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Nancy Laboratory for Rabies and Wildlife

UNIT : Lyssavirus

European Union Reference Laboratory

for Rabies

PROFICIENCY TEST REPORT
Diag-06-2014-V1-EN

INTER-LABORATORY TEST FOR RABIES DIAGNOSIS

Session : 06-2014

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APPROVAL	CLIQUET Florence	Coordinator	2014-09-21	
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1. INTRODUCTION

An inter-laboratory trial on rabies diagnosis techniques is organized annually by the European Union Reference Laboratory (EURL) for Rabies. The objective is to assess the technical performance of laboratories based on the rabies diagnosis reference techniques namely the Fluorescent Antibody Test (FAT) (Dean et al., 1996) and the Rabies Tissue Culture Infection test (RTCIT) (Webster and Casey, 1996) but also to compare results obtained with the current biological molecular techniques represented by the conventional (Tordo et al., 1996) and Real Time (Wakeley et al., 2005) RT-PCR. This work is undertaken in the frame of the Commission Regulation (EU) No 415/2013 of 6 May 2013 laying down additional responsibilities and tasks of the EURL for Rabies and amending Regulation (EC) No 737/2008 designating the EURL for Rabies.

2. GENERAL INFORMATION

2.1 IDENTIFICATION OF COORDINATOR AND STAFF INVOLVED IN THE STUDY

Coordinator: F. Cliquet, Head of Lyssavirus Unit and Director of the EURL for Rabies
Technical Manager: E. Robardet, Project manager of the EURL for Rabies
Technical Staff: J. Rieder, Technician of the EURL for Rabies
Administrative Staff: S. Tourdiat, Administrative secretary of the EURL for Rabies

2.2 INSTRUCTION TO PARTICIPANTS

The call for participation in the inter-laboratory test for rabies diagnosis was sent to laboratories by e-mail in December 2013.
A panel of 9 coded samples to be tested, the acknowledgment form (checking the satisfactory condition of reception of the samples) and the result forms were sent on May 26th 2014 by a specialized carrier. Each sample contained 1ml of homogenate of brains (mouse or fox origin) and was susceptible to be infected by a RABV, EBLV-1, EBLV-2, DUVV or ABLV strain. The shipment of the panel was achieved at 4°C temperature by an international agreed carrier and under UN3373 conditions to avoid exposure hazards as specified by the International Air Transport Association (IATA, 2009) and the “Accord européen relatif au transport international des marchandises Dangereuses par Route” (ADR, 2009) regulation. All the panels were received by the participating laboratories during the time range where stability was ensured (76% of the samples were received within 3 days). Before use, it was recommended to laboratories to store the panel at 4°C as soon as received.
Laboratories were invited to test the samples with the technique(s) of his choice: FAT (Fluorescent Antibody test), RTCIT (Rabies Tissue Culture Infection test), RT-PCR, Real Time RT-PCR using their own procedures performed in routine. Expected results were positive or negative for both FAT and RTCIT while RT-PCR and Real Time RT-PCR were intended to identify virus species. The deadline for result reception was on June 30th 2013.
In parallel to the test, it was asked to participating laboratories to fill an online technical questionnaire for each tested technique. The knowledge of the methods used by the laboratories allows firstly to examine

variations between laboratory methods and, secondly, to assist in the interpretation of discordances of results when occurring in a laboratory.

3. PROFICIENCY TEST ITEMS

3.1 PREPARATION OF THE PROFICIENCY TEST ITEMS

The virus batches used in this study were produced by intra-cerebral inoculation of mice to reproduce as much as possible standard conditions of a diagnosis process. Viruses were produced according to the animal experimentation directives issued by the French Ethic Committee and virus production was continued until the death of the animal to collect a maximum amount of virus. For each batch of virus, brains were excised after the death of the animals then mixed, homogenized, aliquoted into 1ml tubes and then freeze-dried. The samples of the panel were constituted of 9 blindly coded samples of freeze-dried homogenized brains infected with various rabies virus species (Table 1).

The strains involved in the inter-laboratory test were:

- CVS27: a RABV fixed strain.
- Cn Viv Estonie: a RABV raccoon dog strain from Estonia.
- RABV Macédoine: a RABV fox strain from former Yugoslav Republic of Macedonia
- EBLV-1a: a bat strain from France.
- EBLV-2: a bat strain from United Kingdom.
- GS7: a RABV fox strain from France.

One negative sample as well as two diluted GS7 samples (dilution 1/30 and 1/50) were also included in the panel. The material used as negative batch and as dilution material was confirmed negative red foxes brains collected in the field in France.

Table 1: Composition of the panel test of the inter-laboratory test on rabies diagnosis.

ID	Batch name	Passaged on	Strain Origin	Species	Country	Year od isolation	Infected species
1	CVS 27 18-13	Mouse	CVS 27 14-10	RABV	/	/	*Fixed strain*
2	Cn Viv Estonie 15-13	Mouse	Cn Viv Estonie 10-12	RABV	Estonia	2006	*Nyctereutes procyonoides*
3	RABV former Yugoslav Republic of Macedonia 10-13	Mouse	Macedonia 37-12	RABV	former Yugoslav Republic of Macedonia	2011	*Vulpes vulpes*
4	EBLV-1a 08-14	Mouse	122938	EBLV-1	France	2002	*Eptesicus serotinus*
6	EBLV-2 13-13	Mouse	EBL2 RV1787	EBLV-2	United Kingdom	2004	*Myotis daubentonii*
6	GS7 18-13	Mouse	GS7	RABV	France	1986	*Vulpes vulpes*

7	GS7diluted 1/30	Mouse	GS7	RABV	France	1986	*Vulpes vulpes*
8	GS7diluted 1/50	Mouse	GS7	RABV	France	1986	*Vulpes vulpes*
9	Negative 17-13	Red fox	/	/	France	2012	*Vulpes vulpes*

3.2 IDENTIFICATION OF THE PROFICIENCY TEST ITEMS

For each panel, all items were coded randomly. The code was constituted by the date of the inter-laboratory test campaign, the identification of the laboratory and the unique specific code of the item. Each item code was carefully checked and then sticked on the corresponding vial.

3.3 HOMOGENEITY

The evaluation of the homogeneity was undertaken for all batches by analyzing 3 randomly chosen samples by each technique (FAT, RTCIT, RT-PCR Real Time PCR) for the positive batches (CVS 27 18-13; CnViv Estonia 15-13; RABV Macedonia 10-13; EBLV-1a 08-14; EBLV-2 13-13; GS7 18-13; GS7diluted 1/30; GS7diluted 1/50) and 10 randomly chosen samples for the negative batch. The batches were considered homogeneous when all the results were concordant to the expectations.

3.4 STABILITY

Stability of the panel was tested after 7 and 14 days at room temperature ensuring the positive samples remained positive for FAT, RTCIT and RT-PCR techniques and the negative samples remained negative.

3.5 PARTICIPATING LABORATORIES

In 2014, fifty laboratories randomly coded from L1 to L51 participated in the inter-laboratory test for rabies diagnosis. Participating laboratories included 25 National Reference Laboratories (NRLs) from the European Union and 25 laboratories from third countries.

Fifty laboratories performed the FAT, 29 performed the RTCIT, 33 performed the conventional RT-PCR and 25 performed the Real Time RT-PCR (Table 2). Proportion of participating laboratories for the different tests was 100% for the FAT, 58% for the RTCIT, 66% for the conventional RT-PCR and 50% for the Real Time RT-PCR.

The detailed list of participating laboratories for each technique is presented in Table 3.

Table 2: Number of participating laboratories for the different inter-laboratory tests of 2014.

	European NRLs	Third country laboratories	**Total**
FAT	25	25	**50**
RTCIT	20	10	**30**
RT-PCR	16	15	**31**
Real Time	19	7	**26**
Total Laboratories	25	25	**50**

Table 3: List of registered participants for each technique; pink box = registered has participating laboratory.

Continent	Country	Laboratory	Contact Name	FAT	RTCIT	Con. RT-PCR	Real Time RT-PCR
Europe	**Austria**	AGES, Institute for Veterinary Disease Control	Dr. Elisabeth Vanek	y	y	y	y
(NRLs)	**Belgium**	Rabies Laboratory | Communicable and Infectious Diseases	Dr. Bernard Brochier	y	n	n	y
	Bulgaria	National Diagnostic and Veterinary Research Institute	Dr. Darinka Ilieva	y	n	n	n
	Croatia	Croatian Veterinary Institute, Laboratory for Rabies	Dr. Tomislav Bedeković	y	y	n	y
	Cyprus	Animal Health Laboratories	Dr. Vasiliki Christodoulou	y	n	n	y
	Czech Republic	State Veterinary Institute Prague	Dr. Miroslav Tomči	y	y	y	n
	Denmark	DTU National Veterinary Institute	Dr. Thomas Bruun Rasmussen	y	y	n	y
	Estonia	Estonian Veterinary and Food Laboratory	Dr. Katrin Mähar	y	y	n	y
	Finland	Finnish Food Safety Authority Evira	Dr. Tiina Nokireki	y	y	y	y
	France	ANSES LRFSN	Dr. Florence Cliquet	y	y	y	y
	Germany	Federal Research Institute for Animal Health	Dr. Thomas Müller	y	y	y	y
	Greece	Institute of Infectious & Parasitic Diseases. Athens Centre of Veterinary Institutes	Dr. Konstantia Tasioudi	y	n	y	y
	Hungary	CAO VDD	Dr. Hakos Hornyak	y	y	y	y
	Italy	Istituto Zooprofilattico Sperimentale delle Venezie	Dr. Franco Mutinelli	y	y	y	y
	Latvia	Institute of Food Safety	Dr. Zita Muizniece	y	y	y	n
	Lithuania	National Food and Veterinary Risk Assessment Institute	Dr. Ingrida Jaceviciene	y	y	y	n
	Poland	National Veterinary Research Institute	Dr. Marcin Smreczak	y	y	y	y
	Portugal	Laboratório Nacional de Investigação Veterinária (LNIV)	Dr. Miguel Fevereiro; Dr. Isabel Almeida	y	y	y	n
	Romania	Institute for Diagnosis and Animal Health	Dr. Dragos Boncea	y	y	y	n
	Slovakia	State Veterinary Institute Zvolen	Dr. Slavomir Jerg	y	y	n	y
	Slovenia	VF / National Veterinary Institute	Dr. Peter Hostnik	y	y	y	y
	Spain	Centro Nacional de Microbiología, Instituto de Salud Carlos III	Dr. Juan E. Echevarría	y	y	y	y
	Sweden	Statens Veterinärmedecinska	Dr. Louise Treiberg Berndtsson	y	y	n	y
	The Netherlands	Central Veterinary Institute – WUR, Lelystad	Dr. Bart Kooi	y	n	n	y
	United Kingdom	Animal Health and Veterinary Laboratories Agency	Dr. Trudy Goddard	y	y	y	y
Total NRLs				**25**	**20**	**16**	**19**
Europe	**Albania**	Instituti i Sigurise Ushqimore dhe Veterinarise	Dr. Liljana Lufo	y	y	n	n
(NRLs from third countries)	**Bosnia and Herzegovina**	Public Veterinary Institute of Republic of Srpska "Dr Vaso Butozan" Banja Luka	Dr. Violeta Santrac	y	n	n	n
	Kosovo	Food and Veterinary Laboratory	Dr. Xhemajl Deervishi	y	n	n	n
	Former Yugoslav Republic of Macedonia	University Ss Cyril and Methodius in Skopje	Dr. Slavcho Mrenoshki	y	y	y	y

	Montenegro	Diagnostic Veterinary Laboratory	Dr. Nikola Pejovic	y	n	n	n
	Norway	Norwegian Veterinary Institute	Dr. Irene Ørpetveit	y	n	y	y
	Serbia	Institute of Veterinary Medicine of Serbia	Dr. Vesna Milicevic	y	y	y	y
	Serbia	Pasteur Institute Novi Sad	Dr. Dusan Lalosevic	y	n	n	n
Total European third countries				**8**	**3**	**3**	**3**
America	**Chile**	Instituto de salud publica de Chile	Dr. Veronica Yung	y	n	y	n
	Mexico	Centro Nacional de Servicios de Diagnostico en Salud Animal	Dr. Juan Antonio Montano Hirose	y	y	y	y
	U.S.A.	Center for Disease Control and Prevention	Dr. Cathleen A Hanlon	y	n	y	n
	U.S.A.	Kansas State University	Dr. Rolan Davis	y	n	y	n
	Peru	Lab. de Zoonosis Virales-Instituto Nacional de Salud	Dr. Ricardo Luis Lopez Ingunza	y	y	y	n
Total America				**5**	**2**	**5**	**1**
Africa	**Burkina Faso**	Laboratoire National d'Elevage	Dr. Germaine L. Compaore /Minoungou	y	n	y	n
	Morocco	Laboratoire Régional d'Ananalyses et de Recherches de Fès	Dr. Essaleh Lahcen	y	n	n	n
	Morocco	ONSSA-DPIV-Rabbat	Dr. Nadia Aboulfidaa	y	y	n	n
	Morocco	Laboratoire régional d'analyses et de recherches de Marrakech	Dr. Mohamed Faydi	y	n	n	y
	Nigeria	National Veterinary Research Institute	Dr. Chika Nwosuh	y	n	y	n
	Republic of South Africa	Onderstepoort Veterinary Institute	Dr. Claude Sabeta	y	y	y	n
	Republic of South Africa	Center for emerging and zoonotic diseases	Dr. Jacqueline Weyer	y	n	n	y
Total Africa				**7**	**2**	**3**	**2**
Asia	**Israel**	Kimron Veterinary Institute	Dr. Dan David	y	y	y	n
	Taiwan	National Rabies Diagnosis Laboratory	Dr. Shu-Hwae Lee	y	n	y	n
	Turkey	Etlik Central Veterinary Control and Research Institute	Dr. Nil Unal	y	n	n	n
Total Asia				**3**	**1**	**2**	**0**
Oceania	**Australia**	CSIRO Australian Animal Health Laboratory	Dr. David Williams	y	y	y	n
Total Oceania				**1**	**1**	**1**	**0**
Others	**France**	National Reference Centre for Rabies	Dr. Hervé Bourhy	y	y	y	y
Total Others				**1**	**1**	**1**	**1**
Total				**50**	**30**	**31**	**26**

4. RESULTS

4.1 THE FLUORESCENT ANTI BODY TEST

4.1.1 Results of participating laboratories (Table 4 and table 5)

In 2014, fifty laboratories participated in the test. Seven laboratories (14%) harboured discordant results. Because one of these laboratories received the panel after a long transport period so that we could no more ensure the stability of the samples duration and because we could not conclude on the reason of the failure, we have not considered results of this laboratory (L01) in the report.

Three tests on 49 (represented by **L13**, **L15** and **L18**) were identified as false positive results (6.1% of true negative samples) and 4 tests on 392 (represented by **L10**; **L16**, **L22**) provided false negative or inconclusive results. All the discordant results found in positive samples were found in the diluted samples (n=1 for GS7 1/30, n=3 for GS7 1/50).

Table 4: Results per strain for FAT inter-laboratory test

	n Discordant/ total	Discordant (%)	Interval confidence (%)
Number of laboratories	6/49	**12.2**	[5.1 – 25.5]
Negative samples (false positives)	3/49	**6.1**	[1.6 – 17.9]
Positive samples (false negatives)	4/392	**1.0**	[0.3 – 2.8]
CVS 27	0/49	**0**	[0 – 9.0]
RABV CnViv Estonia	0/49	**0**	[0 – 9.0]
RABV Fox former Yugoslav Republic of Macedonia	0/49	**0**	[0 – 9.0]
GS7	0/49	**0**	[0 – 9.0]
GS7 1/30	1/49	**2.0**	[0.1 – 12.2]
GS7 1/50	3/49	**6.1**	[0.7 – 15.1]
EBLV-1	0/49	**0**	[0 – 9.0]
EBLV-2	0/49	**0**	[0 – 9.0]

4.1.2 Evolution of the discordant results in participating laboratories

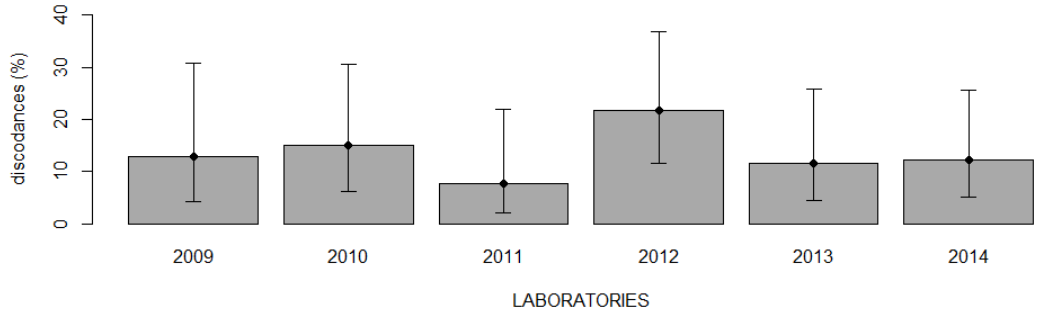


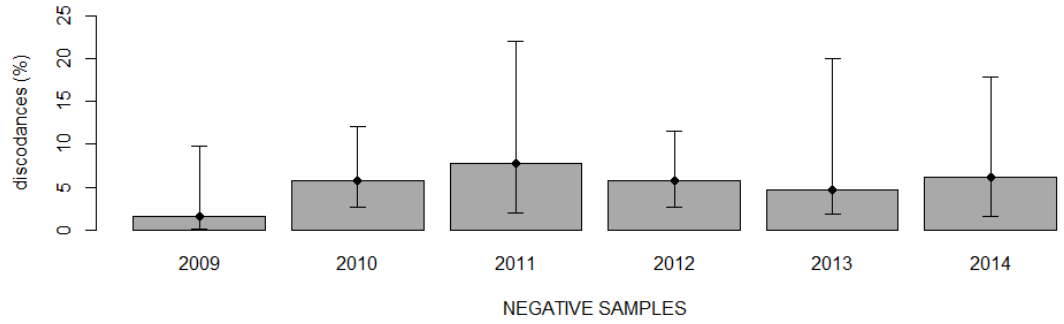
Figure 1: Evolution of the proportion of laboratories with false positive and false negative results. (2011: Only the Panel 2 including different rabies virus species is taken into account, Panel 1 including a range of RABV diluted samples is excluded from the analysis)

The proportion of laboratories with discordant results (Figure 1) and the proportion of discordant results according to the different species (Figure 2) remain stable among years.

