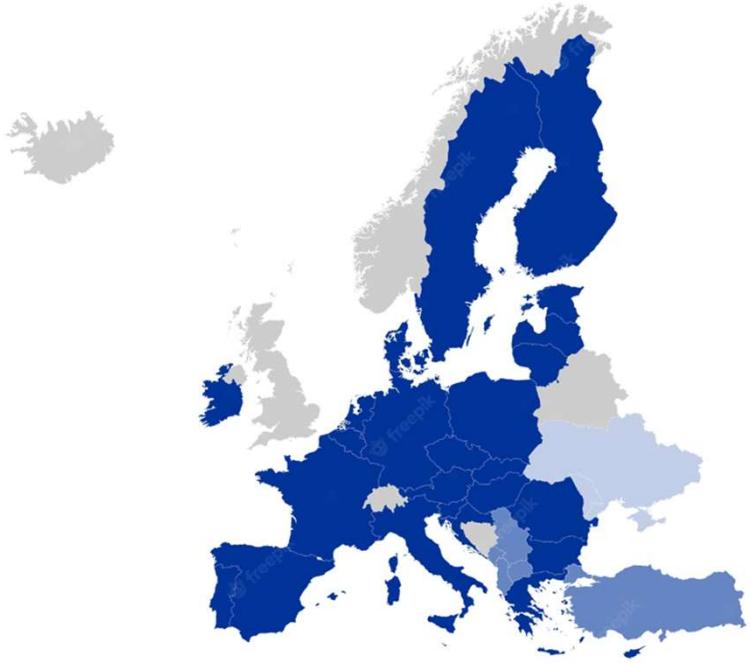
Inter-laboratory assay for performance evaluation of Lyssavirus (rt) RT-PCR techniques: results of year 2022

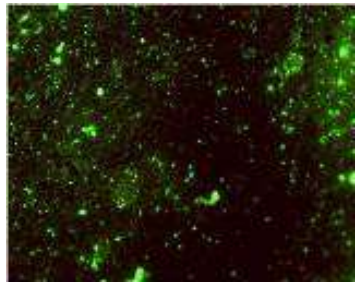


14th Workshop for Rabies Ljubljana, Slovenia, 22 June 2023

Introduction

NOTE

Diagnosis of rabies using molecular methods has been accepted by the WOAH from 2018 for the confirmation of rabies cases, in particularly in situations when the samples are sub-optimal or for ante-mortem diagnosis



In 2018, real-time (rt) and conventional (end-point) RT-PCR were used by 47% and 63% of NRLs, respectively,

In 2021, (rt) RT-PCR was used by 51% of NRLs (~ 5% of the total amount of tests) while the end-point RT-PCR was used by 20% of NRLs (~ 2% of the analyses performed). (rt)RT-PCR was the 2nd technique of choice in addition to FAT in 2021.

(rt) RT-PCR is increasingly used to replace the cell isolation test.

Need to evaluate the (rt) RT-PCR techniques used in NRLs

Aim of the study

Organization of an inter-laboratory assay in 2022 to evaluate the (rt) RT-PCR assays used by NRLs within the European Union

Objectives:

- **Determine whether or not the (rt) RT-PCR methods used by different laboratories provide similar performances (limit of detection of PCR, specificity and sensitivity)**
- **Evaluate potential discrepancies among different unknown samples:**
  - Negative
  - Positive with different levels of positivity: strong, moderate, weak
  - Different Lyssavirus species: RABV and bat lyssaviruses

anses

THE PANEL

Characteristics of samples

ID	Sample nature	Batch name	N°batch	Passaged on	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	Decoy	DUVV	04-21	Mouse	/			Bat
8		Ukraine	05-21	Mouse				Fox
9		Ukraine	06-21	Mouse				Fox
10		Buffer TE		/				/

* GR64C/12, KC011844

A panel 2 – in - 1 was constituted with 21 frozen RNA samples to 1) assess the specificity and sensitivity by species and by positivity level and 2) assess the limit of detection of PCR

Composition of the panel (1)

- **The panel (1) was constituted of 20 frozen coded samples.**

- **Including:**
  - 3 RABV,
  - 3 bat lyssaviruses,
  - and 1 negative sample.

