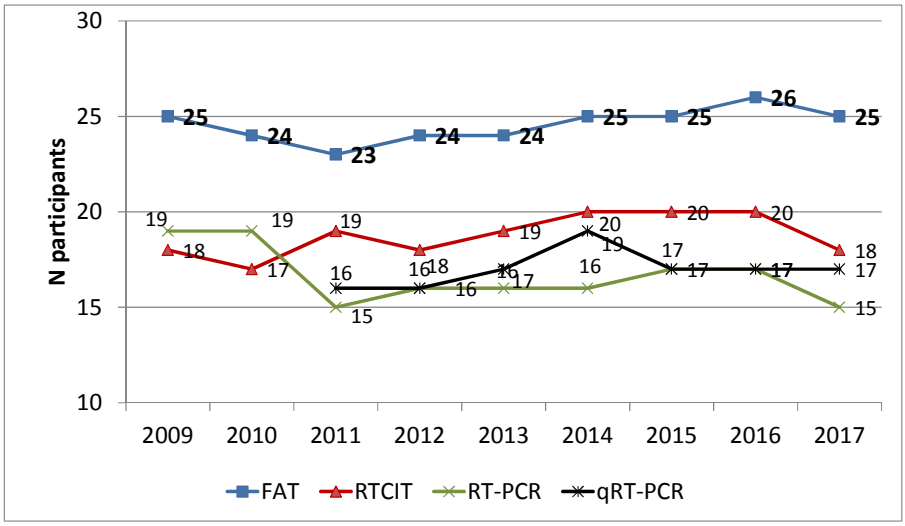

Figure 2: Evolution of the number of participating laboratories in the rabies diagnosis inter-laboratory test from EU countries

This 2017 session presented the best diagnosis performances of laboratories ever observed on positives samples whatever the technique used. The lowest proportion of laboratories producing discrepant results was found in the RT-PCR (0%), closely followed by the gold standard technique, the FAT, with 2.2% of laboratory with discordant results (Table 10). For the third year (*i.e*. 2012 and 2013 sessions), not a single false negative result was notified in the RT-PCR results. RT-PCR trial shows the best diagnosis sensitivity. Wrong overall diagnosis conclusion on positive samples was detected in one laboratory. This laboratory participated in the FAT trial only (using a confirmatory test indeed increase the reliability of the results).

Unexpected results on the negative samples show evidence of a batch contamination and led the evaluation of laboratories on this batch unfeasible. Due to the low number of representative laboratories per category, no factors have unfortunately been highlighted as affecting the probability of providing positive results on the batch 12-15. When different result combination occurred, variable diagnosis conclusions on the batch 12-15 have been given. This raises that harmonization of diagnosis conclusion in case of different result combination could be discussed within the network. We also raise the question on the importance of RT-PCR results on the drawn diagnosis conclusion when neither antigen, either infectious virus has been detected in the sample. RT-PCR results seem indeed having driven most of the diagnosis conclusion, even FAT negative result was observed.

In 2015, an Anses working group designed a questionnaire of satisfaction survey dedicated to participants of inter-laboratory test organised by the Anses laboratories. The EURL used this survey annually, at the end of the inter-laboratory campaign to assess participant satisfaction. The 2017 survey indicated a global good satisfactory level with 20 satisfactory (83.3%), 3 quite satisfactory (12.5%) and 1 unsatisfactory (4.2%). 24/54 recipients of the survey participated in the evaluation (Figure 3).

Table 10: Rabies diagnosis laboratory discrepancies in 2017 session (percentage are given in brackets).

n discrepant/N (%)	FAT	RTCIT	RT-PCR	Overall Diagnosis conclusion
Number of laboratories	1/45 (2.2)	3/29 (10.3)	**0/30 (0)**	1/45 (2.2)
Positive samples (false negatives)	3/270 (1.1)	4/174 (2.3)	**0/180 (0)**	3/270 (1.1)
CVS 27	1/45 (2.2)	**0/29 (0)**	**0/30 (0)**	1/45 (2.2)
RABV GS7	**0/45 (0)**	1/29 (3.4)	**0/30 (0)**	**0/45 (0)**
RABV dog Spain	1/45 (2.2)	2/29 (6.9)	**0/30 (0)**	1/45 (2.2)
Total RABV	2/135 (1.5)	3/124 (2.4)	**0/30 (0)**	2/135 (1.5)
ABLV	1/45 (2.2)	**0/29 (0)**	**0/30 (0)**	1/45 (2.2)
EBLV-1	**0/45 (0)**	**0/29 (0)**	**0/30 (0)**	**0/45 (0)**
EBLV-2	**0/45 (0)**	1/29 (3.4)	**0/30 (0)**	**0/45 (0)**

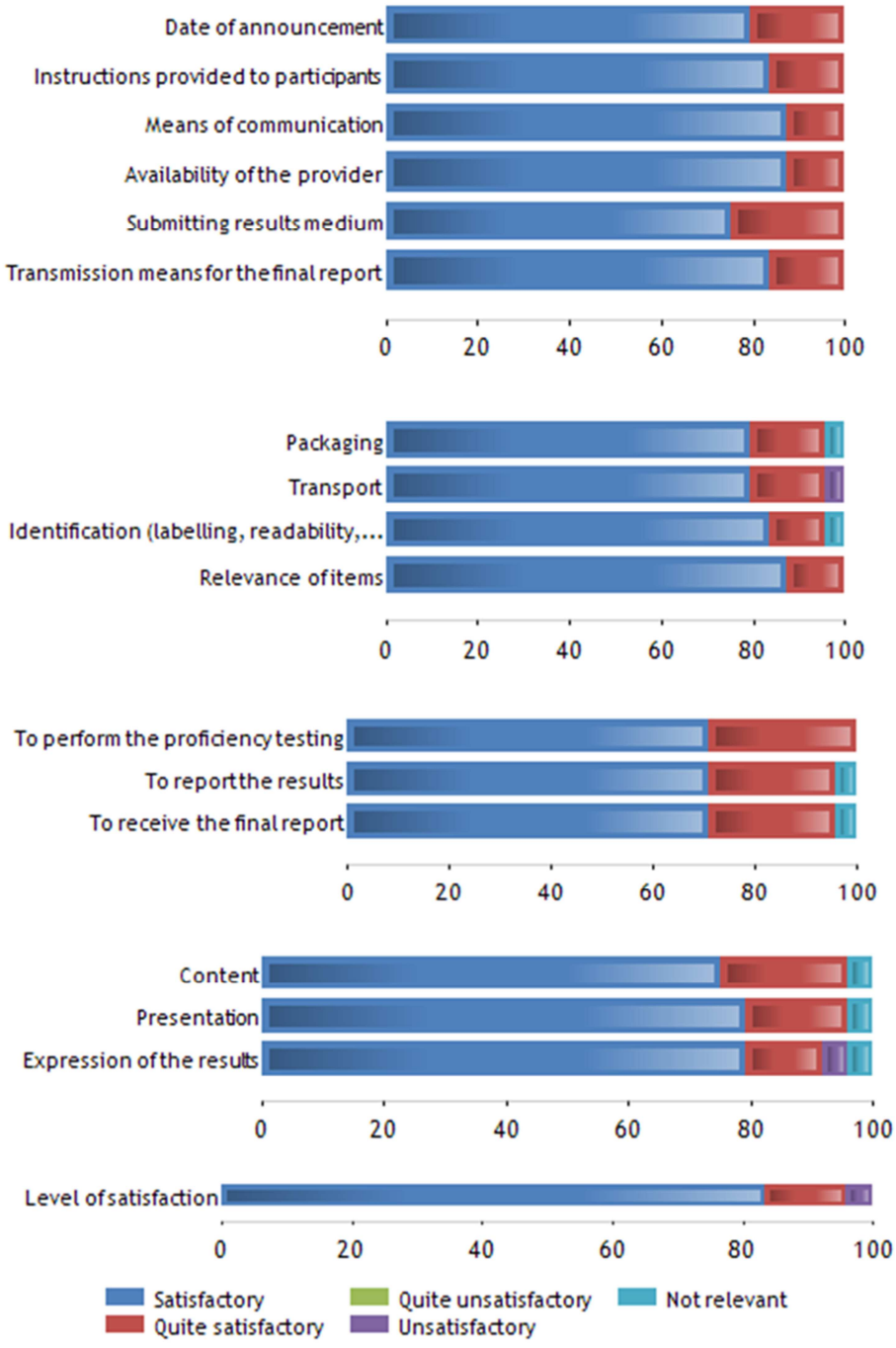

Figure 3: Results of the Anses satisfaction survey regarding the inter-laboratory test for Rabies diagnosis –session 2017 (latest results received on 11/01/2018 corresponding to 24 responses)