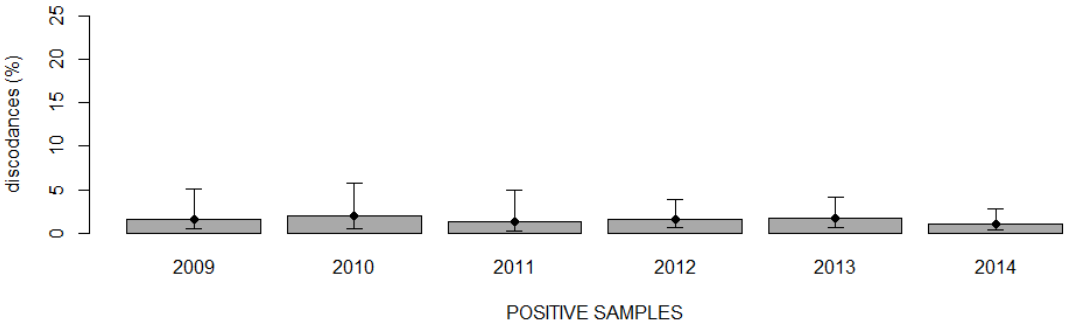
The only observable difference is that no discordant results were observed on bat species, in both EBLV-1 and EBLV-2, for the second year (Figure 2).

Figure 2: Evolution of the proportion of discordant results according to the strain used. A: negative samples; B: positive samples; C: RABV samples (diluted samples excluded); D: EBLV-1 samples, E: EBLV-2 samples.

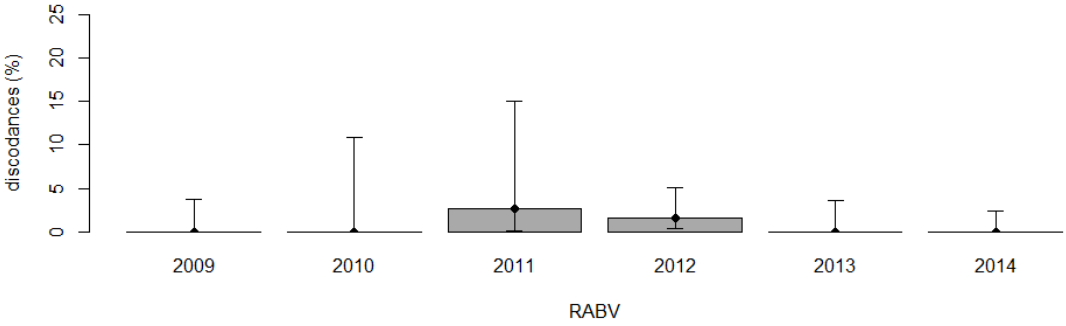
A)



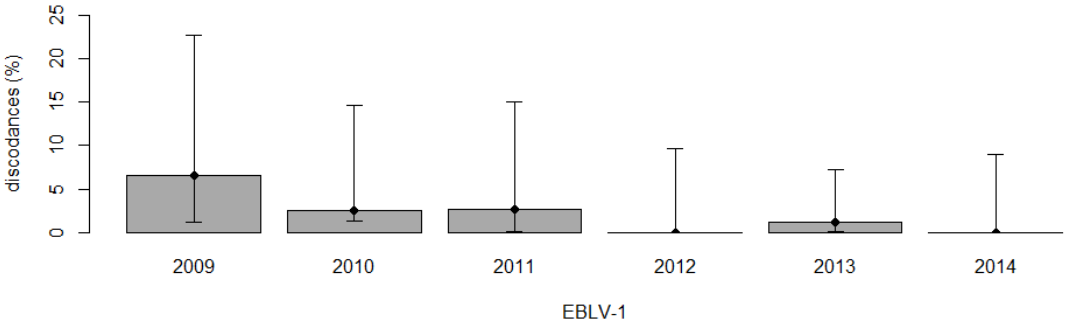
B)



C)



D)



E)

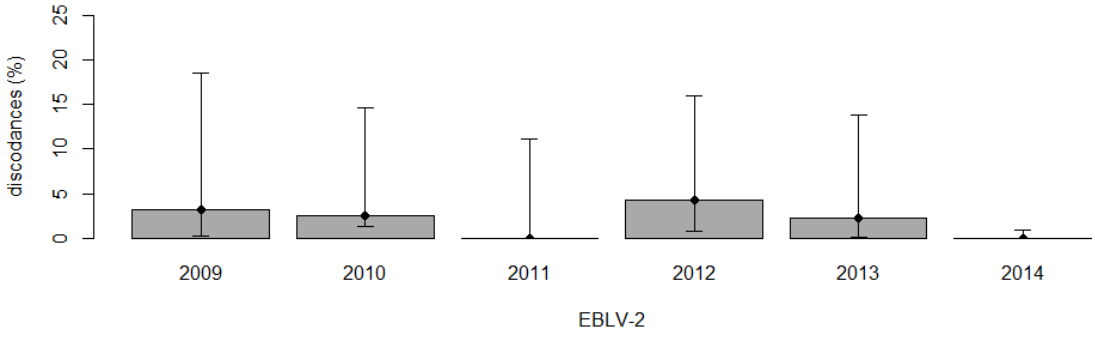


Table 5: Results of the inter-laboratory test for FAT. Coded laboratories coded written in red are those that did not provide satisfactory results. Pink box: negative result; Green box: positive result.

Lab_code	CVS27	result	RABV Cn ViV Estonia	result	RABV FYROM	result	GS7	result	GS7 1/30	result	GS7 1/50	result	EBLV-1	result	EBLV2	result	NEGATIF	result
L01	14050108	pos	14050105	pos	14050111	pos	14050100	pos	14050104	neg	14050147	neg	14050144	pos	14050152	pos	14050137	neg
L02	14050259	pos	14050213	pos	14050284	pos	14050257	pos	14050288	pos	14050233	pos	14050212	pos	14050214	pos	14050245	neg
L03	14050301	pos	14050323	pos	14050354	pos	14050397	pos	14050319	pos	14050341	pos	14050312	pos	14050357	pos	14050307	neg
L04	14050471	pos	14050457	pos	14050492	pos	14050417	pos	14050456	pos	14050428	pos	14050415	pos	14050486	pos	14050459	neg
L05	14050541	pos	14050554	pos	14050538	pos	14050532	pos	14050543	pos	14050590	pos	14050542	pos	14050530	pos	14050529	neg
L06	14050640	pos	14050662	pos	14050607	pos	14050613	pos	14050683	pos	14050690	pos	14050631	pos	14050689	pos	14050696	neg
L07	14050704	pos	14050760	pos	14050730	pos	14050786	pos	14050711	pos	14050770	pos	14050759	pos	14050718	pos	14050756	neg
L08	14050827	pos	14050883	pos	14050870	pos	14050851	pos	14050823	pos	14050897	pos	14050894	pos	14050831	pos	14050819	neg
L09	14050911	pos	14050945	pos	14050974	pos	14050978	pos	14050910	pos	14050909	pos	14050915	pos	14050905	pos	14050981	neg
L10	14051047	pos	14051085	pos	14051034	pos	14051060	pos	14051064	pos	14051074	inc	14051017	pos	14051052	pos	14051035	neg
L11	14051115	pos	14051142	pos	14051158	pos	14051100	pos	14051155	pos	14051113	pos	14051117	pos	14051124	pos	14051134	neg
L12	14051215	pos	14051210	pos	14051278	pos	14051281	pos	14051258	pos	14051245	pos	14051257	pos	14051295	pos	14051216	neg
L13	14051304	pos	14051375	pos	14051394	pos	14051393	pos	14051395	pos	14051392	pos	14051380	pos	14051311	pos	14051348	pos
L14	14051403	pos	14051487	pos	14051421	pos	14051477	pos	14051417	pos	14051427	pos	14051481	pos	14051468	pos	14051458	neg
L15	14051590	pos	14051544	pos	14051550	pos	14051513	pos	14051537	pos	14051520	pos	14051510	pos	14051500	pos	14051501	pos
L16	14051662	pos	14051664	pos	14051607	pos	14051689	pos	14051622	pos	14051605	inc	14051670	pos	14051685	pos	14051635	neg
L17	14051773	pos	14051702	pos	14051725	pos	14051726	pos	14051778	pos	14051709	pos	14051724	pos	14051797	pos	14051727	neg
L18	14051805	pos	14051840	pos	14051831	pos	14051812	pos	14051860	pos	14051816	pos	14051885	pos	14051849	pos	14051855	pos
L19	14051938	pos	14051907	pos	14051969	pos	14051947	pos	14051917	pos	14051909	pos	14051919	pos	14051997	pos	14051932	neg
L20	14052095	pos	14052097	pos	14052048	pos	14052030	pos	14052085	pos	14052087	pos	14052084	pos	14052057	pos	14052060	neg
L21	14052143	pos	14052140	pos	14052105	pos	14052147	pos	14052166	pos	14052185	pos	14052135	pos	14052179	pos	14052149	neg
L22	14052234	pos	14052212	pos	14052204	pos	14052226	pos	14052287	neg	14052285	neg	14052216	pos	14052261	pos	14052230	neg
L23	14052321	pos	14052359	pos	14052383	pos	14052353	pos	14052345	pos	14052346	pos	14052329	pos	14052324	pos	14052398	neg
L24	14052498	pos	14052476	pos	14052450	pos	14052417	pos	14052442	pos	14052404	pos	14052495	pos	14052471	pos	14052458	neg
L25	14052542	pos	14052529	pos	14052578	pos	14052563	pos	14052593	pos	14052545	pos	14052587	pos	14052548	pos	14052553	neg
L26	14052625	pos	14052665	pos	14052644	pos	14052659	pos	14052615	pos	14052676	pos	14052623	pos	14052620	pos	14052624	neg
L27	14052778	pos	14052702	pos	14052761	pos	14052764	pos	14052773	pos	14052732	pos	14052779	pos	14052762	pos	14052727	neg
L28	14052898	pos	14052848	pos	14052809	pos	14052891	pos	14052832	pos	14052833	pos	14052812	pos	14052844	pos	14052845	neg
L29	14052922	pos	14052942	pos	14052936	pos	14052907	pos	14052950	pos	14052974	pos	14052952	pos	14052932	pos	14052958	neg
L30	14053003	pos	14053068	pos	14053070	pos	14053056	pos	14053091	pos	14053004	pos	14053028	pos	14053048	pos	14053001	neg
L31	14053110	pos	14053125	pos	14053118	pos	14053160	pos	14053189	pos	14053193	pos	14053131	pos	14053141	pos	14053192	neg
L32	14053268	pos	14053262	pos	14053252	pos	14053232	pos	14053293	pos	14053203	pos	14053260	pos	14053243	pos	14053245	neg
L33	14053309	pos	14053343	pos	14053366	pos	14053312	pos	14053392	pos	14053398	pos	14053378	pos	14053374	pos	14053375	neg
L34	14053448	pos	14053414	pos	14053442	pos	14053457	pos	14053445	pos	14053489	pos	14053427	pos	14053415	pos	14053447	neg
L35	14053503	pos	14053501	pos	14053592	pos	14053531	pos	14053525	pos	14053575	pos	14053520	pos	14053554	pos	14053560	neg
L36	14053625	pos	14053691	pos	14053696	pos	14053666	pos	14053609	pos	14053659	pos	14053693	pos	14053651	pos	14053667	neg
L38	14053845	pos	14053890	pos	14053869	pos	14053819	pos	14053848	pos	14053888	pos	14053825	pos	14053856	pos	14053875	neg
L39	14053951	pos	14053933	pos	14053997	pos	14053928	pos	14053911	pos	14053956	pos	14053918	pos	14053932	pos	14053962	neg
L40	14054028	pos	14054018	pos	14054038	pos	14054097	pos	14054005	pos	14054024	pos	14054039	pos	14054080	pos	14054019	neg
L41	14054115	pos	14054190	pos	14054195	pos	14054145	pos	14054155	pos	14054117	pos	14054139	pos	14054154	pos	14054184	neg
L42	14054201	pos	14054239	pos	14054262	pos	14054214	pos	14054234	pos	14054218	pos	14054278	pos	14054266	pos	14054283	neg
L43	14054322	pos	14054316	pos	14054380	pos	14054383	pos	14054368	pos	14054342	pos	14054396	pos	14054315	pos	14054381	neg
L44	14054468	pos	14054461	pos	14054497	pos	14054498	pos	14054414	pos	14054499	pos	14054428	pos	14054477	pos	14054440	neg
L45	14054543	pos	14054535	pos	14054568	pos	14054594	pos	14054534	pos	14054507	pos	14054599	pos	14054574	pos	14054503	neg
L46	14054618	pos	14054662	pos	14054600	pos	14054672	pos	14054671	pos	14054697	pos	14054680	pos	14054606	pos	14054629	neg
L47	14054769	pos	14054747	pos	14054725	pos	14054756	pos	14054755	pos	14054733	pos	14054732	pos	14054718	pos	14054723	neg
L48	14054816	pos	14054818	pos	14054859	pos	14054806	pos	14054898	pos	14054830	pos	14054895	pos	14054831	pos	14054838	neg
L49	14054955	pos	14054961	pos	14054973	pos	14054984	pos	14054942	pos	14054963	pos	14054968	pos	14054975	pos	14054952	neg
L50	14055004	pos	14055021	pos	14055069	pos	14055038	pos	14055095	pos	14055058	pos	14055061	pos	14055056	pos	14055091	neg
L51	14055113	pos	14055167	pos	14055153	pos	14055180	pos	14055137	pos	14055109	pos	14055135	pos	14055110	pos	14055188	neg

4.1.3 Answers of the participating laboratories to the FAT technical questionnaire

Item	Number of laboratories/ number of total answers	EU laboratory answers	EURL Recommendation
Diagnostic technique for FAT (routine diagnosis)			
Slides are degreased	20/37 (54%)	9/20 (45%)	YES
Impression technique	36/44 (82%)	18/24 (75%)	
Smear technique	21/39 (54%)	14/21 (67%)	
Which brain sections are routinely examined?			
Ammon's horn	45/47 (98%)	25/24 (100%)	YES (or Cerebellum)
Cerebellum	46/57 (98%)	23/24 (96%)	YES (or Ammon's horn)
Cortex	33/43 (78%)	18/23 (78%)	
Medulla oblongata	39/43 (91%)	21/24 (88%)	YES
Thalamus	17/34 (50%)	12/22 (54%)	
Salivary glands			
Drying			
Drying step performed before fixation	45/48 (94%)	22/25 (88%)	YES
Drying step duration (in min)	≤ 5 : 11/45 (25%) 10 : 12/45 (27%) 15 : 12/45 (27%) 20 : 2/45 (4%) 30 : 3/45 (7%) >30 : 2/45 (4%)	≤ 5 : 5/22 (23%) 10 : 5/22 (23%) 15 : 6/22 (27%) 20 : 2/22 (9%) 30 : 1/22 (5%) >30 : 2/22 (9%)	15-30 min
Drying temperature (in °C)	RT: 41/44 (93%)	RT: 20/21 (95%)	RT
Fixation			
Heat fixation technique	10/36 (28%)	6/19 (32%)	
Acetone fixation technqiue	45/47 (96%)	22/24 (92%)	YES
Acetone percentage (in %)	80%: 4/45 (9%) 99%: 9/45 (20%) 100%: 31/45 (69%)	80: 3/22 (14%) 99: 4/22 (18%) 100: 15/22 (68%)	99-100%
Acetone fixation duration (in min)	<30: 4/46 (9%) 30: 28/46 (6%) 40: 1/46 (2%) 60: 12/46 (26%) >120: 1/46 (2%)	<30:3/23 (13%) 30:16/23 (70%) 40 : 1/20 (5%) 60:3/19 (16%)	30 min
Acetone fixation temperature (in °C)	-20: 31/45 (69%) 4: 1/45 (2%) RT: 7/45 (16%)	-20: 16/22 (73%) 4: 1/22 (5%) RT: 5/22 (23%)	-20°C
Acetone bath changed after every positive detected case	14/44 (32%)	11/22 (50%)	YES
Controls and the sample slides placed in separate acetone bath	28/41 (68%)	14/19 (74%)	YES

