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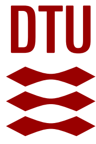

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Molecular double-check strategy for the identification and characterization of European Lyssaviruses

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The "gold standard" for post-mortem rabies diagnosis is the direct fluorescent antibody test (FAT). However, in the case of ante-mortem non-neural sample material or decomposed tissues, the FAT reaches its limit, and the use of molecular techniques can be advantageous. In this study, we developed and validated a reverse transcription PCR cascade protocol feasible for the classification of samples, even those for which there is no epidemiological background knowledge. This study emphasises on the most relevant European lyssaviruses.

In a first step, two independent N- and L-gene based pan-lyssavirus intercalating dye assays are performed in a double-check application to increase the method's diagnostic safety. For the second step, characterization of the lyssavirus positive samples via two independent multiplex PCR-systems was performed. Both assays were probe-based, species-specific multiplex PCR-systems for *Rabies virus*, *European bat lyssavirus* type 1 and 2 as well as *Bokeloh bat lyssavirus*. All assays were validated successfully with a comprehensive panel of lyssavirus positive samples, as well as negative material from various host species.

This double-check strategy allows for both safe and sensitive screening, detection and characterization of all lyssavirus species of humans and animals, as well as the rapid identification of currently unknown lyssaviruses in bats in Europe.

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1. Introduction

Although rabies has been known since antiquity, it still represents one of the most important zoonotic diseases with an immense public health impact, especially in developing countries (Knobel et al., 2005; Anderson and Shwiff, 2013). The causative agents of this fatal encephalitis are lyssaviruses (order *Mononegavirales*, family *Rhabdoviridae*), with the prototype species being *Rabies virus* (RABV). Further members of the genus *Lyssavirus* are *Lagos bat virus* (LBV), *Mokola virus* (MOKV), *Duvenhage virus* (DUVV), *European bat lyssavirus* type 1 and 2 (EBLV-1 & -2), *Australian bat lyssavirus* (ABLV), *Aravan virus* (ARAV), *Khujand virus* (KHUV), *Irkut virus* (IRKV), *West Caucasian bat virus* (WCBV), and *Shimoni bat virus* (SHIBV) (Dietzgen et al., 2012). The two most recently discovered lyssaviruses, *Bokeloh bat lyssavirus* (BBLV) and *Ikoma virus* (IKOV) (Freuling et al., 2011; Marston et al., 2012), are approved as new lyssavirus species and are awaiting ratification by the International Committee on Taxonomy of Viruses. Bats appear to be the original reservoir for lyssaviruses, and many of those bat-associated viruses have caused human fatalities worldwide (Johnson et al., 2010; Banyard et al., 2011). However, the vast majority of human exposures and consequently fatalities are related to RABV, which is transmitted by dog bites (WHO, 2013).

The "gold standard" method for rabies diagnosis that is recommended by the World Health Organization (WHO) and the Organization for Animal Health (OIE) is the direct fluorescent

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antibody test (FAT; Dean et al., 1996; OIE, 2008). This method facilitates the detection of lyssavirus antigens in post-mortem brain specimens (Dürr et al., 2008; Fooks et al., 2009). Though, for ante-mortem diagnosis in humans using non-neural sample material (e.g., saliva, cerebrospinal fluid, skin biopsies) or in the case of decomposed tissues, the FAT reaches its limits. Additionally, with respect to passive bat rabies surveillance, post-mortem laboratory diagnosis is often hampered by such difficulties as obtaining sufficient and good quality brain material. Furthermore, serial and fully automated testing of larger sample numbers, e.g., for screening purposes in bats, is not possible using the FAT.

Although not yet recommended for routine post-mortem diagnosis of rabies, in such situations, including epidemiological surveys, the use of molecular methods, such as reverse transcription PCR (RT-PCR) and other amplification techniques, can be advantageous (David et al., 2002; Dacheux et al., 2010; WHO, 2013). As a result, numerous pan-lyssavirus or lyssavirus species-specific conventional and real-time RT-PCRs have been developed (for details see Fooks et al., 2009; Coertse et al., 2010; Hoffmann et al., 2010). Because of the ability of real-time PCRs, to detect small amounts of viral RNA, these assays have become the method of choice for molecular diagnostics of infectious agents, including rabies, over the past decade (Hoffmann et al., 2009; Coertse et al., 2010).

To improve the overall diagnostic possibilities, we developed a cascade protocol for molecular lyssavirus diagnostics with an emphasis on the most relevant European lyssaviruses. As a first step, two independent pan-lyssavirus assays based on the detection of an intercalating dye are performed in a double-check application to increase diagnostic reliability. If a lyssavirus is recognized ("positive result"), as a second step, two probe-based (TaqMan), species-specific multiplex systems for RABV, EBLV-1, EBLV-2 and BBLV detection are applied to determine the virus species. Additionally, using this procedure, samples without any further epidemiological background information can be detected and classified.

2. Material and methods

2.1. Pan-lyssavirus and species-specific PCRs

To detect various lyssavirus species reliably, one pan-lyssavirus (pan-Lyssa) real-time RT-PCR (RT-qPCR) described recently by Hayman et al. (2011) and one newly developed RT-qPCR, with both using the OneStep RT-PCR Kit (Qiagen, Hilden, Germany), targeting regions in the nucleoprotein (N) and large (L, RNA dependent polymerase) gene, respectively, were applied in parallel. The assays were optimized for limited sample material using a total reaction volume of 12.5 μl. For one reaction, 5.5 μl RNase-free water, 2.5 μl 5x OneStep RT-PCR Buffer, 0.5 μl OneStep RT-PCR Enzyme Mix, 0.5 μl dNTP Mix (10 mM each), 0.5 μl ResoLight Dye (Roche, Mannheim, Germany), 0.25 μl of each primer (Table 1) and 2.5 μl RNA template or RNase free water for the no template control (NTC) was used. Functionality of amplification was tested in an extra tube using the heterologous internal control (IC) system developed by Hoffmann et al. (2006) in combination with the endogenous housekeeping assay according to Toussaint et al. (2007; Table 1) as extraction control. For the heterologous internal control, 0.25 μl IC2 RNA (Hoffmann et al., 2006) per reaction was spiked in the PCR reaction mix. The following thermal program was applied: 1 cycle of 50 °C for 30 min and 95 °C for 15 min, followed by 45 cycles of 94 °C for 30 s, 56 °C for 30 s and 72 °C for 30 s. Subsequently, melt curve analysis was performed (1 min 95 °C, 1 min 55 °C, increase 0.5 °C per cycle for 10 s; 55–95 °C) for the verification of positive results (Supplementary Table 1). Validity ranges of the melting temperature (T_m) were defined for the pan-Lyssa N-gene RT-qPCR (T_m 77.5 ± 1.5 °C) and the pan-Lyssa L-gene RT-qPCR (T_m 82 ± 1.5 °C). Final confirmation was achieved by sequencing, using the respective primers and the Big Dye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, Carlsbad, USA).

For lyssavirus species-specific detection, the AgPath-ID^{TM} One-Step RT-PCR Kit (Applied Biosystems, Carlsbad, USA) was used by combining the assay developed by Wakeley et al. (2005; R13 MP) with a novel FLI multiplex (R14 MP) assay both targeting the N-gene of lyssaviruses. Both assays were optimized using a total reaction volume of 12.5 μl. Furthermore, the published assay by Wakeley et al. (2005) was extended by an additional BBLV probe (Table 1). For one single reaction, 2.75 μl (R13 MP) or 1.25 μl (R14 MP) RNase-free water, 6.25 μl 2x RT-PCR buffer, 0.5 μl 25x RT-PCR enzyme mix, 2.5 μl RNA template or RNase free water for the no template control (NTC) and 0.5 μl of the according primer–probe mixes (Table 1) were combined. The following thermal program was applied: 1 cycle of 45 °C for 10 min and 95 °C for 10 min, followed by 45 cycles of 95 °C for 15 s, 56 °C for 20 s, and 72 °C for 30 s.