Sub Activity 3.2: Collecting data and information on the methods of rabies diagnosis (reference and molecular biology techniques) used by laboratories

Work plan: In parallel to their participation in the inter-laboratory test, the procedures used by Member States for rabies diagnosis (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. On the basis of the inter-laboratory test results and the synthesis of procedures used in Member States, recommendations on key points to consider in each step of the procedures will be included in the inter-laboratory report. The objective of the EURL is to ensure a satisfactory level of rabies diagnosis performance into the EU and to propose recommendations in case of detection of critical gap in the technique used.

Work carried out in 2016 and 2017: 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 provided precious information to highlight mismatch in laboratory procedures and helped 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 for rabies diagnosis: Session 09-2017***" and "***Inter-laboratory test for rabies diagnosis: Session 06-2016***". The reports have been sent to participants on September 2016 and 2017 respectively and are available in the part under restrictive access of the EURL website (https://eurl-rabies.anses.fr/en/minisite/rabies/inter-laboratory-trials).

Sub Activity 3.3: Inter-laboratory test to evaluate serological tests used by NRLs for the follow-up of ORV campaigns

Work plan: The EURL will organise a proficiency test on serological techniques performed by the NRLs by using wildlife samples collected in the field. The main objective of this proficiency test will be to have a global overview of the performances of the techniques and protocols undertaken by the NRLs to titrate the rabies antibodies in wildlife samples. Considering the number of different techniques in use in the EU (seroneutralisation tests and ELISA tests), an ultimate objective for next years would be to try to get a better harmonisation of results obtained by the NRLs.

Work carried out in 2016:

The EURL has organised an inter-laboratory study on serological techniques performed by the NRLs by using wildlife samples collected in the field. The panel was composed of 18 items: eight negative samples and ten positive samples. The naïve samples were obtained from field and caged foxes and caged

raccoon dogs. Moreover 2 naïve serum samples from dog origin, used as reference negative control in the international trade, were added to the panel to validate the specificity of the serological test used by the participating laboratories irrespective of the status and biological state of fox and raccoon dog samples. The positive samples were obtained from caged fox and caged raccoon dog. Each blood sample taken from caged animal (vaccinated or unvaccinated) was divided in two parts to obtain clear serum samples and haemolysed serum samples (obtained by freezing the blood sample). Each sample constituting the panel was previously tested several times by FAVN test and by BioPro ELISA to assess the reliability and the consistency of the panel before aliquoting. Then, the panel was checked for homogeneity and stability. Finally, the panel was sent to participants in October 2016. Two methods of serum titration were used by the participating laboratories either the ELISA test or the seroneutralisation test (FAVN test). For the ELISA test, 13 laboratories took part in this inter-laboratory test and for the seroneutralisation test, 2 laboratories took part in this inter-laboratory test. The technical report has been sent by e-mail to the participating laboratories in January 2017.

Sub Activity 3.4: Inter-laboratory tests to evaluate tetracycline and age determination techniques *(Planned for 2017)*

Work plan: The technique of tetracycline (TTC) and age determination is widely used within the EU in the frame of oral vaccination follow-up. Most of vaccine baits include tetracycline to provide a life-long marking of bones and teeth of the bait consumers. When applying oral rabies vaccination, international institutions (WHO, OIE, EC) recommend controlling the vaccination effectiveness by notably analysing the presence of fluorescence in fox and raccoon dog teeth. To evaluate the performance of NRLs following the first (2010), second (2012) and third inter-laboratory test (2014), a third inter-laboratory test will be conducted.

The different steps of the trials are the followings:

- Contacting all EU laboratories (and possibly some from third countries after consultation and agreement of the EC) to establish a list of interested laboratories;
- Collecting positive and negative reference materials (red fox jaws issued from vaccinated areas);
- Testing half jaws to characterize the sample (positive, negative for TTC, age determination);
- Constituting a panel with the remaining half jaws;
- Distributing a panel of characterised samples for inter-laboratory comparison and validation;
- Collecting the methods used by participating laboratories using an online technical questionnaire;
- Interpreting all results of participating laboratories and analysis of the techniques used, then writing and dispatching a synthesis report.

Work carried out in 2017:

The inter-laboratory comparison started on 02 October 2017. At this date, the samples and result forms were dispatched by an international specialised courier. Six half jaws were sent to participants to evaluate results on tetracycline detection and age determination and 13 laboratories participated in the test. As for previous years, online technical questionnaires were used to collect information on the procedure used by participants for both tetracycline detection and age determination. The survey and statistics software "Sphinx iQ" was used and answer of laboratories were used to estimate the origin of laboratory discrepancies.

The fourth inter-laboratory session has revealed that 12/13 participating laboratories (92%) presented 100% concordant results in the tetracycline detection test. This is the best results ever obtained in inter-laboratory comparison on tetracycline detection. The proportion of discordant results in the 2017 session (1%) was found comparable to 2012 and 2014 (2% and 4%) and significantly lower compared to the proportion of discordant results observed in 2010 (26%) (Figure 4). Considering the age determination of the samples, 9/13 laboratories (69%) estimated a correct age class on the whole panel. This is also the best results ever obtained in inter-laboratory comparison on age determination. Five discordant results (6%) were detected on a total of 78 samples analysed for age estimation while 13% was detected in 2010, 30% in 2012 and in 2014 (Figure 5).

These results demonstrate a constant high level of performance of the laboratories in the detection of tetracycline and a high improvement in age determination. They are encouraging and demonstrate the laboratories capacity and the satisfactory results comparability for bait uptake estimations performed at EU level in the frame of oral vaccination campaigns.

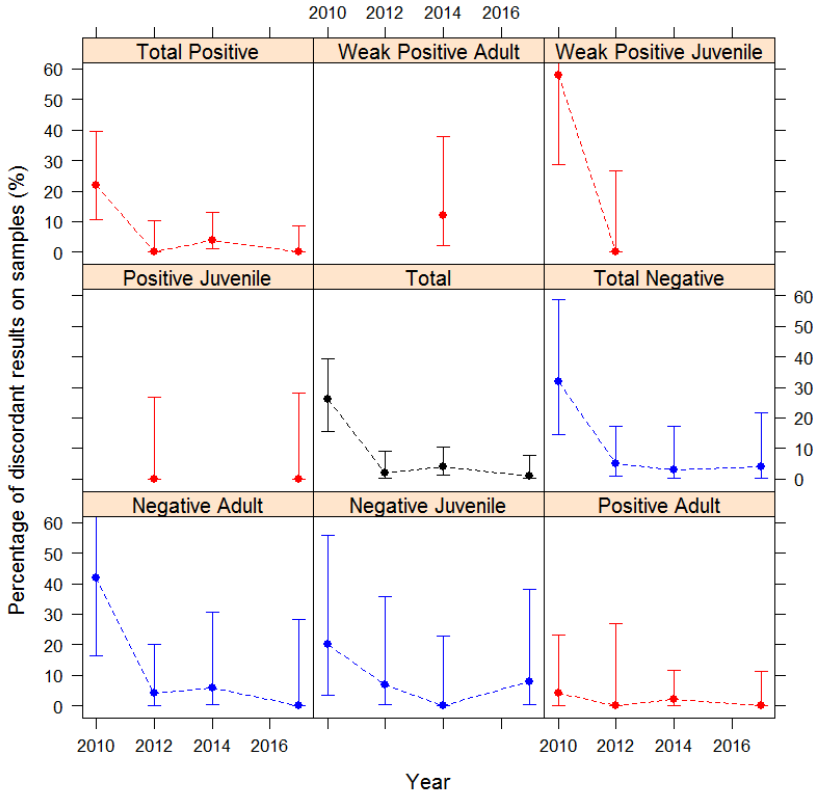

Figure 4: Evolution of discordant results in inter-laboratory comparisons on tetracycline detection by sample category.

