Lyssavirus Unit

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REPORT

INTER-LABORATORY ASSAY FOR PERFORMANCE CHARACTERISTICS EVALUATION OF LYSSAVIRUS REAL TIME RT-PCR TECHNIQUES

Year 2022

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1. INTRODUCTION

The EURL for rabies organised in early 2022 a specific inter-laboratory assay to evaluate the real-time RT-PCR assays used by NRLs within the European Union. The inter-laboratory evaluation of the techniques was performed using a panel of viral RNAs bringing together the following species: RABV and 3 other bat lyssaviruses currently isolated in bats in Europe (EBLV-1, EBLV-2 and BBLV). The aim of the present study was to evaluate the sensitivity of the Real-Time RT-PCR methods, which is increasingly used in NRLs in replacement to the cell-isolation test. The aim was also to determine whether or not the real-time PCR methods performed by different laboratories provide similar performances and to evaluate potential discrepancies among negative and different positive levels for different Lyssavirus species (RABV and other lyssaviruses).

2. PROFICIENCY TEST ITEMS

2.1 PREPARATION OF SAMPLES

Characteristics of samples

Five different lyssavirus strains were used for the constitution of the panel (Table 1):

- CVS-27: a RABV fixed strain
- EBLV-1b: a bat strain from France
- RABV (Fox, Greece): RABV fox strain from Greece
- EBLV-2: a bat strain from United Kingdom
- BBLV: a bat strain from France.

The material used as “negative” batch was collected from chicken brains that were provided by Dr Pelzer from the departmental veterinary and food laboratory of Meurthe et Moselle (France).

One additional sample was included in the panel test to avoid collusion (named decoy). This sample differs from one laboratory to another. The sample was originated from either RNA extracted from virus batches produced by the EURL for rabies in the frame of the ILTs (DUVV lot 04-21, Ukraine Lot 05-21 or Ukraine lot 06-21) or corresponds to a negative control (Buffer TE). This sample was not included in the analysis of raw data.

Table 1: Characteristic of virus stocks, negative samples and samples used to avoid collusion used for preparing the panel.

ID	Sample nature	Batch name	Passaged on	Origin	Lyssavirus species	Country	Year of isolation	Species
1	Virus	CVS 27 11-14	Mouse		RABV	/	/	*Fixed strain*
2		Greece 36-12	Mouse		RABV	Greece	2012	*Fox*
3		EBLV-1b 03-08	Mouse		EBLV-1	France	2000	*Bat*
4		EBLV-2 03-12	Mouse		EBLV-2	UK	2004	*Bat*
5		BBLV 35-18	Mouse		BBLV	France	2012	*Bat*
6	Negative	Chicken 02-19	/	/	/	France	/	*Chicken*
7	Samples used to avoid collusion (decoy)	DUVV lot 04-21	Mouse	/	/			*Bat*
8		Ukraine Lot 05-21	Mouse					*Fox*
9		Ukraine lot 06-21	Mouse					*Fox*
10		Buffer TE	/	/				/

Composition of the panel

The panel was constituted of 21 RNA samples with (1) 20 blindly frozen coded samples and (2) one tube with a known status: CVS-27 RNA (Batch 11-14).

Panel 1: The panel detailed in Table 2 included different lyssavirus RNAs: two RABVs (a fixed RABV challenge strain CVS-27 RNA in duplicate and a field RABV RNA from Greece) and three European bat Lyssaviruses RNA (EBLV-1, EBLV-2 and BBLV). Each lyssavirus RNA was provided with three different RNA levels: highly positive (18<Ct≤23), moderately positive (23<Ct≤28) and weak positive (28<Ct≤35).

Table 2: Characteristics of samples included in the panel 1.

ID	Strain	Class of dilutions		Ct values	Nb. of copies/µL RNA
1	Greece	Strongly positive	18<Ct≤23	20.76±0.04	3.49E+04
2		Moderate positive	23<Ct≤28	25.52±0.08	1.33E+03
3		Weak positive	28<Ct≤35	30.98±0.31	3.14E+01
4	CVS (1)	Strongly positive	18<Ct≤23	20.91±0.04	3.14E+04
5		Moderate positive	23<Ct≤28	25.98±0.17	9.73E+02
6		Weak positive	28<Ct≤35	32.22±1.22	1.34E+01
7	CVS (2)	Strongly positive	18<Ct≤23	21.09±0.04	2.77E+04
8		Moderate positive	23<Ct≤28	25.67±0.08	1.20E+03
9		Weak positive	28<Ct≤35	31.54±0.13	2.14E+01
10	EBLV-1b	Strongly positive	18<Ct≤23	20.24±0.1	4.95E+04
11		Moderate positive	23<Ct≤28	25.5±0.12	1.35E+03
12		Weak positive	28<Ct≤35	30.3±0.31	5.01E+01
13	EBLV-2	Strongly positive	16<Ct≤21	18 ± 0.1	2.35E+05
14		Moderate positive	21<Ct≤26	23.18±0.1	6.61E+03
15		Weak positive	26<Ct≤31	28.38±0.16	1.88E+02

16	BBLV	Strongly positive	16<Ct≤21	19.84±0.01	6.52E+04
17		Moderate positive	21<Ct≤26	25.24±0.19	1.61E+03
18		Weak positive	26<Ct≤31	30.1±0	5.77E+01
19	Chicken	/	No Ct (CT>45)	No Ct (CT>45)	0
20	Decoy	Mix (Strongly positive or Negative)	Mix (Strongly positive or Negative)	Mix (Strongly positive or Negative)	Mix (Strongly positive or Negative)

Panel 2: The tube of known status (CVS-27 RNA) which corresponds to the real-time RT-PCR positive control was provided at a concentration of 10^7 copies/µL of RNA. Table 3 details the characteristics of the positive control CVS-27 RNA. This positive control was used for the generation of standard curves for the determination of the limit of detection of PCR and efficiency of PCR.

Table 3: Characteristics of the positive control CVS-27 RNA included in the panel 2 for the generation of the standard curve.

ID	Dilution (log)	Ct values	Nb of copies/µL RNA	95% CI
1	1	16.09±0.16	8.56E+05	[2.881E+05 . 2.543E+06]
2	2	19.6±0.0.02	7.69E+04	[3.265E+04 . 1.811E+05]
3	3	22.84±0.12	8.34E+03	[3.807E+03 . 1.825E+04]
4	4	26.6±0.24	6.35E+02	[2.549E+02 . 1.582E+03]
5	5	29.19±0.26	1.07E+02	[3.575E+01 . 3.221E+02]
6	6	32±0.39	1.57E+01	[4.063E+00 . 6.035E+01]

Preparation of samples

The virus batches used in the 2022 inter-laboratory test were produced by intra-cerebral inoculation of mice. Stock of viruses were produced according to the animal experimentation directives issued by the French Ethic Committee and virus production was continued until animals harbored symptoms suggestive of rabies development (stage 3/5) to collect a maximum amount of virus.

Based on a collection of 10 brains, a virus stock was constituted for each strain tested in the panel. After the death of the inoculated animal, brains were excised then stabilized in a volume of 10 mL of RNA Later buffer and then stored at < -18°C before use.