Drying (Acetone fixation only)			
Drying step performed before staining	44/45 (98%)	21/22 (95%)	YES
Drying step duration (in min)	<5 : 11/44 (25%) 10 : 14/44 (32%) 15 : 10/44 (23%) 20 : 1/44 (2%) >30 : 4/44 (9%)	<5 : 4/21 (19%) 10 : 6/21 (29%) 15 : 6/21 (29%) 60 : 4/21 (19%)	15-30 min
Drying step temperature (in °C)	RT: 41/44 (93%)	RT: 21/21 (100%)	RT
Conjugate			
Use of a commercially prepared conjugate	38/40 (95%)	22/22 (100%)	
Manufacturer of the conjugate	Bio-Rad: 14/49 (29%) Bioveta: 2/49 (4%) Fujirebio: 21/49 (43%) Millipore: 3/49 (6%) Sifin : 5/49 (10%)	Bio-Rad : 9/26 (35%) Bioveta: 2/26 (8%) Fujirebio: 11/26 (42%) Sifin: 3/26 (12%)	See Report of the collaborative study on antirabies conjugates (2012)
Are the conjugate dilution manufacturer instructions strictly followed?	40/46 (87%)	21/26 (81%)	
Use of home made conjugate	2/49 (10%)	0/23 (0%)	
The conjugate is composed by polyclonal anti-bodies	20/38 (53%)	12/21 (57%)	
The conjugate is composed by a pool of monoclonal anti-bodies	28/39 (72%)	14/20 (70%)	
Use of another conjugate	15/49 (31%)	10/26 (38%)	
In case of	-each inconclusive -suspect cases -always	-each inconclusive -suspect cases -always	
Evan's blue added to the conjugate preparation	19/48 (40%)	9/22 (32%)	
Do you use Rabies fixed virus Mouse Brain suspension (RMB) as diluents to control non specific fluorescence?	4/47 (9%)	2/25 (8%)	
Do you use Normal Mouse Brain Suspension (NMB) as diluent to control non specific fluorescence?	7/46 (15%)	4/25 (16%)	
Staining			
Volume of conjugate deposited on the slide (in µl)	20 : 5/49 (10%) 30 : 10/49 (20%) 50 : 15/49 (31%) 100 : 11/49 (22%) 150 : 4/49 (8%) >150 : 3/49 (6%)	20 : 3/26 (12%) 30 : 4/26 (15%) 50 : 8/26 (31%) 100 : 8/26 (31%) 150 : 0/26 (0%) >150 : 2/26 (8%)	

Incubation duration (in min)	30 : 36/40 (90%) 40 : 2/40 (5%) 60 : 2/40 (5%)	30 : 20/22 (91%) 40 : 1/22 (5%) 60 : 1/22 (5%)	30 min
Incubation temperature (in °C)	20 : 1/40 (3%) 35 : 1/40 (3%) 37 : 38/40 (95%)	20 : 1/22 (5%) 37 : 21/22 (95%)	37°C
Washing			
Slides washed with water	19/42 (45%)	10/21 (48%)	
Slides washed with PBS	45/48 (94%)	23/25 (92%)	
Slides washed with another product	2/38 (5%)	1/18 (6%)	
Product	Elisa buffer, Natrii Chloridi Infundibile 0.9%	Elisa buffer	
Slides washed by soaking	41/49 (84%)	24/26 (92%)	YES
Slides washed in running liquid	5/46 (11%)	3/24 (13%)	
Drying			
Mounting done on dry slides	30/40 (75%)	15/22 (68%)	YES
Drying step duration (in min)	<10 : 8/25 (32%) 15-20 : 12/25 (48%) 30 : 5/25 (20%)	<10 : 3/14 (21%) 20 : 6/14 (43%) 30 : 5/14 (36%)	15-30 min
Drying step temperature (in °C)	RT : 24/26 (92%) 37 : 1/26 (4%) 45 : 1/26 (4%)	RT: 13/13 (100%)	RT
Mounting medium			
pH of the mounting medium	>8.5 : 20/39 (51%) <8.5 : 16/39 (41%)	>8,5 : 14/20 (70%) <8,5 : 6/20 (30%)	≥8.5
Use of home made mounting medium	24/24 (71%)	11/17 (65%)	YES
Use of commercially prepared mounting medium	9/31 (29%)	5/16 (31%)	
Percentage of glycerol in the preparation	10 : 3/38 (8%) 20 : 8/38 (21%) 30 : 2/38 (5%) 50 : 16/38 (42%) 80 : 3/38 (8%) 90 : 1/38 (3%)	20 : 6/19 (32%) 30 : 1/19 (5%) 50 : 8/19 (42%) 80 : 1/19 (5%)	Avoid strong concentration
Observation of fluorescence			
Microscope equipped with mercury lamp	31/40 (78%)	14/22 (64%)	
Microscope equipped with halogen lamp	18/37 (49%)	10/19 (53%)	
Microscope equipped with LED lamp	6/31 (19%)	4/17 (24%)	
Excitation filter (in nm)	450-495 : 34/40 (85%) Other : 6/40 (15%)	485-495 : 15/20 (75%) Other : 5/20 (25%)	Fluorescein excitation: 490 nm
Stop filter (in nm)	510-520: 26/31 (84%) Other: 5/31 (16%)	510-520: 12/16 (75%) Other : 4/16 (25%)	Fluorescein emission: 520 nm

General magnification, including intermediate pieces (x...)	200 : 8/43 (19%) 400 : 29/43 (67%) 1000 : 2/43 (47%)	200 : 1/21 (48%) 400 : 16/21 (76%) 1000 : 2/21 (95%)	from 200 to 400 nm
Number of persons examining separately the slides at each session:	1 : 6/49 (12%) 2 : 39/49 (80%) 3 : 3/49 (6%) 4 : 1/49 (2%)	1 : 5/26 (19%) 2 : 19/26 (73%) 3 : 2/26 (7%)	2 independent readers
Controls			
Negative control included in the test	41/49 (84%)	21/26 (81%)	YES
Positive controls included in the test	47/49 (96%)	25/26 (96%)	YES
Positive control from field strain origin	26/38 (68%)	14/20 (70%)	YES
Positive control from CVS mouse brain origin	19/32 (59%)	10/19 (53%)	
Positive control from EBLV-1 mouse brain origin	23/28 (82%)	13/17 (76%)	
Positive control from EBLV-2 mouse brain origin	26/27 (96%)	16/17 (94%)	
Positive control from other origin	3/30 (10%) (ABLV, CVS from cells)	2/15 (13%) (CVS from cells)	

4.1.4 Interpretation of the discordant results

False positive results (**L13**, **L15** and **L18**) could be explained by cross-contamination between samples. All the tests of the trials (FAT, RTCIT, RT-PCR and Real Time PCR) were performed on a single panel and the multiplicity of testing on the same samples may increase the probability of cross-contamination between samples leading to false positive results. Preferably, we recommend to L13 the separation of acetone bath of controls and samples to eliminate potential risk of cross contamination during this step.

False negative results have been identified in three laboratories (**L10**; **L16**; **L22**). Potential explanation of these discordances has been done by analysing the technical questionnaires returned by laboratories.

L10; L16 and L22:
pH of the mounting medium should be checked. At a pH <8.5, the performance of the FITC fluorescence is much lower and in acid medium, conjugate can disconnect from antigen and then be washed away, inducing false negative results (Durham et al., 1986; Pital and Janowitz, 1963).

L16:
Analysis should be preferably performed on degreased slides.

L10:
Acetone fixation should be preferentially used than heat fixation as demonstrated by Upcott and Markson (1971).

L22:
The acetone fixation conditions should follow the optimized technique, i.e. in 100% acetone during 30 minutes at -20°C as demonstrated by Roehe et al., 2002 and described by Dean et al., 1996.

4.2 THE RABIES TISSUE CULTURE INFECTION TEST

Analysis of laboratory results revealed that 6 laboratories (21.4%) obtained discordant results in the RTCIT (Table 6 and Table 7). Three false positive results (10.7% of true negative samples) were obtained by 3 participating laboratories (**L13**, **L14** and **L18**) while three false negative results (1.3% of true positive samples) were found by 3 laboratories (**L6**, **L12** and **L51**). False negative results were detected in RABV strains only (two in RABV from former Yugoslav Republic of Macedonia and one in RABV from Estonia).

Table 6: Results per strain for the RTCIT inter-laboratory test

	n Discordant/ total	Discordant (%)	Interval confidence (%)
Number of laboratories	6/28	**21.4**	[9.0 – 41.4]
Negative samples (false positives)	3/28	**10.7**	[2.8 – 29.4]
Positive samples (false negatives)	3/224	**1.3**	[0.3 – 4.2]
CVS 27	0/28	**0**	[0 – 15.0]
RABV CnViv Estonia	1/28	**3.6**	[0.2 – 20.2]
RABV Fox former Yugoslav Republic of Macedonia	2/28	**7.1**	[1.3 – 25.0]
GS7	0/28	**0**	[0 – 15.0]
GS7 1/30	0/28	**0**	[0 – 15.0]
GS7 1/50	0/28	**0**	[0 – 15.0]
EBLV-1	0/28	**0**	[0 – 15.0]
EBLV-2	0/28	**0**	[0 – 15.0]

4.2.1 Evolution of the discordant results in participating laboratories

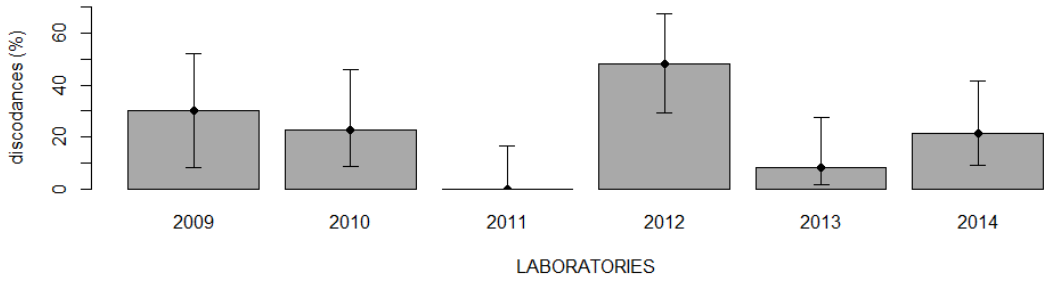
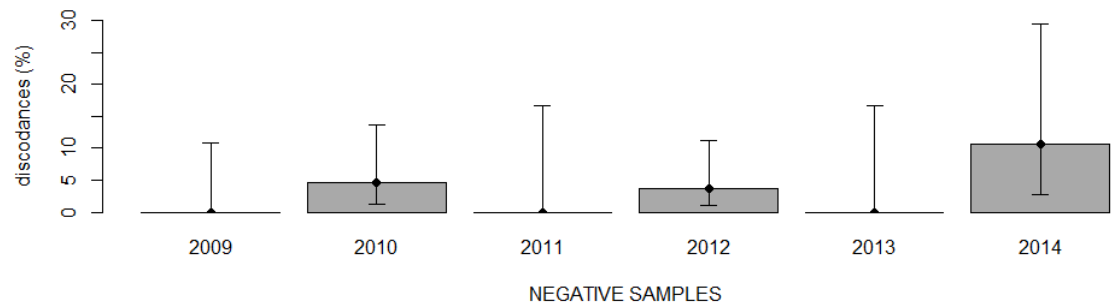


Figure 3: Evolution of the proportion of laboratories harbouring discordant results. (2011: Only the Panel 2 including different rabies virus species is taken into account, Panel 1 including only French RABV diluted samples is excluded from the analysis)

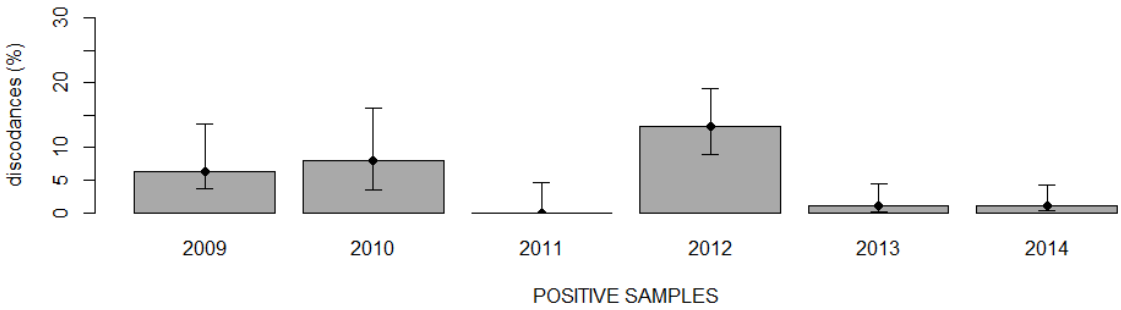
The proportion of laboratories with discordant results is comparable to 2013 (Figure 3). In 2014, no discordant results were observed on bat species, both EBLV-1 and EBLV-2 (Figure 4).

Figure 4: Evolution of the proportion of discordant results according to the strain used. A: negative samples; B: positive samples; C: RABV samples; D: EBLV-1 samples, E: EBLV-2 samples.

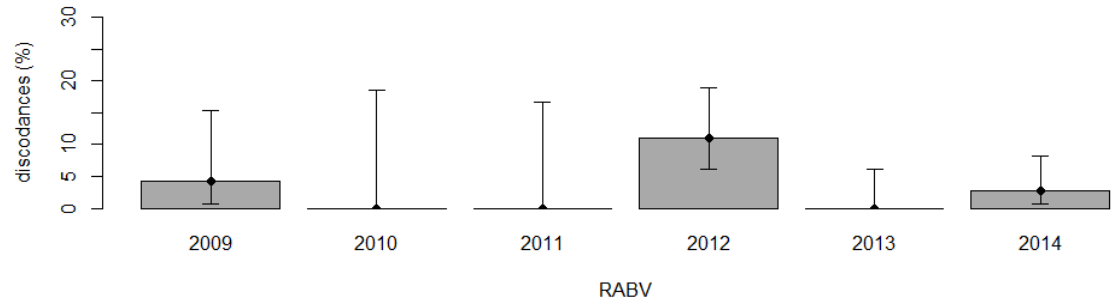
A)



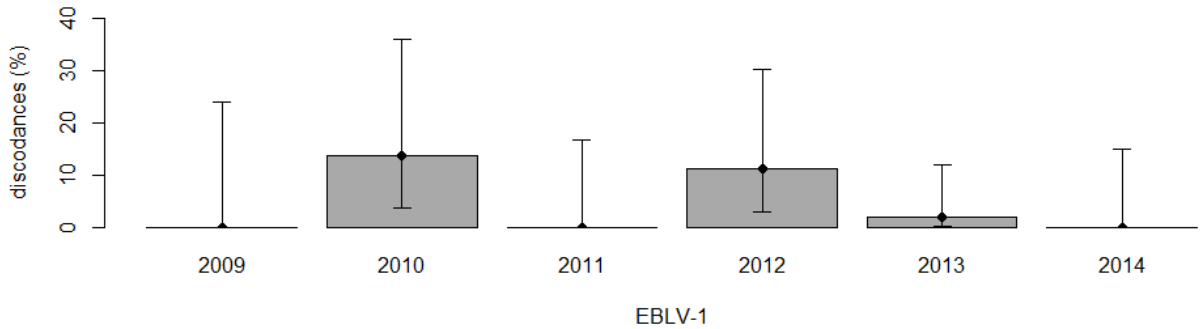
B)



C)



D)



E)

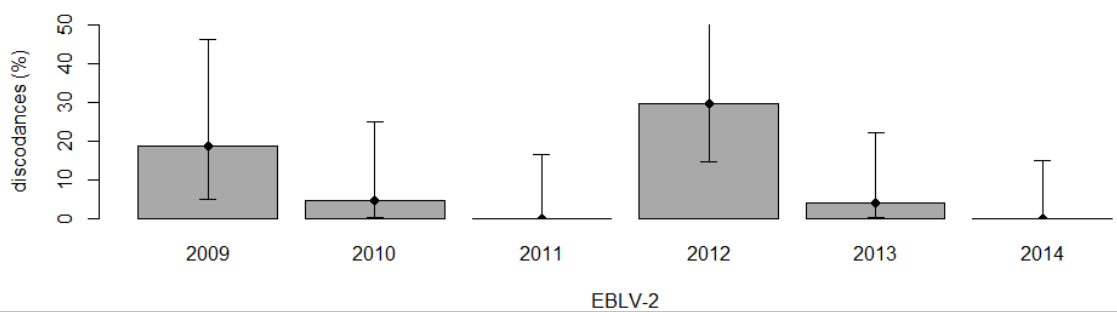


Table 7: Results of the inter-laboratory test on RTCIT. pos: positive result; neg: negative result; NA: missing data.