All reactions were carried out as technical duplicates in Bio-Rad 96-well PCR plates using a CFX96 quantitative PCR system (Bio-Rad Laboratories, Hercules, USA). For each RT-qPCR, a quantification cycle number (C_q) was determined according to the PCR cycle number at which the fluorescence of the reaction crosses a value that is statistically higher than the background which is determined by the respective software associated with each system. Finally, mean C_q-values were calculated from the technical duplicates. A cut off >42 was defined for negative results.

2.2. Generation of a synthetic positive control

For repeatability analysis, a synthetic gene was designed including positive controls for all lyssavirus assays used in this study (Supplementary Table 2). The construct was synthesized by GeneArt (GeneArt, Regensburg, Germany) with the vector pMA-7-Ar as a backbone (Supplementary Fig. 1). Plasmids were amplified in *Escherichia coli* DH10B (Invitrogen, Carlsbad, USA) and purified by Qiagen Plasmid Mini and Midi Kits (Qiagen, Hilden, Germany) according to standard protocols. The identity of the plasmids was confirmed by *Not*I-digestion, gel electrophoresis and sequencing using the Big Dye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, Carlsbad, USA). Linearized and purified plasmid DNA was in vitro transcribed with the RiboMAX Large Scale RNA Production Systems (Promega, Mannheim, Germany), and a DNase I digestion was performed subsequently using the SP6/T7 Transcription Kit (Roche, Mannheim, Germany) according to the manufacturer's instructions. During purification of the in vitro transcribed RNA using the RNeasy Mini Kit (Qiagen, Hilden, Germany), a second on-column DNase I digestion according to the manufacturer's recommendations was implemented. The exact number of RNA molecules was calculated as described (Hoffmann et al., 2005), and a $\log_{10}$ dilution series (2×10^6 to 2×10^{-1} copies per μl) was prepared in RNA-safe buffer (RSB; Hoffmann et al., 2006). To investigate matrix effects, a 20% (w/v) suspension of negative wolf brain was prepared, and RNA was extracted in two independent runs using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The artificial positive control was spiked in the extracted matrix as final $\log_{10}$ dilutions of 2×10^6 to 2×10^{-1} copies per μl. RNA was stored until use at −20 °C.

2.3. Validation

Analytical sensitivity was determined for the pan-lyssavirus RT-qPCR systems by using a defined positive spiking control (Tables 2A and 2B). Therefore, full genome RABV (sample ID 5989) was spiked in 20% (w/v) negative wolf brain suspension

Table 1
Oligonucleotide sequences and composition of primer–probe mixes used in this study.

PCR assay	Primer/Probe	Sequence (5′–3′)	Position	Concentration [μM][b]	Reference[c]
Pan-lyssavirus					
N-gene PCR	JW12	ATG TAA CAC CYC TAC AAT G	55–73[d]	20	Hayman et al. (2011)
	N165-146	GCA GGG TAY TTR TAC TCA TA	165–146[d]	20	
L-gene PCR	Pan-Lyssa-7531F	TTC TTC GCT YTR ATG TCW TGG AA	7074–7096[d]	20	This study
	Pan-Lyssa-7749R	ATG RTT GTT CCA CTT YTC ATA RTC	7292–7269[d]	20	
Lyssavirus-specific					
R13 MP	JW12	ATG TAA CAC CYC TAC AAT G	55–73[d]	10	Wakeley et al. (2005) mod.
	N165-146	GCA GGG TAY TTR TAC TCA TA	165–146[d]	10	
	LysGT1-FAM	FAM-ACA AGA TTG TAT TCA AAG TCA ATA ATC AG-BHQ1	81–109[d]	2.5	
	LysGT5-HEX	HEX-AA CAR GGT TGT TTT YAA GGT CCA TAA-BHQ1	80–105[d]	2.5	
	LysGT6-Cy5	Cy5-ACA RAA TTG TCT TCA ARG TCC ATA ATC AG-BHQ3	81–109[d]	2.5	
	BBLV-1TEX	TEX-CTC TGA CAA GAT TGT CTT CAA AGT C-BHQ2	76–101[d]	2.5	
R14 MP	RV-N-196-F	GAT CCT GAT GAY GTA TGT TCC TA	266–288[d]	10	Hoffmann et al. (2010)
	RV-N-283-R	RGA TTC CGT AGC TRG TCC A	353–335[d]	10	
	RabGT1-B-FAM	FAM-CAG CAA TGC AGT TYT TTG AGG GGA C-BHQ1	297–321[d]	2.5	
R14 MP	EBLV1-353F	GCT CAA ACR GGA GGT CAA GA	431–450[e]	10	this study
	EBLV1-440R	AGA CAR AGA AGA AGT CCW ACC A	510–489[e]	10	
	EBLV1-392HEX	HEX-ACC CTA CRA CAC CTG AAC ATG CAT CT-BHQ1	462–487[e]	2.5	
R14 MP	EBLV2-42F	RGT GTC TGT AAA RCC AGA AG	112–131[f]	15	Schatz et al. (2013) mod.
	EBLV2-173R	GAC AGA ATR GAC TTA TAA GCT CT	243–221[f]	15	
	EBLV2N Probe	Cy5-TCG GAA AAA ACC CAG CAT AAC CCT-BHQ2	175–198[f]	2.5	
R14 MP	BBLV-2F	CCT TGG TRA ACA TTC AGA GAA CG	390–412[g]	10	Freuling et al. (2013) mod.
	BBLV-2R	GGC CAC AGT TGG ATC CCT TG	475–456[g]	10	
	BBLV-2TEX_as	TEX-TCC TCC GGT CAA GGC CCA RTT GCC-BHQ2	422–445[g]	2.5	
Internal controls[a]					
IC_h	EGFP1-F	GAC CAC TAC CAG CAG AAC AC	637–656[h]	2.5	Hoffmann et al. (2006)
	EGFP2-R	GAA CTC CAG CAG GAC CAT G	768–750[h]	2.5	
	EGFP-Probe 1	FAM-AGC ACC CAG TCC GCC CTG AGC A-BHQ1	703–724[h]	1.25	
IC_e	ACT-1005-F	CAG CAC AAT GAA GAT CAA GAT CAT C	1005–1029[i]	2.5	Toussaint et al. (2007)
	ACT-1135-R	CGG ACT CAT CGT ACT CCT GCT T	1135–1114[i]	2.5	
	ACT-1081-HEX	HEX-TCG CTG TCC ACC TTC CAG CAG ATG T-BHQ1	1081–1105[i]	1.25	

[a] IC_h: heterologous internal control, IC_e: endogenous internal control.
[b] Applied concentration for the reaction.
[c] mod.: original assay was modified for this study.
[d] Reference Pasteur virus (M13215).
[e] Reference EBLV-1 (EF157976).
[f] Reference EBLV-2 (EF157977).
[g] Reference BBLV (JF311903).
[h] Reference cloning vector pEGFP-1 (U55761).
[i] Reference beta actin sequences (AY141970, U39357).

as final $\log_{10}$ dilutions of $1 \times 10^{4.45}$ to $1 \times 10^{-3.45}$ 50% tissue culture infectious doses per ml ($TCID_{50}\,ml^{-1}$). RNA was extracted in two independent runs per sample (biological duplicates), eluted in 100 μl RNase free water and stored until use at −20 °C. Biological duplicates were tested by each pan-lyssavirus RT-qPCR system in three independent runs. The limit of detection (LOD) was defined as the virus titer, giving a positive RT-qPCR result in 95% of the repeats (Vandemeulebroucke et al., 2010). Coefficient of determination (R^2) values were calculated by the CFX manager software 3.0 (Bio-Rad Laboratories, Hercules, USA). The coefficient of variation (CV) was calculated according to Vandemeulebroucke et al. (2010) [CV = standard deviation/mean C_q value of all replicates]. Furthermore, for the pan-lyssavirus RT-qPCR systems, the positive spiking control was compared to the artificial positive control spiked in extracted negative brain matrix or diluted in RSB (Supplementary Table 3).