For each batch of virus produced, one brain fixed in RNA later was cleaned in a tube containing 4 ml of PBS buffer, then transferred in a tube containing 9 ml of PBS buffer before grinding and centrifugation for 5 minutes at 5,000 g. The clarified supernatant was collected and aliquoted in DNAse-RNase free tubes of 3 ml into different then stored at < -65°C until RNA extraction.

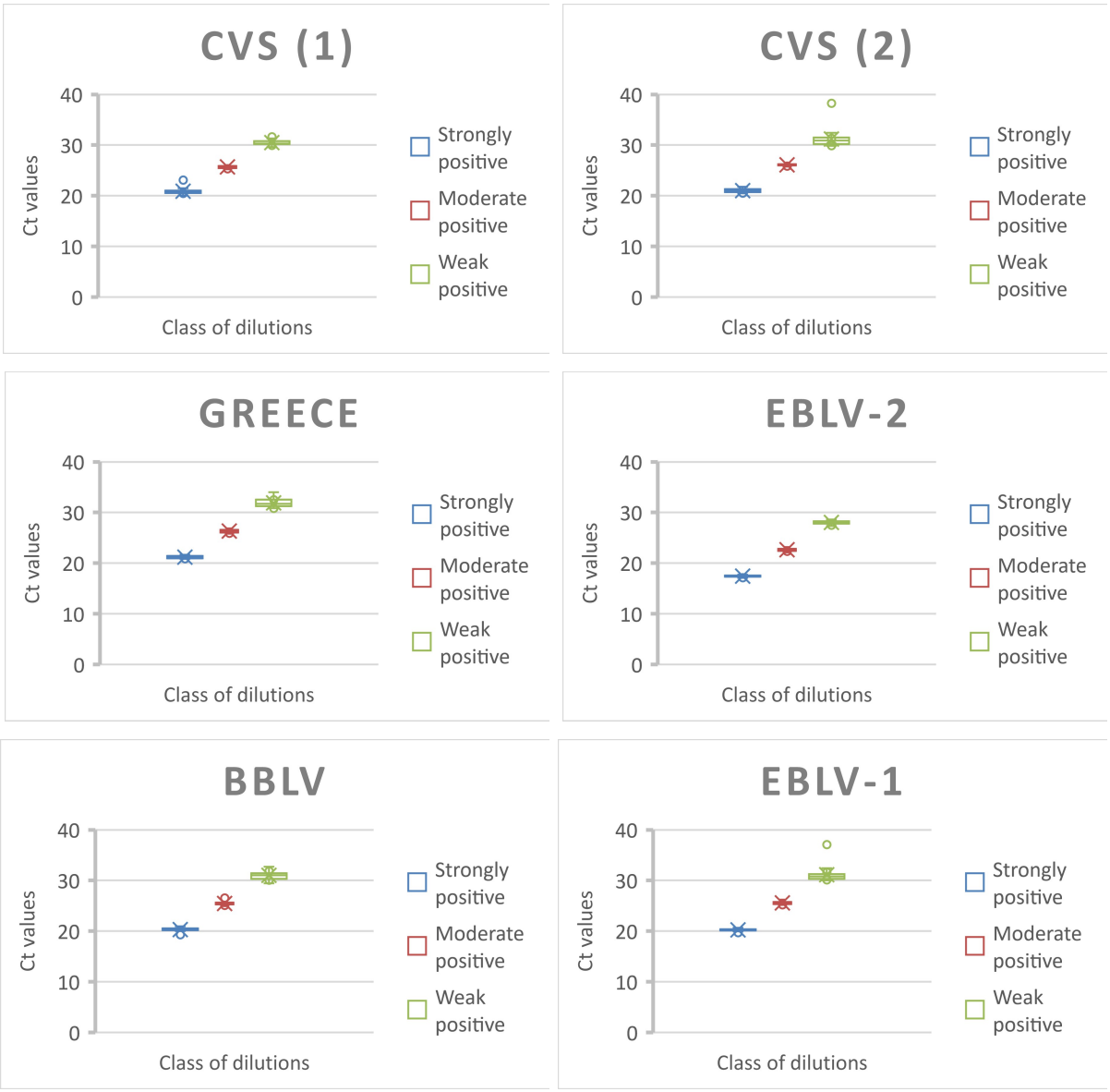
The RNA extraction was carried out for each RNA batch using the QIAmp Viral RNA mini kit from QIAGEN (Les Ulys, France) according to the manufacturer's instructions. Briefly, 3 ml of clarified supernatant was added in a tube containing 12 ml of lysis buffer AVL (provided by the kit), mixed by pulse vortexing for 15 s, then incubated at room temperature for 10 minutes. The lysis buffer tube was briefly centrifuged, mixed with 12 ml of absolute ethanol before mixing and centrifugation to remove drops from inside the lid. Twenty QIAmp mini columns were used to process all volume of lysis buffer, with a volume of 630 µL transferred by mini column. RNA was eluted in a volume of 100 µL of Buffer AVE (provided by the kit) then centrifuged for 1 min at 6,000 g. A pool of 2 ml of RNA extract was carried out for each batch then aliquoted in two tubes of 50 µL for controls and one tube of 1.8 ml for the preparation of samples. RNA was stored at < -65°C.

Three classes of dilutions were performed by lyssavirus strain tested from a stock S1 of RNA with 15<Ct values<20. Serial dilutions of S1 were carried out in TE buffer to obtain highly positive samples (18<Ct≤23 ; dilution of 32), moderately positive samples (23<Ct≤28 ; dilution of 1072) and weak positive samples (28<Ct≤35 ; dilution of 35.000).

Homogeneity

The evaluation of the homogeneity was undertaken by one operator by selecting randomly 20 samples per batch and by analysing them by SYBR Green RT-PCR. The batches were considered homogeneous as all results were concordant to the expectations for all samples (< 1 Ct value for strong (18<Ct≤23) and moderate (23<Ct≤28) positives), and < 2 for weak positives (28<Ct≤35) (Figure 1).

