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Perspectives on molecular detection methods of lyssaviruses

Perspektiven auf dem Gebiet molekularer Methoden zum Nachweis von Lyssaviren

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Summary

Lyssaviruses belong to the family *Rhabdoviridae* within the order *Mononegavirales*. Their genomes consist of a negative stranded linear RNA. Although dog transmitted rabies causes the majority of the estimated 55 000 worldwide fatalities per year, bat lyssaviruses have also caused human cases. Based on a broad range of different host species, their geographical distribution and the evolutionary age lyssaviruses display a high degree of genetic variability. Thus the development of molecular diagnostics for the reliable detection and identification of members of the genus *Lyssavirus* (pan-lyssavirus assay) is a challenge, but is valuable for the fight against rabies worldwide. In this study different published PCR-systems or primers for the detection of a wide range of lyssaviruses were tested as real-time RT-PCRs with different one-step and two-step assays. Comparison of different two-step assays by varying the RT-chemistry revealed differences in sensitivity. Nevertheless, most of the tested one-step systems provided an improved performance, including a reduced assay time and a reduced risk of cross-contaminations, when compared to the optimized two-step assay. Finally, we also provide an overview of additional state-of-the-art molecular methods to detect and differentiate lyssavirus species in general.

Keywords: lyssavirus, rabies, real-time RT-PCR, pyro-sequencing, SYBRGreen, molecular diagnostics

Zusammenfassung

Lyssaviren gehören der Ordnung *Mononegavirales* innerhalb der Familie *Rhabdoviridae* an. Es handelt sich um einzelsträngige RNA-Viren mit einem linearen, negativ orientierten Genom. Obwohl die durch Hundebisse übertragene Tollwut den Großteil der etwa 55 000 weltweiten Todesfälle pro Jahr darstellt, können auch fast alle Fledermaus-Lyssaviren diese Krankheit beim Menschen hervorrufen. Basierend auf einem breiten Wirtsspektrum, ihrer geografischen Verteilung und dem evolutionären Alter besitzen Lyssaviren einen erheblichen Grad genetischer Variabilität. Daher ist die Entwicklung molekular-diagnostischer Verfahren zum verlässlichen Nachweis und zur Identifikation der Mitglieder des Genus *Lyssavirus* (pan-lyssavirus assay) eine Herausforderung, andererseits aber sehr nützlich im Kampf gegen die Tollwut und Tollwut-ähnliche Erkrankungen weltweit. In dieser Arbeit wurden unterschiedliche publizierte PCR Systeme oder Primer zum Nachweis einer großen Bandbreite von Lyssaviren als real-time RT-PCR mit verschiedenen one-step und two-step Systemen getestet. Ein Vergleich verschiedener two-step Systeme mittels Variation der RT-Chemie zeigte Unterschiede in der Sensitivität. Dennoch zeigten die meisten der getesteten one-step Methoden bessere Ergebnisse bei gleichzeitiger Verkürzung der Versuchsdauer und Reduktion des Risikos von Kreuzkontaminationen im Vergleich zum optimierten two-step System. Schließlich geben wir einen Überblick über weitere aktuelle molekulare Methoden zum Lyssavirus-Nachweis sowie zur Differenzierung der unterschiedlichen Spezies.

Schlüsselwörter: Lyssaviren, Tollwut, real-time RT-PCR, Pyro-Sequenzierung, SYBRGreen, molekulare Diagnostik

Introduction

Lyssavirus phylogeny and association with rabies

Lyssaviruses belong to the family *Rhabdoviridae* within the order *Mononegavirales*. Their genome consists of a linear, negatively sensed single stranded RNA. The *Lyssavirus* genus within the family *Rhabdoviridae* includes the virus species rabies virus (RABV), Lagos bat virus (LBV), Mokola virus (MOKV), Duvenhage virus (DUVV), European bat lyssavirus type 1 and 2 (EBLV-1, EBLV-2), Australian bat lyssavirus (ABLV), Aravan virus (ARAV), Khujand virus (KHUV), Irkut virus (IRKV), and West Caucasian bat virus (WCBV) (ICTV, 2009). Furthermore, novel and yet unassigned virus species, like the Shimoni bat virus (SHIBV) and the Bokeloh bat lyssavirus (BBLV) have recently been isolated from the lesser horseshoe bat (*Hipposideros commersoni*) and the Natterer's bat (*Myotis nattererii*), respectively (Kuzmin et al., 2010; Freuling et al., 2011). A significant genetic variability of lyssavirus genomes can be ascertained based on the broad range of viral hosts – from bats to wildlife and domestic animals and even humans – the geographical distribution all over the world and the evolutionary age (Bourhy et al., 2008; Nadin-Davis and Real, 2011).