Moreover, assay efficiencies of the pan-lyssavirus RT-qPCR systems were determined for RABV, EBLV-1, EBLV-2

Table 2A
Evaluation of the analytical sensitivity of the positive spiking control (RABV 5989) regarding the pan-Lyssa N-gene RT-qPCR.

Titer[a]	Mean (n = 12)[b]	SD[c]	CV (%)[d]
$1 \times 10^{4.45}$	19.5	0.23	1.20
$1 \times 10^{3.45}$	22.8	0.26	1.16
$1 \times 10^{2.45}$	26.2	0.14	0.55
$1 \times 10^{1.45}$	29.8	0.30	1.01
$1 \times 10^{0.45}$	33.1	0.27	0.82
$1 \times 10^{-1.45}$	36.7	0.52	1.41
$1 \times 10^{-2.45}$	no C_q	–	–
$1 \times 10^{-3.45}$	no C_q	–	–

[a] Titer of positive spiking control in $TCID_{50}\,ml^{-1}$.
[b] Mean value of 12 replicates (biological and technical replicates of three runs), no C_q: no C_q-value detected.
[c] SD: standard deviation.
[d] CV: coefficient of variation calculated according to Vandemeulebroucke et al., 2010; –: no value available.

Table 2B
Evaluation of the analytical sensitivity of the positive spiking control (RABV 5989) regarding the pan-Lyssa L-gene RT-qPCR.

Titer[a]	Mean (n = 12)[b]	SD[c]	CV (%)[d]
$1 \times 10^{4.45}$	22.6	0.24	1.06
$1 \times 10^{3.45}$	26.4	0.25	0.95
$1 \times 10^{2.45}$	29.6	0.25	0.85
$1 \times 10^{1.45}$	34.3	0.22	0.65
$1 \times 10^{0.45}$	37.5	0.28	0.74
$1 \times 10^{-1.45}$	40.3	0.33	0.82
$1 \times 10^{-2.45}$	no C_q	–	–
$1 \times 10^{-3.45}$	no C_q	–	–

[a] Titer of positive spiking control in $TCID_{50}\,ml^{-1}$.
[b] Mean value of 12 replicates (biological and technical replicates of three runs), no C_q: no C_q-value detected.
[c] SD: standard deviation.
[d] CV: coefficient of variation calculated according to Vandemeulebroucke et al., 2010; –: no value available.

Table 3A
Double-check analysis of viral genome detection via pan-lyssavirus RT-qPCRs.

Sample ID[a]	Virus species	Material[b]	Host[c,d]	Year of isolation[c]	Origin	pan-Lyssa N-gene PCR[e]	pan-Lyssa L-gene PCR[e]	IC_h[f]	IC_e[g]
122	RABV	BS	fox	2005	Romania	28.9	29.6	25.6	38.1
132	RABV	BS	domestic cat	2005	Romania	25.9	27.1	25.7	39.0
1390	RABV	TCS	fox	1998	Germany	35.0	40.0	26.2	40.2
5989	RABV	BS	dog	2002	Azerbaijan	26.3	27.5	25.8	36.7
11164	RABV	BS	fox	2005	Germany	25.6	26.4	25.5	39.9
11240	RABV	BS	human	2005	Germany	29.3	31.7	25.7	38.9
11317	RABV	BS	raccoon dog	1990	Estonia	31.9	33.0	25.8	37.0
11318	RABV	BS	fox	1981	Norway	25.1	29.9	25.8	38.5
11321	RABV	BS	fox	1995	Bulgaria	28.8	30.7	25.9	37.1
11322	RABV	BS	dog	1979	Pakistan	25.3	25.9	25.9	35.1
11329	RABV	BS	dog	1988	Nigeria	23.1	26.6	26.0	37.4
11332	RABV	BS	dog	1982	India	21.6	21.8	25.9	38.5
11333	RABV	BS	dog	1981	Mexico	24.5	26.3	25.7	38.1
12887	RABV	BS	raccoon dog	1986	Poland	34.9	39.3	25.8	41.8
12909	RABV	BS	fox	1993	Poland	25.5	26.1	25.7	40.2
12952	RABV	BS	fox	2001	Estonia	36.1	35.2	25.6	39.3
13001	RABV	TCS	raccoon dog	1991	Estonia	35.7	35.3	25.9	41.2
13044	RABV	TCS	fox	1990	Saudi Arabia	36.2	33.0	25.8	N/A
13056	RABV	BS	dog	1984	Turkey	28.2	30.2	25.7	36.8
13077	RABV	BS	fox	1995	Bulgaria	32.5	33.7	25.9	38.5
13078	RABV	BS	human	1995	Bulgaria	25.1	25.0	25.8	36.4
13079	RABV	BS	fox	1995	Bulgaria	33.0	36.1	25.8	39.6
13088	RABV	BS	dog	1979	Pakistan	23.8	24.7	25.8	35.1
13118	RABV	BS	dog	1983	Algeria	34.1	30.9	25.9	41.9
13160	RABV	TCS	sheep	1991	Iran	31.4	29.5	25.9	N/A
13164	RABV	TCS	hyaena	1991	Iran	39.9	35.6	25.8	N/A
13213	RABV	TCS	skunk	1981	USA	29.0	30.2	25.8	40.3
13240	RABV	BS	–	1986	Canada	32.3	28.1	25.8	38.5
FN	RABV	BS	fox	–	Romania	27.6	29.2	25.6	36.8
12865	EBLV-1	BS	bat (E. serotinus)	1968	Germany	36.3	28.8	25.6	35.3
25438	EBLV-1	OS (p.m.)	bat	1997	Germany	34.9	32.2	25.6	35.0
25768	EBLV-1	OS (bladder)	bat (E. serotinus)	2011	Germany	40.4	37.2	25.9	37.2
25969	EBLV-1	OS (s.g.)	bat (E. serotinus)	2009	Germany	23.6	21.0	25.9	38.5
25978	EBLV-1	OS (p.m.)	bat (E. serotinus)	2010	Germany	38.9	34.5	25.5	34.4
27932	EBLV-1	OS (heart)	bat (E. serotinus)	2006	Germany	35.9	29.9	25.2	36.2
27987	EBLV-1	OS (testis)	bat (E. serotinus)	2011	Germany	33.0	31.5	25.6	35.4
27988	EBLV-1	OS (tongue)	bat (E. serotinus)	2011	Germany	28.9	28.8	25.5	36.2
28113	EBLV-1	BS	bat (E. serotinus)	–	Denmark	34.2	25.6	25.6	36.4
28119	EBLV-1	TCS	–	1993	Denmark	27.4	18.4	25.7	39.0
28120	EBLV-1	TCS	–	1994	Denmark	34.4	28.2	25.8	41.6
13034	EBLV-1	BS	bat (E. serotinus)	1987	The Netherlands	34.8	32.3	25.8	35.6
13037	EBLV-1	BS	bat (E. serotinus)	1987	The Netherlands	27.0	25.6	25.8	37.6
12950	EBLV-2	BS	human	1985	Finland	30.0	37.6	25.6	38.9
25538	EBLV-2	TCS	bat (M. daubentonii)	2006	Germany	35.3	40.8	25.8	41.6
13035	EBLV-2	BS	bat (E. serotinus)	1987	The Netherlands	27.1	33.3	25.6	36.5
13036	EBLV-2	BS	bat (M. dasycneme)	1987	The Netherlands	24.3	32.8	25.5	34.3
BH 51/10	BBLV	BS	bat (M. nattereri)	2010	Germany	28.9	26.5	25.9	33.5
29029	BBLV	TCS	bat (M. nattereri)	2012	Germany	41.0	31.1	25.8	34.3
GH235	LBV	BS	bat (E. helvum)	2012	Ghana	36.5	28.2	25.6	34.6
12858	MOKV	TCS	dog	1992	Ethiopia	41.3	29.6	25.6	39.6
12869	DUVV	TCS	–	1984	Germany	31.8	30.1	25.7	N/A
13849	ABLV	TCS	bat		Australia	31.9	36.5	25.6	40.5
BH 62/13-28	neg.	BS	fox	2011	Germany	N/A	N/A	25.4	32.3
BH 62/13-29	neg.	BS	badger	2009	Germany	N/A	N/A	25.5	32.4
BH 62/13-53	neg.	BS	polecat (fitch)	2009	Germany	N/A	N/A	25.6	30.5
BH 62/13-54	neg.	BS	raccoon dog	2010	Germany	N/A	N/A	25.7	33.0
BH 62/13-113	neg.	BS	mink	2010	Germany	N/A	N/A	25.8	31.5
BH 62/13-114	neg.	BS	pine marten	2010	Germany	N/A	N/A	25.6	32.3
BH 62/13-144	neg.	BS	stone marten	2011	Germany	N/A	N/A	25.4	30.3
BH 23/13-8	neg.	serum	raccoon	2012	Germany	N/A	N/A	26.1	31.9
25371	neg.	OS (tongue)	bat (P. auritus)	2011	Germany	N/A	N/A	25.8	37.5
25372	neg.	OS (heart)	bat (P. auritus)	2011	Germany	N/A	N/A	25.7	35.3
GH233	neg.	BS	bat (E. helvum)	2012	Ghana	N/A	N/A	25.8	34.6