Figure 1. Results of the homogeneity assessment

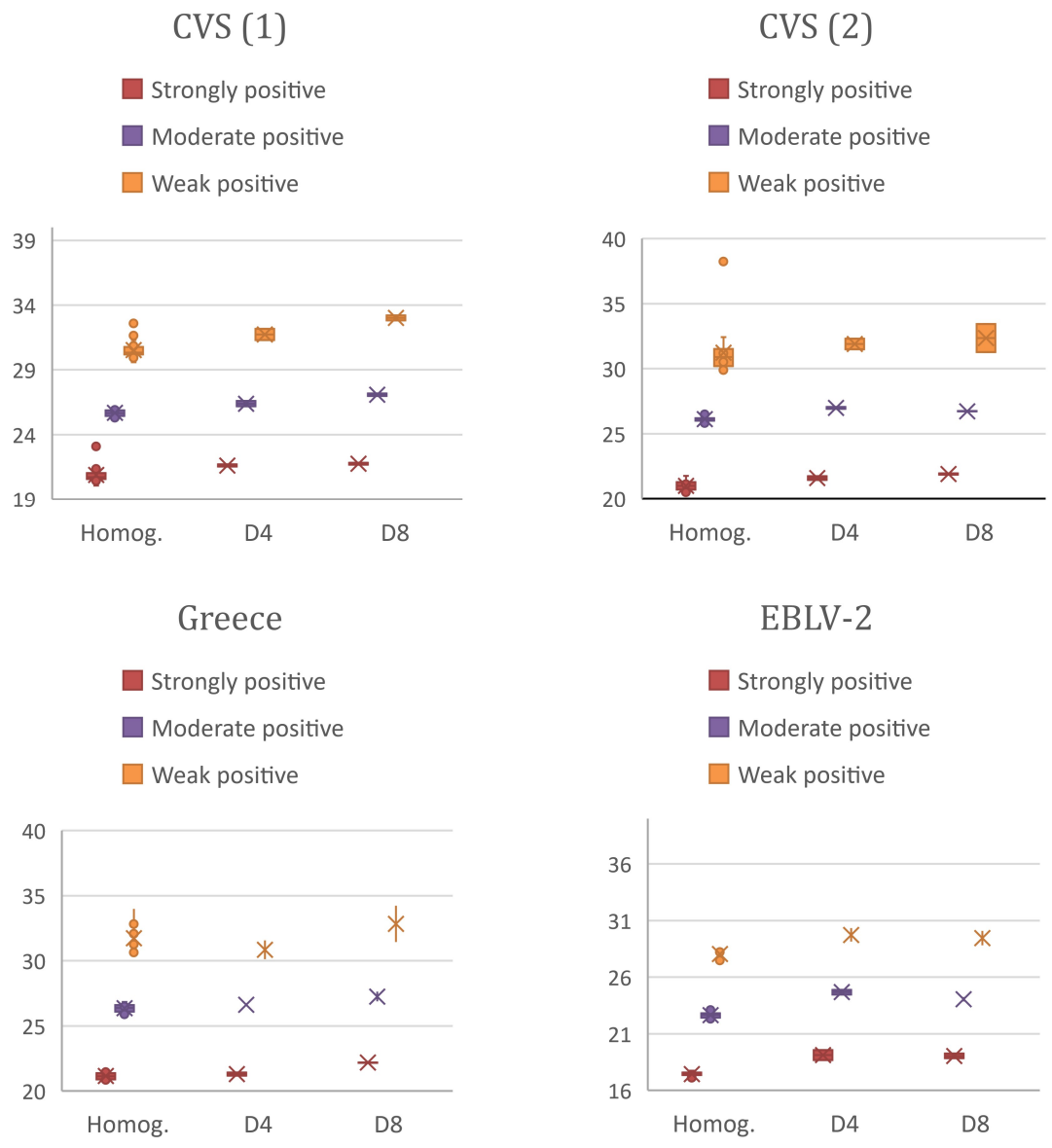


Stability

The stability of the panel was tested after 4 and 8 days at a temperature < -18°C (Figure 2) and following 4 successive cycles of freezing-thawing of samples (Figure 3).

All tested conditions were shown satisfactory and confirmed the stability of all batches submitted in the stability test. The batches were considered stable as all results were concordant to the expectations for all samples (< 1 Ct value for strong (18<Ct≤23) and moderate (23<Ct≤28) positives), and < 2 for weak positives (28<Ct≤35).

Figure 2. Results of the stability test after 4 and 8 days at < -18°C.



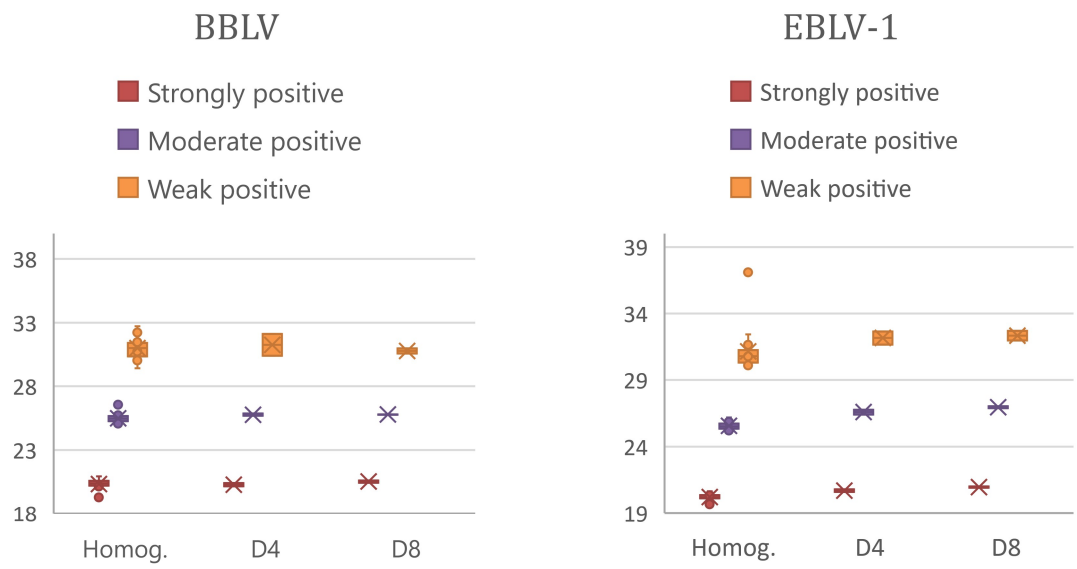
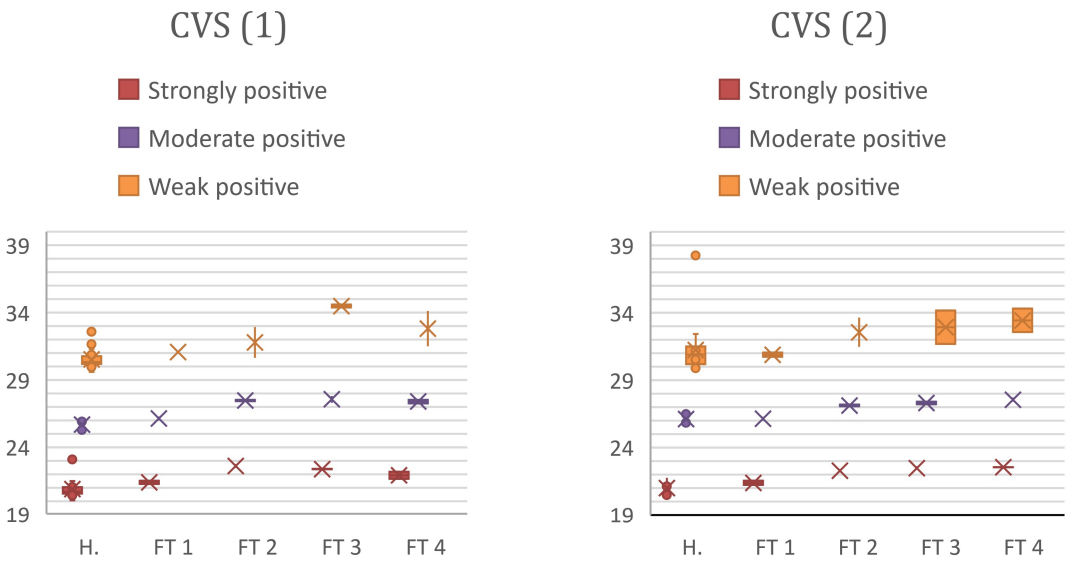
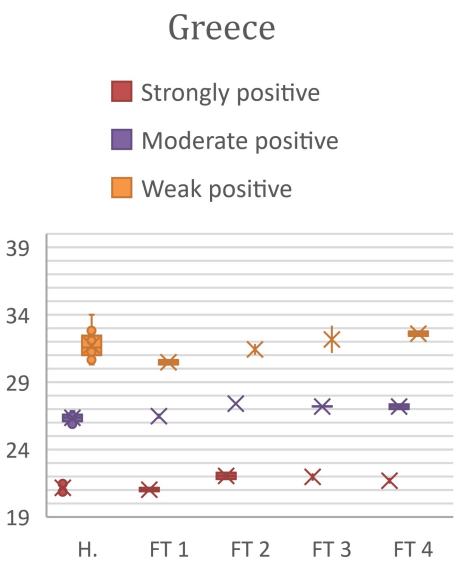
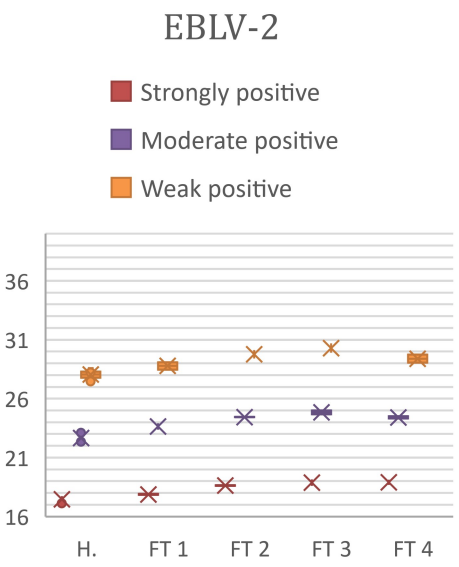
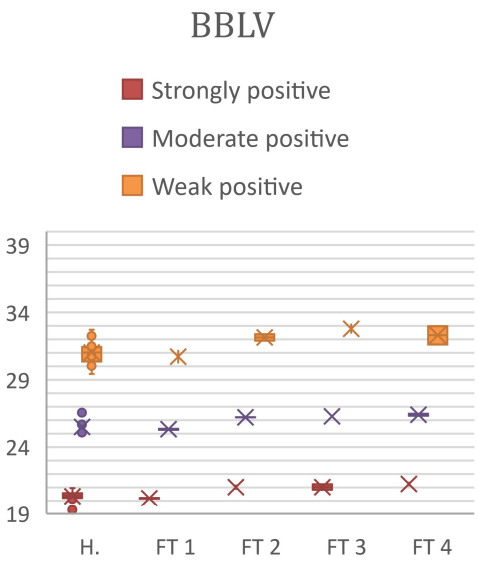