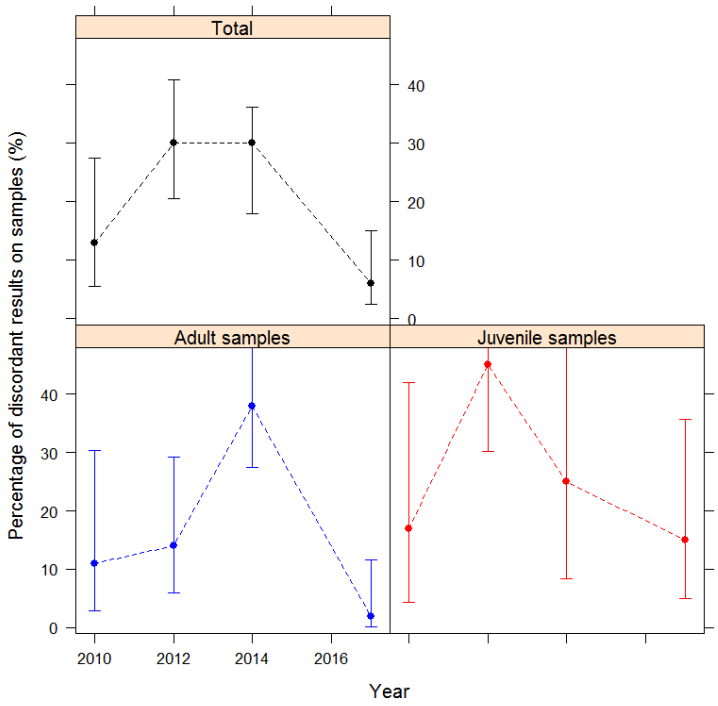

Figure 5: Evolution of discordant results in inter-laboratory comparisons on age determination by true age category.

Sub Activity 3.5: Collecting data on tests carried out in the EC

Work plan: Every year, the EURL will organise an annual survey on tests and analysis performed in each NRL. This will help to evaluate the number of tests performed in EU Member States for diagnosis, typing, virus titration, serology, tetracycline detection and age determination and to report their results at an European level.

Work carried out in 2016 and 2017: An online annual activity questionnaire was sent to all NRLs in February 2016 and 2017 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, analysed and reviews entitled "***Review of the analysis related to rabies diagnosis and follow-up of oral vaccination performed in NRLs in 2016***" and "***Review of the analysis related to rabies diagnosis and follow-up of oral vaccination performed in NRLs in 2015***" have been produced. These documents have been sent by e-mail to the network in June 2016 and 2017 respectively and are available in the part under restrictive access of the EURL website (https://eurl-rabies.anses.fr/en/minisite/rabies/technical-reports).

Activity 4: Meeting, Workshop and Network Management

Sub-activity 4.1: Organising an annual workshop for NRLs

Work plan: On an annual basis, the EURL for rabies organises a workshop for gathering all EU National Reference Laboratories for rabies and several laboratories from certain third countries after consultation and agreement of the EC. The workshop is the opportunity to share information on rabies actualities and on the work that has been carried out during the year. Participants might be invited to deliver a presentation especially for participants from countries where rabies still occurs.

In 2016, the workshop will take place in France in April 2016 (date yet to be determined). The workshop will focus on:

- Proficiency tests for rabies diagnosis: We will present the last NRLs inter-laboratory tests for rabies diagnosis (2015 session). For the first time, the lowest proportion of laboratories with discordant results was found in Real Time PCR and not in the FAT (gold standard technique). Laboratories are more and more performant in molecular biology techniques and definitely more performant with these novel tools than with the RTCIT (which is recommended as a confirmatory test by both OIE and WHO while molecular techniques are still not We would like to open a discussion on such results, the possible change in the designation of reference techniques and their implication in the routine diagnosis of rabies.

- Rabies conjugates: During last summer, the laboratories had to face a shortage of anti-rabies conjugate (Fujirebio conjugate). The new produced batches received by laboratories were not able to detect EBLV strains properly. The information has been shared within the network. We would like to discuss on the impact and the different measures taken in the different NLRs following this incident.
- Presentations from NRLs: Lithuania: After being declared rabies free country according to the OIE criteria, one case has been detected in last October and a second case this month. We will invite the NRL to give an update of the rabies surveillance situation in Lithuania. Norway: We will invite the NRL from Norway to discuss on the first detection of a bat rabies strain on their territory (EBLV-2 isolated in 2015, confirmed by the EURL). Romania: Romanian NRL has isolated a rabies vaccine induced case in a cow (case confirmed by the EURL). This is the first identification on such case on a domestic animal (cow). We would like to invite our Romanian colleague to discuss on this particular case. France: Two significant events have occurred in 2015 on the French territory. The coming workshop will be the opportunity to share information on these two events: rabies detection in a dog in Guyana and rabies case importation from Algeria (via Hungary).

The progress in the evaluation of the rabies qPCR techniques performed in the EURL in 2015 will also be presented. Recent rabies activities (laboratory techniques and rabies surveillance) will also be presented by some participating laboratories and discussed within the network.

In spring 2017, the workshop will take place in Spain and will focus on:

- Proficiency tests for rabies serology: In 2016 will be held the first inter-laboratory test on the serological techniques used for the follow-up of oral rabies vaccination (ORV) campaigns. Currently, a wide range of techniques and material are used to assess the herd immunity level in the ORV targeted population. The 2017 workshop will be the opportunity to conclude on this study and on the comparability of the techniques. Further steps to investigate, if any, will be debated with all NRLs.
- Proficiency tests for rabies diagnosis: WHO is currently opening a discussion to potentially include the real Time PCR as a reference technique (WHO meeting of 09 December 2015). We would like to discuss on this matter and to share on WHO expert meeting outcomes in 2017.

  Presentations from NRLs: In the 2017 workshop will be discussed the key points of the rabies laboratory activities and the significant rabies events of 2016.
- Work programme 2018-2019: Because it will be the end of the two year program of the EURL, including the end of the real time PCR evaluation project, 2017 workshop would be the opportunity to discuss on the new challenges in rabies diagnosis and techniques of ORV follow-up, needs and collaborative study opportunities of the laboratory network.

Work carried out in 2016:

The eighth workshop for rabies was held on 16 June 2016 in Strasbourg, France. The annual rabies EURL meeting gathered a total of 44 participants, among them 30 from the Member States and 10 from the non-European Union bordering countries (countries bordering EU countries).

As usual, the meeting gave the opportunity for rabies scientists to meet all together ending with fruitful exchanges.

A file including a booklet containing the abstracts of the talks, the list and details of each participant, the agenda, documentation on Strasbourg, and practical details has been provided to every participant.