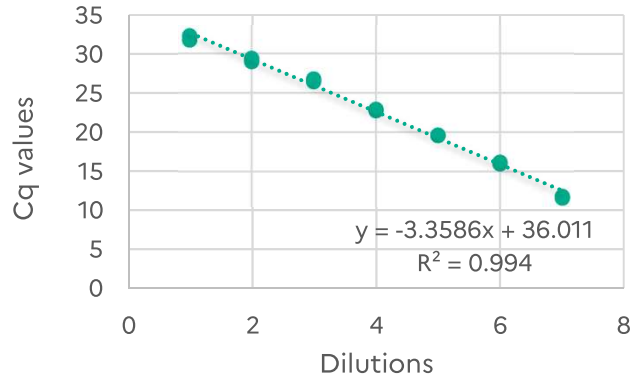
- **Each Lyssavirus RNA sample was provided with 3 different levels of positivity:**
  - **strong** (18<Ct≤23),
  - **moderate** (23<Ct≤28),
  - **weak** (28<Ct≤35).

ID	Strain	Class	Cq values	Nb. of copies/µL RNA
1	Greece	Strong	20.76 (± 0.04)	3.49E+04
2		Mod.	25.52 (±0.08)	1.33E+03
3		Weak	30.98 (±0.31)	3.14E+01
4	CVS (1)	Strong	20.91 (±0.04)	3.14E+04
5		Mod.	25.98 (±0.17)	9.73E+02
6		Weak	32.22 (±1.22)	1.34E+01
7	CVS (2)	Strong	21.09 (±0.04)	2.77E+04
8		Mod.	25.67 (±0.08)	1.20E+03
9		Weak	31.54 (±0.13)	2.14E+01
10	EBLV-1b	Strong	20.24 (±0.1)	4.95E+04
11		Mod.	25.5 (±0.12)	1.35E+03
12		Weak	30.3 (±0.31)	5.01E+01
13	EBLV-2	Strong	18 (± 0.1)	2.35E+05
14		Mod.	23.18 (±0.1)	6.61E+03
15		Weak	28.38 (±0.16)	1.88E+02
16	BBLV	Strong	19.84 (±0.01)	6.52E+04
17		Mod.	25.24 (±0.19)	1.61E+03
18		Weak	30.1 (±0)	5.77E+01
19	Chicken	/	No Ct	0
20	Decoy	Mix (Neg or Strong.)	Mix (Neg or Strong.)	Mix (Neg or Strong.)

Composition of the panel (2)

- The panel (2) was constituted of one tube with a known status.
- CVS-27 RNA was supplied at a concentration of 10^7 copies/µL of RNA for the generation of a standard curve (6 dilutions at 1 log, each).

ID	Batch	Dilution (log)	Cq values		Nb of copies/µL RNA
1	CVS-27 batch 11-14	1	16.09	(±0.16)	8.56E+05
2		2	19.6	(±0.02)	7.69E+04
3		3	22.84	(±0.12)	8.34E+03
4		4	26.6	(±0.24)	6.35E+02
5		5	29.19	(±0.26)	1.07E+02
6		6	32	(±0.39)	1.57E+01



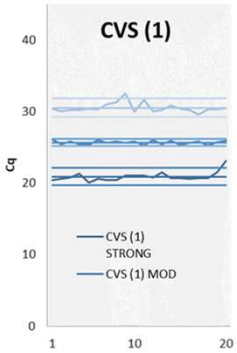
Panel validation: Homogeneity assessment

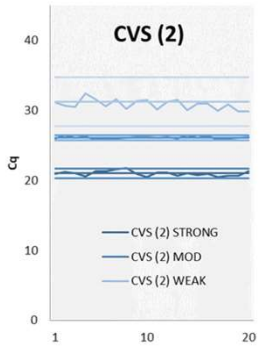
Batch name	Species
CVS 27 11-14*	RABV
RABV 36-12	
EBLV-1 03-08	EBLV-1
EBLV-2 03-12	EBLV-2
BBLV 35-18	BBLV
Negative 02-19	/