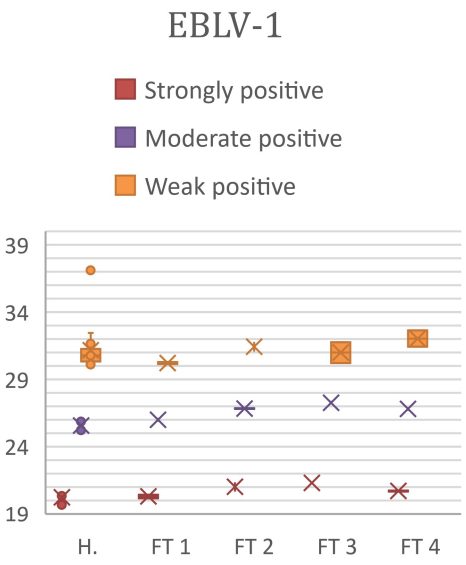
Figure 3. Results of the stability test after 4 successive cycles of freezing-thawing of samples.




Greece
Strongly positive
Moderate positive
Weak positive
39
34
29
24
19
H.
FT 1
FT 2
FT 3
FT 4


EBLV-2
Strongly positive
Moderate positive
Weak positive
36
31
26
21
16
H.
FT 1
FT 2
FT 3
FT 4


BBLV
Strongly positive
Moderate positive
Weak positive
39
34
29
24
19
H.
FT 1
FT 2
FT 3
FT 4


EBLV-1
Strongly positive
Moderate positive
Weak positive
39
34
29
24
19
H.
FT 1
FT 2
FT 3
FT 4

2.2 PARTICIPATING LABORATORIES

Twenty-five laboratories from EU participated in the inter-laboratory test.
The list of participating laboratories used is presented in Table 4.

Table 4. List of participating laboratories**.**

Country	Laboratory
Austria	Institute for Veterinary Disease Control, AGES, Robert Koch-Gasse 17, 2340 Mödling,
Belgium	Sciensano - National reference center/laboratory for rabies, Engelandstraat 642, 1180 Brussels
Bulgaria	Bulgarian Food Safety Agency, National Diagnostic and Research Veterinary Institute. NRL Anthrax and Rabies, Sofia 1606, 15 Pencho Slaveykov Blvd.
Republic of Croatia	Laboratory for rabies and general virology, Savska cesta 143, 10000 Zagreb
Cyprus	Laboratory for Animal Health - Virology Section, Veterinary Services, State Veterinary Laboratories, 1417 Nicosia
Czech Republic	State Veterinary Institute Prague, Sídlištní 136/24, 165 03, Praha 6 – Lysolaje
Denmark	Statens Serum Institute, SSI, Artillerivej 5, DK-2300 Copenhagen
Estonia	Friedrich Reinhold Kreutzwaldi 30, 51006 Tartu
Finland	Finnish Food Authority, Virology unit, Mustialankatu 3, 00790 Helsinki
France	Anses - Nancy Laboratory for Rabies and Wildlife, Technopole agricole et Vétérinaire, 54220 Malzeville
Germany	Friedrich-Loeffler-Institut, Institute of Molecular Virology and Cell Biology, Südufer 10, 17493 Greifswald-Insel Riems
Greece	Virology Laboratory, Department of Molecular Diagnostics, FMD, Virological, Ricketsial and Exotic diseases, 25, Neapoleos str, PC15341, Agia Paraskevi
Hungary	National Food Chain Safety Office, Veterinary Diagnostic Directorate, Virology Laboratory, 1143 Budapest, Tábornok utca 2
Ireland	Virology Division, Central Veterinary Research Laboratory, Stacumny Lane, Backweston, Celbridge, Co. Kildare
Italy	Istituto Zooprofilattico Sperimentale delle Venezie, Viale dell'Università 10, 35020 Legnaro (Padova)
Latvia	Institute of Food Safety, Animal Health and Environment BIOR, Lejupes iela 3 Riga LV-1076
Lithuania	National Food and Veterinary Risk Assessment Institute, J. Kairiukscio str. 10, LT - 08409, Vilnius
Netherlands	Wageningen Bioveterinary Research (WBVR), Houtribweg 39 8221RA Lelystad
Poland	NATIONAL VETERINARY RESEARCH INSTITUTE 57, Partyzantow Avenue 24-100 Pulawy
Portugal	Instituto Nacional de Investigação Agrária e Veterinária, I.P (INIAV, IP)
Romania	Institute for Diagnosis and Animal Health, str dr staicovici nr 63 050557 Bucharest
Slovakia	State Veterinary and Food Institute - Veterinary Institute Zvolen, Pod Dráhami 918, 960 86 Zvolen
Slovenia	VF / National veterinary institute Gerbičeva 60, 1000 Ljubljana
Spain	Instituto de Salud Carlos III, Centro Nacional de Microbiología, Ctra. Majadahonda-Pozuelo s/n 28220-Majadahonda Comunidad de Madrid
Sweden	Statens veterinärmedicinska anstalt SE-751 89 Uppsala