The total number of participants was in decrease compared to the previous years. The attendant rate of both EU and EU bordering countries consequently proportionally lowered. This can be explained because the bordering countries did not include the North African countries as it used to be the previous years.

Additionally to the representative of the European Commission, a representative of the DG Santé (Unit D4 – Food safety programme, emergency funding) (G5 Unit of EC DG Santé), the EURL team was pleased to welcome for the first time and also representatives speakers from the Food and Agriculture Organization of the United Nations (FAO) and from the French Directorate General for Food (DGAl) attended the meeting and presented a talk. The analysis of the results of in the proficiency tests-laboratory tests for rabies diagnosis organized in 2015 by the EURL for rabies was presented with an open discussion on the subject. The EURL for rabies also presented on one hand a performance study of disinfectants used in the context of molecular biology techniques, and on the other hand the progress of the study on the evaluation methods of Real Time RT-PCR. The Western Balkan region was in the spotlight this during this workshop year, and several laboratories attended the meeting (from Albania, Bosnia and Herzegovina, Republic of Kosovo, Former Yugoslav Republic of Macedonia and Serbia) as well as other eastward countries such as Moldova, Ukraine and Turkey. Each of them presented both the rabies situation and the rabies programmes in progress in their country.

Summary of the workshop and results of satisfaction questionnaires filled in by participants during these days have been reported in the document entitled **"8th workshop for rabies report"**. Agenda and abstract of the presentations were available in the booklet provided during this workshop. Workshop booklet, workshop report and support of presentations are available online in the part under restrictive access of the EURL website (https://eurl-rabies.anses.fr/en/minisite/rabies/workshops).

At the end of the meeting day, a satisfaction questionnaire was distributed to participants to explore satisfaction of NRLs regarding the different EURL tasks and potential improvement proposals.

In 2016, 78% of the surveyed laboratories answered the questionnaire (31 questionnaires collected). Three to five questions were asked per topic concerned, excepted for the workshop with 8 questions (yes/no answer). Global satisfaction reached 100% (Figure 6).

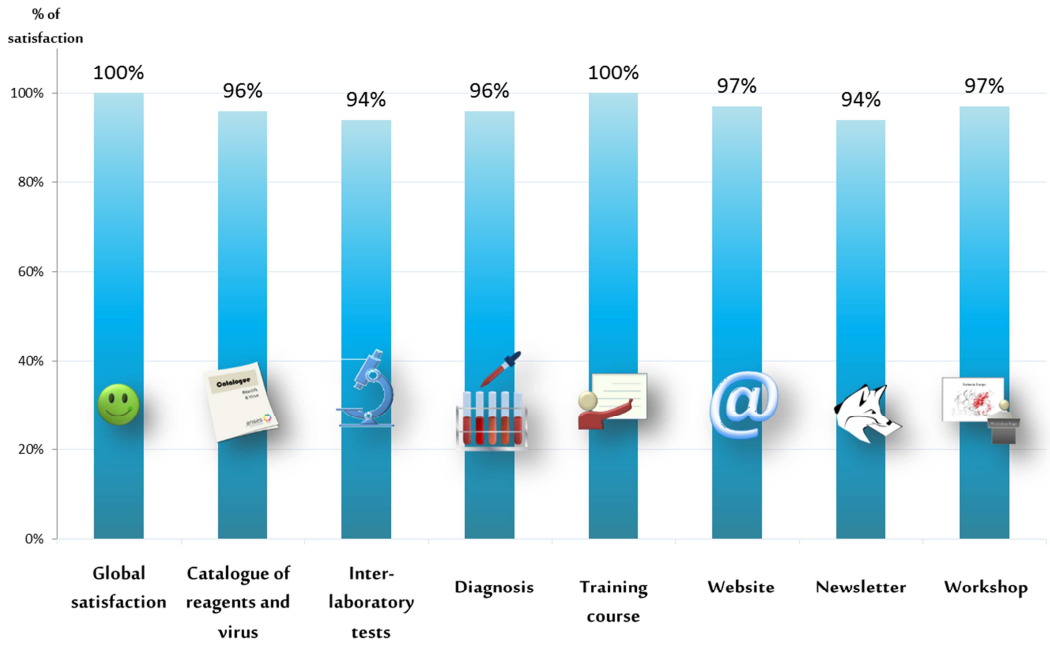

Figure 6: Results of the rabies EURL satisfaction survey - 2016 (31 participant laboratories)

Work carried out in 2017:

The 9th workshop for rabies was held on 13 and 14 June 2017 in Budapest, Hungary. The initial workshop localisation announced in Spain was not realised because of financial aspect. The annual rabies European Union Reference Laboratory (EURL) meeting gathered a total of 89 participants, among them 38 from the European Union Member States (EUMS) and 11 from European Union bordering countries supported by TAIEX and 30 from other laboratories from all over the world. As usual, the meeting gave the opportunity for rabies scientists to meet all together ending with fruitful exchanges. This year, the meeting was held just before the European Union for rabies serology meeting, and scientists of this second meeting were invited to join the 9th workshop for rabies explaining the substantial increase in the proportion of participants out of EU members states compared to the previous years (Figure 7).

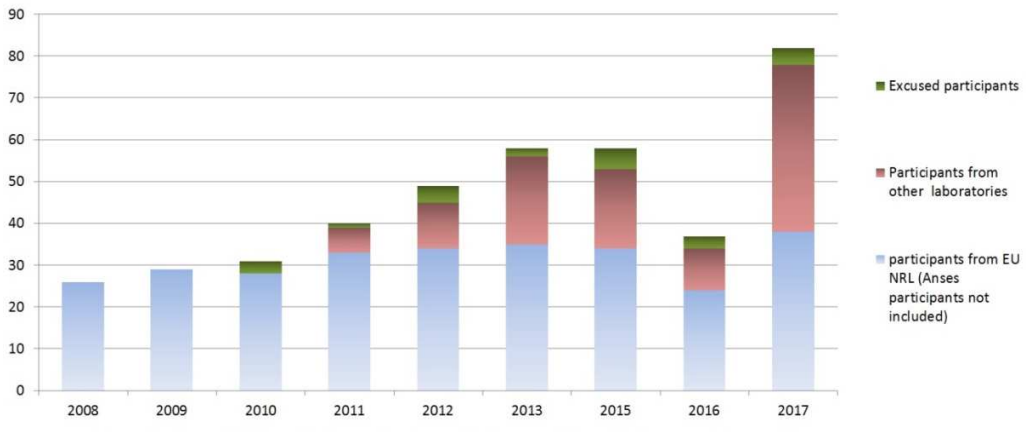

Figure 7: Evolution of the number of workshop participants

Additionally to the representative of the European Commission (EC), a representative of the DG Health and Food Safety (Unit D4 – Food safety programme, emergency funding), a second representative of the EC (DG Health and Food Safety, Ireland) participated in the meeting. The EURL team was also pleased to welcome the OIE (World Organisation for Animal Health) and a representative from the Food and Agriculture Organization of the United Nations (FAO) who attended the meeting and presented a talk.
In 2017, the EURL invited the wildlife biologist Dr Chipman from United States Department of Agriculture to introduce to the audience challenges of serologic analysis in wild meso-carnivores for monitoring oral rabies vaccination program in the U.S. The presentations provided by the EURL team were related to 2016 rabies diagnosis proficiency test results, first inter-laboratory comparison on wildlife serological tests, update on impending modifications in OIE manual and results on qPCR methods evaluation:

- *Robardet E, Servat A, Picard-Meyer E, Wasniewski M, Cliquet F. Proficiency test on rabies diagnosis/ 2016 session results and outcome of satisfaction questionnaires.*
- *Wasniewski M, Cliquet F. First inter-laboratory study based on the rabies antibody detection in wildlife samples in Europe.*
- *Servat A, Picard-Meyer E, Cliquet F. Update on new rabies diagnosis techniques.*
- *Picard-Meyer E, Peytavin de Garam C, Robardet E, Cliquet F. Comparisons of different real-time PCR methods: Results of the evaluation of SYBR Green and TaqMan RT-PCR*.