[a] Standard face 1:1000 dilution, bold face 1:100, italics 1:5 dilution of original RNA.
[b] BS: brain sample, TCS: tissue culture supernatant, OS: organ suspension, p.m.: pectoral muscle, s.g.: salivary gland.
[c] –: no information available.
[d] *E. helvum*: *Eidolon helvum*; *E. serotinus*: *Eptesicus serotinus*; *M. dasycneme*: *Myotis dasycneme*; *M. daubentonii*: *Myotis daubentonii*; *M. nattereri*: *Myotis nattereri*; *P. auritus*: *Plecotus auritus*.
[e] N/A: no C_q-value available.
[f] IC_h: heterologous internal control.
[g] IC_e: endogenous internal control; columns 7–10 represent mean C_q-values from duplicates.

Table 3B
Virus characterization via double-check multiplex RT-qPCR systems.

Sample ID[a]	Virus species[b]	R13 MP[c]				R14 MP[c]			
		RABV	EBLV-1	EBLV-2	BBLV	RABV	EBLV-1	EBLV-2	BBLV
122	RABV	26.6	N/A	N/A	N/A	26.6	N/A	N/A	N/A
132	RABV	24.1	N/A	N/A	N/A	24.7	N/A	N/A	N/A
1390	RABV	32.3	N/A	N/A	N/A	31.8	N/A	N/A	N/A
5989	RABV	25.7	N/A	N/A	N/A	25.9	N/A	N/A	N/A
11164	RABV	25.6	N/A	N/A	N/A	23.3	N/A	N/A	N/A
11240	RABV	27.9	N/A	N/A	N/A	30.4	N/A	N/A	N/A
11317	RABV	30.5	N/A	N/A	N/A	30.9	N/A	N/A	N/A
11318	RABV	23.9	N/A	N/A	N/A	28.0	N/A	N/A	N/A
11321	RABV	27.5	N/A	N/A	N/A	27.7	N/A	N/A	N/A
11322	RABV	24.2	N/A	N/A	N/A	29.6	N/A	N/A	N/A
11329	RABV	20.9	N/A	N/A	N/A	N/A	N/A	N/A	N/A
11332	RABV	20.7	N/A	N/A	N/A	26.1	N/A	N/A	N/A
11333	RABV	22.8	N/A	N/A	N/A	23.7	N/A	N/A	N/A
12887	RABV	33.1	N/A	N/A	N/A	34.6	N/A	N/A	N/A
12909	RABV	25.7	N/A	N/A	N/A	29.2	N/A	N/A	N/A
12952	RABV	34.1	N/A	N/A	N/A	33.6	N/A	N/A	N/A
13001	RABV	33.7	N/A	N/A	N/A	33.8	N/A	N/A	N/A
13044	RABV	31.3	N/A	N/A	N/A	31.4	N/A	N/A	N/A
13056	RABV	26.2	N/A	N/A	N/A	28.3	N/A	N/A	N/A
13077	RABV	30.3	N/A	N/A	N/A	30.1	N/A	N/A	N/A
13078	RABV	24.0	N/A	N/A	N/A	23.8	N/A	N/A	N/A
13079	RABV	31.7	N/A	N/A	N/A	31.3	N/A	N/A	N/A
13088	RABV	22.9	N/A	N/A	N/A	28.2	N/A	N/A	N/A
13118	RABV	30.8	N/A	N/A	N/A	29.4	N/A	N/A	N/A
13160	RABV	28.9	N/A	N/A	N/A	28.0	N/A	N/A	N/A
13164	RABV	36.2	N/A	N/A	N/A	37.8	N/A	N/A	N/A
13213	RABV	26.5	N/A	N/A	N/A	26.3	N/A	N/A	N/A
13240	RABV	N/A	N/A	N/A	N/A	30.1	N/A	N/A	N/A
FN	RABV	26.1	N/A	N/A	N/A	26.4	N/A	N/A	N/A
12865	EBLV-1	N/A	30.2	N/A	N/A	N/A	28.2	N/A	N/A
25438	EBLV-1	N/A	30.6	N/A	N/A	N/A	29.9	N/A	N/A
25768	EBLV-1	N/A	34.4	N/A	N/A	N/A	33.8	N/A	N/A
25969	EBLV-1	N/A	20.7	N/A	N/A	N/A	20.3	N/A	N/A
25978	EBLV-1	N/A	33.3	N/A	N/A	N/A	32.2	N/A	N/A
27932	EBLV-1	N/A	28.7	N/A	N/A	N/A	27.3	N/A	N/A
27987	EBLV-1	N/A	30.2	N/A	N/A	N/A	28.5	N/A	N/A
27988	EBLV-1	N/A	27.0	N/A	N/A	N/A	25.8	N/A	N/A
28113	EBLV-1	N/A	26.7	N/A	N/A	N/A	22.6	N/A	N/A
28119	EBLV-1	N/A	21.3	N/A	N/A	N/A	16.2	N/A	N/A
28120	EBLV-1	N/A	27.8	N/A	N/A	N/A	23.6	N/A	N/A
13034	EBLV-1	N/A	30.2	N/A	N/A	N/A	29.2	N/A	N/A
13037	EBLV-1	N/A	23.8	N/A	N/A	N/A	23.4	N/A	N/A
12950	EBLV-2	N/A	N/A	28.4	N/A	N/A	N/A	28.7	N/A
25538	EBLV-2	N/A	N/A	34.0	N/A	N/A	N/A	33.4	N/A
13035	EBLV-2	N/A	N/A	25.9	N/A	N/A	N/A	26.1	N/A
13036	EBLV-2	N/A	N/A	22.8	N/A	N/A	N/A	23.5	N/A
BH 51/10	BBLV	27.4[d]	N/A	27.4[d]	26.7	N/A	N/A	N/A	23.0
29029	BBLV	34.6[d]	N/A	34.5[d]	33.8	N/A	N/A	N/A	27.2
GH235	LBV	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
12858	MOKV	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
12869	DUVV	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
13849	ABLV	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
BH 62/13-28	neg.	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
BH 62/13-29	neg.	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
BH 62/13-53	neg.	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
BH 62/13-54	neg.	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
BH 62/13-113	neg.	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
BH 62/13-114	neg.	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
BH 62/13-144	neg.	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
BH 23/13-8	neg.	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
25371	neg.	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
25372	neg.	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
GH233	neg.	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A