Although dog transmitted rabies cause the majority of the estimated 55 000 fatalities per year worldwide, most bat lyssaviruses have also the potential to induce human diseases (Johnson et al., 2010). Generally, rabies is still a severe public health risk especially in developing countries, and is therefore regarded a neglected zoonotic disease (Knobel et al., 2005; World Health Organisation, 2005). In the clinical phase infected patients suffer from acute, progressive, viral encephalitis (Bourhy et al., 2008). A post-exposure prophylaxis (PEP – inactivated vaccine in combination with passive immunization) should be initiated as soon as possible, and can only be helpful before the onset of clinical symptoms like malaise, loss of appetite, fatigue, headache, fever, insomnia and depression (Harkess and Fooks, 2011).

Molecular methods

Besides immuno-based diagnostic assays, like the "gold standard" direct fluorescent antibody test (DFA) or the verification of viral replication via the rabies tissue culture infection test (RTCIT), nucleic acid-based molecular-diagnostic methods have recently been developed for rabies diagnosis. The rapidity and high sensitivity of the polymerase chain reaction (PCR, summarized in Tab. 1) facilitated new prospects of the intra vitam diagnosis being superior to conventional techniques (Crepin et al., 1998; Smith et al., 2003). Another nucleic acid-based molecular-diagnostic method are microarray-ased assays (Fooks et al., 2009; Gurrala et al., 2009). Generally, viral RNA can be extracted from any sample type, e. g. fluids (saliva, tears, urine) or even decomposed tissue samples (Gurrala et al., 2009; Dacheux et al., 2010) where RTCIT has only a very small chance to be successful.

However, intra vitam diagnostic of rabies is often hampered by intermittent shedding of virus. Although serial sampling is required, a negative test does therefore not rule out the possibility of rabies (World Health Organisation, 2005).

Polymerase chain reaction (PCR)

As a general prerequisite of lyssavirus RT-PCR-analysis, extraction of viral RNA is required, followed by a reverse transcription (RT) step to generate double stranded complementary DNA (cDNA) which is specifically amplified by PCR using pre-defined primers. Subsequent detection of the amplification product is enabled by two different well established principles: the conventional PCR using" endpoint detection" (e. g. by agarose gel), and the "real-time PCR" (qPCR) detecting amplification product formation while the reaction is in progress (Harkess and Fooks, 2011).

Conventional PCR techniques for lyssavirus genome detection (summarized in Tab. 1A) like hemi-nested PCR – the most widely employed method, or nested PCR are able to sensitively detect viral RNA, but the ability to quantify RNA loads is very limited (Coertse et al., 2010; Harkess and Fooks, 2011). Furthermore conventional PCR methods are rather time-consuming as the detection of the amplification product is an additional step and the handling of positive samples, being not contained, implements an increased risk of cross-contaminations (Belak and Thoren, 2001). Conventional two-step reverse transcriptase (RT)-PCR protocols comprise two steps: first performing the reverse transcription step and afterwards performing the PCR in another tube. If a one-step strategy is used then the RT-step and the PCR-step take place in the same tube. In general, strategies that manage amplification and detection without further handling of possibly positive samples are preferable.

