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Networks

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

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

Introduction

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

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

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Materials and Methods

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

1. Participating laboratories

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

2. Constitution and composition of the panels to be tested

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

3. Control and stability of the panels

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

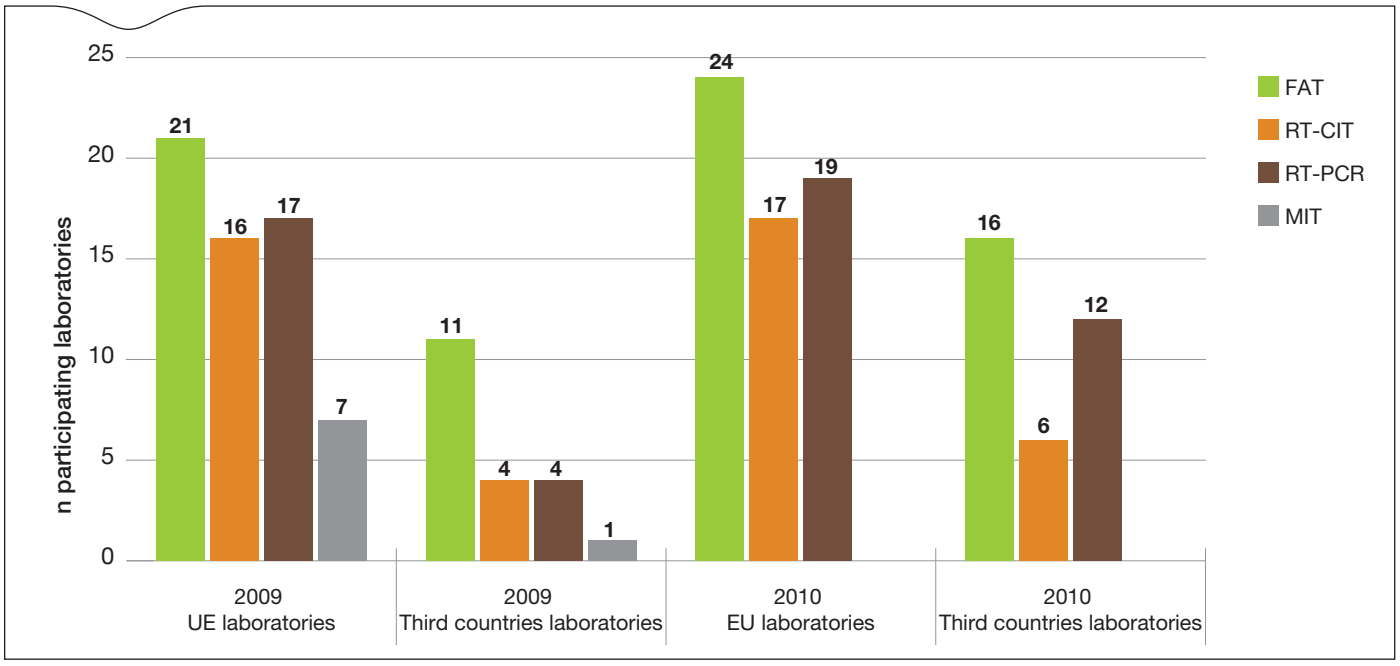

Figure 1: Number laboratories from EU Member States and from third countries participating in the 2009 and 2010 rabies diagnosis inter-laboratories trials.

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

4. Shipment conditions of the panels

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

5. Technical questionnaire

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

Results

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

1. Fluorescent antibody test (FAT) results

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

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

2. Rabies tissue culture infection test (RTCIT) results

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

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

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

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

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

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

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

3. Mice inoculation test (MIT) results

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

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4. PCR results

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

Discussion

1. Fluorescent antibody test (FAT)

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

Table 2. Recommendations of the EURL for the FAT

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

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

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

2. Rabies Tissue Culture Infection Test (RTCIT)

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

Table 3. Recommendations of the EURL for the RTCIT

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

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

3. Mouse Inoculation Test (MIT)

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

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

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

5. Techniques comparisons

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

6. Harmonization scheme

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

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

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References

ADR. (2009) European Agreement concerning the International Carriage of Dangerous Goods by Road, 146 pp. edition (ECE/TRANS/202, Vol. I and II), as amended by document ECE/TRANS/WP.15/199, annex 1. ed.

Barrat, J., Barrat, M.J., Picard, M., Aubert, M.F.A., Gerard, Y., Patron, C., Ambert, J. and Quillou, B. (1988) Diagnostic de la rage sur culture cellulaire: Comparaison des resultats de l'inoculation au neuroblastome murin et de l'inoculation a la souris. Comparative Immunology, Microbiology and Infectious Diseases 11(3-4), 207-214.

Bingham, J. and van der Merwe, M. (2002) Distribution of rabies antigen in infected brain material: determining the reliability of different regions of the brain for the rabies fluorescent antibody test. Journal of Virological Methods 101(1-2), 85-94.

Cliquet, F., Freuling, C., Smreczak, M., Van der Poel, W.H.M., Horton, D., Fooks, A.R., Robardet, E., Picard-Meyer, E. and Müller, T. (2010) Development of harmonized schemes for monitoring and reporting of rabies in animals in the European Union. Rabies Bulletin Europe 34 (2). 7-8.