Lab_code	CVS27	result	Cn ViV estonie	result	RABV FYROM	result	GS7	result	GS7 1/30	result	GS7 1/50	result	EBLV-1a	result	EBLV-2	result	NEGATIVE	result
L03	14050301	pos	14050323	pos	14050354	pos	14050397	pos	14050319	pos	14050341	pos	14050312	pos	14050357	pos	14050307	neg
L05	14050541	pos	14050554	pos	14050538	pos	14050532	pos	14050543	pos	14050590	pos	14050542	pos	14050530	pos	14050529	neg
L06	14050640	pos	14050662	pos	14050607	neg	14050613	pos	14050683	pos	14050690	pos	14050631	pos	14050689	pos	14050696	neg
L07	14050704	pos	14050760	pos	14050730	pos	14050786	pos	14050711	pos	14050770	pos	14050759	pos	14050718	pos	14050756	neg
L09	14050911	pos	14050945	pos	14050974	pos	14050978	pos	14050910	pos	14050909	pos	14050915	pos	14050905	pos	14050981	neg
L10	14051047	pos	14051085	pos	14051034	pos	14051060	pos	14051064	pos	14051074	pos	14051017	pos	14051052	pos	14051035	neg
L11	14051115	pos	14051142	pos	14051158	pos	14051100	pos	14051155	pos	14051113	pos	14051117	pos	14051124	pos	14051134	neg
L12	14051215	pos	14051210	neg	14051278	pos	14051281	pos	14051258	pos	14051245	pos	14051257	pos	14051295	pos	14051216	neg
L13	14051304	pos	14051375	pos	14051394	pos	14051393	pos	14051395	pos	14051392	pos	14051380	pos	14051311	pos	14051348	pos
L14	14051403	pos	14051487	pos	14051421	pos	14051477	pos	14051417	pos	14051427	pos	14051481	pos	14051468	pos	14051458	pos
L15	14051590	pos	14051544	pos	14051550	pos	14051513	pos	14051537	pos	14051520	pos	14051510	pos	14051500	pos	14051501	neg
L16	14051662	pos	14051664	pos	14051607	pos	14051689	pos	14051622	pos	14051605	pos	14051670	pos	14051685	pos	14051635	neg
L18	14051805	pos	14051840	pos	14051831	pos	14051812	pos	14051860	pos	14051816	pos	14051885	pos	14051849	pos	14051855	pos
L19	14051938	pos	14051907	pos	14051969	pos	14051947	pos	14051917	pos	14051909	pos	14051919	pos	14051997	pos	14051932	neg
L21	14052143	pos	14052140	pos	14052105	pos	14052147	pos	14052166	pos	14052185	pos	14052135	pos	14052179	pos	14052149	neg
L23	14052321	pos	14052359	pos	14052383	pos	14052353	pos	14052345	pos	14052346	pos	14052329	pos	14052324	pos	14052398	neg
L25	14052542	pos	14052529	pos	14052578	pos	14052563	pos	14052593	pos	14052545	pos	14052587	pos	14052548	pos	14052553	neg
L28	14052898	pos	14052848	pos	14052809	pos	14052891	pos	14052832	pos	14052833	pos	14052812	pos	14052844	pos	14052845	neg
L29	14052922	pos	14052942	pos	14052936	pos	14052907	pos	14052950	pos	14052974	pos	14052952	pos	14052932	pos	14052958	neg
L30	14053003	pos	14053068	pos	14053070	pos	14053056	pos	14053091	pos	14053004	pos	14053028	pos	14053048	pos	14053001	neg
L38	14053845	pos	14053890	pos	14053869	pos	14053819	pos	14053848	pos	14053888	pos	14053825	pos	14053856	pos	14053875	neg
L39	14053951	pos	14053933	pos	14053997	pos	14053928	pos	14053911	pos	14053956	pos	14053918	pos	14053932	pos	14053962	neg
L41	14054115	pos	14054190	pos	14054195	pos	14054145	pos	14054155	pos	14054117	pos	14054139	pos	14054154	pos	14054184	neg
L43	14054322	pos	14054316	pos	14054380	pos	14054383	pos	14054368	pos	14054342	pos	14054396	pos	14054315	pos	14054381	neg
L46	14054618	pos	14054662	pos	14054600	pos	14054672	pos	14054671	pos	14054697	pos	14054680	pos	14054606	pos	14054629	neg
L48	14054816	pos	14054818	pos	14054859	pos	14054806	pos	14054898	pos	14054830	pos	14054895	pos	14054831	pos	14054838	neg
L49	14054955	pos	14054961	pos	14054973	pos	14054984	pos	14054942	pos	14054963	pos	14054968	pos	14054975	pos	14054952	neg
L51	14055113	pos	14055167	pos	14055153	neg	14055180	pos	14055137	pos	14055109	pos	14055135	pos	14055110	pos	14055188	neg

4.2.2 Answers of the participating laboratories to the RTCIT technical questionnaire

Item	Number of laboratories/ number of total answers	EU laboratory answers	EURL Recommendation
Brain sections routinely examined			
Ammon's horn	26/26 (100%)	20/20 (100%)	YES (or Cerebellum)
Cerebellum	25/26 (96%)	18/19 (95%)	YES (or Ammon's horn)
Cortex	19/23 (83%)	16/19 (84%)	
Medulla oblongata	23/26 (88%)	19/21 (90%)	YES
Thalamus	15/23 (65%)	13/19 (28%)	
Salivary glands	18/20 (90%)	15/17 (88%)	
Grinding medium			
Cell type	N2a: 18/28 (64%) N2a ; BHK 21: 3/28 (11%) NA 42/13: 4/28 (14%) NA 42/13 ; BHK 21: 2/28 (7%)	N2a: 14/21 (58%) N2a ; BHK 21: 1/21 (5%) NA 42/13: 4/21 (19%) NA 42/13 ; BHK 21: 2/21 (10%)	Neuroblastoma
Grinding medium used	DMEM: 16/28 (57%) EMEM: 4/28 (14%) GMEM: 2/28 (7%) PBS: 5/28 (18%)	DMEM: 15/21 (71%) EMEM: 2/21 (10%) GMEM: 2/21 (10%) PBS: 2/21 (10%)	Medium+ 10% FBS + Antibiotics
Serum added to the grinding medium	15/28 (54%)	12/21 (57%)	YES
Proportion added (in %)	10: 8/15 (53%) 40: 3/15 (20%) 50: 4/15 (27%)	10: 6/12 (50%) 40: 3/12 (25%) 50: 3/12 (25%)	
Antibiotics added to the grinding medium	24/28 (86%)	18/21 (86%)	YES
Percentage of added antibiotic (in %)	1: 14/24 (58%) 5: 5/24 (21%)	1: 9/18 (50%) 5: 5/18 (28%)	
Plasmocin added to the grinding medium	2/28 (7%)	1/21 (5%)	
Percentage of added plasmocin (in %)	1: 1/1 (100%)	1: 1/1 (100%)	
Final suspension after adding the grinding medium to the tissue (in %)	10: 18/28 (64%) 20: 8/28 (29%) 50: 1/28 (4%)	10: 13/21 (62%) 20: 6/21 (29%) 50: 1/21 (5%)	10%
Freezing/ defrosting step included in the procedure	11/23 (48%)	7/17 (41%)	YES

Tissue suspension centrifuged	22/23 (96%)	16/17 (94%)	YES
Cells suspension			
Type of cells used	DMEM: 16/28 (57%) EMEM: 8/28 (29%) GMEM: 3/28 (11%) HMEM: 1/28 (3.5%)	DMEM: 15/21 (71%) EMEM: 5/21 (24%) GMEM : 1/21 (5%)	Medium +antibiotics
Serum added to the cells suspension	28/28 (100%)	21/21 (100%)	
Proportion added (in %)	5 : 2/28 (7%) 10: 22/28 (79%) 40: 1/28 (4%)	5 : 2/21 (10%) 10: 16/21 (76%) 40: 1/21 (48%)	
Antibiotics added to the cells suspension	25/28 (89%)	18/21 (86%)	YES
Percentage of added antibiotics (in %)	<1 : 4/25 (16%) 1: 16/25 (64%) 2: 1/25 (4%) 5: 2/25 (8%)	<1 : 5/18 (28%) 1: 11/18 (61%) 5: 2/18 (11%)	
Plasmocin added to the cells suspension	5/28 (18%)	3/21 (14%)	
Percentage of added plasmocin (in %)	1%: 3/5 (60%)	1% : 2/3 (67%)	
DEAE dextran added to the cell culture medium	4/28 (14%)	3/21 (14%)	
Concentration (in µg/ml)	100: 3/3 (100%)	100: 2/2 (100%)	
Substrate			
Technique performed on Labteck	10/28 (36%)	7/21 (35%)	
Technique performed on mi-croplate	17/28 (64%)	15/21 (69%)	
Number of wells for microplates	4: 2/16 (13%) 24: 5/16 (31%) 96: 8/16 (50%)	4: 2/14 (14%) 24: 4/14 (29%) 96: 7/14 (50%)	

Technique on Labteck chamber slides			
Inoculation			
Cell density of the substrate (in cells/ml)	$10x10^4$: 3/10 (30%) $40x10^4$: 1/10 (10%) $50x10^4$: 2/10 (20%) $80x10^4$: 1/10 (10%) $10x10^5$: 1/10 (10%) $2x10^5$: 1/10 (10%) $6x10^5$: 1/10 (10%)	$10x10^4$: 1/7 (14%) $40x10^4$: 1/7 (14%) $50x10^4$: 2/7 (29%) $80x10^4$: 1/7 (14%) $6x10^5$: 1/11 (9%)	Before inoculation ensure that monolayer is minimaly 80% confluent
Volume of cell suspension added (in µl)	200: 1/10 (10%) 300: 1/10 (10%) 350: 1/10 (10%) 400: 7/10 (70%)	300: 1/7 (14%) 350: 1/7 (14%) 400: 4/7 (57%)	400 µl
Volume of inoculums (in µl)	50: 8/10 (80%) 100: 1/10 (10%)	50: 6/7 (86%) 100 : 1/7 (14%)	50 µl
Incubation			
Duration of incubation (in days)	1: 1/10 (10%) 2: 3/10 (30%) 3: 4/10 (40%) 4: 1/10 (10%)	1: 1/7 (14%) 2: 2/7 (29%) 3: 3/7 (43%) 4: 1/7 (14%)	from 2 to 4 days
Temperature of incubation (in °C)	35: 3/10 (30%) 36: 2/10 (20%) 37: 5/10 (50%)	35: 2/7 (29%) 36: 1/7 (14%) 37: 4/7 (57%)	36 +-2°C
Incubation made in a CO2 incubator	10/10 (100%)	7/7 (100%)	YES
Percentage (in %)	5: 8/10 (80%) 3: 2/10 (20%)	5: 5/7 (71%) 3: 2/7 (29%)	5%
change of medium 24 hours later	4/10 (40%)	3/7 (43%)	
change of medium 72 hours later	0/6 (0%)	0/4 (0%)	YES
Washing			
Washing step before fixation	3/10 (30%)	2/7 (29%)	YES
Washing performed by soaking	3/3 (100%)	2/2 (100%)	YES
Washing performed in running liquid	0/3 (0%)	0/2 (0%)	
Drying			
Drying step is included before fixation	7/11 (64%)	4/7 (57%)	YES
The usual drying duration (in min)	10 : 2/6 (33%) 15 : 2/6 (33%) 20 : 1/6 (17%) 30 : 1/6 (17%)	10 : 2/4 (25%) 15 : 0/4 (25%) 20 : 1/4 (25%) 30 : 1/4 (25%)	15-30 min
The usual drying temperature (in °C)	RT : 6/6 (100%)	RT : 4/4 (100%)	RT
Fixation			
Slides heat fixed	1/9 (11%)	1/6 (17%)	
Fixation made in acetone	9/10 (90%)	6/7(86%)	YES

Acetone percentage (in %)	80 : 5/9 (56%) 99 : 4/9 (45%)	80 : 4/6 (67%) 99 : 2/6 (33%)	99-100% for Labtek
Fixing duration (in min)	20 : 1/9 (11%) 30 : 7/9 (78%) 60 : 1/9 (11%)	30 : 6/11 (55%) 60 : 1/11 (9%)	30 min
Fixing temperature (in C°)	-20 : 5/9 (56%) 4 : 1/9 (11%) RT : 3/9 (33%)	-20 : 3/6 (50%) 4 : 1/6 (17%) RT : 2/6 (33%)	-20°C
Acetone bath changed after every positive detected case	3/9 (33%)	2/6 (33%)	YES
Control and sample slides placed in separate acetone bath	6/8 (75%)	3/5 (60%)	YES
Drying (Acetone fixation only)			
Drying step performed before staining	9/10 (90%)	6/7 (86%)	YES
Drying duration (in min)	5 : 1/9 (11%) 10 : 2/9 (22%) 15 : 2/9 (22%) 20 : 2/9 (22%) 30 : 1/9 (11%)	10 : 2/6 (33%) 20 : 2/6 (33%) 30 : 1/6 (17%)	15-30 min
Drying temperature (in C°)	RT : 9/9 (89%)	RT: 6/6 (100%)	RT
Conjugate			
Use of commercially prepared conjugate	10/10 (100%)	7/7 (100%)	
Manufacturer of the conjugate	Biorad : 4/10 (40%) Fujirebio : 3/10 (30%) Millipore : 1/10 (10%) Sifin : 2/10 (20%)	Biorad : 4/7 (57%) Fujirebio : 1/7 (14%) Millipore : 0/7 (0%) Sifin : 2/7 (29%)	
Conjugate diluted as indicated by the producer	9/10 (90%)	6/6 (100%)	
composed by polyclonal anti-bodies	4/7 (57%)	4/5 (80%)	
composed by monoclonal anti-bodies	6/8 (75%)	3/5 (60%)	
Use of another conjugate	1/10 (10%)	1/7 (14%)	
In which case?	if usual conjugate not available	if usual conjugate not available	
RMB used as diluents to control non specific fluorescence	0/10 (0%)	0/7 (0%)	
NMB used as diluent to control non specific fluorescence	0/10 (0%)	0/7 (0%)	
Staining			

Volume of conjugate deposited on the slide (in µl)	40 : 2/10 (20%) 50 : 6/10 (60%) 100 : 2/10 (20%)	50 : 5/7 (71%) 100 : 2/7 (29%)	
Duration of incubation (in min)	30 : 9/10 (90%) 60 : 1/10 (10%)	30 : 6/7 (86%) 60 : 1/7 (14%)	30 min
Temperature (in °C)	37 : 10/10 (100%)	37: 7/7 (100%)	37°C
Washing			
With PBS	10/10 (100%)	7/7 (100%)	YES
With water	3/7 (43%)	2/5 (40%)	
Washing performed by soaking	10/10 (100%)	7/7 (100%)	YES
Washing performed in running liquid	1/9 (11%)	1/7 (14%)	
Drying			
Mounting done on dry slides	9/10 (90%)	6/7 (86%)	YES
Drying duration (in min)	<5 : 1/9 (11%) 10 : 3/9 (22%) 15 : 3/9 (22%) 20 : 1/9 (11%) 30 : 1/9 (11%)	<5 : 1/6 (17%) 10 : 2/6 (33%) 15 : 1/6 (17%) 20 : 1/6 (17%) 30 : 1/6 (17%)	15-30 min
Drying temperature (in °)	RT: 8/9 (89%)	RT: 5/6 (83%)	RT
Mounting medium			
pH of mounting medium (pH=...)	<8.5 : 4/9 (45%) >8.5 : 4/9 (45%)	<8.5 : 3/6 (50%) >8.5 : 2/6 (33%)	≥8.5
Use of home made mounting medium	5/6 (83%)	2/3 (67%)	
Use of commercially prepared mounting medium	4/7 (57%)	4/5 (80%)	
Percentage of glycerol in the preparation (in %)	20 : 2/7 (29%) 30 : 2/7 (29%) 50 : 1/7 (14%) 80 : 1/7 (14%)	20 : 1/4 (25%) 30 : 1/4 (25%) 50 : 0/4 (0%) 80 : 1/4 (25%)	Avoid strong concentration

Technique on microplate			
Inoculation			
Volume of inoculums (in µl)	30: 1/16 (6%) 40: 1/16 (6%) 50: 5/16 (31%) 100: 2/16 (13%) 200:2/16 (13%) 500: 3/16 (19%) 1000: 1/16 (6%)	30: 1/14 (7%) 50: 4/14 (29%) 100: 2/14 (14%) 200:2/14 (14%) 500: 3/14 (21%) 1000: 2/14 (14%)	
Cell density of the substrate (in cells/ml)	$10x10^4$: 2/15 (13%) $20x10^4$: 2/15 (13%) $30x10^4$: 2/15 (13%) $40x10^4$: 1/15 (7%) $50x10^4$: 2/15 (13%) $60x10^4$: 1/15 (7%) $12x10^5$: 1/15 (7%) $20x10^5$: 1/15 (7%) $1x10^6$: 2/15 (13%)	$10x10^4$: 2/15 (13%) $20x10^4$: 2/15 (13%) $30x10^4$: 2/15 (13%) $40x10^4$: 1/15 (7%) $50x10^4$: 1/15 (13%) $60x10^4$: 1/15 (7%) $12x10^5$: 1/15 (7%) $20x10^5$: 1/15 (7%) $1x10^6$: 2/15 (13%)	Before inoculation ensure that monolayer is at least 80% confluent
Volume of cell suspension added (in µl)	100 : 5/17 (29%) 200 : 4/17 (24%) 500 : 2/17 (12%) 750 : 1/17 (6%) 1000 : 5/17 (29%)	100 : 4/15 (29%) 200 : 4/15 (24%) 500 : 4/15 (12%) 750 : 2/15 (6%) 1000 : 1/15 (29%)	
Incubation			
Duration of incubation (in days)	2 : 1/17 (6%) 3 : 11/17 (65%) 4 : 4/17 (24%) 5 : 1/17 (6%)	2 : 1/11 (9%) 3 : 10/11 (91%) 4 : 3/11 (27%) 5 : 1/11 (9%)	From 48 to 96 h
Temperature of incubation (in °C)	35 : 1/17 (6%) 36 : 2/17 (12%) 37 : 14/17 (82%)	35 : 1/15 (7%) 36 : 2/15 (13%) 37 : 12/15 (80%)	36°C+-2°C
Incubation made in a CO2 incubator	17/17 (100%)	15/15 (100%)	YES
Percentage (in %)	5: 17/17 (100%)	5: 15/15 (100%)	5%
24 hours later?	4/17 (24%)	4/15 (27%)	
72 hours later?	1/16 (6%)	1/14 (7%)	YES
Washing			
Water	0	0	
PBS	6/9 (67%)	5/8 (63%)	YES
Acetone	5/9 (56%)	5/8 (63%)	
Percentage of acetone (in %)	60: 1/5 (20%) 80: 4/5 (80%)	60: 1/5 (20%) 80: 4/5 (80%)	
Washing performed by soaking	7/10 (70%)	6/9 (67%)	YES
Washing performed in running liquid	3/11 (27%)	2/10 (20%)	
Drying			
Drying step before fixation	4/17 (24%)	3/15 (20%)	YES