3. RESULTS

3.1 Review of methods used

Out of the 25 participating laboratories, 19 (76%) used *pan-Lyssavirus* RT-PCR method (SYBR Green and/or TaqMan) and 6 (24%) used the Lyssavirus specific Probe based RT-PCR only for the specific detection of RABV, EBLV-1, EBLV-2, and/or BBLV, respectively.
Of the 19 laboratories that used the *pan-Lyssavirus* method, 12 laboratories used SYBR Green RT-PCR, and 7 *pan-Lyssavirus* Probe based RT-PCR.

The list of the techniques used is presented in Table 5.

Table 5. Details of real-time RT-PCR techniques used by participating laboratory.

Code laboratory	SYBR Green Pan *Lyssavirus* RT-PCR	*Lyssavirus* specific Probe based RT-PCR	*Pan-Lyssavirus* Probe based RT-PCR
L1	x	x	
L2	x		
L3		x	
L4	x		
L5			x
L6	x		
L7	x		
L8	x		
L9			x
L10		x	
L11	x	x	
L12	x		
L13			x
L14			x
L15	x		
L16	x		
L17	x	x	
L18		x	
L19		x	x
L20		x	
L21	x (1)		x
L22			x
L23		x	
L24		x	
L25	x		
Total nb. lab	**12 + 1**	**10**	**7**

(1) the test was dedicated to the CVS amplification, only.

3.2 Delivery time and receipt of parcels

The 25 packages were delivered within the timeframe tested by the stability assessment (<7 days). All packages were received between 1 and 2 days after shipment, except two laboratories that received their packages 4 days after shipment.

3.3 Part I: Results of the specificity and sensitivity analysis by species and by positivity level

All results were sent back within the fixed time limit of the inter-laboratory test.

All three methods, Lyssavirus specific Probe based RT-PCR *Pan-Lyssavirus* Probe RT-PCR and SYBR Green RT-PCR were performed with 10, 7 and 13 tests performed respectively.

Of the 13 tests performed by SYBR Green RT-PCR, 1 was performed with primers dedicated for the specific CVS strain amplification.

Raw data submitted by each laboratory are detailed in Appendix A:

- S Table 1 details the SYBR Green Pan Lyssavirus RT-PCR results with the C_T values, Tm (°C) of the PCR product and the conclusion given by the laboratory ;
- S Table 2 details the TaqMan specific Probe_RT-PCR results with the C_T values and the conclusion given by the laboratory ;
- and S Table 3 details the Pan Lyssavirus TaqMan RT-PCR results (C_T values and the conclusion given by the laboratory).

3.3.1. Discordance on negative samples

Discordant results were shown regardless of the method used on the negative sample as well as on the positive samples (Tables 6-8).

A false positive result was reported on the negative sample regardless of the method used: one–fold by SYBR Green RT-PCR (8.3% discrepant results), one fold by TaqMan specific Probe_RT-PCR (10% discrepant results), and one-fold also by *Pan-Lyssavirus* Probe RT-PCR (14.3% discrepant results). These results could be explained either by a cross-contamination of samples, an error during the transcription of the samples during the analysis or a reporting error in results.

3.3.2. Discordance on positive samples

Discordances in the positive samples were found variable according to the methods used, the strain tested and the level of positivity tested.

Out of all tested samples, no discrepancies were reported for the following samples:

- RABV strongly positive, regardless of the method used
- RABV moderate positive, regardless of the method used

- EBLV-1 strongly positive, regardless of the method used.

Globally, the SYBR Green RT-PCR yielded more frequently false negatives results (10% discrepant results) compared to the method based on TaqMan Probes (4% and 5% for the *Pan-Lyssavirus* Probe and TaqMan specific Probe RT-PCR, respectively). When considering the different level of positivity, the majority of discrepancies were shown for weakly positive samples regardless of the method used: 24.7 % for SYBR Green RT-PCR, 7.1 % for pan-Lyssavirus and 15 % for the TaqMan specific Probe RT-PCR). Highest discrepancies frequencies were noted for the weakly positive RABV strain using SYBR Green RT-PCR (26.3% of discrepant results) and TaqMan RABV specific Probe RT-PCR (23.3% of discrepancies).

The highest frequency of discordant results was observed for the lyssavirus BBLV sample with the Pan-Lyssavirus TaqMan RT-PCR (23.8%). The proportion of discrepancies increased on weak BBLV samples (42.8%).

It is to note that few TaqMan RT-PCR assays specific to the BBLV strain were performed by the participating laboratories. The results "Not detected" given for the BBLV strain and obtained by using a TaqMan RT-PCR that did not target BBLV were hence not counted in discrepancies. The same principle was applied for the analysis of the results of the EBLV-2, EBLV-1 and RABV samples.

Raw data analyzes tend to show cross-reactions between the BBLV strain and TaqMan Probes (RABV, EBLV-1, EBLV-2) for two TaqMan specific Probe RT-PCR tests of 10 tests performed.

Table 6. SYBR Green RT-PCR Results by samples tested

	SYBR Green RT-PCR results		
Sample tested	**n tests carried out**	**n Discrepant**	**Discrepant (%)**
Negative samples	12*	**1**	**8.3 (0.2 - 38.5)**
Positive samples by lyssavirus species	**221**	**22**	**10 (6.3. – 14.6)**
RABV	113	**10**	**8.8 (4.3. – 15.7)**
EBLV-1	36	**4**	**11.1 (3.1. – 26.1)**
EBLV-2	36	**5**	**13.9 (4.7- 29.5)**
BBLV	36	**3**	**8.3 (1.8-22.5)**
Positive samples by class of positivity	**222**	**22**	**10 (6.3-14.7)**
strongly positive	74	**1**	**1.4 (0-7.3)**
moderate positive	74	**3**	**4.1 (0.8-11.4)**
weak positive	74	**18**	**24.3 (15.3-36.1)**
Positive samples by species and by class of positivity	**221**	**22**	**10 (6.3- 14.7)**
RABV strongly positive	38	0	0 (0-9.3)
RABV moderate positive	38	0	0 (0.9.3)
RABV weak positive	37	**10**	**27 (13.8-44.1)**
EBLV-1 strongly positive	12	0	0 (0-26.5)
EBLV-1 moderate positive	12	**1**	**8.3 (0.2-38.5)**
EBLV-1 weak positive	12	**3**	**25 (5.5-57.2)**
EBLV-2 strongly positive	12	**1**	**8.3 (0.2-38.5)**
EBLV-2 moderate positive	12	**2**	**16.7 (2.1 – 48.4)**
EBLV-2 weak positive	12	**2**	**16.7 (2.1-48.4)**
BBLV strongly positive	12	0	0 (0-9.3)
BBLV moderate positive	12	0	0 (0-9.3)
BBLV weak positive	12	**3**	**25 (**0-9.3)

* Of the 13 tests performed by SYBR Green RT-PCR, only one was dedicated to the CVS amplification. Negative and positive samples (BBLV, EBLV-1, EBLV-2, RABV) were hence not tested.