Rabies surveillance system, epidemiological situation and control programme in progress were presented by NRLs from Poland, Greece and Slovakia. Colleagues from the German NRL also presented a comparative study on different rapid diagnosis kits.

At the end of the meeting day, a proposed workprogamme was discussed and it was notably decided to change the frequency of rabies diagnosis proficiency test from annual to one test every two years and to launch an inter-laboratory test on rabies rapid diagnosis kit in 2018. A satisfaction questionnaire with potential improvement proposals was distributed to participants to explore satisfaction of NRLs regarding

the workshop organisation but also for the different EURL activities. In 2017, 61% of the participants answered the questionnaire (54 questionnaires collected). Globally, 100% of satisfaction was declared for workshop organisation, destination, conference centre choice and timing. Evaluation of documentation provided reach 96% while scientific content 88% of satisfaction (Figure 8: one question per item, answers yes, no or NA). Global satisfaction of EURL activities reached 100% and all the topics evaluated had satisfaction evaluation up to 94% (Figure 9: Three to five questions asked per topic concerned, answers yes, no or NA).

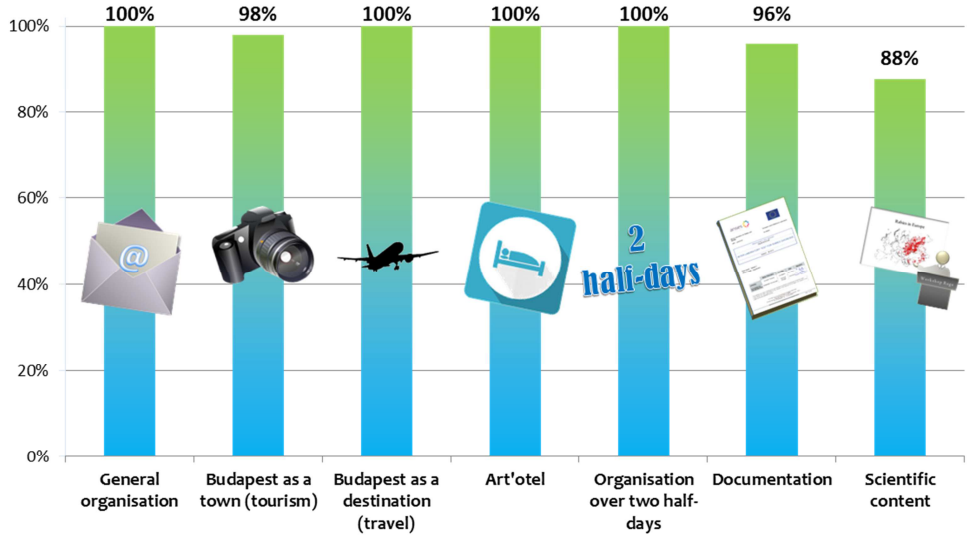

Figure 8: Results of the rabies EURL satisfaction survey on the 9th workshop for rabies

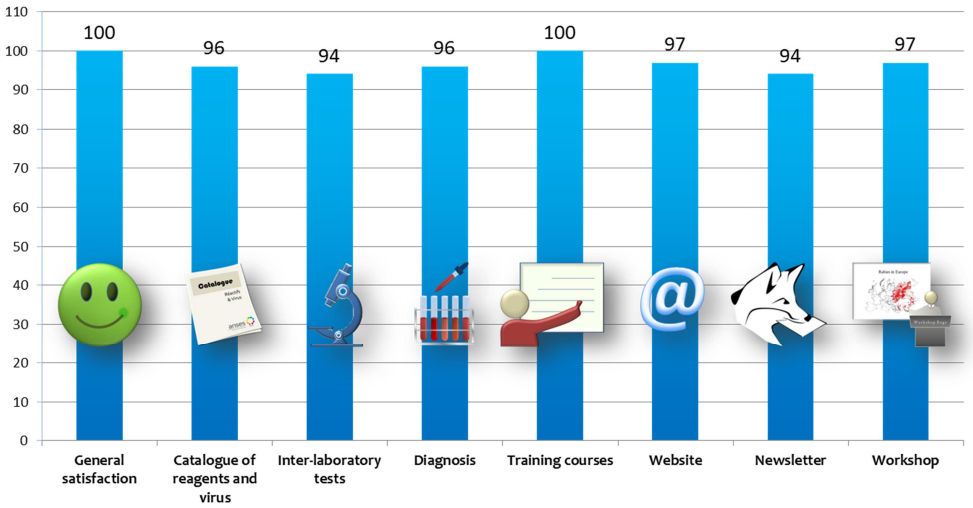

Figure 9: Results of the 2017 rabies EURL satisfaction survey

Summary of the workshop and results of satisfaction questionnaires filled in by participants during these days have been reported in the document entitled **"9th workshop for rabies report"**. Agenda and abstract of the presentations were available in the booklet provided during this workshop. Workshop booklet, workshop report and support of presentations are available online in the part under restrictive access of the EURL website (https://eurl-rabies.anses.fr/en/minisite/rabies/workshops).

Sub-activity 4.2: Keeping abreast of development in surveillance, epidemiology and prevention of rabies throughout the world

Work plan: The EURL will attend and participate in the annual international rabies conference in 2016 and in 2017 (RITA congress) to share its experience in epidemiology and virology in regards to rabies.
The EURL will also provide the European Commission and NRLs with scientific advice and technical assistance at his request.

Work carried out in 2016: One expert of the EURL for rabies participated in the "Rabies in the Americas (RITA)" congress that held in Belem (Brazil) from 23th to 28st October 2016 (http://rita2016.iec.gov.br/rita2016/)
The RITA congress is an annual event that has been held since 1990 hosted in many countries across the Americas. To date, this is the only worldwide rabies expert meeting. The congress provides the 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 a poster presentation entitled "Animal Welfare: the 3R's applied to rabies across the years (27th International Meeting of Rabies in the Americas (RITA), 2016).

Work carried out in 2017: Two experts of the EURL for rabies participated in the "28th Rabies In the Americas meeting" that has been held in Calgary, Canada, from 22 to 26 October 2017 (www.ritaconference.org).
The RITA congress is an annual event that has been held since 1990 hosted in many countries across the Americas. To date, this is the only worldwide rabies expert meeting. The congress provides the 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 present two posters entitled *"Robardet E., Rieder J., Servat A., Picard-Meyer E., Cliquet F. Performance evaluation of laboratories in rabies diagnosis test: Which techniques lead to the most reliable results in practice?"* and *"Servat A, Picard-Meyer E, Cliquet F. Update on new rabies diagnosis techniques" and an oral*

presentation entitled: "Robardet E., Borel C., Moinet M., Jouan D., Wasniewski M., Barrat J., Boué F., Monchâtre-Leroy E., Servat A., Gimenez O., Cliquet F, Picard-Meyer E., Long-term population surveys of two serotine bat (Eptesicus serotinus) colonies exposed to EBLV-1 (European Bat Lyssavirus Type 1): assessment of rabies transmission using capture-recapture models".

Sub-activity 4.3: Website management

Work plan: An internet website dedicated to the NRLs network went online in 2014. The website is hosted at https://eurl-rabies.anses.fr and allows consultation of news and events dealing with rabies in the EU, the NRL network presentation, the EURL activities and reports, workshop presentations, including the work programmes and technical reports. Each NRL has received a login and password giving an access to the documentation, training list, reagent catalogue, etc...
The website will be regularly updated and a monthly newsletter including the news of the website will be prepared and sent to the NRL network.