[a] Standard face 1:1000 dilution, bold face 1:100, italics 1:5 dilution of original RNA.
[b] neg.: lyssavirus negative material.
[c] N/A: no C_q-value available.
[d] Cross-reactivity for BBLV samples; columns 3–10 represent mean C_q-values from duplicates.

and BBLV. Therefore, the positive spiking control ($1 \times 10^{3.45}$ to $1 \times 10^{-4.45}$ $TCID_{50}\,ml^{-1}$), EBLV-1 ($1 \times 10^{3.75}$ to $1 \times 10^{-4.75}$ $TCID_{50}\,ml^{-1}$) and EBLV-2 ($1 \times 10^{3.17}$ to $1 \times 10^{-4.17}$ $TCID_{50}\,ml^{-1}$) samples extracted from brain suspension spiked with full genome virus as well as BBLV RNA (1×10^{-2} to 1×10^{-8}) diluted in RSB were analyzed (Supplementary Fig. 2). The respective efficiencies were calculated according to Vandemeulebroucke et al. (2010) [PCR efficiency (%) = $100 \times (10^{1/\text{slope}} - 1)$], where the slope was

Table 4
Results of the inter-laboratory validation.

Sample ID (lab[a])	Virus species	Material[b]	Host	Year of isolation	Origin[c]	Dilution[d]	pan-Lyssa N-gene PCR	pan-Lyssa L-gene PCR	IC_h[e]	IC_e[f]	R13 MP[g]				R14 MP[g]			
											RABV	EBLV-1	EBLV-2	BBLV	RABV	EBLV-1	EBLV-2	BBLV
08017359 (CVI)	EBLV-1	BS	bat	2008	NL	undil.	17.8	13.2	25.0	32.1	N/A	17.8	N/A	N/A	N/A	14.6	N/A	N/A
11014118 (CVI)	EBLV-1	BS	bat	2011	NL	undil.	17.6	14.2	25.0	28.5	N/A	16.7	N/A	N/A	N/A	16.0	N/A	N/A
11014798 (CVI)	EBLV-1	BS	bat	2011	NL	undil.	18.2	13.5	24.7	29.5	N/A	17.0	N/A	N/A	N/A	15.0	N/A	N/A
11014831 (CVI)	EBLV-1	BS	bat	2011	NL	undil.	15.0	13.1	25.2	27.0	N/A	13.6	N/A	N/A	N/A	14.5	N/A	N/A
12009493 (CVI)	EBLV-1	BS	bat	2012	NL	undil.	17.7	14.9	24.6	28.2	N/A	16.7	N/A	N/A	N/A	15.5	N/A	N/A
12014060 (CVI)	EBLV-1	BS	bat	2012	NL	undil.	15.5	11.5	24.7	27.2	N/A	14.9	N/A	N/A	N/A	12.3	N/A	N/A
12014718 (CVI)	EBLV-1	BS	bat	2012	NL	undil.	15.7	11.9	24.6	27.6	N/A	15.2	N/A	N/A	N/A	13.4	N/A	N/A
12019333 (CVI)	EBLV-1	BS	bat	2012	NL	undil.	17.9	13.3	24.9	29.3	N/A	19.5	N/A	N/A	N/A	15.2	N/A	N/A
PC 2×10^4	–	IVT	–	–	–	2×10^4	21.5	24.1	25.0	–	22.6	21.7	21.9	21.4	21.3	21.2	20.6	21.8
PC 2×10^3	–	IVT	–	–	–	2×10^3	24.3	27.4	25.6	–	25.4	24.2	24.6	24.0	24.1	24.4	23.4	24.6
PC 2×10^2	–	IVT	–	–	–	2×10^2	28.2	31.0	24.9	–	28.8	27.6	28.2	27.8	27.6	27.6	26.5	27.9
Gra 7/10 (DTU)	RABV	BS	polar fox[h]	2010	GL	undil.	11.1	17.4	24.6	19.3	18.8	N/A	N/A	N/A	14.6	N/A	N/A	N/A
Gra 1/13 (DTU)	RABV	BS	polar fox[h]	2013	GL	undil.	13.8	15.7	25.1	19.0	19.1	N/A	N/A	N/A	19.2	N/A	N/A	N/A
Gra 2/13 (DTU)	RABV	BS	polar fox[h]	2013	GL	undil.	12.5	15.3	25.1	18.8	17.0	N/A	N/A	N/A	17.5	N/A	N/A	N/A
Gra 3/13 (DTU)	RABV	BS	polar fox[h]	2013	GL	undil.	13.4	14.7	24.8	20.8	17.4	N/A	N/A	N/A	18.1	N/A	N/A	N/A
Ra 8/06 (DTU)	EBLV-1	BS	bat	2006	DK	1:100	23.4	23.2	24.8	29.3	N/A	23.9	N/A	N/A	N/A	22.6	N/A	N/A
Ra 8/06 (DTU)	EBLV-1	BS	bat	2006	DK	1:1000	27.3	27.6	25.2	32.8	N/A	27.9	N/A	N/A	N/A	26.0	N/A	N/A
Ra 32/03 (DTU)	EBLV-1	BS	bat	2003	DK	undil.	26.9	22.9	24.6	31.1	N/A	28.5	N/A	N/A	N/A	26.3	N/A	N/A
Ra 34/03 (DTU)	EBLV-1	BS	bat	2003	DK	undil.	28.1	23.7	24.8	31.0	N/A	28.6	N/A	N/A	N/A	26.7	N/A	N/A
Ra 5/09 (DTU)	EBLV-1	BS	bat	2009	DK	undil.	11.8	11.2	24.8	23.3	N/A	13.7	N/A	N/A	N/A	13.1	N/A	N/A
PC 2×10^4	–	IVT	–	–	–	2×10^4	21.5	24.4	25.1	–	23.7	24.5	21.9	22.7	22.1	23.7	20.9	23.5
PC 2×10^3	–	IVT	–	–	–	2×10^3	24.5	27.9	24.8	–	27.0	27.5	25.2	25.9	25.2	26.4	23.7	26.3
PC 2×10^2	–	IVT	–	–	–	2×10^2	27.7	31.2	25.0	–	29.4	29.6	27.7	28.2	28.0	29.1	26.2	28.5

[a] CVI: Central Veterinary Institute of Wageningen UR, DTU: Technical University of Denmark – National Veterinary Institute, PC: artificial positive control.
[b] BS: brain sample, IVT: in vitro transcript.
[c] NL: The Netherlands, GL: Greenland, DK: Denmark.
[d] undil.: undiluted, dilution of PC given as copies per μl.
[e] IC_h: heterologous internal control.
[f] IC_e: endogenous internal control.
[g] N/A: no C_q-value available; –: no information available; columns 8–19 represent mean C_q-values from duplicates.
[h] Polar fox (*Vulpes lagopus*).

evaluated by the CFX manager software 3.0. Additionally, the intra-assay repeatability of all applied systems was determined using four replicates of the $\log_{10}$ dilution series of the artificial positive control (2×10^6 to 2×10^{-1} copies per μl). The inter-assay repeatability was assessed for the same dilution series on four separate days. Standard deviations (SD) and box plots were generated by R software (R Foundation for Statistical Computing, 2011).