TABLE 1A: *List of existing conventional PCRs* for lyssavirus detection

Literature	Detected species	Amplified region	Type of assay	Forward primer (5'-3')	Reverse primer (5'-3')
De Benedictis et al., 2011	RABV, LBV, MOKV, DUVV, EBLV-1+2, ABLV	*N gene*	pyro-sequencing	AAC ACY YCT ACA ATG GA	TCC AAT TNG CAC ACA TTT TGT G TCC ART TAG CGC ACA TYT TAT G TCC AGT TGG CRC ACA TCT TRT G
Dacheaux et al., 2008	RABV, LBV, MOKV, DUVV, EBLV-1, ABLV	*L gene*	hn RT-PCR	ATG ACA GAC AAY YTG AAC AA	1) GGT CTG ATC TRT CWG ARY AAT A 2) TGA CCA TTC CAR CAR GTN G
Vazquez-Moron et al., 2006	RABV, LBV, MOKV, DUVV, EBLV-1+2, ABLV	*N gene*	nested RT-PCR	1) AAR ATN GTR GAR CAY CAC AC 2) AAR ATG TGY GCI AAY TGG AG	1) GCR TTS GAN GAR TAA GGA GA 2) TCY TGH CCI GGC TCR AAC AT
Picard-Meyer et al., 2004	EBLV-1	*N gene*	hn RT-PCR	ATG TAA CAC CYC TAC AAT G	1) CAR TTV GCR CAC ATY TTR TG 2) GTC CCG AGT GAG ATC TTG A
Heaton et al., 1997	RABV, LBV, MOKV, DUVV, EBLV-1+2	*N gene*	hn RT-PCR	ATG TAA CAC CYC TAC AAT TG	1) CAA TTC GCA CAC ATT TTG TG 1) CAG TTG GCA CAC ATC TTG TG 1) CAG TTA GCG CAC ATC TTA TG 2) GTC ATC AAA GTG TGR TGC TC 2) GTC ATC AAT GTG TGR TGT TC 2) GTC ATT AGA GTA TGG TGT TC

Rabies virus (RABV), Lagos bat virus (LBV), Mokola virus (MOKV), Duvenhage virus (DUVV), European bat lyssavirus type 1 and 2 (EBLV-1, EBLV-2).

1) primary amplification; 2) second round amplification.

The conventional RT-PCR system developed by Heaton et al. (1997; Tab. 1A) is capable of recognizing RABV, LBV, MOKV, DUVV, EBLV-1 and -2. This RT-PCR was also able to detect the yet unassigned BBLV (Freuling et al., 2011).

In contrast to the conventional PCR techniques, the real-time quantitative PCR (qPCR) is based on PCR-product detection by fluorescence signaling. Two different systems are widely used at the moment: 1) non-specific fluorescent dyes (e. g. SYBRGreen I or ResoLight®) intercalating with double-stranded DNA, and 2) sequence specific DNA probes consisting of oligonucleotides that are labeled with a fluorescent reporter, which permits the detection only after hybridization of the probe with its complementary DNA target. The most frequently used probes are the so-called TaqMan® probes which also allow a certain degree of mismatching between target and probe. Up to four mismatches did e. g. not affect the detection efficiency for RABV (Coertse et al., 2010). However, three and even two mismatches, respectively, in the probe binding region resulted in a detection failure by another RABV real-time PCR assay (Hoffmann et al., 2010). In addition, the combination of different primer sets and different fluorophors in one assay allows a species identification even by multiplex qPCR.

In general, RT-qPCR is a quick, cheap, sensitive, specific and reliable high-throughput system to detect and identify notifiable diseases including lyssaviruses (Hoffmann et al., 2009).

The first real-time system for lyssavirus detection was developed in 2002 by Black and co-workers as a two-step system (Tab. 1B). The assay requires five different primers and eight probes for the detection of six different lyssavirus species. The target region for the probe is located within the nucleoprotein gene (Black et al., 2002). Wakeley et al. (2005) developed a one-step assay, which detects RABV, EBLV-1 and EBLV-2 using one probe for each species (Tab. 1B). Recently, Coertse et al. (2010) established a RT-qPCR assay especially for the detection of RABV and the African lyssaviruses LBV, MOKV, DUVV using a novel set of primers (Tab. 1B). Also in 2010, Hoffmann et al. published a double check strategy specific for the sensitive and reliable RABV identification by combining a newly developed and a previously described assay. With this method, 93 dif-