Work carried out in 2016 and 2017: The website has been updated each month by including the news of the network, agenda of activities and reports. A newsletter, linked with these activities was also produced and sent to the network monthly.
The newsletter includes news related to the EURL activities, news from laboratories & International Institutions, a focus on rabies activities of a NRL network member, agenda of the forthcoming international events, meetings linked to rabies, rabies notifications in Europe (Relayed from Pro-MED and Ec.europa.eu website) and a selection of last month edited publications on laboratory techniques and epidemiology in Europe regarding rabies.

Activity 5: Research Activities

Sub-activity 5.1: Comparison and evaluation of different Real Time PCR methods

Work plan:

The EURL demonstrated in 2015 an identical sensitivity of detection for six one-step TaqMan qRT-PCR kits currently used in NRLs, regardless of the machine used. We found a limit of detection at 95% (LOD95%) of approximately 20 copies/µl of RNA for the 6 tested kits, using validated rabies primers and TaqMan probe. Based on these previous results, we propose to continue the comparison of real-time PCR methods with the evaluation of four published primers/labelled probes currently used in laboratories for the amplification of specific RABV. In a first step, we will determine the performance of the method (LOD95%) using two synthetic RNA controls (a Greek fox isolate and the fixed rabies strain CVS-27), the four selected couples of primers/TaqMan probes and the most efficient one-step Taqman kit (RNA

UltraSense). Following this preliminary study, the specificity of the four couples of primers/probes will be undertaken on a collection of rabies virus held in the EURL laboratory. An additional collection of rabies virus fixed on FTA paper will be performed and will complete the specificity study.

The overall objective of this activity is to ensure the development of new high quality/state of art analytical methods for possible use in a next future for routine rabies diagnosis, as such methods are still not recommended as reference tests.

Work carried out in 2016:

Comparison of TaqMan RT-PCR methods with the evaluation of six published models of primers and labelled probes was performed. These models are currently used in the National Reference Laboratories for the specific amplification of RABV. The six studied models were the models respectively described by: Wakeley et al., in 2005, Hoffman et al., in 2010, Nadin-Davis et al., in 2009 and Orlowska et al., in 2008.

The study was undertaken with the RNA UltraSenseTM One-step Quantitative RT-PCR kit (Invitrogen Life Technologies, France) shown as the most efficient kit in our previous study undertaken on the TaqMan kits comparison (LOD95% of 18 copies/µL of RNA). Four synthetic RNAs (1353-bp) were generated for the determination of the most efficient model of primers/probe using molecular genetic methods. The RABV isolates selected for this study were: a laboratory fixed strain CVS-27, a vaccine strain SAD B19, a field strain GS7 isolated from a fox in France in 1986 and a field strain isolated from a fox in Greece in 2012.

RT-PCR was optimised for each assay regarding the concentrations of primers/probe and the thermocycling conditions. All assays were performed in triplicates using seven ten-fold dilutions (i.e., 106 to 1 copies/µL) of each RNA controls for generating standard curves, reaction efficiency and the limit of detection for each assay.

Of the six validated models and the four synthetic RNAs tested, the Wakeley model yielded greater sensitivity and resulted in a detection of at least 5 copies/µL regardless of the RNA control tested. The study demonstrated that the optimised assays performed with the models described by Hoffman and Nadin Davis yielded good sensitivity with the detection of at least 100 copies/µL of RNA. The Orlowska's model yielded a detection between 25 copies/µL and 104 copies/µL for the amplification of fixed strains and field isolates, respectively. The results showed that the PCR sensitivity varied among the six TaqMan models tested and the four synthesized RNA controls tested, showing the influence of the primers/probe as well as the positive RNA control used in the real-time PCR. All studied models of primers/probe enable the detection of RABV, albeit not with the same sensitivity.

In addition to the study of sensitivity, we started in 2016 the study of specificity for the six published models. Preliminary results performed on rabies isolates from the North East of Europe showed the amplification of all tested isolates regardless of the model tested, with an identical sensitivity expressed in number of copies/µL of RNA for all assays.

Work carried out in 2017:

The evaluation of six TaqMan qRT-PCR methods currently used in NRLs for the specific amplification of the classical rabies virus (RABV) was completed in 2017 with a study of specificity. The study was performed with the six published models of primers and labelled probes evaluated in 2016 in terms of the sensitivity. The six studied models were the models respectively described by: Wakeley et al., in 2005 (1 model), Hoffman et al., in 2010 (1 model), Nadin-Davis et al., in 2009 (3 models) and Orlowska et al., in 2008 (1 model). All models amplify a part of the nucleoprotein gene. Briefly, we previously showed that the six models of primers/probe enable the detection of RABV, albeit not with the same sensitivity of detection. Of the six models tested, the Wakeley model yielded greater sensitivity with a limit of detection of PCR of at least 5 copies/µL, while the sensitivity of the 5 other models varied between 25 copies/µL and 10^4 copies/µL (Orlowska's model). Preliminary results performed on rabies isolates from the North East of Europe showed the amplification of all tested isolates, irrespective of the model tested.

The study of specificity was performed with negative (n=35) and positive samples (n=113) isolated in different parts of the world. The panel of positive samples selected for the study were constituted by RABV (n=105) and 8 *Lyssaviruses* spp. isolated in bats, that are: EBLV-1 (n=3), EBLV-2 (n=1), ABLV (n=1), BBLV (n=1), DUVV (n=1) and LLEBV (n=1). Positive samples were from Europe (n=53), Asia (n=29), Africa (n=20) and the Americas (n=3). The positive samples were obtained from the collection of isolates held in the EURL laboratory (n=85) and from a specific collection established for the study (n=27). All the 27 samples were impregnated on FTA paper to inactivate the infectivity of samples, this technology being previously validated as a mean to store rabies nucleic acids. Fixed samples on FTA paper were classically sent by normal routes to the EURL Laboratory for analysis.
The study was undertaken with the RNA UltraSense™ One-step Quantitative RT-PCR kit (Invitrogen Life Technologies, France) shown as the most efficient kit in our previous study undertaken on the TaqMan kits comparison (LOD95% of 18 copies/µL of RNA).

Of the six models tested, the Nadin-Davis model N°1 (RABVD1 forw/RABVD1 rev/TQ RABVD1) yielded the greater results with 100% of specificity. The 105 positive samples tested were amplified, regardless of the origin of isolation. While the Wakeley model (JW12/N165-N146/LysGT1) yielded the greater sensitivity of detection out of the six models tested, it gave in the study only 99% of specificity (104 samples amplified/105). All tested samples were amplified, except one sample from Africa. This sample isolated from a dog in 1991 in Tunisia was amplified with the five other models tested. The two Nadin-Davis models -N°2 and N°3- gave 91% and 93% of specificity, respectively. The Nadin-Davis N°2 model did not amplify samples from Nigeria (5 negative samples/5 tested) and did not detect 2 out of 7 samples from Azerbaijan, 1 out of 5 samples from Bulgaria and did not detected a sample from Serbia. The Nadin-Davis N°3 model did not detect 7 out of 10 viruses from Taiwan. The Orlowska and Hoffmann models gave the lowest specificity with 77% and 74% of specificity, respectively. The Orlowska model did not amplify RABV from Mali, Nigeria and Sri Lanka and detected only 1 out of 3 samples from Brazil and 8 samples out of 10

samples from Taiwan. The Hoffmann model did not detect RABV from Mali, Nigeria and Taiwan, and detected only 4 out of 9 samples from Sri Lanka. The study of specificity demonstrated that all samples from Europe were amplified irrespective of the model used, excepted for one model (Nadin-Davis N°2) on two samples (Bulgaria and Serbia). All negative samples showed negative results, irrespective of the model used, while the testing of the lyssaviruses species isolated in bats showed false positive results irrespective of the models used. The Nadin-Davis N°1 model detected ABLV with a Ct of 15.65 ± 0.06 but did not detect the other *Lyssaviruses* spp. The models Wakeley, Orlowska and Hoffmann detected BBLV and DUVV. The Nadin-Davis N°2 model detected EBLV-1 and EBLV-2 (Ct value of 20.4) while the Nadin-Davis N°3 model detected all *Lyssaviruses* spp. tested except LLEBV.