For determination of diagnostic specificity, a reference panel of 52 selected lyssavirus positive samples, consisting of 29 RABV strains from different countries and different host species as well as LBV, MOKV, DUVV, ABLV, BBLV, EBLV-1 and EBLV-2 samples (Table 3A), was used. Furthermore, specificity evaluation was performed with lyssavirus negative bat and carnivore sample materials as well as cell culture material of related rhabdoviruses, i.e., Vesicular stomatitis Indiana virus (VSV Ind), Vesicular stomatitis New Jersey virus (VSV NJ), Bovine ephemeral fever virus (BEFV) and Viral hemorrhagic septicemia virus (VHSV) (Supplementary Table 4). According to sample quantity, RNA was extracted manually using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions or was extracted using the King Fisher 96 Flex (Thermo Scientific, Braunschweig, Germany) in combination with the MagAttract Virus Mini M48 Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. RNA was eluted in 100 μl RNase free water and stored until use at −20 °C. Dilutions of the original RNA were performed in RSB, ensuring stability of the nucleic acid (Fischer et al., 2013; Table 3B).

Finally, an inter-laboratory validation was performed in two additional cooperating laboratories, located in the Netherlands and in Denmark, using samples from their respective archives and $\log_{10}$ dilutions (2×10^4 to 2×10^2 copies per μl) of the synthetic positive control (Table 4).

3. Results

In the first step of the diagnostic cascade, both applied pan-lyssavirus RT-qPCR systems were able to recognize all lyssavirus positive samples (Table 3A; Supplementary Fig. 3). However, differences in the sensitivity were recognized between the two systems. Notably, the pan-Lyssa N-gene RT-qPCR displayed a superior sensitivity for the RABV samples "1390" from Germany and "11318" from Norway as well as all tested EBLV-2 and ABLV isolates. Whereas, for RABV samples from the Middle East countries Saudi Arabia ("13044") and Iran ("13160", "13164") as well as from Canada ("13240"), the pan-Lyssa L-gene RT-qPCR seemed to be more sensitive. Furthermore, the pan-Lyssa L-gene RT-qPCR demonstrated a considerably increased sensitivity for all analyzed EBLV-1, BBLV, MOKV and DUVV samples in contrast to the pan-Lyssa N-gene RT-qPCR. Both pan-Lyssa RT-qPCR systems scored negative for all of the negative material in the validation panel (Supplementary Fig. 3). Furthermore, rhabdovirus isolates of VSV NJ, VSV Ind, BEFV and VHSV were analyzed for specificity testing using both pan-lyssavirus RT-qPCRs. The pan-Lyssa N-gene RT-qPCR scored negative for all tested isolates. The pan-Lyssa L-gene RT-qPCR displayed a positive result solely for the VSV NJ isolate (Supplementary Table 4).

The application of the heterologous internal control (IC_h, Table 3A) according to Hoffmann et al. (2006) demonstrated the functionality of amplification during PCR. The heterologous RNA was detected with approximately 26 C_q-values, as described previously (Hoffmann et al., 2006), which indicates that there were no PCR inhibitors present. The endogenous internal control (IC_e) was not analysable for the test panel due to the pre-dilution (100–1000-fold) of the original RNA, producing artificial results. However, the endogenous internal control system was validated preliminarily for different sample matrices (e.g., brain or liver) using various undiluted negative carnivore sample materials (Table 5). Obtained data were analyzed according to matrix types. For the matrices of liver, spleen, kidney and lung, a C_q range of 23–26 was defined. Brain material yielded a C_q range of 28–30, indicating minor inhibitory effects for undiluted samples. This issue was also observed for both pan-lyssavirus RT-qPCR systems by comparing a $\log_{10}$ dilution series of the artificial positive control diluted in RSB to a dilution series of the artificial positive control spiked in negative brain matrix, yielding increased C_q-values for the positive control spiked in matrix material compared to the positive control without matrix background (Supplementary Table 3).

Table 5
Validation of the endogenous internal control system.

Different material types[a]	C_q range beta actin
Liver ($n = 327$)	23–26
Spleen ($n = 1$)	23–26
Kidney ($n = 2$)	23–26
Lung ($n = 1$)	23–26
Brain ($n = 158$)	28–30
Samples $\sum n = 489$	

[a] Sample material was obtained from 9 different species: fox (*Vulpes vulpes*, $n = 192$), badger (*Meles meles*, $n = 27$), raccoon (*Procyon lotor*, $n = 28$), raccoon dog (*Nyctereutes procyonoides*, $n = 39$), marten (*Martes*, $n = 8$), stone marten (*Martes foina*, $n = 20$), pine marten (*Martes martes*, $n = 30$), mink (*Mustela lutreola*, $n = 26$), polecat (*Mustela putorius*, $n = 2$).

In the second line, the multiplex assays R13 MP and R14 MP displayed a widely comparable sensitivity for RABV isolates, except for samples "11322" and "13088" from Pakistan, "11332" from India, "11318" from Norway and "12909" from Poland, where the R13 MP system was superior (Table 3B). In exchange, the R14 MP assay demonstrated a higher sensitivity for the recognition of EBLV-1 and BBLV. Some of the RABV-isolates were only recognized by one system (sample "11329" – R13 MP only; sample "13240" – R14 MP only). Furthermore, both systems displayed equal sensitivities for EBLV-2 detection (e.g., sample "12950": 28.4 in R13 MP and 28.7 in R14 MP; sample "13035": 25.9 in R13 MP and 26.1 in R14 MP). Additionally, differences in the BBLV differentiation capabilities became obvious as the RABV and EBLV-2 specific probes of the R13 MP assay showed cross-reactivity with both BBLV-isolates whereas the R14 MP assay displayed an optimal specificity (reaction occurred solely with the BBLV probe). Both multiplex PCR-systems scored negative for LBV, MOKV, DUVV, ABLV, and all negative control materials.

Concerning analytical sensitivity, both pan-Lyssa RT-qPCR assays displayed a detection limit of $1 \times 10^{-1.45}$ $TCID_{50}$ ml^{-1} for the positive spiking control (Tables 2A and 2B). Furthermore, standard deviations were low (0.14–0.52 for the pan-Lyssa N-gene RT-qPCR and 0.22–0.33 for the pan-Lyssa L-gene RT-qPCR), and the coefficient of variation (CV) ranged from 0.55% to 1.41% (pan-Lyssa N-gene RT-qPCR) or 0.65% to 1.06% (pan-Lyssa L-gene RT-qPCR), indicating low variation between the replicates. The coefficient of determination (R^2) and assay efficiency (E) were determined for three independent runs as 0.993 to 0.996 (R^2) and 92% to 94% (E) for the pan-Lyssa N-gene RT-qPCR as well as 0.993 to 0.998 (R^2) and 85% to 89% (E) for the pan-Lyssa L-gene RT-qPCR. Furthermore, assay efficiencies determined for EBLV-1, EBLV-2 and BBLV, ranged from 80% to 89% for the pan-Lyssa N-gene RT-qPCR and from 74% to 88% for the pan-Lyssa L-gene RT-qPCR (amplification plots see Supplementary Fig. 2).