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Table 7. Pan Lyssavirus Probe RT-PCR Results by samples tested

	***Pan-Lyssavirus* Probe RT-PCR**		
Sample tested	**n tests carried out**	**n Discrepant**	**Discrepant (%)**
Negative samples	7	**1**	14.3 (0.4-57.9)
Positive samples by lyssavirus species	**126**	**5**	**4 (1.3-9.0)**
RABV	63	0	0 (0.0 – 5.7)
EBLV-1	21	0	0 (0.0-16.1)
EBLV-2	21	0	0 (0.0-16.1)
BBLV	21	**5**	**23.8 (8.2-47.2)**
Positive samples by class of positivity	**126**	**5**	**4 (1.3-9.0)**
strongly positive	42	**1**	**2.4 (0.0 – 12.6)**
moderate positive	42	**1**	**2.4(0.0 – 12.6)**
weak positive	42	**3**	**7.1 (1.5 – 19.5)**
Positive samples by species and by class of positivity	**126**	**5**	**4 (1.3-9.0)**
RABV strongly positive	21	0	0 (0.0-16.1)
RABV moderate positive	21	0	0 (0.0-16.1)
RABV weak positive	21	0	0 (0.0-16.1)
EBLV-1 strongly positive	7	0	0 (0.0-16.1)
EBLV-1 moderate positive	7	0	0 (0.0-16.1)
EBLV-1 weak positive	7	0	0 (0.0-16.1)
EBLV-2 strongly positive	7	0	0 (0.0-16.1)
EBLV-2 moderate positive	7	0	0 (0.0-16.1)
EBLV-2 weak positive	7	0	0 (0.0-16.1)
BBLV strongly positive	7	**1**	**14.3 (0.4-57.9)**
BBLV moderate positive	7	**1**	**14.3 (0.4-57.9)**
BBLV weak positive	7	**3**	**42.9 (9.9-81.6)**

Table 8. Lyssavirus specific Probe RT-PCR Results by samples tested

	TaqMan specific Probe RT-PCR		
Sample tested	**n tests carried out**	**n Discrepant**	**Discrepant (%)**
Negative samples	10	**1**	**10 (0.3-44.5)**
Positive samples by lyssavirus species	**180**	**9**	**5 (2.3-9.3)**
RABV	90	7	**7.8 (3.2-15.4)**
EBLV-1	30	2	**6.7 (0.8-22.1)**
EBLV-2	30	0	0 (0.0-11.6)
BBLV	30	0	0 (0.0-11.6)
Positive samples by class of positivity	**180**	**9**	**5 (2.3-9.3)**
strongly positive	60	0	0 (0.0 – 6.0)
moderate positive	60	0	0 (0.0 – 6.0)
weak positive	60	**9**	**15 (7.1 – 26.6)**
Positive samples by species and by class of positivity	**180**	**9**	**5 (2.3-9.3)**
RABV strongly positive	30	0	0 (0.0-11.6)
RABV moderate positive	30	0	0 (0.0 – 11.6)
RABV weak positive	30	**7**	**23.3 (10.0 – 42.3)**
EBLV-1 strongly positive	10	0	0 (0.0-30.8)
EBLV-1 moderate positive	10	0	0 (0.0-30.8)
EBLV-1 weak positive	10	**2**	**20 (3.5-55.8)**
EBLV-2 strongly positive	10	0	0 (0.0-30.8)
EBLV-2 moderate positive	10	0	0 (0.0-30.8)
EBLV-2 weak positive	10	0	0 (0.0-30.8)
BBLV strongly positive	10	0	0 (0.0-30.8)
BBLV moderate positive	10	0	0 (0.0-30.8)
BBLV weak positive	10	0	0 (0.0-30.8)

3.3 Part 2: Evaluation results of the PCR's limit of detection

All the 25 participating laboratories sent back their results.

All three methods, Lyssavirus specific Probe based RT-PCR, *Pan-Lyssavirus* Probe RT-PCR and SYBR Green RT-PCR were tested with 10, 6 and 13 tests performed respectively.

A total of 11 tests was performed using the Lyssavirus specific probe RT-PCR by the participating laboratories. One laboratory submission was not considered for the analysis of raw data because only one dilution was tested 5 times, which does not allow the calculation of the slope of the linear regression and the efficiency. This result could be explained either by a misunderstanding of instructions given or errors in recording results.

Raw data submitted by each laboratory are detailed in Appendix (except the 11th laboratory submission).

- S Table 4 details the SYBR Green Pan Lyssavirus RT-PCR results with the C_T values and Tm (°C) of the PCR product; S Table 5 details the TaqMan specific Probe_RT-PCR results with the C_T values; and S Table 6 details the *Pan-Lyssavirus* TaqMan RT-PCR results with C_T values. The dilution giving 100% of positive results are underlined in green in the Tables.

The standard curve analysis gave preliminary results on the estimation of PCR efficiency as well as on an estimate of the detection limit of the three real-time RT-PCR methods.

On the basis that the limit of detection was either equal or above to the dilution giving 100% of positive results, the estimated limit of detection of PCR are as follows for the three techniques (Table 9).

- **SYBR Green RT-PCR**
    - The limit of detection of PCR was shown varying between 1 copy/µL (n=5 assays), 10 copies/µL (5 assays) and 100 copies (2 assays). One assay showed a LD ~ 1000 copies/µL.
- **Lyssavirus specific TaqMan RT-PCR**
    - The limit of detection vary between 1 copy/µL (3 assays), 10 copies/µL (3 assays) and 100 copies/µL (4 l assays).
- ***Pan-Lyssavirus* RT-PCR**
    - The limit of detection vary between 1 copy/µL (1 assay) and 10 copies/µL (4 assays). One assay showed a LD ~ 100 copies/µL.

Table 9. Estimation of the limit of detection of PCR by method used.

		Limit of detection of PCR		
		n assays performed		
Dilution (log)	**Nb of copies/µL RNA**	**SYBR Green pan-lyssavirus RT-PCR**	**Lyssavirus specific Probe RT-PCR**	***Pan-Lyssavirus* Probe based RT-PCR**
1	1. 10^6	0	0	0
2	1. 10^5	0	0	0
3	1. 10^4	0	0	0
4	1. 10^3	**1**	0	0
5	1. 10^2	**2**	**4**	**1**
6	1. 10^1	**5**	**3**	**4**
7	1.	**5**	**3**	**2**
Total tests carried out :		**13**	**10***	**7**

** A total of 11 tests were performed by Lyssavirus specific Probe RT-PCR. Of the 11 tests carried out, one was not considered for the analysis (misunderstanding of instructions or errors in recording results).