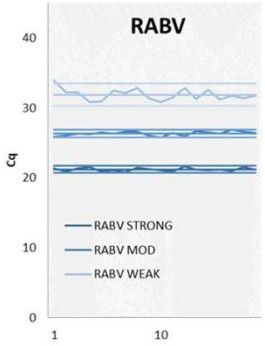
* duplicate

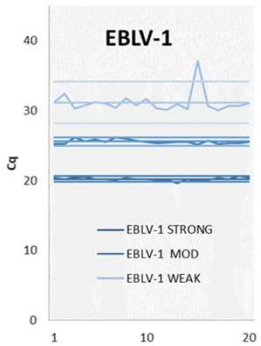
- Testing of 10 samples per batch for each class of positivity (strong, moderate, weak)
- Testing in duplicates by SYBR Green RT-PCR

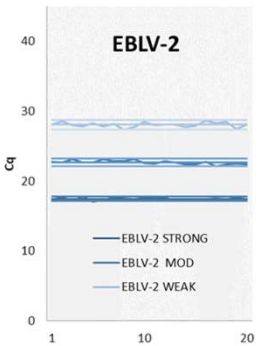
All batches were tested, shown homogeneous
- < 1 Cq value for strong and moderate
- < 2 Cq values for weak positives

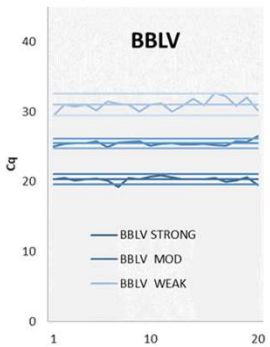


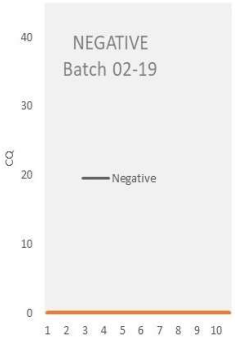












Panel validation: stability assessment

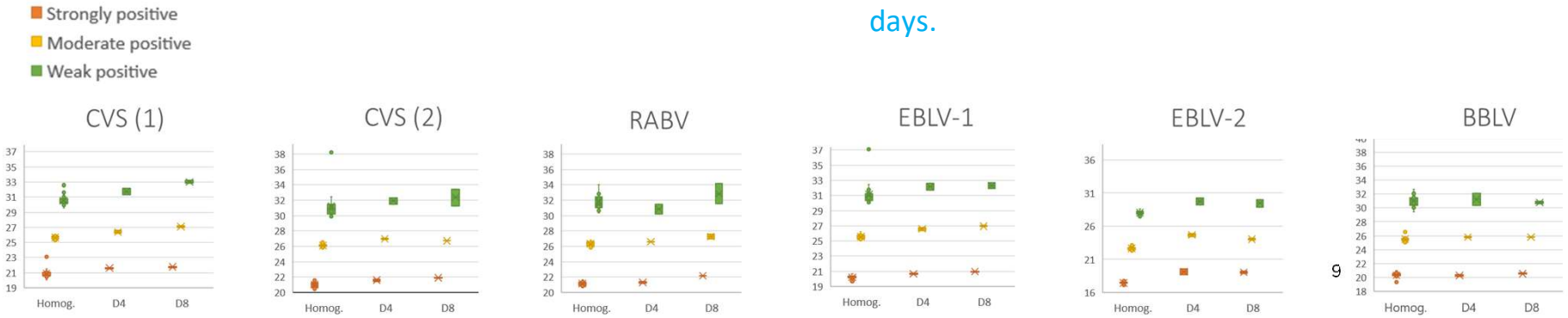
- The panel was tested at D+4 and D+8 at a temperature < -18C
- The panel was then tested with 4 successive cycles of freezing-thawing of samples

Each sample was tested in duplicate by SYBR Green RT-PCR

Expected results :