The usual drying duration (in min)	15-30 : 4/4 (100%)	15-30 : 3/3 (100%)	15-30 min
The usual drying temperature (in °C)	RT: 4/4 (100%)	RT: 3/3 (100%)	RT
Fixation			
Fixation made in acetone	17/17 (100%)	15/15 (100%)	YES
Acetone percentage (in %)	60 : 2/17 (12%) 80 : 15/17 (88%)	60 : 2/15 (13%) 80 : 13/15 (87%)	80% for microplates
Fixing duration (in min)	15 : 2/17 (12%) 30 : 13/17 (76%) 60 : 1/17 (6%)	15 : 2/15 (12%) 30 : 12/15 (76%) 60 : 1/15 (6%)	30 min
Fixing temperature (in C°)	-20: 5/17 (30%) 4: 4/17 (26%) RT: 8/17 (47%)	-20: 4/15 (27%) 4: 4/15 (27%) RT: 7/15 (47%)	
Drying			
Drying step performed before staining	13/15 (87%)	10/12 (83%)	YES
Drying duration (in min)	<5 : 4/16 (25%) 10 : 1/16 (6%) 15 : 4/16 (25%) 20 : 3/16 (19%) 30 : 2/16 (13%)	<5 : 4/14 (29%) 10 : 1/14 (7%) 15 : 2/14 (14%) 20 : 3/14 (21%) 30 : 2/14 (14%)	15-30 min
Drying temperature (in C°)	RT: 15/16 (94%)	RT: 14/14 (100%)	RT
Conjugate			
Use of commercially prepared conjugate	16/17 (94%)	14/15 (100%)	
Manufacturer of the conjugate	BioRad : 4/17 (24%) Fujirebio : 9/17 (53%) Sifin : 2/17 (12%)	BioRad : 3/15 (20%) Fujirebio : 7/15 (47%) Sifin : 1/15 (7%)	
Conjugate diluted as indicated by the producer	14/16 (88%)	13/15 (87%)	
Use of home made conjugate	1/17 (6%)	0/15 (0%)	
composed by polyclonal anti-bodies	6/14 (43%)	5/12 (42%)	
composed by monoclonal anti-bodies	10/15 (67%)	9/13 (69%)	
Use of another conjugate	6/17 (35%)	5/15 (33%)	
In which case?	In case of doubtful ; routinely	In case of doubtful ; routinely	
Evan's blue added to the conjugate preparation	6/17 (35%)	5/15 (33%)	
RMB used as diluents to control non specific fluorescence	0	0	

NMB used as diluent to control non specific fluorescence	0	0	
Staining			
Volume of conjugate deposited on the slide (in µl)	30 : 1/17 (6%) 40 : 1/17 (6%) 50 : 7/17 (41%) 100 : 2/17 (12%) 150 : 1/17 (6%) 200 : 4/17 (24%)	30 : 1/15 (7%) 40 : 1/15 (7%) 50 : 7/15 (47%) 100 : 1/15 (12%) 150 : 1/15 (7%) 200 : 4/15 (27%)	
Duration of incubation (in min)	30 : 12/17 (71%) 40 : 1/17 (7%) 60 : 3/17 (7%)	30 : 12/15 (80%) 40 : 1/15 (9%) 60 : 2/15 (9%)	30 min
Temperature (in °C)	RT : 2/17 (12%) 37 : 15/17 (88%)	20 : 1/15 (7%) 37 : 14/15 (93%)	37°C
Washing			
With PBS	14/16 (88%)	12/14 (86%)	YES
With water	7/12 (58%)	6/10 (60%)	
Washing performed by soaking	12/17 (71%)	10/15 (67%)	YES
Washing performed in running liquid	3/17 (18%)	3/15 (20%)	
Drying			
Drying step included before reading	9/17 (53%)	7/15 (47%)	YES
Drying duration (in min)	<15 : 4/9 (44%) 16-30 : 4/9 (44%) >60 : 1/9 (11%)	<15 : 3/7 (43%) 16-30 : 3/7 (43%) >60 : 1/7 (14%)	15-30 min
Temperature (in °C)	RT: 8/9 (89%)	RT: 7/7 (100%)	RT

Observation of fluorescence			
Mercury lamp	18/26 (69%)	12/19 (63%)	
Halogen lamp	14/24 (58%)	11/18 (61%)	
LED lamp	3/21 (14%)	3/16 (19%)	
Excitation filter (in mn)	450-495 : 22/24 (92%)	450-495 : 15/17 (88%)	Fluorescein excitation: 490 nm
Stop filter (in mn)	510-520 : 15/20 (75%)	510-520 : 11/15 (73%)	Fluorescein emission: 520 nm
General magnification(including interme-diate pieces) (x ...)	200 : 5/25 (20%) 400 : 14/25 (56%)	200 : 2/18 (11%) 400 : 10/18 (56%)	from 200 to 400
Number of persons examining separately the slides at each session	1 : 6/28 (21%) 2 : 19/28 (68%) 3 : 3/28 (11%)	1 : 6/21 (29%) 2 : 12/21 (57%) 3 : 3/21 (14%)	2 independent trained readers
Controls			

Negative controls included in RTCIT	26/28 (93%)	15/16 (94%)	YES
Positive controls included in RTCIT	28/28 (100%)	21/21 (100%)	YES
Positive control from field strain	15/24 (63%)	10/18 (56%)	YES
CVS	16/23 (70%)	11/18 (61%)	
EBLV-1 mouse brain	5/15 (38%)	4/11 (36%)	
EBLV-2 mouse brain	1/17 (6%)	1/14 (7%)	
Miscellaneous			
Number of successive passages performed in case of negative results	1 : 11/28 (39%) 2 : 8/28 (29%) 3 : 5/28 (18%) 4 : 1/28 (4%)	1 : 7/21 (33%) 2 : 6/21 (29%) 3 : 4/21 (19%) 4 : 1/21 (5%)	

4.2.3 Interpretation of the discordant results

False positive results (**L13**, **L14** and **L18**) could be explained by cross-contamination between samples. All the tests of the trials (FAT, RTCIT, RT-PCR and Real Time PCR) were performed on a single panel and the multiplicity of testing on the same samples may increase the probability of cross-contamination between samples leading to false positive results.

False negative results have been identified in three laboratories (**L06**; **L12**; **L51**). Probable explanation of these discordances has been done by analysing the technical questionnaires returned by laboratories.

Various clones of neuroblastoma cells exist and their variations in their sensitivities to rabies virus have been well demonstrated. Tsiang et al, (1983) have also found evidence of variable sensitivities to rabies virus in different clones of the same parent cell line while Rudd et al (1989) have detected a more permissive growth of the N2a (ATCC CCL-131) compare to its subclone the NA cell line C-1300. In consequence, it is recommended to **L06** and **L51** to use preferable the N2a cell lines (ATCC 131) to avoid any loss of technique sensitivity.
When using the microplate technique, Webster et al. have found a better sensitivity when using a cell density of 5 $x10^5$ cells /ml (Webster and Casey, 1996), an incubation period of 3-4 days (Webster, 1987) and an acetone fixation in 70-80% acetone for 30 minutes at room temperature (Webster and Casey, 1996)

L**12** did not used conventional technique on labtek or microplate. It is recommended to laboratories to follow the recommended techniques described in guidelines (WHO, 1996; OIE 2013) or the EURL recommendation presented in inter-laboratory reports (Robardet et al., 2013).

4.3 THE CONVENTIONAL REVERSE TRANSCRIPTASE POLYMERASE CHAIN REACTION

Discordant results were identified in 6 laboratories (18.2% of the participating laboratories) (**L13**, **L14**, **L18**, **L21**, **L40**, **L44**) (Table 8 and Table 9). The discordant results included 4 false positive results (12.1% of true negative samples) provided by **L13**, **L14**, **L18** and **L21**. When the laboratory announced in the result form that he could not amplify certain species in the result form and when the corresponding species were found negative (ex: L44 for bats species EBLV-1 and EBLV-2) the results were not taken into account (a false negative results occur only when the technique used is validated to amplify the concerned species). Consequently, two false negative results were identified in 254 true positive samples (0.8%). The false negative results were identified in a RABV species by **L40** and **L44**.

No laboratory failed to indicate the correct strain species of a positive sample.

Table 8: Results per strain for the RT-PCR inter-laboratory test

	n Discordant/ total	Discordant (%)	Interval confidence (%)
Number of laboratories	6/33	**18.2**	[7.6 – 36.1]
Negative samples (false positives)	4/33	**12.1**	[4.0 – 29.1]
Positive samples (false negatives)	2/254	**0.8**	[0.1 – 3.1]
CVS 27	0/32	**0**	[0 – 13.3]
RABV CnViv Estonia	1/32	**3.1**	[0.2 – 18.0]
RABV Fox former Yugoslav Republic of Macedonia	1/32	**3.1**	[0.2 – 18.0]
GS7	0/32	**0**	[0 – 13.3]
GS7 1/30	0/32	**0**	[0 – 13.3]
GS7 1/50	0/32	**0**	[0 – 13.3]
EBLV-1	0/31	**0**	[0 – 13.3]
EBLV-2	0/31	**0**	[0 – 13.3]

4.3.1 Evolution of the discordant results in participating laboratories

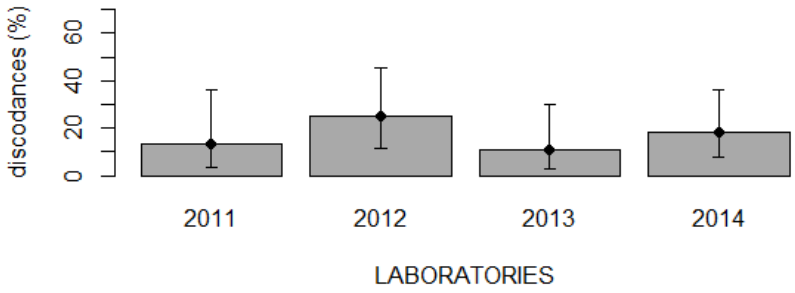
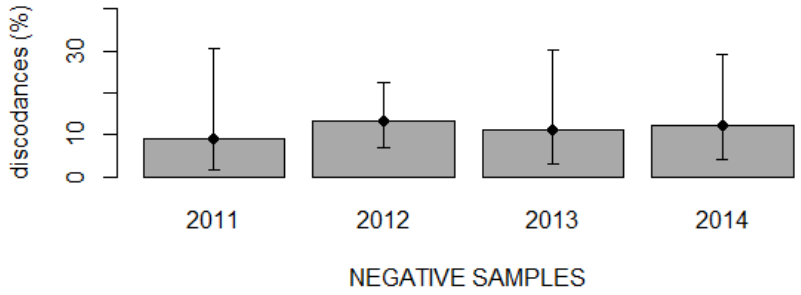


Figure 5: Evolution of the proportion of laboratories harbouring discordant results. (2011: Only the Panel 2 including different rabies virus species is taken into account, Panel 1 including only French RABV diluted samples is excluded from the analysis)

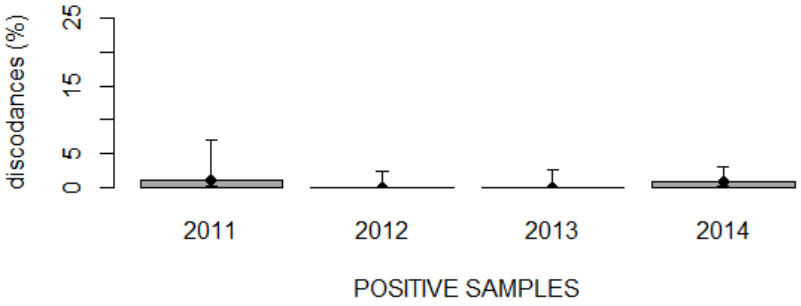
The proportion of laboratories with discordant results (Figure 5) and the proportion of discordant results within the different species (Figure 6) remains stable among years.

Figure 6: Evolution of the proportion of discordant results according to the strain used. A: negative samples; B: positive samples; C: RABV samples; D: EBLV-1 samples, E: EBLV-2 samples.

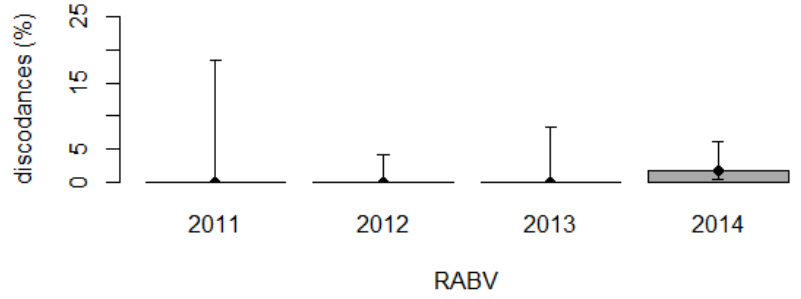
A)



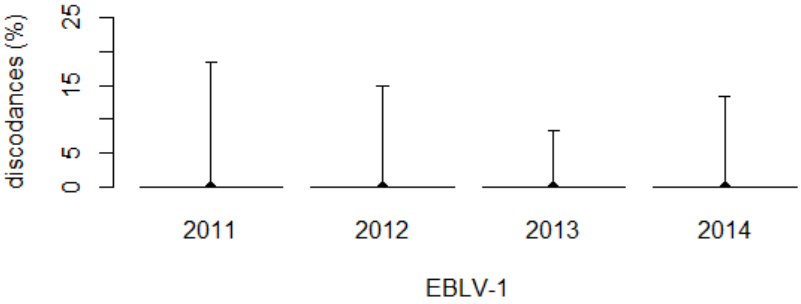
B)



C)



D)



E)

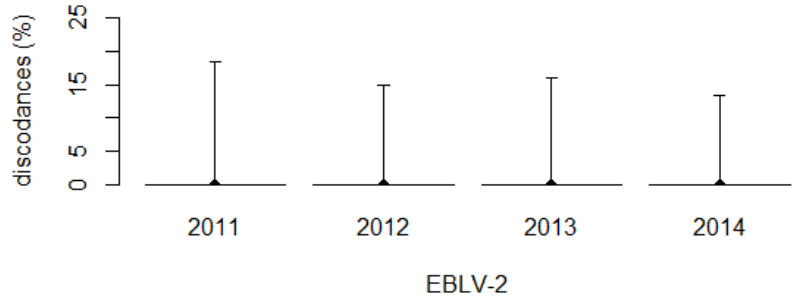


Table 9: Results of the inter-laboratory test on RT-PCR. pos: positive result; neg: negative result. grey: the technique used did not allow to detect the virus species, the result is consequently not taken into account for the analysis (each laboratory had to declare its ability to detect the different rabies species via online filled technical questionnaires). Police in red: misidentification of the strain. NA: data not available.

Lab_code	CVS27	result (pos/neg)	species	Cn ViV estonie	result (pos/neg)	species	RABV FYROM	result (pos/neg)	species	GS7	result (pos/neg)	species	GS7 1/30	result (pos/neg)	species
L02	14050259	pos	RABV	14050213	pos	RABV	14050284	pos	RABV	14050257	pos	RABV	14050288	pos	RABV
L03	14050301	pos	RABV	14050323	pos	RABV	14050354	pos	RABV	14050397	pos	RABV	14050319	pos	RABV
L05	14050541	pos	RABV	14050554	pos	RABV	14050538	pos	RABV	14050532	pos	NA	14050543	pos	NA
L07	14050704	pos	RABV	14050760	pos	RABV	14050730	pos	RABV	14050786	pos	RABV	14050711	pos	RABV
L10	14051047	pos	RABV	14051085	pos	RABV	14051034	pos	RABV	14051060	pos	RABV	14051064	pos	RABV
L11	14051115	pos	RABV	14051142	pos	RABV	14051158	pos	RABV	14051100	pos	RABV	14051155	pos	RABV
L12	14051215	pos	RABV	14051210	pos	RABV	14051278	pos	RABV	14051281	pos	RABV	14051258	pos	RABV
L13	14051304	pos	NA	14051375	pos	NA	14051394	pos	NA	14051393	pos	NA	14051395	pos	NA
L14	14051403	pos	NA	14051487	pos	NA	14051421	pos	NA	14051477	pos	NA	14051417	pos	NA
L15	14051590	NA	NA	14051544	NA	NA	14051550	NA	NA	14051513	NA	NA	14051537	NA	NA
L16	14051662	pos	RABV	14051664	pos	RABV	14051607	pos	RABV	14051689	pos	RABV	14051622	pos	RABV
L17	14051773	NA	NA	14051702	NA	NA	14051725	NA	NA	14051726	NA	NA	14051778	NA	NA
L18	14051805	pos	RABV	14051840	pos	RABV	14051831	pos	RABV	14051812	pos	RABV	14051860	pos	RABV
L19	14051938	pos	RABV	14051907	pos	RABV	14051969	pos	RABV	14051947	pos	RABV	14051917	pos	RABV
L21	14052143	pos	RABV	14052140	pos	RABV	14052105	pos	RABV	14052147	pos	RABV	14052166	pos	RABV
L22	14052234	pos	RABV	14052212	pos	RABV	14052204	pos	RABV	14052226	pos	RABV	14052287	pos	RABV
L23	14052321	pos	RABV	14052359	pos	RABV	14052383	pos	RABV	14052353	pos	RABV	14052345	pos	RABV
L25	14052542	pos	RABV	14052529	pos	RABV	14052578	pos	RABV	14052563	pos	RABV	14052593	pos	RABV
L27	14052778	pos	RABV	14052702	pos	RABV	14052761	pos	RABV	14052764	pos	RABV	14052773	pos	RABV
L29	14052922	pos	NA	14052942	pos	NA	14052936	pos	NA	14052907	pos	NA	14052950	pos	NA
L30	14053003	pos	RABV	14053068	pos	RABV	14053070	pos	RABV	14053056	pos	RABV	14053091	pos	RABV
L31	14053110	pos	RABV	14053125	pos	RABV	14053118	pos	RABV	14053160	pos	RABV	14053189	pos	RABV
L32	14053268	pos	RABV	14053262	pos	RABV	14053252	pos	RABV	14053232	pos	RABV	14053293	pos	RABV
L36	14053625	pos	RABV	14053691	pos	RABV	14053696	pos	RABV	14053666	pos	RABV	14053609	pos	RABV
L38	14053845	pos	RABV	14053890	pos	RABV	14053869	pos	RABV	14053819	pos	RABV	14053848	pos	RABV
L39	14053951	pos	RABV	14053933	pos	RABV	14053997	pos	RABV	14053928	pos	RABV	14053911	pos	RABV
L40	14054028	pos	RABV	14054018	pos	RABV	14054038	neg		14054097	pos	RABV	14054005	pos	RABV
L41	14054115	pos	RABV	14054190	pos	RABV	14054195	pos	RABV	14054145	pos	RABV	14054155	pos	RABV
L42	14054201	pos	RABV	14054239	pos	RABV	14054262	pos	RABV	14054214	pos	RABV	14054234	pos	RABV
L44	14054468	pos	NA	14054461	neg		14054497	pos	NA	14054498	pos	NA	14054414	pos	NA
L46	14054618	pos	RABV	14054662	pos	RABV	14054600	pos	RABV	14054672	pos	RABV	14054671	pos	RABV
L47	14054769	pos	NA	14054747	pos	NA	14054725	pos	NA	14054756	pos	NA	14054755	pos	NA
L48	14054816	pos	RABV	14054818	pos	RABV	14054859	pos	RABV	14054806	pos	RABV	14054898	pos	RABV
L49	14054955	pos	RABV	14054961	pos	RABV	14054973	pos	RABV	14054984	pos	RABV	14054942	pos	RABV

Table 9: Results of the inter-laboratory test on RT-PCR. pos: positive result; neg: negative result. grey: the technique used did not allow to detect the virus species, the result is consequently not taken into account for the analysis (each laboratory had to declare its ability to detect the different rabies species via online filled technical questionnaires). Police in red: misidentification of the strain. NA: data not available.