Dacheux L.; Wacharapluesadee S.; Hemachudha T.; Meslin F.X.,Buchy P.; Reynes J.M.; Bourhy H. (2010). More accurate insight into the Incidence of human rabies in developing countries through validated laboratory techniques. PLoS Negl .Trop. Dis 4 (11), 5 p

David, D., Yakobson, B., Rotenberg, D., Dveres, N., Davidson, I. and Stram, Y. (2002) Rabies virus detection by RT-PCR in decomposed naturally infected brains. Veterinary Microbiology 87(2), 111-118.

Dean, D., Abelseth, M.K. and Atanasiu, P. (1996) The fluorescent antibody test. In: F.X. Meslin, M.M. Kaplan and H. Koprowski (Eds), Laboratory techniques in rabies, pp. 88-95. Vol. Fourth edition. World Health Organization, Geneva.

Durham, T.M., Smith, J.S. and Reid, F.L. (1986) Stability of immunofluorescence reactions produced by polyclonal and monoclonal antibody conjugates for rabies virus. Journal of Clinical Microbiology 24(2), 301-303.

European Comission. (2008) COMMISSION REGULATION (EC) No 737/2008 of 28 July 2008 designating the Community Reference Laboratories for crustacean diseases, rabies and bovine tuberculosis, laying down additional responsibilities and tasks for the Community Reference Laboratories for rabies and bovine tuberculosis and amending Annex VII to Regulation (EC) No 882/2004 of the European Parliament and of the Council.

Fooks, A.R., Johnson, N., Freuling, C.M., Wakeley, P.R., Banyard, A.C., McElhinney, L.M., Marston, D.A., Dastjerdi, A., Wright, E., Weiss, R.A. and Müller, T. (2009) Emerging technologies for the detection of rabies virus: Challenges and hopes in the 21st century. PLoS Neglected Tropical Diseases 3(9).

IATA. (2009) (International Air Transport Association) Infectious Substances Shipping Guidelines, 187 pp.

Koprowski, H. (1996) The mouse inoculation test, Laboratory techniques in rabies, pp. 476. Vol. Fourth edition. World Health Organization, Geneva.

Lewis, V.J., Thacker, W.L. and Engelman, H.M. (1973) Evaluation of the interference filter for use in rabies diagnosis by the fluorescent antibody test. Journal of Applied Microbiology 26(3), 429-430.

Lopes, M.C., Venditti, L.L.R. and Queiroz, L.H. (2010) Comparison between RT-PCR and the mouse inoculation test for detection of rabies virus in samples kept for long periods under different conditions. Journal of Virological Methods 164(1-2), 19-23.

McElhinney, L., Fooks, A.R. and Radford, A.D. (2008) Diagnostic tools for the detection of rabies virus. EJCAP 18(3), 224-230.

OIE. (2011) Manual of Diagnostic Tests and Vaccines for Terrestrial Animals. 7th ed, Paris.

Picard-Meyer, E., Barrat, J. and Cliquet, F. (2007) Use of filter paper (FTA®) technology for sampling, recovery and molecular characterisation of rabies viruses. Journal of Virological Methods 140(1-2), 174-182.

Pital, A. and Janowitz, S.L. (1963) Enhancement of staining intensity in the fluorescent antibody reaction. J. Bacteriol. 86, 888-889.

Robardet, E., Cliquet, F. (2010) Inter-laboratory trial 2009: Fluorescent antibody test (FAT), Rabies Tissue Culture Infection test (RTCIT), Mouse Inoculation Test (MIT). Report of the Community Reference Laboratory for Rabies, January 2010, 43p.

Robardet, E., Andrieu, S., Rasmussen, T.B., Dobrostana, M., Horton, D.L., Hostnik, P., Jaceviciene, I., Juhasz, T., Muller, T., Mutinelli, F., Servat, A., Smreczak, M., Vanek, E., Vazquez-Moron, S., Cliquet, F., Demerson, J.M., Picard-Meyer, E., Moroz, D., Trotsenko, Z., Drozhzhe, Z., Biarnais, M. and Solodchuk, V. (2013) Comparative assay of fluorescent antibody test results among twelve European National Reference Laboratories using various anti-rabies conjugates. Journal of Virological Methods. 191, 88-94

Rudd, R.J., Smith, J.S., Yager, P.A., Orciari, L.A. and Trimarchi, C.V. (2005) A need for standardized rabies-virus diagnostic procedures: Effect of cover-glass mountant on the reliability of antigen detection by the fluorescent antibody test. Virus Research. Rabies in the Americas 111(1), 83-88.

Rudd, R.J. and Trimarchi, C.V. (1989) Development and evaluation of an *in vitro* virus isolation procedure as a replacement for the mouse inoculation test in rabies diagnosis. Journal of Clinical Microbiology 27(11), 2522-2528.

Upcott, D.H. and Markson, L.M. (1971) Some aspects of fixation in the fluorescent antibody test for rabies. Tropical Animal Health and Production 3(2), 83-85.

Webster, W.A. and Casey, G.A. (1996) Virus Isolation in neuroblastoma cell culture, Laboratory techniques in rabies. Vol. Fourth Edition. World Health Organization. pp. 476.

WHO. (1996) Laboratory techniques in rabies, 4th edition ed, edited by F. Meslin, C. Kaplan and H. Koprowski. Geneva. 476 pp.

WHO. (2005) WHO Expert Consultation on rabies. World Health Organization technical report series. 931, 1-88.

WHO, (2010) Rabies Pre and Post exposure Prophylaxis in Humans. World Health Organization. 1-21