A comparison among the positive spiking control and the artificial positive control with and without brain matrix background revealed a high concordance among C_q-values obtained from dilutions of the positive spiking control and the artificial positive control with matrix background in both pan-Lyssa RT-qPCR assays (Supplementary Table 3). The artificial positive control without matrix background yielded lower C_q-values. The overall detection

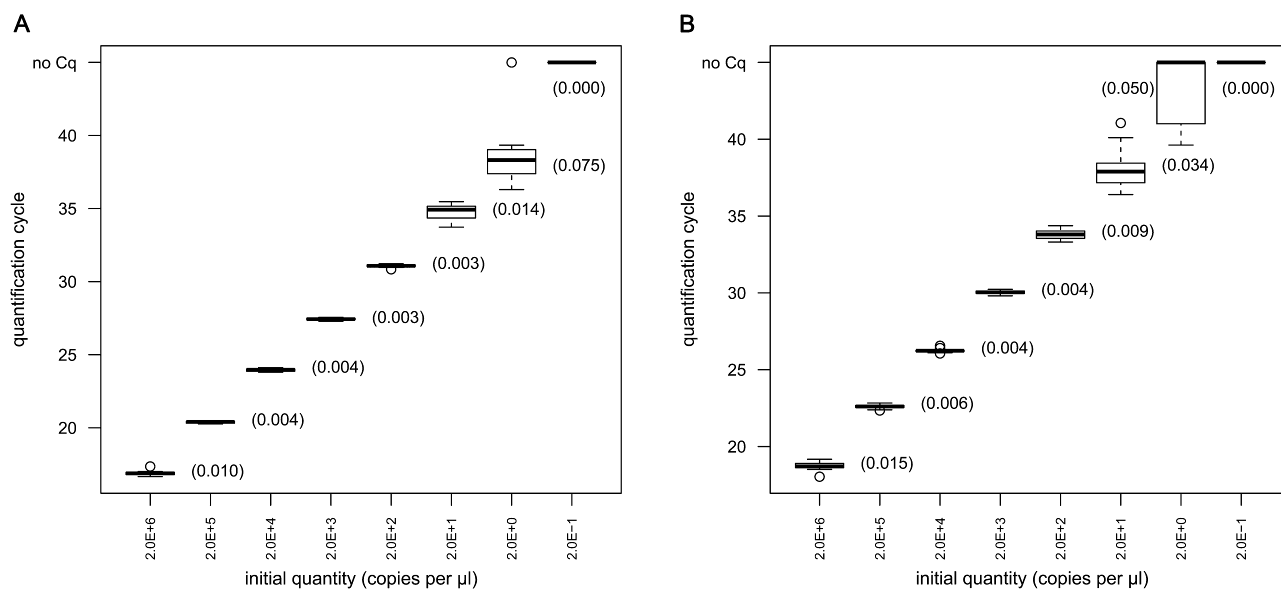

Fig. 1. Repeatability analysis of the pan-lyssavirus real-time RT-PCR assays. (A) pan-Lyssa N-gene RT-qPCR and (B) pan-Lyssa L-gene RT-qPCR were investigated using a $\log_{10}$ dilution series of the positive control. C_q-Values from 16 replicates are shown. Standard deviations are stated in parentheses. Box plots were designed supported by R software (R Foundation for Statistical Computing, 2011).

ranges however, were the same for the artificial positive control with and without brain matrix background (2×10^6 to 2×10^0 copies per µl for the pan-Lyssa N-gene RT-qPCR and 2×10^6 to 2×10^1 copies per µl for the pan-Lyssa L-gene RT-qPCR). Repeatability analysis was performed for all assays using the artificial positive control. Mean C_q-values and standard deviations for intra-assay and inter-assay repeatability are shown in Figs. 1 and 2.

In general, all applied systems displayed excellent repeatability. Regarding the multiplex assays, detection ranges from 2×10^6 to 2 copies per µl for RABV or EBLV-2 as well as 2×10^6 to 20 copies per µl for EBLV-1 or BBLV were observed (Fig. 2).

The samples applied for the inter-laboratory validation (Table 4), predominantly EBLV-1 specimens, were detected correctly by both pan-lyssavirus RT-qPCR systems. As observed before,

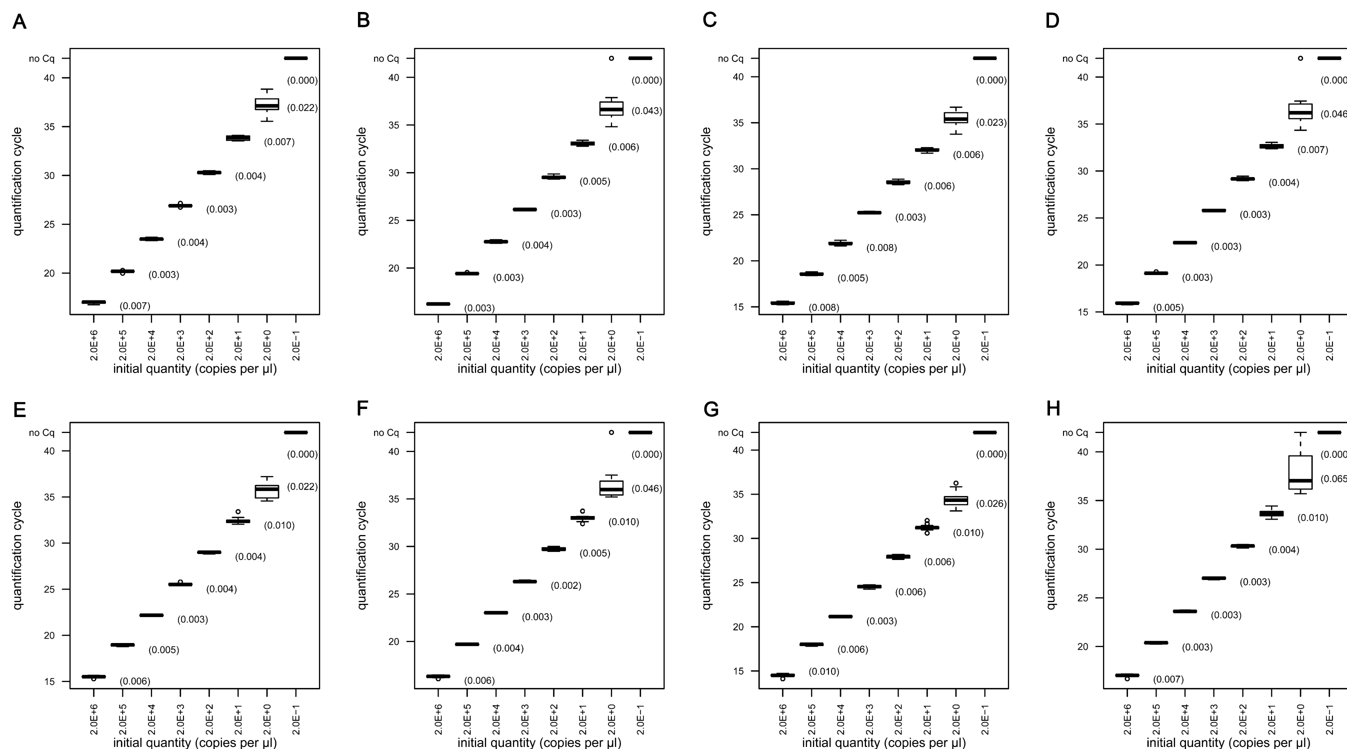

Fig. 2. Repeatability analysis of the applied species-specific multiplex real-time RT-PCR systems. (A–D) R13 MP and (E-H) R14 MP. The detection of RABV (A + E), EBLV-1 (B + F), EBLV-2 (C + G) and BBLV (D + H) was investigated using a $\log_{10}$ dilution series of the positive control. C_q-Values from 16 replicates are shown each. Standard deviations are stated in parentheses. Box plots were designed supported by R software (R Foundation for Statistical Computing, 2011).