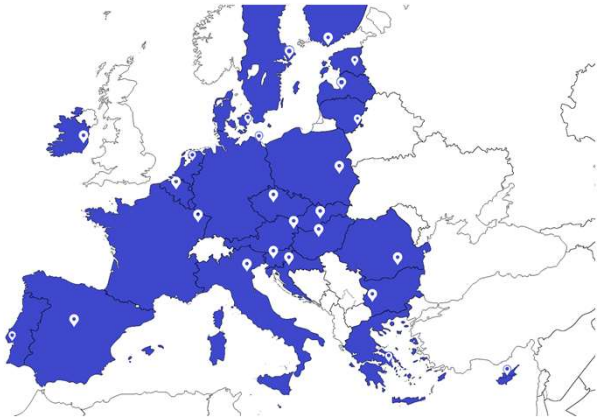
- < 1 Cq value for strong and moderate ; < 2 Cq values for weak positives

All tested conditions were shown satisfactory and confirmed the stability of all batches submitted in the successive cycles of freezing-thawing, as well at < -18°C during 4/8 days.



PARTICIPANTS-METHODS USED

Participating laboratories



25 labs. participated in the inter-laboratory test

Delivery time and receipt of panels

- 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.

Methods used

Three methods were used:

- **Pan-Lyssavirus SYBR Green RT-PCR** (Hayman et al, 2011)
- **Pan-Lyssavirus Real-Time (Probe) RT-PCR** (Gigante et al., 2018)
- **Lyssavirus specific probe RT-PCR** (Wakeley et al., 2005 ; Fischer et al., 2014)

- **Of 25 participating lab., 19 used pan-Lyssavirus RT-PCR method and 10 used the Lyssavirus specific Probe RT-PCR.**
- **Of the 19 lab. that used the pan-Lyssavirus method, 12 lab. (63%) used SYBR Green RT-PCR and 7 (37%) the pan-Lyssavirus Probe RT-PCR.**
- **4 participants used both SYBR Green and Probe PCR, and 1 used both the pan-Lyssavirus Probe RT-PCR and the specific probe RT-PCR.**

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

* dedicated to CVS, only.

RESULTS

PART I: RESULTS OF THE SPECIFICITY AND SENSITIVITY ANALYSIS BY SPECIES AND BY POSITIVITY LEVEL

SYBR Green RT-PCR

- **Negative samples:**

  Discrepancy noted once (8%)

  11/12 negative

- **Positive samples:**

  Discrepancies noted for

  - RABV weakly pos. (27%)
  - EBLV-1 moderate (8%) and weakly pos. (25%)
  - EBLV-2 strong (8%) , moderate (17%) and weak pos (2%)
  - BBLV weak pos. (25%)

Total of 10% false negatives

100% detection on:
strong and mod. RABV
strong EBLV-1
strong and mod. BBLV

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)

TaqMan specific Probe RT-PCR

- **Negative samples:**

  Discrepancy noted once (10%)

  9/10 negative

- **Positive samples:**

  Discrepancies noted for

  - **RABV weakly pos. (23%)**
  - **EBLV-1 weakly pos. (20%)**

Total of 5% false negatives

100% detection on:
strong and mod. RABV
strong and mod. EBLV-1
strong, mod., weak EBLV-2
strong, mod., weak BBLV

Sample tested	TaqMan specific Probe RT-PCR 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)

Pan-Lyssavirus Probe RT-PCR

- **Negative samples:**

  Discrepancy noted once (14%)

  6/7 negative

- **Positive samples:**

  Discrepancies noted for

  - **BBLV strong (14%), mod. (14%) and weakly pos. (43%)**

Total of 4% false negatives

100% detection on:
strong, mod. and weak RABV,
strong, mod. and weak EBLV-1,
strong, mod. and weak EBLV-2,

	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)**

PART 2: EVALUATION RESULTS OF THE PCR'S LIMIT OF DETECTION

Limit of detection of PCR

		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

73%

SYBR Green RT-PCR: 1 to 1000 copies/µL

Lyssavirus specific Probe RT-PCR : 1 to 100 copies/µL

Pan-Lyssavirus Probe RT-PCR: 1 to 100 copies/µL

Efficiency of PCR

1. SYBR Green RT-PCR

- **ranged between 63% and 107% (n=13 labs)**
  - 77%: 90-110% (10/13) / 23%: 65-77% (3/13)