In conclusion, all RABV samples from Europe were detected by TaqMan RT-PCR, irrespective of the model of primers/probe used, showing that all the six models tested here are suitable. Although not yet recommended for routine post-mortem diagnosis of rabies due partly to the high sensitivity of the real-time method, real-time RT-PCR methods yield the advantage to be rapid, sensitive and a useful technique for rabies diagnosis. The combination of SYBR Green and TaqMan RT-PCR assays, represent a useful method for the rapid amplification of all *Lyssaviruses* RNA and for their rapid characterization.

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

1. Research programmes and cooperation projects

The laboratory took part in different research and cooperation projects on rabies epidemiology in countries infected with canine and wildlife rabies.

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

From the beginning of 2016, 8 training courses counting a total of 10 participants were organised 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	Number of participants	Laboratory	Training title	Training place	Starting date	Ending date
Morocco	1	ONSSA – Service du Contrôle et des Expertises – Division de la Pharmacie et des Intrants Vétérinaires – Direction des Services Vétérinaires	Training course on rabies diagnosis (RTCIT) and molecular biology techniques (PCR, qPCR)	ANSES Nancy	29/02/2016	11/03/2016
Morocco	1	ONSSA - Division de la Pharmacie et des Intrants Vétérinaires – Diagnostic de la rage et de l'influenza aviaire	Training course on rabies diagnosis (RTCIT) and molecular biology techniques (PCR, qPCR)	ANSES Nancy	29/02/2016	11/03/2016
United Kingdom	2	APHA Weybridge	FAVN Training	ANSES Nancy	18/04/2016	21/04/2016
Tunisia	1	National Center of Zoosanitary Vigilance	Epidemiological analysis of rabies	ANSES Nancy	09/05/2016	13/05/2016
Croatia	1	Croatian Veterinary Institute – Laboratory for rabies and general virology	Training course on molecular biology techniques (PCR, qPCR) and FAVN test	ANSES Nancy	23/05/2016	06/06/2016

Estonia	1	Veterinary and Food Laboratory	Training course on molecular biology (RT-PCR)	ANSES Nancy	27/06/2016	01/07/2016
Ukraine	1	State Science Control Institute of Biotechnology and Strains of Microorganisms	Training course on rabies vaccine titration	ANSES Nancy	22/08/2016	26/08/2016
Luxembourg	2	Laboratoire de Médecine Vétérinaire de l'Etat	Training course on rabies diagnosis (FAT)	ANSES Nancy	21/11/2016	23/11/2016

From the beginning of 2017, 6 training courses were organised in the ANSES Nancy laboratory in the frame of the rabies activities (Rabies diagnosis, typing, virus production and titration, cell culture maintenance, serology, tetracycline detection and epidemiology, etc.):

Number of participants	**Country**	**Subject of the training**	**Dates**
1	United Kingdom	Rabies epidemiology and modelling	from 26/01 to 31/01/2017
1	Romania	Molecular biology and sequencing	from 01/02 to 03/04/2017
1	Argentina	Training course in a foreign laboratory as part of the veterinary studies course- All rabies activities	from 20/02 to 24/03/2017
1	Sri-Lanka	CVS-11 virus production, cell culture and FAVN test	from 27/03 to 14/04/2017
1	Sri-Lanka	Training course on molecular sequencing of Lyssavirus	from 15/05 to 19/05/2017
1	Niger	CVS-11 virus production, cell culture and FAVN test	from 18/08 to 29/09/2017

3. Participation and presentation in international congress and meetings

1. **Wasniewski M**, Labadie A, Rieder J, Schereffer JL, **Cliquet F**. Rabies serology proficiency tests: compliance with the ISO/CEI 17043 international standard. Rabies Serology Meeting; 14-15 June 2017; Budapest, Hungary..

2. Laurentie M, **Wasniewski M**, **Cliquet F**. Assessment of the laboratories and statistical approach in proficiency testing: how combine international standard and statistic. Rabies Serology Meeting; 14-15 June 2017; Budapest, Hungary..
3. Klein H, **Wasniewski M**, Labadie A, Rieder J, Tribout L, **Cliquet F**. Survey about the activities of approved rabies testing laboratories with regard to movements of dogs, cats and ferrets from non EU-countries. Rabies Serology Meeting; 14-15 June 2017; Budapest, Hungary..

4. **Cliquet F**, **Servat A**, **Wasniewski M**. Proficiency testing and rabies serology as alternatives to quarantine: overview of the key steps in Europe. Rabies Serology Meeting; 14-15 June 2017; Budapest, Hungary..

5. **Cliquet F**. Campagnes de vaccination – Monitoring post-vaccination – Prise en compte des chiens difficiles. Atelier sur le programme national de lutte contre la rage animale du ministère de l'agriculture en Tunisie : remontée des équipes de terrain, état des lieux et voies d'amélioration. 25-26 September 2017, Tunis, Tunisia.

6. **Cliquet F**. Dog rabies control Vaccination of dogs Pasteur Institute International Workshop on Surveillance and Control of Rabies, 08-19 October 2017, Tehran, Iran.

7. **Wasniewski M**., Laurentie M., **Cliquet F**. Assessment of the laboratories and statistical approach in rabies serology proficiency testing: how to combine international standard and statistics? 9th Eurachem workshop on Proficiency testing in Analytical Chemistry, Microbiology and Laboratory Medicine, 09 - 12 October 2017, Portoroz, Slovenia.

8. **Cliquet F**. Private expert at EU Task Force on rabies eradication, 02 - 04 October 2017, Bucharest, Romania.

9. **Robardet E**., Borel C., Moinet M., Jouan D., **Wasniewski M**., Barrat J., Boué F., Montchâtre-Leroy E., **Servat A**., Gimenez O., **Cliquet F**., **Picard-Meyer E**. Long-term population surveys of two serotine bat (*Eptesicus serotinus*) colonies exposed to EBLV-1 (European Bat Lyssavirus Type 1): assessment of

rabies transmission using capture-recapture models. 28th Rabies In the Americas meeting, 22 - 26 October 2017, Calgary, Canada.

10. **Cliquet F**. Rabies: virus characteristics, pathogenesis and diagnosis. Workshop on rabies eradication in wildlife: Oral rabies vaccination (ORV) campaigns, 08 - 10 November 2017, Belgrade, Serbia.

11. **Cliquet F**. Vaccines for wildlife, domestic animals and prevention procedures in humans. Workshop on rabies eradication in wildlife: Oral rabies vaccination (ORV) campaigns, 08 - 10 November 2017, Belgrade, Serbia.

12. **Cliquet F**. The EU co-financing system for eradication programs (EU regulations, EC decisions, templates for program submission, approval of programs, Task Force, list of indicators, guidance document, how to obtain the status of free Country). Workshop on rabies eradication in wildlife: Oral rabies vaccination (ORV) campaigns, 08- 10 November 2017, Belgrade, Serbia.

13. Englund L., **Cliquet F**. Overview report of SANTE recent audits on rabies eradication in the EU. Workshop on rabies eradication in wildlife: Oral rabies vaccination (ORV) campaigns, 08 - 10 November 2017, Belgrade, Serbia.