Lab_code	GS7 1/50	result (pos/neg)	species	EBLV-1a	result (pos/neg)	species	EBLV-2	result (pos/neg)	species	NEGATIVE	result (pos/neg)	species
L02	14050233	pos	RABV	14050212	pos	EBLV-1	14050214	Pos	EBLV-2	14050245	neg	
L03	14050341	pos	RABV	14050312	pos	EBLV-1	14050357	Pos	EBLV-2	14050307	neg	
L05	14050590	pos	NA	14050542	pos	EBLV-1	14050530	Pos	EBLV-2	14050529	neg	
L07	14050770	pos	RABV	14050759	pos	EBLV-1	14050718	Pos	EBLV-2	14050756	neg	
L10	14051074	pos	RABV	14051017	pos	EBLV-1	14051052	Pos	EBLV-2	14051035	neg	
L11	14051113	pos	RABV	14051117	pos	EBLV-1	14051124	Pos	EBLV-2	14051134	neg	
L12	14051245	pos	RABV	14051257	pos	EBLV-1	14051295	Pos	EBLV-2	14051216	neg	
L13	14051392	pos	NA	14051380	pos	NA	14051311	Pos	NA	14051348	pos	
L14	14051427	pos	NA	14051481	pos	NA	14051468	Pos	NA	14051458	pos	
L15	14051520	NA	NA	14051510	NA	NA	14051500	NA	NA	14051501	neg	
L16	14051605	pos	RABV	14051670	pos	EBLV-1	14051685	Pos	EBLV-2	14051635	neg	
L17	14051709	NA	NA	14051724	NA	NA	14051797	NA	NA	14051727	NA	NA
L18	14051816	pos	RABV	14051885	pos	EBLV-1	14051849	Pos	EBLV-2	14051855	pos	**EBLV-2**
L19	14051909	pos	RABV	14051919	pos	EBLV-1	14051997	Pos	EBLV-2	14051932	neg	
L21	14052185	pos	RABV	14052135	pos	EBLV-1	14052179	Pos	EBLV-2	14052149	pos	**RABV**
L22	14052285	pos	RABV	14052216	pos	EBLV-1	14052261	Pos	EBLV-2	14052230	neg	
L23	14052346	pos	RABV	14052329	pos	EBLV-1	14052324	Pos	EBLV-2	14052398	neg	
L25	14052545	pos	RABV	14052587	pos	EBLV-1	14052548	Pos	EBLV-2	14052553	neg	
L27	14052732	pos	RABV	14052779	pos	EBLV-1	14052762	Pos	EBLV-2	14052727	neg	
L29	14052974	pos	NA	14052952	pos	NA	14052932	Pos	NA	14052958	neg	
L30	14053004	pos	RABV	14053028	pos	EBLV-1	14053048	Pos	EBLV-2	14053001	neg	
L31	14053193	pos	RABV	14053131	pos	EBLV-1	14053141	Pos	EBLV-2	14053192	neg	
L32	14053203	pos	RABV	14053260	pos	EBLV-1	14053243	Pos	EBLV-2	14053245	neg	
L36	14053659	pos	RABV	14053693	pos	EBLV-1	14053651	Pos	EBLV-2	14053667	neg	
L38	14053888	pos	RABV	14053825	pos	EBLV-1	14053856	Pos	EBLV-2	14053875	neg	
L39	14053956	pos	RABV	14053918	pos	EBLV-1	14053932	Pos	EBLV-2	14053962	neg	
L40	14054024	pos	RABV	14054039	pos	EBLV-1	14054080	Pos	EBLV-2	14054019	neg	
L41	14054117	pos	RABV	14054139	pos	EBLV-1	14054154	Pos	EBLV-2	14054184	neg	
L42	14054218	pos	RABV	14054278	pos	EBLV-1	14054266	Pos	EBLV-2	14054283	neg	
L44	14054499	pos	NA	14054428	neg		14054477	neg		14054440	neg	
L46	14054697	pos	RABV	14054680	pos	EBLV-1	14054606	Pos	EBLV-2	14054629	neg	
L47	14054733	pos	NA	14054732	pos	NA	14054718	Pos	NA	14054723	neg	
L48	14054830	pos	RABV	14054895	pos	EBLV-1	14054831	Pos	EBLV-2	14054838	neg	
L49	14054963	pos	RABV	14054968	pos	EBLV-1	14054975	Pos	EBLV-2	14054952	neg	

4.3.2 Answers of the participating laboratories to the conventional RT-PCR technical questionnaire

Item	Number of laboratories/ number of total answers	EU laboratory answers
Techniques used		
RT-PCR alone	20/27 (74%)	7/12 (63%)
Nested RT-PCR	13/24 (54%)	8/14 (57%)
Published technique	28/32 (57%)	17/18 (95%)
RNA Extraction		
RNA extraction performed manually	16/22 (73%)	10/13 (76)
RNA extraction performed with automated RNA purification	8/21 (38%)	3/11 (27)
Viral RNA extracted	16/21 (76%)	9/11 (81)
Total RNA extracted	16/23 (70%)	8/13 (61)
Commercial kit used to perform the extraction	16/23 (70%)	9/13 (59)
Name of the extraction kit	- **Qiagen** QIAamp Viral RNA Mini Kit: 7/20 (35%) - **Macherey-Nagel** NucleoSpin RNA II: 3/20 (15%) - **Invitrogen** iPrep PureLink Kit: 2/20 (10%) - **Roche** Magna pure compact Nucleic acid isolation kit: 2/20 (10%) - **Qiagen** Rneasy Mini Kit: 2/16 (13%) - **Ambion** Pure LinkTM RNA Mini Kit: 1/20 (5%) - **BioMerieux** Nuclisense reagents: 1/20 (5%)	- **Qiagen** QIAamp Viral RNA Mini Kit: 5/14 (36%) - **Macherey-Nagel** NucleoSpin RNA II: 3/14 (21%) - **Invitrogen** iPrep PureLink Kit: 2/14 (14%) - **Qiagen** Rneasy Mini Kit: 2/14 (14%) - **Ambion** Pure LinkTM RNA Mini Kit: 1/14 (7%) - **BioMerieux** Nuclisense reagents: 1/14 (5%)
Trizol	8/20 (40%)	3/14 (21%)
Guanidium thiocyanate	1/20 (5%)	1/14 (7%)
CsCl gradient	0 (0%)	0 (0%)
Quantity of RNA in samples measured after the extraction step	5/32 (16%)	1/18 (6%)
RT-PCR		
One step RT-PCR	18/24 (75%)	11/13 (85)
Two step RT-PCR	7/24 (29%)	3/13 (23)
One step RT-PCR		
Commercial kit used to perform the one step RT-PCR	16/18 (89%)	9/11 (82)

Name of the kit	- **Qiagen** OneStep RT-PCR Kit: 9/15 (60%) - **Invitrogen** SuperScript III RT-PCR with Platinum Taq: 6/15 (40%)	- **Qiagen** OneStep RT-PCR Kit: 7/9 (78%) - **Invitrogen** SuperScript III RT-PCR with Platinum Taq: 2/9 (22%)
N region	17/18 (94%)	10/11 (91)
Others	3/11 (27%)	2/9 (22)
If other, genome region used	G gene: 2/3 (67%) M gene: 1/3 (33%)	G gene: 1/2 (50%) M gene: 1/2 (50%)
Nucleotide localisation of the primers used for RT-PCR (based on PV virus)	- **JW12**: 55-74; **JW6**: 641-660: 14/19 (74%) - **Lys001**:1-16; **304:** 1514-1533: 1/19 (5%) - **GRAB1F:** 538-557; **GRAB1R:** 911-892: 2/19 (11%) - **RabForPyro:** 59–75; **RabRevPyro:** 662–641: 1/19 (5%) - **N-113F**: 1013-1029; **N-304R**: 1514-1533: 1/19 (5%)	- **JW12**: 55-74; **JW6**: 641-660: 10/13 (77%) - **GRAB1F:** 538-557; **GRAB1R:** 911-892: 2/13 (15%) - **RabForPyro:** 59–75; **RabRevPyro:** 662–641: 1/13 (8%) - **N-113F**: 1013-1029; **N-304R**: 1514-1533: 1/13 (8%)
Pair of primers published	20/21 (95%)	14/14 (100%)
Size of amplified product	606 pb : 14/19 (74%) 373 pb : 2/19 (11%) 603 pb : 1/19 (5%) 521 pb : 1/19 (5%) 1566 pb : 1/19 (5%)	606 pb : 10/13 (77%) 373 pb : 2/13 (15%) 603 pb : 1/13 (8%)
Total reactional volume (in µl)	20 : 3/20 (15%) 25 : 9/20 (45%) 50 : 7/20 (35%)	20 : 2/13 (15%) 25 : 5/13 (38%) 50 : 5/13 (38%)
RNA final volume per tube (in µl)	2: 4/19 (21%) 5: 11/19 (58%)	2: 3/12 (25%) 5: 7/12 (58%)
Quantity of RNA per tube (in ng)	0 : 1/12 (8%) 50 : 3/12 (25%) 250 : 3/12 (25%) No quantifed : 5/12 (42%)	0 : 1/7 (14%) 50 : 1/7 (14%) 250 : 3/7 (43%) No quantifed : 2/7 (29%)
Two step RT-PCR: RT reaction		
Rabies specific primer	8/10 (80%)	2/3 (67%)
Universal primer	5/12 (42%)	3/4 (75%)
If universal primer is used, please specify (pdN6, etc.)	**Random Hexanucleotide**: 4/5 (80%)	**Random Hexanucleotide**: 3/3 (100%)
Commercial kit used to perform the RT	5/11 (45%)	3/4 (75%)
Name of the kit	**Invitrogen** Superscript III: 3/5 (60%) **Applied biosystems**: 1/5 (20%)	**Invitrogen** Superscript III: 2/3 (7%) **Applied biosystems**: 1/3 (33%)
Nucleotide localisation of the primers used	- 54-73: 4/8 (50%) - 0-15 and 647-666: 1/8 (13%) - 66-82: 1/8 (13%) - 1157-1476 : 1/8 (13%) - 55-19 ;1-16 ; 647-666 ; 1136-1155 : 1/8 (13%)	54-73: 2/2 (100%)
Primer used in the RT published	8/10 (80%)	2/3 (67%)
AMV	4/10 (40%)	0/4 (0%)
M-MuLV	3/10 (30%)	1/4 (25%)

Other	4/10 (40%)	4/2 (50%)
Duration of incubation (in min)	45: 1/12 (8%) 50: 3/12 (25%) 60: 1/12 (8%) 90: 5/12(42%) 120 : 2/12 (17%)	50: 2/4 (25%) 60: 1/4 (8%) 90: 1/4(42%)
Temperature of incubation (in °C)	37: 1/12 (8%) 42: 5/12 (42%) 50: 3/12 (25%) 55: 2/12 (17%) 65 : 1/12 (8%)	42: 2/4 (50%) 50: 3/6 (50%)
Quantity of RNA per tube (in ng)	<1500: 2/4 (50%) >1500: 2/4 (50%)	<1500: 1/2 (50%) >1500: 1/2 (50%)
RNA final volume per tube (in µl)	2: 4/11 (36%) 5: 3/11 (27%) 6: 1/11 (9%) 7: 1/11 (9%) >10: 2/11 (18%)	2: 2/4 (50%) 5: 2/4 (50%)
Total reactional volume (in µl)	20: 10/12 (83%) 30 : 1/12 (8%)	20: 3/4 (75%) 30 : 1/4 (25%)
Two step RT-PCR: PCR amplification		
N region	12/12 (100%)	4/4 (100%)
Others	3/8 (38%)	1/3 (33%)
If other, please specify the region:	G: 1/2 (50%) L: 1/2 (50%)	L: 1/1 (100%)
Nucleotide localisation of the primers used for RT-PCR (based on PV virus)	- **JW12**: 55-74; **JW6**: 660-641: 6/11 (55%) - ~ 1060 and N-L intergenic region: 1/11 (9%) -550F, 304: 1/11 (9%) - For gene N = N127:55-74/N8m:1572-1590, for gene L =PVO5m:7170/PVO9:7489: 1/11 (9%) - Jw12-304: 1/11 (9%) - lyss001: 1-16/550B: 647-666; 550F: 647-666/1066B:1136-1155; 921F:991-1011/304: 1514-1533; 1066F:1136-1155/304:1514-1533: 1/11 (9%)	**JW12**: 55-74; **JW6**: 660-641: 3/4 (75%)
Pair of primers published	10/11 (90%)	4/4 (100%)
Size of amplified product (in bp)	606: 5/12 (42%) 320 : 1/12 (8%) 582 : 1/12 (8%) 612 : 1/12 (8%) 1353 : 1/12 (8%) 1536 : 1/12 (8%)	606: 2/4 (50%) 582: 1/4 (25%) 1536: 1/4 (25%)
Two step RT-PCR: PCR 2		

Name of the product	- **Applied biosystems** – AmpliTaq: 5/14 (36%) - **Invitrogen** - Platinum Taq DNA polymerase: 2/14 (14%) - **Qiagen** - Taq DNA polymerase: 2/14 (14%) - **Feramentas** - Recombinant Taq: 1/14 (7%) - **Bioline** - BioTaq DNA Polymerase: 1/14 (7%) - **Gotaq** green master mix Promega: 1/14 (7%) - **Promega**-GoTaq DNA Polymerase: 1/14 (7%) - **Qiagen** HotStar Plus : 1/14 (7%)	- **Applied biosystems** – AmpliTaq: 3/9 (33%) - **Invitrogen** - Platinum Taq DNA polymerase: 1/9 (11%) - **Qiagen** - Taq DNA polymerase: 2/9 (22%) - **Fermentas** - Recombinant Taq: 1/9 (11%) - **Bioline** - BioTaq DNA Polymerase: 1/9 (11%) - **Promega**-GoTaq DNA Polymerase: 1/9 (11%)
Quantity of polymerase included per tube (in U/µl)	0.02 : 1/11 (9%) 0.1 : 1/11 (9%) 1 : 1/11 (9%) 1.25 :5/11 (45%) 5 : 2/11 (18%)	0.02 : 1/9 (11%) 0.1 : 1/9 (11%) 1 : 1/9 (11%) 1.25 :4/9 (45%) 5 : 2/9 (2%)
Nucleotide localisation of the primers used for PCR 2 (based on PV virus)	- **JW12**: 55-74; **JW6**: 660-641: 2/14 (14%) - **JW12**: 54-73; **JW10**: 617-636: 1/7 (57%) - For gene N = **N127:55-74/N829**:871-889, for gene L =**PVO5m**:7170-7189/PVO8:7419-7398: 1/14 (7%) - **GENRAB2F** 574-593/**GENRAB2R** 833-814: 1/14 (7%) - **Lys001**: 1-16/1066: 1136-1155 and **550Fw**: 647-666/304 1514-1533: 1/14 (7%) - **lys001**:1-16/520B:620-636; **550F**:647-666/921B:991-1011; **1066F**:1136-1155/304:1514-1533 **1087F**:1157-1173/304: 1514-1533 : 1/14 (7%)	- **JW12**: 55-74; **JW6**: 660-641: 2/9 (22%) - **JW12**: 54-73; **JW10**: 617-636: 5/9 (55%) - For gene N = **N127:55-74/N829**:871-889, for gene L =**PVO5m**:7170-7189/PVO8:7419-7398: 1/9 (11%) - **GENRAB2F** 574-593/**GENRAB2R** 833-814: 1/9 (11%)
Pair of primers published	11/13 (85%)	9/9 (100%)
Size of amplified product (in bp)	589 : 8/14 (57%) 259 : 1/14 (7%) 498 : 1/14 (7%) 586 : 1/14 (7%) 1174 and 886 : 1/14 (7%)	589 : 6/9 (66%) 259 : 1/9 (11%) 586 : 1/9 (11%)
RNA final volume per tube (in µl)	1: 6/14 (43%) 2: 4/14 (29%) 5: 3/14 (21%)	1: 5/9 (56%) 2: 2/9 (22%) 5: 2/9 (22%)
Total reactional volume (in µl)	20: 3/14 (21%) 25: 7/14 (50%) 50: 3/14 (21%)	20: 3/9 (33%) 25: 4/9 (44%) 50: 2/9 (22%)

Controls		
Additionally to tested sample, negative samples are intercalated within the panel	26/32 (81%)	16/18 (89%)
Negative brain	12/24 (50%)	6/15 (40%)
Water	21/26 (81%)	12/16 (75%)
A saline buffer as PBS	3/21 (14%)	2/14 (14%)
Negative controls included in the RT-PCR	30/32 (94%)	17/18 (94%)
RNA negative extraction control	19/26 (73%)	11/15 (73%)
RT negative control	9/22 (41%)	7/14 (50%)
PCR1 negative control	17/26 (65%)	11/16 (69%)
PCR2 negative control	9/22 (41%)	7/14 (50%)
Other	6/19 (32%)	2/11 (18%)
Positive controls included in the RT-PCR	30/32 (94%)	17/18 (94%)
RNA positive extraction control	13/24 (54%)	6/15 (40%)
RT positive negative control	9/22 (41%)	6/14 (43%)
PCR1 positive control	18/26 (69%)	10/15 (67%)
PCR2 positive control	7/23 (30%)	6/15 (40%)
Other	4/20 (20%)	2/13 (15%)
Ubiquitous gene amplified to validate the RT-PCR results is (as 18S mouse rRNA)	7/30 (23%)	5/17 (29%)

4.3.3 Interpretation of the discordant results

Discrepant results of conventional RT-PCR consisted of four false positive results in four laboratories (**L13**; **L14**; **L18** and **L21**) and two false negative results in two laboratories (**L40** and **L44**).