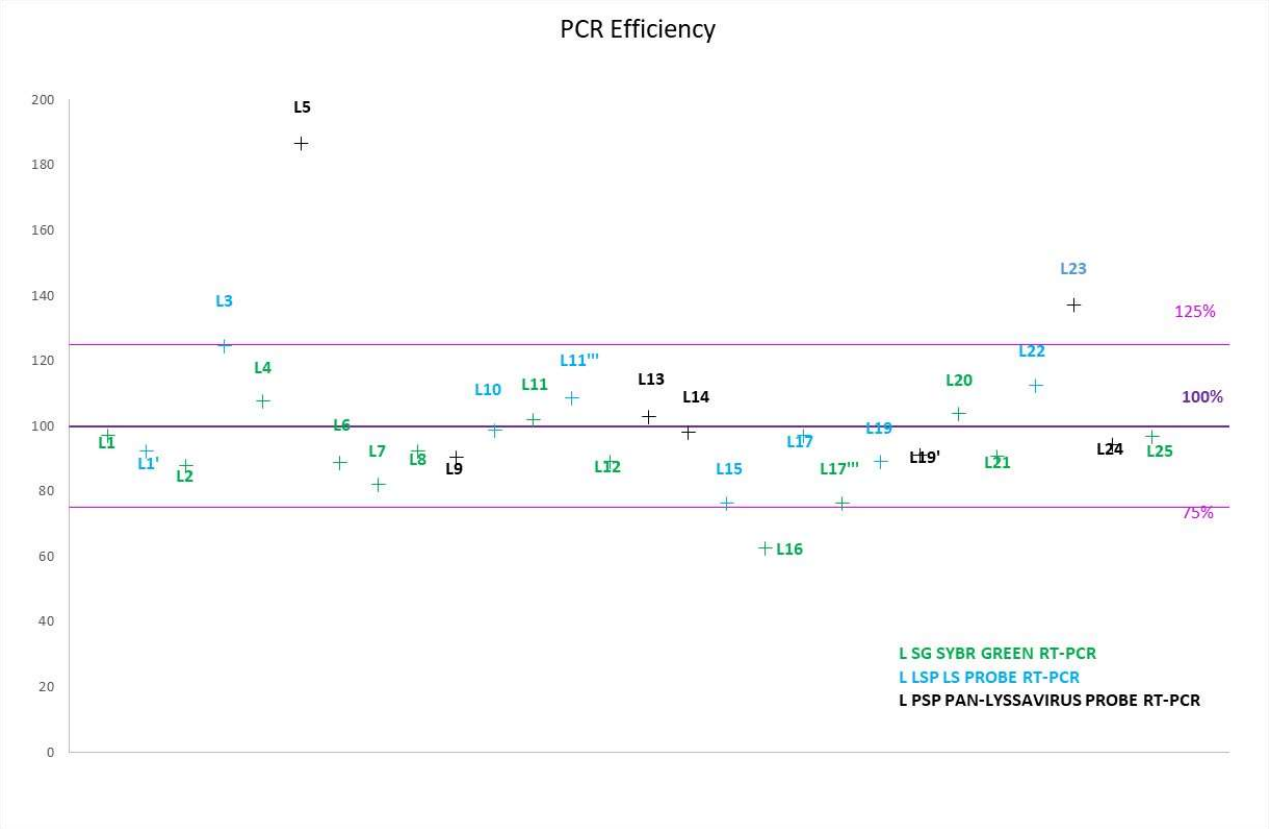
Analysis of the raw data showed that the limit of detection of PCR vary between 1 copy/µL and 10 copies/µL in 77% (10/13x100) of SYBR Green RT-PCR assays, 60% for Lyssavirus specific Probe RT-PCR (6/10*100) and 86% for *pan-Lyssavirus* Probe RT-PCR (6/7*100). Few assays showed a LD PCR ~ 1000 copies/µL (1 assay/13 by SYBR Green RT-PCR) and ~100 copies (4 assays/10 for the Lyssavirus specific Probe RT-PCR and 1 by *pan-Lyssavirus* RT-PCR). These results could be explained by the fast cycles conditions used for RT (600 sec) and/or for PCR (cycle in 2 step) compared to the other assays that used "conventional" cycles conditions: RT (1800 sec) and PCR (cycle in 3 steps).

The PCR efficiency was calculated from the slopes of standard curves using the equation: $E(\%) = [10^{(-1/slope)} -1] \times 100$. The slope is determined as the slope of the standard curve that was plotted with the y axis as Ct values and the x axis as log(quantity) of standard tested.

In general, a slope is generally between -4.115 and -2.839, which corresponds to a PCR efficiency between 75% and 125% (NF-U-47-600). The ideal reaction efficiency for a real-time PCR is 100%, but typically, the widely accepted range is 90–110%.

Graphical repartition of the PCR efficiency calculated by type of method (SYBR Green RT-PCR, *Pan-Lyssavirus* TaqMan RT-PCR and Lyssavirus specific Probe RT-PCR) is shown in Figure 4.

Figure 4. Graphical repartition of the PCR efficiency calculated from the range of dilutions by laboratory and by type of PCR: SYBR Green RT-PCR, *Pan-Lyssavirus* TaqMan RT-PCR and Lyssavirus specific Probe RT-PCR.



PCR efficiency ranged between 75% - 107% for 92% of the SYBR Green assays that were performed (12/13x100), 89.3% - 108.7% for Lyssavirus specific RT-PCR (7/9 assays) and 90.6%-112.6% for *Pan-Lyssavirus* RT-PCR (5/6 assays). Four assays out of 13 SYBR Green RT-PCR assays showed efficiencies values outside of the given range of 90-110% (62.7%; 76.3%, 76.4% and 82%). Only 2 and 1 assays carried out with the Lyssavirus specific Probe and the *Pan-Lyssavirus* RT-PCR showed efficiencies values outside of 90-110% with values of 124.2-137.2% and 186.6%, respectively.

Four assays showed efficiency either greater than 125% or less than 75%: two correspond to the Lyssavirus specific Probe RT-PCR assays (124.8 % and 137.2%), one to the *Pan-Lyssavirus* TaqMan RT-PCR (186.6%) and one to the SYBR Green RT-PCR (62.7%).

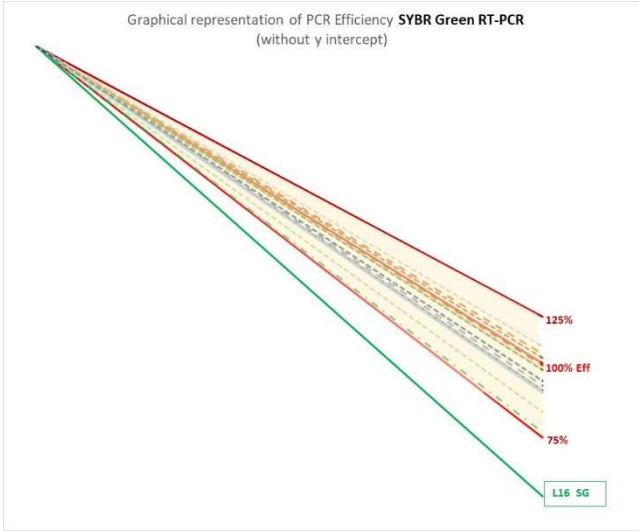
An efficiency above 110% tend to indicate that inhibition is occurring in the PCR reaction. Causes of inhibition are multiple and can be due to poor nucleic acids quality, high template concentration, chaotropic salts, ... Inhibition is normally less common than poor reaction efficiency, with an efficiency below 90%. Multiple causes can also explain an E< 90% and can be due to suboptimal reagent concentrations with mainly primers, magnesium, and/or Taq DNA polymerase. Other factors contributing to poor reaction efficiency include primer and/or suboptimal thermocycling conditions.