14. **Robardet E**., **Cliquet F**. Rabies Surveillance and Control: The EU Experience Rabies surveillance – Local workshop. 10 November 2017, Taichung, Taiwan.

15. **Robardet E**., **Servat A**., **Picard-Meyer E**., **Wasniewski M**., **Cliquet F**. The Application of antigen & antibody diagnostic techniques in wildlife rabies management in the EU. APEC Meeting, 13-17 November 2017, Taipei, Taiwan.

4. International publications related to rabies published in 2016 and 2017

5. Darkaoui S., Fassi Fihri O., Schereffer J.L., Aboulfidaa N., **Wasniewski M**., Zouine K., Bouslikhane M., Id Sidi Yaga K. and **Cliquet F**.- Immunogenicity and efficacy of Rabivac vaccine for animal rabies control in Morocco. Clin. Exp. Vacc. Res., 2016, 5, pp 60-69.

6. Ribadeau-Dumas F., **Cliquet F**., Gautret P., **Robardet E**., Le Pen C. and Bourhy H.- Travel-associated rabies in pets and residual rabies risk, Western Europe. Emerg. Infect. Dis., 2016, 22, pp 1268-1271.

7. **Robardet E**., **Picard-Meyer E**., Dobrostana M., Jaceviciene I., Mahar K., Muizniece Z., Pridotkas G., Masiulis M., Niin E., Olsevskis E. and **Cliquet F**.- Rabies in the Baltic States: Decoding a process of control and elimination. PLoS Negl. Trop. Dis., 2016, 10, p e0004432.

8. Traore A., **Picard-Meyer E**., Mauti S., **Biarnais M**., Balmer O., Samaké K., Kamissoko B., Tembely S., Sery A., Traore A.K., Coulibaly A.P., **Robardet E**., Zinsstag J. and **Cliquet F**.- Molecular characterization of canine rabies virus, Mali, 2006–2013. Emerg. Infect. Dis., 2016, 22, pp 866-870.

9. Vuta V., **Picard-Meyer E**., **Robardet E**., Barboi G., Motiu R., Barbuceanu F., Vlagioiu, C. and **Cliquet F**.-Vaccine-induced rabies case in a cow (Bos taurus): Molecular characterisation of vaccine strain in brain tissue. Vaccine, 2016, 34, pp 5021-5025.

10. **Wasniewski M**., Almeida I., Baur A., Bedekovic T., Boncea D., Chaves L., David D., De Benedictis P., Dobrostana M., Giraud P., Hostnik P., Jaceviciene I., Kenklies S., Konig M., Mahar K., Mojzis M., Moore S., Mrenoski S., Muller T., Ngoepe E., Nishimura M., Nokireki T., Pejovic N., Smreczak M., Strandbygaard B., Wodak E. and **Cliquet F**.- First international collaborative study to evaluate rabies antibody detection method for use in monitoring the effectiveness of oral vaccination programmes in fox and raccoon dog in Europe. J. Virol. Methods, 2016, 238, pp 77-85.

11. **Picard-Meyer E**, **Servat A**, **Wasniewski M**, Gaillard M, Borel C, **Cliquet F**. Bat rabies surveillance in France: first report of unusual mortality among serotine bats. BMC Vet Res. 2017 Dec 13;13(1):387. doi: 10.1186/s12917-017-1303-1. PMID: 29237469

12. Fooks AR, **Cliquet F**, Finke S, Freuling C, Hemachudha T, Mani RS, Müller T, Nadin-Davis S, **Picard-Meyer E**, Wilde H, Banyard AC. Rabies. Nat Rev Dis Primers. 2017 Nov 30;3:17091. doi: 10.1038/nrdp.2017.91. Review. PMID: 29188797

13. Troupin C, **Picard-Meyer E**, Dellicour S, Casademont I, Kergoat L, Lepelletier A, Dacheux L, Baele G, Monchâtre-Leroy E, **Cliquet F**, Lemey P, Bourhy H. Host Genetic Variation Does Not Determine Spatio-Temporal Patterns of European Bat 1 Lyssavirus. Genome Biol Evol. 2017 Nov 1;9(11):3202-3213. doi: 10.1093/gbe/evx236. PMID: 29165566

14. **Robardet E**, Borel C, Moinet M, Jouan D, **Wasniewski M**, Barrat J, Boué F, Montchâtre-Leroy E, **Servat A**, Gimenez O, **Cliquet F**, **Picard-Meyer E**. Longitudinal survey of two serotine bat (*Eptesicus serotinus*) maternity colonies exposed to EBLV-1 (European Bat Lyssavirus type 1): Assessment of survival and serological status variations using capture-recapture models.PLoS Negl Trop Dis. 2017 Nov 17;11(11):e0006048. doi: 10.1371/journal.pntd.0006048. eCollection 2017 Nov.PMID: 29149215

15. Hsu AP, Tseng CH, Barrat J, Lee SH, Shih YH, **Wasniewski M**, Mähl P, Chang CC, Lin CT, Chen RS, Tu WJ, **Cliquet F**, Tsai HJ. Safety, efficacy and immunogenicity evaluation of the SAG2 oral rabies vaccine in Formosan ferret badgers. PLoS One. 2017 Oct 4;12(10):e0184831. doi: 10.1371/journal.pone.0184831. eCollection 2017. PMID: 28977009

16. Maki J, Guiot AL, Aubert M, Brochier B, **Cliquet F**, Hanlon CA, King R, Oertli EH, Rupprecht CE, Schumacher C, Slate D, Yakobson B, Wohlers A, Lankau EW. Oral vaccination of wildlife using a vaccinia-rabies-glycoprotein recombinant virus vaccine (RABORAL V-RG®): a global review. Vet Res. 2017 Sep 22;48(1):57. doi: 10.1186/s13567-017-0459-9. Review. PMID: 28938920

17. Moldal T, Vikøren T, **Cliquet F**, Marston DA, van der Kooij J, Madslien K, Ørpetveit I. First detection of European bat lyssavirus type 2 (EBLV-2) in Norway. BMC Vet Res. 2017 Jul 11;13(1):216. doi: 10.1186/s12917-017-1135-z. PMID: 28693578

18. Seo W, **Servat A**, **Cliquet F**, Akinbowale J, Prehaud C, Lafon M, Sabeta C. Comparison of G protein sequences of South African street rabies viruses showing distinct progression of the disease in a mouse model of experimental rabies. Microbes Infect. 2017 Sep - Oct;19(9-10):485-491. doi: 10.1016/j.micinf.2017.05.005. Epub 2017 Jun 13. PMID: 28627433

19. Stuckey MJ, Boulouis HJ, **Cliquet F**, **Picard-Meyer E**, **Servat A**, Aréchiga-Ceballos N, Echevarría JE, Chomel BB. Potentially Zoonotic Bartonella in Bats from France and Spain. Emerg Infect Dis. 2017 Mar;23(3):539-541. doi: 10.3201/eid2303.160934.

20. Mauti S, Traoré A, Hattendorf J, Schelling E, **Wasniewski M**, Schereffer JL, Zinsstag J, **Cliquet F**. Factors associated with dog rabies immunisation status in Bamako, Mali. Acta Trop. 2017 Jan; 165:194-202. doi: 10.1016/j.actatropica.2015.10.016. Epub 2015 Dec 12.

21. Darkaoui S, **Cliquet F**, **Wasniewski M**, **Robardet E**, Aboulfidaa N, Bouslikhane M, et al. A century spent combating rabies in Morocco (1911–2015): How much longer? Front Vet Sci. 2017;4(78):1-16. doi: 10.3389/fvets.2017.00078.