Possible explanations of the discrepant results were assessed by analysing the technical questionnaires fulfilled by participating laboratories (L13, L14, L18, L21, L40). Negative controls of RNA extraction and RT-PCR were included in the conventional RT-PCR by laboratories L13, L14, L18 and L21. Negative samples were intercalated within the samples to test in order to check the absence of contamination throughout sample processing (L13, L14, L18 and L21). Of the four laboratories showing false positive results, three laboratories performed the RT-PCR in one-step and one laboratory used the two-step method. Two laboratories used an automated RNA purification and two laboratories performed manually the RNA extraction. All laboratories used commercial kits and validated primers.

Based on the analysis of the technical questionnaires, discordant results might have occurred either during the sample preparation (during the addition of water in the freeze dried tubes) or during the step of RNA extraction. The same discrepant results were also recorded in real-time RT-PCR in the same laboratories (L13, L14 and L21; L18 did not performed the analysis) suggesting the cross contamination of the negative sample by a positive sample.

4.4 THE REAL TIME REVERSE TRANSCRIPTASE POLYMERASE CHAIN REACTION

Discordant results were identified in 5 laboratories (20.0%) (Table 10 and Table 11). The discordant results included three false positive results (12.0%) (**L13**; **L14** and **L21**) and 5 false negative results (26% of true positive samples) (**L5**; **L21**; **L33**). False negative results were detected on GS7 (1 sample), GS7 1/30 (1sample), GS7 1/50 (1 sample), EBLV-1 (2 samples).

All laboratories that indicated the species of the positive samples succeeded in the species identification.

Table 10: Results per strain for the Real Time RT-PCR inter-laboratory test

	n Discordant/ total	Discordant (%)	Interval confidence (%)
Number of laboratories	5/25	**20.0**	[7.6 – 41.3]
Negative samples (false positives)	3/25	**12.0**	[3.1 – 32.3]
Positive samples (false negatives)	5/192	**2.6**	[1.0 – 6.3]
CVS 27	0/25	**0**	[0 – 16.6]
RABV CnViv Estonia	0/25	**0**	[0 – 16.6]
RABV Fox former Yugoslav Republic of Macedonia	0/25	**0**	[0 – 16.6]
GS7	1/25	**4.0**	[0 – 16.6]
GS7 1/30	1/25	**4.0**	[0.2 – 22.3]
GS7 1/50	1/25	**4.0**	[0.2 – 22.3]
EBLV-1	2/21	**9.5**	[1.7 – 31.8]
EBLV-2	0/21	**0**	[0 – 19.2]

4.4.1 Evolution of the discordant results in participating laboratories

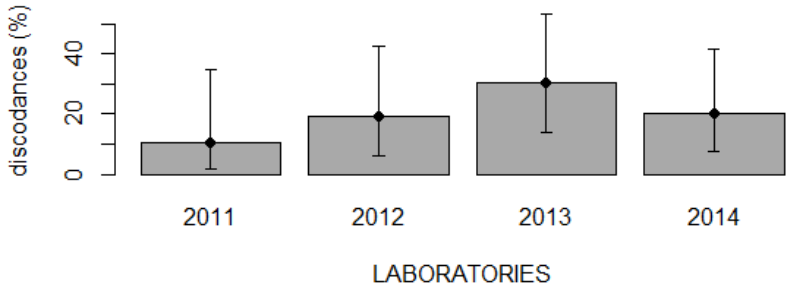


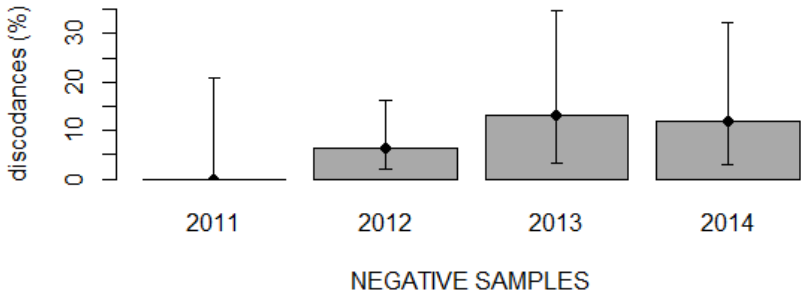
Figure 7: Evolution of the proportion of laboratories harbouring discordant results in Real Time PCR trials. (2011: Only the Panel 2 including different rabies virus species is taken into account, Panel 1 including only French RABV diluted samples is excluded from the analysis)

The proportion of laboratories with discordant results (Figure 7) and the proportion of discordant results within the different species (Figure 8) remain stable among years.

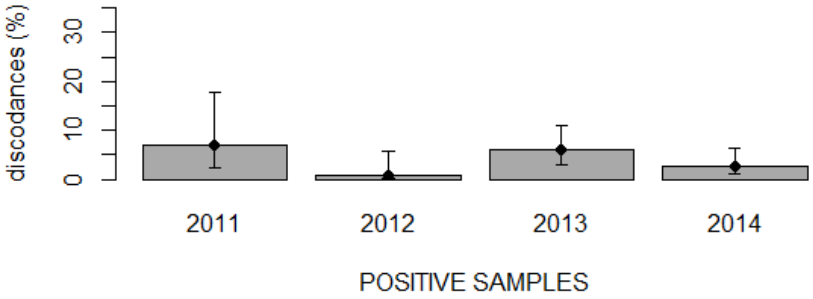
The only observable difference is that no discordant results were observed in 2014 on EBLV-2 bat species (Figure 8).

Figure 8: Evolution of the proportion of discordant results according to the strain used in Real Time PCR trials. A: negative samples; B: positive samples; C: RABV samples; D: EBLV-1 samples, E: EBLV-2 samples.

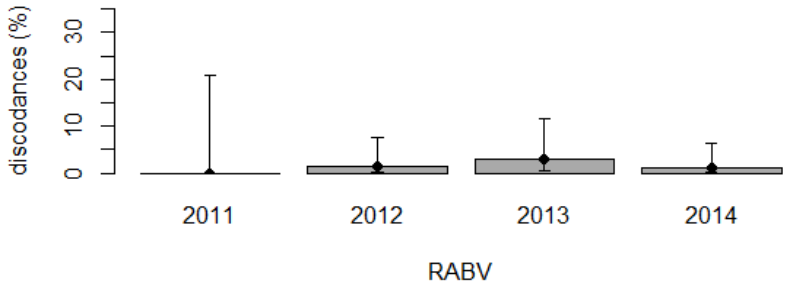
A)



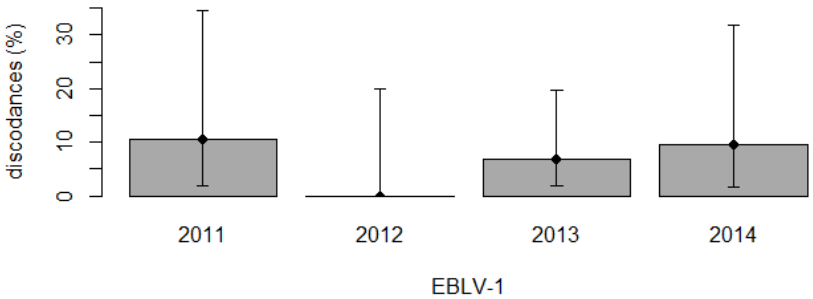
B)



C)



D)



E)

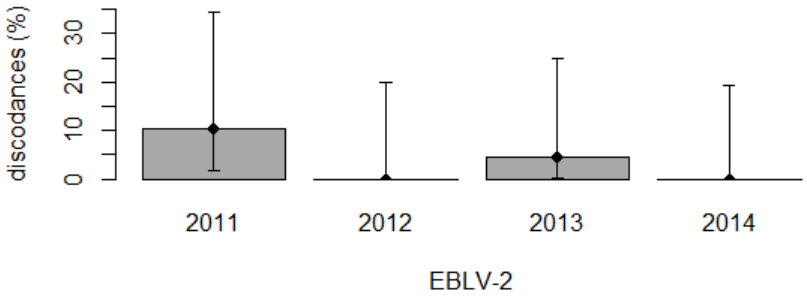


Table 11: Results of the inter-laboratory test on Real Time RT-PCR. pos: positive result; neg: negative result; orange: misidentification of the strain; grey: the technique used did not allow to detect the virus species, the result is consequently not taken into account for the analysis (each laboratory have to declared its ability to detect the different rabies species via online filled technical questionnaires).

Lab_code	CVS27	result (pos/neg)	species	Cn ViV estonie	result (pos/neg)	species	RABV FYROM	result (pos/neg)	species	GS7	result (pos/neg)	species	GS7 1/30	result (pos/neg)	species
L04	14050471	pos	RABV	14050457	pos	RABV	14050492	pos	RABV	14050417	pos	RABV	14050456	pos	RABV
L05	14050541	pos	RABV	14050554	pos	RABV	14050538	pos	RABV	14050532	neg		14050543	neg	
L06	14050640	pos	RABV	14050662	pos	RABV	14050607	pos	RABV	14050613	pos	RABV	14050683	pos	RABV
L08	14050827	pos	NA	14050883	pos	NA	14050870	pos	NA	14050851	pos		14050823	pos	NA
L11	14051115	pos	NA	14051142	pos	NA	14051158	pos	NA	14051100	pos		14051155	pos	NA
L13	14051304	pos	NA	14051375	pos	NA	14051394	pos	NA	14051393	pos		14051395	pos	NA
L14	14051403	pos	RABV	14051487	pos	RABV	14051421	pos	RABV	14051477	pos	RABV	14051417	pos	RABV
L15	14051590	pos	RABV	14051544	pos	RABV	14051550	pos	RABV	14051513	pos	RABV	14051537	pos	RABV
L16	14051662	pos	RABV	14051664	pos	RABV	14051607	pos	RABV	14051689	pos	RABV	14051622	pos	RABV
L21	14052143	pos	RABV	14052140	pos	RABV	14052105	pos	RABV	14052147	pos	RABV	14052166	pos	RABV
L22	14052234	pos	RABV	14052212	pos	RABV	14052204	pos	RABV	14052226	pos	RABV	14052287	pos	RABV
L23	14052321	pos	NA	14052359	pos	NA	14052383	pos	NA	14052353	pos		14052345	pos	NA
L28	14052898	pos	RABV	14052848	pos	RABV	14052809	pos	RABV	14052891	pos	RABV	14052832	pos	RABV
L29	14052922	pos	RABV	14052942	pos	RABV	14052936	pos	RABV	14052907	pos	RABV	14052950	pos	RABV
L30	14053003	NA	NA	14053068	NA	NA	14053070	NA	NA	14053056	NA	NA	14053091	NA	NA
L32	14053268	pos	RABV	14053262	pos	RABV	14053252	pos	RABV	14053232	pos	RABV	14053293	pos	RABV
L33	14053309	pos	NA	14053343	pos	NA	14053366	pos	NA	14053312	pos		14053392	pos	NA
L36	14053625	pos	RABV	14053691	pos	RABV	14053696	pos	RABV	14053666	pos	RABV	14053609	pos	RABV
L39	14053951	pos	NA	14053933	pos	NA	14053997	pos	NA	14053928	pos		14053911	pos	NA
L41	14054115	pos	NA	14054190	pos	NA	14054195	pos	NA	14054145	pos		14054155	pos	NA
L43	14054322	pos	NA	14054316	pos	NA	14054380	pos	NA	14054383	pos		14054368	pos	NA
L44	14054468	NA	NA	14054461	NA	NA	14054497	NA	NA	14054498	NA	NA	14054414	NA	NA
L46	14054618	pos	RABV	14054662	pos	RABV	14054600	pos	RABV	14054672	pos	RABV	14054671	pos	RABV
L48	14054816	pos	RABV	14054818	pos	RABV	14054859	pos	RABV	14054806	pos	RABV	14054898	pos	RABV
L49	14054955	pos	RABV	14054961	pos	RABV	14054973	pos	RABV	14054984	pos	RABV	14054942	pos	RABV
L50	14055004	pos	RABV	14055021	pos	RABV	14055069	pos	RABV	14055038	pos	RABV	14055095	pos	RABV
L51	14055113	pos	RABV	14055167	pos	RABV	14055153	pos	RABV	14055180	pos	RABV	14055137	pos	RABV

Table 11: Results of the inter-laboratory test on Real Time RT-PCR. pos: positive result; neg: negative result; orange: misidentification of the strain; grey: the technique used did not allow to detect the virus species, the result is consequently not taken into account for the analysis (each laboratory have to declared its ability to detect the different rabies species via online filled technical questionnaires).

Lab_code	GS7 1/50	result (pos/neg)	species	EBLV-1a	result (pos/neg)	species	EBLV-2	result (pos/neg)	species	NEGATIVE	result (pos/neg)	species
L04	14050428	pos	RABV	14050415	pos	EBLV-1	14050486	pos	EBLV-2	14050459	neg	
L05	14050590	neg		14050542	neg		14050530	neg		14050529	neg	
L06	14050690	pos	RABV	14050631	pos	EBLV-1	14050689	pos	EBLV-2	14050696	neg	RABV
L08	14050897	pos	NA	14050894	pos	NA	14050831	pos		14050819	neg	
L11	14051113	pos	NA	14051117	pos	NA	14051124	pos		14051134	neg	
L13	14051392	pos	NA	14051380	pos	NA	14051311	pos		14051348	pos	
L14	14051427	pos	RABV	14051481	pos	EBLV-1	14051468	pos	EBLV-2	14051458	pos	RABV
L15	14051520	pos	RABV	14051510	pos	EBLV-1	14051500	pos	EBLV-2	14051501	neg	
L16	14051605	pos	RABV	14051670	pos	EBLV-1	14051685	pos	EBLV-2	14051635	neg	
L21	14052185	pos	RABV	14052135	neg		14052179	pos	EBLV-2	14052149	pos	RABV
L22	14052285	pos	RABV	14052216	neg		14052261	neg		14052230	neg	
L23	14052346	pos	NA	14052329	pos	NA	14052324	pos		14052398	neg	
L28	14052833	pos	RABV	14052812	pos	**RABV**	14052844	pos	EBLV-2	14052845	neg	
L29	14052974	pos	RABV	14052952	pos	**RABV**	14052932	pos	EBLV-2	14052958	neg	
L30	14053004	NA	NA	14053028	NA	NA	14053048	NA	NA	14053001	NA	
L32	14053203	pos	RABV	14053260	pos	EBLV-1	14053243	pos	EBLV-2	14053245	neg	
L33	14053398	pos	NA	14053378	neg		14053374	pos		14053375	neg	
L36	14053659	pos	RABV	14053693	pos	EBLV-1	14053651	pos	EBLV-2	14053667	neg	
L39	14053956	pos	NA	14053918	neg		14053932	neg		14053962	neg	
L41	14054117	pos	NA	14054139	pos	NA	14054154	pos		14054184	neg	
L43	14054342	pos	NA	14054396	pos	NA	14054315	pos		14054381	neg	
L44	14054499	NA	NA	14054428	NA	NA	14054477	NA	NA	14054440	NA	
L46	14054697	pos	RABV	14054680	pos	EBLV-1	14054606	pos	EBLV-2	14054629	neg	
L48	14054830	pos	RABV	14054895	pos	EBLV-1	14054831	pos	EBLV-2	14054838	neg	
L49	14054963	pos	RABV	14054968	neg		14054975	neg		14054952	neg	
L50	14055058	pos	RABV	14055061	pos	EBLV-1	14055056	pos	EBLV-2	14055091	neg	
L51	14055109	pos	RABV	14055135	pos	EBLV-1	14055110	pos	EBLV-2	14055188	neg	