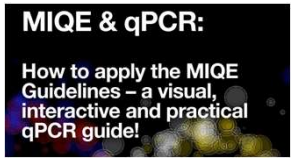
2. Lyssavirus specific probe RT-PCR

- **ranged between 90% and 137% (n=9 labs)**
  - 78%: 90-110% (7/9) / 22%: 124-137%% (2/9)

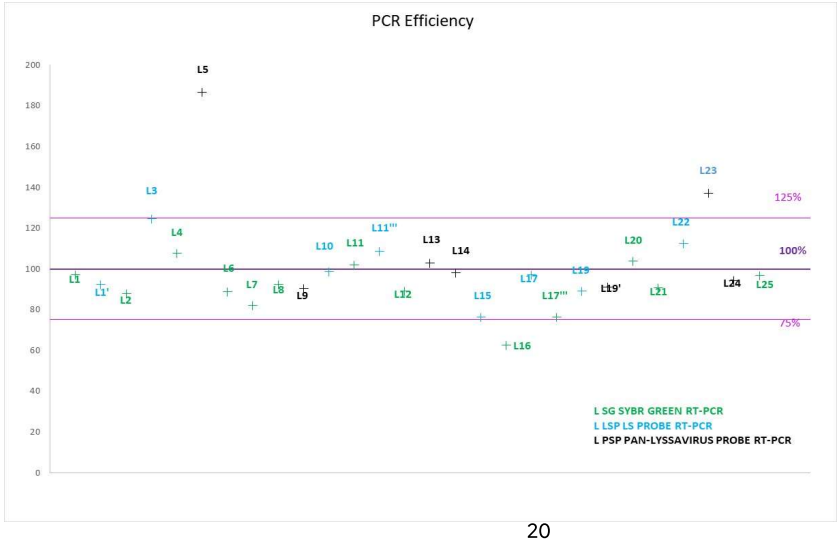
3. Pan-Lyssavirus probe RT-PCR

- **ranged between 91% and 187% (n=6 labs)**
  - 83%: 90-110% (5/6) / 17%: 65-77% (1/6)

NOTE



- Indicates wheter the DNA is doubled in each cycle (10-fold in 3.32 cycles)
- Eff=10^(-1/slope) - 1
- The efficiency of the PCR should be 90-110% according to the MIQE guid



Summary

- **Which methods were the most used?**
  - ❑ The technique the mostly carried out was the pan-Lyssavirus RT-PCR (76%) as recommended by the WOHA. 76% of these assays were based on SYBR Green RT-PCR (63%) followed by pan-Lyssavirus Probe RT-PCR (37%).

- **Specificity and sensitivity analysis by species and by positivity level**
  - ❑ The three methods gave a false positive result in three different laboratories, with respectively a proportion of 8% 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% discrepant results for the pan-Lyssavirus TaqMan RT-PCR (1 out of 7).

Summary (Cont'd)

- **Specificity and sensitivity analysis by species and by positivity level**

- ❑ Regardless of the method carried out, 100% of detection were shown for the strongly positive and moderate samples RABV and strongly positive EBLV-1.
- ❑ False negative results were observed more frequently with SYBR Green RT-PCR (24%) than with TaqMan probe-based assays (7% by Pan-Lyssavirus probe and 15% by Lyssavirus specific probe) 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.
- ❑ 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%).

A variability in the sensitivity of the real-time RT-PCR was identified for detecting weak positive RABV RNA samples with highest discrepancies (~ 20 – 30 %) when using TaqMan RABV RT-PCR or SYBR Green RT-PCR, respectively.

Conclusion

The inter-laboratory assay for performance evaluation of Lyssavirus (rt) RT-PCR techniques held in 2022 showed that the three methods used in NRLs are sensitive and specific, but all 3 methods gave false negatives and false positives in more or less equivalent proportions. In the light of these data (LD, specificity and sensitivity by positivity level), it appears that the pan Lyssavirus RT-PCR is the most effective method for detecting Lyssaviruses RNA (with the possible exception of BBLV?). It would be interesting to investigate more deeply this method in addition to a deeply evaluation of the limit of detection of the three assays.

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- ANSES Nancy laboratory

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