Efficiencies outside the range of 90–110% may artificially skew results and lead to false conclusions. Inhibition and poor efficiency can affect assay sensitivity, leading to a smaller dynamic range.

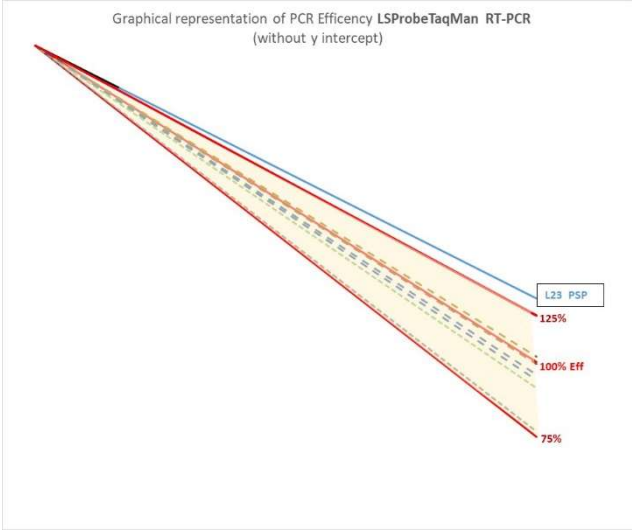
The representation of PCR efficiency in Figure 5 was established considering only the slope of the linear representation for all laboratories (y_intercept=0 for the representation).

Figure5. Graphical representation of the PCR efficiency related to the slope, calculated from the range of dilutions by laboratory and by type of PCR : SYBR Green RT-PCR (A), Lyssavirus specific probe RT-PCR (B) and *Pan-Lyssavirus* RT-PCR (C).

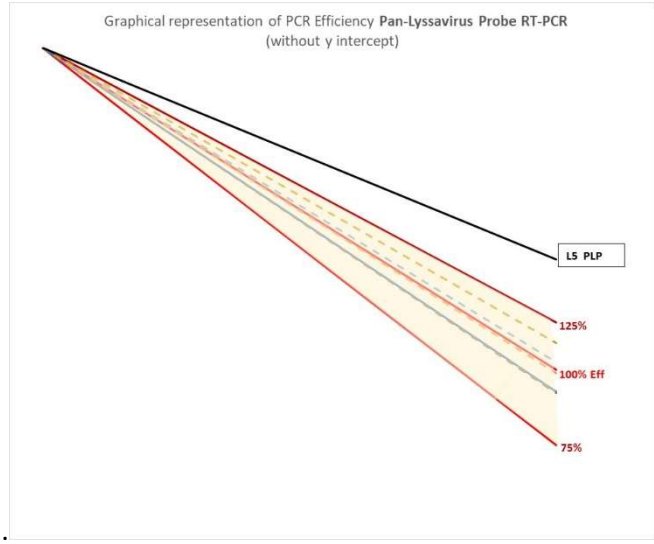
A.



B.



C.



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4. CONCLUSION

- The *pan-Lyssavirus* RT-PCR that is able to detect all lyssavirus species was the technique the mostly carried out in this inter-laboratory test. Seventy-six percent of the assays were indeed *pan-Lyssavirus* RT-PCR while 34% of assays were carried out with TaqMan specific probes. Among *pan-Lyssavirus* RT-PCR, 63% was SYBR Green RT-PCR and 37% was the *pan-Lyssavirus* Probe based RT-PCR.

- The lowest proportion of discrepancies on positive samples was shown for the pan-Lyssavirus TaqMan RT-PCR (4%) compared to the SYBR Green (10%) and the Lyssavirus specific Probe RT-PCR (5%).

- The three methods gave a false positive result on the negative sample, with respectively a proportion of 8.3% discrepant results for the SYBR Green RT-PCR (1 out of 12), 10% discrepant results for the TaqMan specific Probe RT-PCR (1 out of 10), and 14.3% discrepant results for the *pan-Lyssavirus* TaqMan RT-PCR (1 out of 7). The discrepancies were shown in three different laboratories.

- The three methods showed a good sensitivity with 100% of detection for the strongly positive samples RABV and EBLV-1. Regardless of the method carried out, no false negative result was detected on strongly and moderate positive RABV as well as on EBLV-1 strongly positive.

- False negative results were observed more frequently with SYBR Green RT-PCR than with TaqMan probe-based assays on weak positive samples.

- No discordant results were observed on RABV, EBLV-1 and EBLV-2 by *pan-Lyssavirus* TaqMan RT-PCR and on EBLV-2 and BBLV by Lyssavirus specific TaqMan RT-PCR.

- Regardless of the method used, the limit of detection of the PCR techniques varies between laboratories:

    - LD PCR ranges from 1 copy/µL to 1000 copies/µL for SYBR Green RT-PCR assays-

    - LD PCR ranges from 1 copy/µL to 100 copies/µL for Lyssavirus specific Probe RT-PCR assays-

    - LD PCR ranges from 1 copy/µL to 100 copies/µL for *pan-Lyssavirus* Probe RT-PCR assays-

Table 9. Summary of discrepancies in the 3 different Real-time RT-PCR methods

Sample tested	SYBR Green RT-PCR	*Pan-Lyssavirus* Probe based RT-PCR	Lyssavirus specific Probe RT-PCR
n participating laboratories	13*	7	**10**
Negative samples (n/N)	**8.3%** (1/12)	**14.3%** (1/7)	**10%** (1/10)
Positive samples by lyssavirus species	**10%** (22/221)	**4%** (5/126)	**5%** (9/180)
RABV	**8.8%** (10/113)	0% (0/63)	**7.8%** (7/90)
EBLV-1	**11.1%** (4/36)	0% (0/21)	**6.7%** (2/30)
EBLV-2	**13.9%** (5/36)	0% (0/21)	0% (0/30)
BBLV	**8.3%** (3/36)	**23.8%** (5/21)	0% (0/30)
Positive samples by class of positivity	**10%** (22/222)	**4%** (5/126)	**5%** (9/180)
strongly positive	**1.4%** (1/74)	**2.4%** (1/42)	0% (0/60)
moderate positive	**4.1%** (3/74)	**2.4%** (1/42)	0% (0/60)
weak positive	**24.3%** (18/74)	**7.1%** (3/42)	**15%** (9/60)

** Of 13 tests performed by SYBR Green RT-PCR, one was dedicated to the CVS amplification.

Negative and other positive samples (BBLV, EBLV-1, EBLV-2, RABV) were not tested.

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