the pan-lyssavirus L-gene RT-qPCR displayed higher sensitivity for EBLV-1 detection. Application of the endogenous and heterologous internal control systems ensured that no RNA-extraction or amplification failure occurred. Species differentiation using the R13 MP and R14 MP assays was also successful for all samples (Table 4). Furthermore, both laboratories yielded concordant C_q-values for the provided dilutions of the artificial positive control.

4. Discussion

Although numerous molecular techniques have been developed for lyssavirus detection, the diversity of this genus implies certain limitations, especially concerning their diagnostic range (Fooks et al., 2009; Coertse et al., 2010; Hoffmann et al., 2010). Therefore, regarding lyssaviruses, a continuous modification of molecular detection methods is necessary (Coertse et al., 2010). Nevertheless, no ultimate approach or strategy has been established to date, that could facilitate routine rabies diagnosis or epidemiological surveys on molecular grounds. Recent WHO research needs for molecular diagnostic methods for lyssaviruses called for more universal primers, real-time RT-PCR assays and improved protocols to cover the diversity of lyssaviruses and to improve diagnostic reliability (WHO, 2013). Therefore, we developed a novel two level cascade molecular diagnostics protocol using lyssaviruses known to circulate in Europe as a proof of principle. Our study focused on highly sensitive TaqMan real-time reverse transcription PCRs.

Initially, two independent pan-lyssavirus RT-qPCR systems were applied using a double-check strategy. The nucleoprotein (N) gene targeting pan-Lyssa N-gene RT-qPCR is based on the protocol by Hayman et al. (2011) whereas the pan-Lyssa L-gene RT-qPCR is a novel system targeting a conserved region on the polymerase (L) gene spanning the more highly conserved regions at the end and the beginning of the two major functional blocks II and III, respectively (Poch et al., 1990). Both pan-Lyssa RT-qPCRs detected all positive samples of a broad panel with partially different sensitivities (Tables 3A and 4). Interestingly, the pan-Lyssa N-gene RT-qPCR provided better performance for EBLV-2 and ABLV detection, whereas the pan-Lyssa L-gene RT-qPCR was superior for EBLV-1, BBLV, LBV, MOKV and DUVV recognition. This observation strongly supports the idea of the initial application of this double check strategy where one assay is able to balance the impairment of the corresponding assay, thereby increasing both diagnostic sensitivity and reliability. In the case of a doubtful result, melt curve analysis can be consulted to avoid a false positive result due to the formation of primer dimers. Specificity analysis revealed a positive result for a VSV NJ isolate in the pan-Lyssa L-gene RT-qPCR (Supplementary Table 4). Due to this impairment of specificity, we recommend confirmation of positive samples by sequencing. Nevertheless, during the development of this generic L-gene based assay, the focus was on coverage of an extremely broad detection range for lyssaviruses to facilitate the recognition of novel, phylogenetic distant species.

Subsequently, pan-Lyssa RT-qPCR positive samples were subjected to the testing with real-time RT-PCRs for the rapid identification of European lyssavirus species. For species-specific detection of RABV, EBLV-1, EBLV-2 and BBLV, the published multiplex system by Wakeley et al. (2005) was extended by a BBLV-specific probe (R13 MP). The basis for the R14 MP system is the published R14 assay by Hoffmann et al. (2010), which was edited with in-house primer probe sets for EBLV-1, EBLV-2 and BBLV detection (Table 1). The sensitivity of RABV and EBLV-2 recognition was widely comparable between both multiplex PCR-systems (Table 3B). However, differences were observed regarding BBLV differentiation capabilities. The R13 MP assay, using one universal primer pair, appears to be user-friendly; unfortunately, this issue also leads to a limitation of the system. The narrow location of the universal primers confines the sequence region for probe positioning, which adversely affects the specificity and averts an arbitrary expansion of the system. The novel R14 MP assay however, applies a separate primer–probe-set for the detection of each species.

All applied assays scored negative for all of the negative control materials, suggesting notably high specificity of the assays against various host species. Moreover, all four systems displayed excellent repeatability (Figs. 1 and 2). Evaluation of the analytical sensitivity using a RABV positive spiking control revealed high sensitivity for both generic pan-Lyssa RT-qPCR assays with a detection limit of $1 \times 10^{-1.45}$ $TCID_{50}$ ml^{-1} (Tables 2A and 2B). Furthermore, both generic systems displayed sufficient assay efficiencies for RABV, EBLV-1, EBLV-2 and BBLV. Additionally, good concordance, regarding C_q-values, was observed between the positive spiking control and the artificial positive control with matrix background (Supplementary Table 3).

Further advantages of this novel cascade workflow are the flexibility according to the sample materials as well as the rapidity and the reduced risk of cross-contamination due to the closed tube, non-nested format in comparison to nested or hemi-nested RT-PCR protocols (for details see Fooks et al., 2009; Coertse et al., 2010). Thus, the presented cascade workflow combines all known advantages of real-time PCR technology with the improved safety of molecular testing based on a double-check strategy for the first-level screening as well as second-level confirmation assays. The cascade protocol is adapted for the screening, detection and characterization of all lyssavirus species relevant for humans and animals in Europe. However, the described workflow could be modified easily for other parts of the world, e.g., Africa, Asia or Australia, where next to RABV, other bat associated lyssaviruses are known to exist, provided that appropriate and optimized real-time RT-PCRs for the second level cascade are available. For ARAV, KHUV, IRKV and ABLV, real-time RT-PCRs have been described (Foord et al., 2006; Hughes et al., 2006). A one-step quantitative real-time PCR assay utilizing a single primer–probe set for the detection of a diverse panel of four African lyssaviruses (RABV, MOKV, LBV, DUVV) was developed recently (Coertse et al., 2010). However, for individual African lyssaviruses (MOKV, LBV, DUVV, SHIBV, IKOV) and WCBV, species-specific assays are still missing. Under the condition of pan-lyssavirus positive and species-specific real-time RT-PCR negative results, the suggested molecular diagnostic cascade approach might even be able to detect (i) not currently isolated lyssaviruses, such as *Lleida bat lyssavirus* (LLEBV; Aréchiga Ceballos et al., 2013), (ii) lyssaviruses that do not circulate in a particular region, or (iii) unknown bat associated lyssaviruses. Ultimately, this diagnostic cascade applying a real-time RT-PCR double-check strategy represents the highest molecular diagnostic standard available as a routine discriminatory and confirmatory test for rabies diagnosis. Depending on the specific diagnostic question, e.g., bat lyssavirus screening, and depending on the availability of resources, the individually validated assays may be separately used and/or combined to obtain a molecular diagnostic strategy fit for this purpose.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jviromet.2014.03.014.

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