4.4.2 Answers of the participating laboratories to the Real Time RT-PCR technical questionnaire

Item	Number of laboratories/ number of total answers	EU laboratory answers
RNA Extraction		
RNA extraction performed manually	13/21 (62%)	10/16 (63%)
RNA extraction performed with automated RNA purification	8/20 (40%)	6/15 (40%)
If RNA extraction is performed with automated RNA purification, please specify the equipment used:	- **Roche** Magna Pure Compact: 4/10 (20%) - **Invitrogen** IPrep Purification Instrument: 2/10 (20%) - **Biomerieux** NucliSENS easyMag: 1/10 (10%) - **Nordiag** Magnatrix 8000+: 1/10 (10%) - **Qiagen** QIAcube: 1/10 (10%)	- **Roche** Magna Pure Compact: 2/8 (25%) - **Invitrogen** IPrep Purification Instrument: 3/8 (38%) - **Nordiag** Magnatrix 8000+: 1/8 (13%) - **Qiagen** QIAcube: 1/8 (13%)
Viral RNA extracted	17/24 (74%)	13/18 (72%)
Total RNA extracted	17/23 (74%)	11/16 (69%)
Commercial kit used to perform the extraction	21/26 (81%)	15/19 (79%)
Extraction kit used	- **Qiagen** QIAamp Viral RNA Mini Kit: 5/21 (27%) -**Roche** MagNA Pure Total NA Kit: 4/21 (19%) - **Macherey Nagel** NucleoSpin RNA II: 3/21 (14%) - **Invitrogen** iPrep PureLink Virus Kit: 2/21 (10%) - **Qiagen** Viral RNA Mini Kit: 1/20 (5%) - **Qiagen** RNA Blood mini kit: 1/20 (5%) - **Stratec** Invisorb Spin virus RNA mini kit: 1/21 (5%) - **BioMérieux** Nuclisense reagents: 1/20 (5%) - **Diasorin** « Bullet stool » : 1/21 (5%)	- **Qiagen** QIAamp Viral RNA Mini Kit: 3/15 (20%) - **Macherey Nagel** NucleoSpin RNA II: 3/15 (20%) - **Invitrogen** iPrep PureLink Virus Kit: 2/21 (10%) -**Roche** MagNA Pure Total NA Kit: 1/15 (7%) - **Qiagen** Viral RNA Mini Kit: 1/15 (7%) - **Qiagen** RNA Blood mini kit: 1/15 (7%) - **Stratec** Invisorb Spin virus RNA mini kit: 1/15 (7%) - **Diasorin** « Bullet stool » : 1/15 (7%)
Trizol	4/5 (80%)	3/4 (75%)
Guanidium thiocyanate	1/4 (25%)	1/3 (33%)
CsCl gradient	0 (0)	0 (0)
Other	0 (0)	0 (0)
Quantity of RNA in samples measured after the extraction step	4/26 (25%)	2/19 (11%)
qRT-PCR		
One step qRT-PCR	24/26 (92%)	17/19 (89%)
Two step qRT-PCR	3/20 (15%)	2/19 (11%)
One step qRT-PCR		

qRT-PCR cycler used	- **Applied Biosystems** 7500 Real Time PCR System: 5/24 (21%) - **Agilent** Stratagene Mx3005P: 4/24 (17%) - **Qiagen** Rotor Gene 6000: 4/24 (17%) - **BioRad** iQ5 iCycler BioRad: 1/24 (11%) - **Applied Biosystems** 7300 Real Time PCR System: 1/24 (4%) - **Qiagen** Rotor-Gene 3000: 1/24 (4%) - **Qiagen** Rotorgene: 1/24 (4%) - **MJ Research** Chromo4: 1/24 (4%) - **Roche** LichtCycler 2.0: 1/24 (4%) - **Agilent** Stratagene MX3005p: 1/24 (4%) **BioRad** CFX96 Touch CFX96 quantitative PCR system: 1/24 (4%) - **Stratagene** MX 3005P: 1/24 (4%)	- **Applied Biosystems** 7500 Real Time PCR System: 3/17 (18%) - **Agilent** Stratagene Mx3005P: 3/17 (18%) - **Qiagen** Rotor Gene 6000: 4/17 (24%) - **Applied Biosystems** 7300 Real Time PCR System: 1/17 (6%) - **Qiagen** Rotor-Gene 3000: 1/17 (6%) - **Qiagen** Rotorgene: 1/17 (6%) - **MJ Research** Chromo4: 1/17 (6%) - **Agilent** Stratagene MX3005p: 1/17 (6%) **BioRad** CFX96 Touch CFX96 quantitative PCR system: 1/17 (6%) - **Stratagene** MX 3005P: 1/17 (6%)
Commercial kit used to perform the one step RT-PCR	16/19 (84)	11/14 (79)
One step RT-PCR Kit used	- **Qiagen** QuantiTect Probe RT-PCR Kit: 5/21 (24%) - **Ambion** AgPath ID One Step rt PCR kit:3/21 (14%) - **Invitrogen** Superscript III Platinum One-step Quantitative RT-PCR system: 2/21 (10%) - **Roche** LC RNA master Hybridation probe: 2/21 (10%) - **Qiagen** One step RT PCR kit: 2/21 (10%) - **Invitrogen** Ultrasense RNA one-step RT-PCR kit: 1/21 (5%) - **Invitrogen** TaqMan one-step qRT-PCR kit: 1/21 (5%) - **LifeRiver**: 1/21 (5%) - Rotor-Gene SYBR Green RT-PCR Kit: 1/21 (5%) - **Applied Biosystems** TagMan Fast Virus 1-Step Reverse Transcription Kit: 1/21 (5%)	- **Qiagen** QuantiTect Probe RT-PCR Kit: 5/14 (36%) - **Ambion** AgPath ID One Step rt PCR kit: 2/14 (14%) - **Qiagen** One step RT PCR kit: 2/21 (10%) - **Invitrogen** Superscript III Platinum One-step Quantitative RT-PCR system: 1/14 (7%) - **Roche** LC RNA master Hybridation probe: 1/14 (7%) - **Invitrogen** Ultrasense RNA one-step RT-PCR kit: 1/14 (7%) - **Invitrogen** TaqMan one-step qRT-PCR kit: 1/14 (7%) - Rotor-Gene SYBR Green RT-PCR Kit: 1/14 (7%) - **Applied Biosystems** TagMan Fast Virus 1-Step Reverse Transcription Kit: 1/14 (7%)
N region	21/21 (100%)	16/16 (100%)
Others	3/14 (21%)	2/11 (18%)
If other, please specify the genome region:	L: 2/3 (67%)	L: 2/2 (100%)
SYBR Green assay	6/20 (30%)	6/15 (40%)
Taq Man Assay	20/23 (87%)	13/16 (81%)
Pair of primers published	8/23 (35%)	11/13 (85%)
Nucleotide localisation of the primers used for qRT-PCR (based on PV virus):	- **JW12**: 55-73; **N165-146**: 146-165. 8/23 (35%) - 370-463: 1/23 (4%) - **550B**: 646 and **Lys541**: 541: 1/23 (4%) - Hoffmann et al. 2010: 1/23 (4%) - **JW12**; **JW6**: 1/23 (4%) - **RV-N-F**, **RV-N-R** : 1/23 (4%)	- **JW12**: 55-73; **N165-146**: 146-165. 11/17 (35%) - 370-463: 1/17 (6%) - Hoffmann et al. 2010: 1/17 (6%) - **JW12**; **JW6**: 1/17 (6%) - **RV-N-F**, **RV-N-R** : 1/17 (6%)

Nucleotide localisation of the probes used for qRT-PCR (based on PV virus):	- 297-321: 1/15 (7%) - 55-165: 1/15 (7%) - 81 - 109: 1/15 (7%) - 81-109, 80-105, AWgt 1:105-131: 1/15 (7%) - 81-109, 80-105,81-109: 1/15 (7%) - 78-111: 1/15 (7%) - FW 370-391; RV 482-502: 1/15 (7%) - GT1 81-146 ; 297-321: 1/15 (7%) - LysGT1 81-109; LysGT5 80-105; LysGT6 81-109 : 1/15 (7%) - Lyssaprobe 620-645 : 1/15 (7%) - RABV 81-109, EBLV-1 80-105, EBLV-2 81-109 : 1/15 (7%)	- 297-321: 1/11 (9%) - 55-165: 1/11 (9%) - 81-109, 80-105, AWgt 1:105-131: 1/11 (9%) - 81-109, 80-105,81-109: 1/11 (9%) - FW 370-391; RV 482-502: 1/11 (9%) - GT1 81-146 ; 297-321: 1/11 (9%) - LysGT1 81-109; LysGT5 80-105; LysGT6 81-109 : 1/11 (9%) - RABV 81-109, EBLV-1 80-105, EBLV-2 81-109 : 1/11 (9%)
Taq Gt1 (RABV)	14/17 (82)	10/12 (83)
Taq Gt5 (EBLV-1)	11/17 (65)	9/12 (75)
Taq Gt6 (EBLV-2)	11/17 (65)	9/12 (75)
Others	5/17 (29)	3/13 (23)
If other, please specify:	Other Genotypes	Other Genotypes
Quantity of RNA per tube (in ng)	30 : 1/9 (11%) >150 : 1/9 (11%) 2000 : 1/9 (11%) Not measured : 6/9 (67%)	>150 : 1/5 (20%) 2000 : 1/5 (20%) Not measured : 3/5 (60%)
RNA final volume per tube (in µl)	1: 4/24 (17%) 2: 7/24 (29%) 2.5: 1/24 (4%) 4: 2/24 (8%) 5: 10/24 (41%)	1: 2/17 (12%) 2: 5/17 (29%) 2.5: 1/17 (6%) 4: 1/17 (6%) 5: 8/17 (47%)
Total reactional volume (in µl)	12.5 : 1/24 (4%) 15: 1/24 (4%) 20: 5/24 (21%) 25: 15/24 (60%) 50: 2/24 (8%)	12.5 : 1/17 (6%) 15: 1/17 (6%) 20: 2/17 (12%) 25: 11/17 (65%) 50: 2/17 (12%)
Size of amplified product (in bp)	88: 1/20 (5%) 92: 1/20 (5%) 100 : 2/20 (10%) 105 : 1/20 (5%) 110-111: 8/20 (40%) 132: 1/20 (5%) 606: 1/20 (5%)	88: 1/15 (7%) 92 : 1/15 (7%) 100 : 2/15 (13%) 110-111: 6/15 (40%) 132: 1/15 (7%) 606: 2/15 (13%)
Two step qRT-PCR		
Rabies specific primer	0	0
Universal primer	3/3	2/2
If universal primer is used, please specify (pdN6, etc.):	Random Hexamer: 3/3	Random Hexamer: 2/2
Commercial kit used to perform the RT	3/3	2/2
Name of the product	Biorad iscript select cDNA synthesis kit (1/3) Quanta qscrpit cDNA super mix (1/3) High Capacity c DNA Reverse Transcription kits (1/3)	Biorad iscript select cDNA synthesis kit (1/2) Quanta qscrpit cDNA super mix (1/2)
Primer used in the RT published	3	2
AMV	0	0
M-MuLV	3	2
Other	0	0

Duration of incubation (in min)	30 120 5; 30; 5	30 120
Temperature of incubation (in °C)	42 37 25 42 85	42 37
RNA final volume per tube (in µl)	5 10 18	5 18
Total reactional volume (in µl)	20	20
qPCR cycler used	Biorad CFX 96 Biorad IQ5 RotorGene Q MDx	Biorad CFX 96 RotorGene Q MDx
Commercial kit used to perform the one step qPCR	Biorad Sofast evagreen supermix Quanta Sybergreen supermix QuantiTect ™ SYBR® Green PCR	Biorad Sofast evagreen supermix Quanta Sybergreen supermix
N region	3	2
SYBR Green assay	3	2
Taq Man Assay	1/2	1/2
Primers used:	JW12: 55-74 / N165-146:146-165 ; GT1 probe ; GT5 probe ; GT6 probe ; PCR1 = RAB PCR1 F : 572-595 and RAB PCR1 R: 892-914. qPCR = RAB qPCR F : 676-698 and GRAB2R : 814-833	JW12: 55-74 / N165-146:146-165 ; GT1 probe ; GT5 probe ; GT6 probe ; PCR1 = RAB PCR1 F : 572-595 and RAB PCR1 R: 892-914. qPCR = RAB qPCR F : 676-698 and GRAB2R : 814-833
Controls		
Run performed several times per sample	11/26 (42%)	7/19 (37%)
If yes, number of runs	2: 11/11 (100%)	2: 7/7 (100%)
Additionally to tested samples, negative samples are intercalated within the panel	18/19 (95%)	25/26 (96%)
Negative sample is: Negative brain	10/21 (48%)	7/16 (44%)
Water	18/24 (75%)	12/18 (67%)
A saline buffer as PBS	6/21 (29%)	4/16 (25%)
Negative controls included in:	10/21 (48%)	7/16 (44%)
the preparation of samples	23/25 (92%)	16/18 (89%)
qRT-PCR (one step only)	20/22 (91%)	14/16 (88%)
qRT-PCR (two step only)	4/15 (27%)	3/12 (25%)
Other	1/11 (9%)	1/9 (11%)
Negative control for extraction: Negative brain	6/8 (75%)	4/6 (67%)
Water	7/9 (78%)	4/6 (67%)
A saline buffer as PBS	3/8 (38%)	3/7 (43%)
Positive controls included in the qRT-PCR	26/26 (100%)	19/19 (100%)
RNA positive extraction control	15/24 (63%)	11/18 (61%)
RT positive negative control	10/22 (45%)	7/17 (41%)
qPCR positive control	15/23 (65%)	10/17 (59%)
Pre-extracted and know CT value	13/22 (59%)	8/16 (50%)
Ubiquitous gene amplified to validate the RT-PCR results (as 18S mouse rRNA)	9/26 (35%)	7/19 (37%)

4.4.3 Interpretation of the discordant results

Discrepant results of real-time RT-PCR consisted of three false positive results in three laboratories (**L13**, **L14** and **L21**) and five false negative results in three laboratories (three discrepant results in L05, one in L21 and one in **L33**).

As for the conventional RT-PCR, possible explanations of the discrepant results were assessed by analysing the technical questionnaires fulfilled by participating laboratories (**L05**, **L13**, **L14**, **L21**, **L33**).

False positive results might have occurred either during the sample preparation (by the addition of water in the freeze dried tubes) or during the step of RNA extraction. The same discrepant results of real-time RT-PCR and RT-PCR recorded in the same laboratories (**L13**, **L14** and **L21**) suggest a cross-contamination of the negative sample by a positive sample during the samples processing.

5. CONCLUSIONS OF THE 2014 PERFORMACE TRIAL

The lowest proportion of laboratories producing discordant results was found in FAT (12.2%) as regularly observed among the successive inter-laboratory test sessions. The amount of laboratories with a failure for FAT was then followed by the conventional RT-PCR (18.2%) then the Real Time RT-PCR (20.0%) and the RTCIT (21.4%) (Table 12).

Sensitivity appeared higher in RT-PCR (0.8% of false negative identified) closely followed by the FAT (1.0% of false negatives) and the RTCIT (1.3% of false negatives) and the Real Time RT-PCR (2.6%).

Specificity was higher in FAT (6.1 % of false positives) followed by RTCIT (10.7% of false positives), Real Time RT-PCR (12.0% of false positives) and RT-PCR (12.1% of false positives).

It should be noted that no error was notified for the undiluted RABV samples in FAT and no error in bats species for FAT, RTCIT, and conventional RT-PCR.

Table 12: Overall comparison of laboratory performances in 2014 for rabies diagnosis techniques.

discordant results n/N (%)	FAT	RTCIT	RT-PCR	Real Time
Number of laboratories	6/49 (12.2)	6/28 (21.4)	6/33 (18.2)	5/25 (20.0)
Negative samples (false positives)	3/49 (6.1)	3/28 (10.7)	4/33 (12.1)	3/25 (12.0)
Positive samples (false negatives)	4/392 (1.0)	3/224 (1.3)	2/254 (0.8)	5/192 (2.6)
CVS 27	0/49 (0)	0/28 (0)	0/32 (0)	0/25 (0)
RABV Rac. Dog Estonia	0/49 (0)	1/28 (3.6)	1/32 (3.1)	0/25 (0)
RABV Fox former Yugoslav Republic of Macedonia	0/49 (0)	2/28 (7.1)	1/32 (3.1)	0/25 (0)
GS7	0/49 (0)	0/28 (0)	0/32 (0)	1/25 (4.0)
Total RABV	0/196 (0)	3/112 (2.7)	2/128 (1.6)	1/100 (1.0)
GS7 1/30	1/49 (0)	0/28 (0)	0/32 (0)	1/25 (4.0)
GS7 1/50	3/49 (0)	0/28 (0)	0/32 (0)	1/25 (4.0)
Total diluted RABV	4/147 (2.7)	0/56 (0)	0/64 (0)	2/50 (4.0)
EBLV-1	0/49 (0)	0/28 (0)	0/31 (0)	2/21 (9.5)
EBLV-2	0/49 (0)	0/28 (0)	0/31 (0)	0/21 (4.5)

6. REFERENCES

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