TABLE 1B: *List of existing real-time PCR assays for lyssavirus detection*

Literature	Detected species	Amplified region	Type of assay	Forward primer (5'-3')	Reverse primer (5'-3')	Probe sequence (5'-3')
Hayman et al., 2011	all species	*N gene*	SYBRGreen	ATG TAA CAC CYC TAC AAT G	GCA GGG TAY TTR TAC TCA TA	
Hoffmann et al., 2010	RABV	*N gene*	Taqman	GAT CCT GAT GAY GTA TGT TCC TA	RGA TTC CGT AGC TRG TCC A	FAM-CAG CAA TGC AGT TYT TTG AGG GGA C-TAMRA
Coertse et al., 2010	RABV, LBV, MOKV, DUVV	*N gene*	Taqman	CAC MGS NAA YTA YAA RAC NAA	GTR CTC CAR TTA GCR CAC AT	FAM-CAT CAC ACC TTG ATG ACA ACT CAC AA-BHQ-1
Nadin-Davis et al., 2009	RABV	*N gene*	Taqman	ATG TAA CAC CYC TAC AAT G	AAT CCA GAG GCT CAT CCT GGT	FAM-CGC GTA GAA CTG TGA CAA CAA CGC TGA-TAMRA
Nadin-Davis et al., 2009	RABV	*N gene*	Taqman	TRA TGA CAA CYC ACA ARA TGT	TCA GTC GCT AGA GGA AAA TGG	
Nadin-Davis et al., 2009	RABV	*N gene*	Taqman	AYT TCT TCC AYA ARA ACT TYG A		
Wacharapluesadee et al., 2008	RABV	*N gene*	Taqman	CTG GCA GAC GAC GGA ACC	CAT CCR ACA AAG TGR ATG AG	FAM-TGY CCY GGC TCR AAC ATY CTY CTT AT-BHQ1
Saengseesom et al., 2007	RABV	*N gene*	SYBRGreen	1) GAC ATG TCC GGA AGA CTG G	1) GTA TTG CCT CTC TAG CGG TG	
Saengseesom et al., 2007	RABV	*N gene*	SYBRGreen	2) GTA ACA CCT CTA CAA TGG ATG C	2) TCA AAT CTT TGA TGG CAG GGT A	
Nagaraj et al., 2006	RABV	*N gene*	SYBRGreen	CTA CAA TGG ATG CCG AC	CCT AGA GTT ATA CAG GGC T	
Wakeley et al., 2005	RABV	*N gene*	Taqman	ATG TAA CAC CYC TAC AAT G	GCA GGG TAY TTR TAC TCA TA	FAM-ACA AGA TTG TAT TCA AAG TCA ATA ATC AG -TAMRA
Wakeley et al., 2005	EBLV-1	*N gene*	Taqman			HEX-AAC ARG GTT GTT TTY AAG GTC CAT AA-BHQ1
Wakeley et al., 2005	EBLV-2	*N gene*	Taqman			Cy5-ACA RAA TTG TCT TCA ARG TCC ATA ATC AG-BHQ2
Black et al., 2002	RABV	*N gene*	Taqman	GAT CAR TAT GAG TAY AAA TAT CC	CAA TTC CGA CAC ATT TTG TG	FAM-CCC AAT TCC CTT CTA CAT CAG TAC GT-TAMRA
Black et al., 2002	RABV	*N gene*	Taqman		CAG TTA GCG CAC ATC TTA TG	FAM-CCC AGT TCC CTT CTA CAT CAG TAC GT-TAMRA
Black et al., 2002	RABV	*N gene*	Taqman		CAG TTG GCA CAC ATC TTG TG	FAM- CCC AAT TTC CTT CTA CAT CAG TAC GT-TAMRA
Black et al., 2002	LBV	*N gene*	Taqman			FAM-ACA GAT GGG AAG AAA CCT GGT-TAMRA
Black et al., 2002	MOKV	*N gene*	Taqman			FAM-TAG ATG GAA AGA AAC CAG GGA TAA C-TAMRA
Black et al., 2002	DUVV	*N gene*	Taqman			FAM-TGT GTG TCC CGA AGA TTG GGT T-TAMRA
Black et al., 2002	EBLV-1	*N gene*	Taqman			FAM-TTT ACG TGG ACG CAT GGT CTT GT-TAMRA
Black et al., 2002	EBLV-2	*N gene*	Taqman			FAM-AGA GCT ACG GGA TTC TCA TTG CT-TAMRA

Rabies virus (RABV), Lagos bat virus (LBV), Mokola virus (MOKV), Duvenhage virus (DUVV), European bat lyssavirus type 1 and 2 (EBLV-1, EBLV-2).

1) first round RT-PCR; 2) second round PCR.

TABLE 2: *Panel of samples used for assay comparison*

Sample no.	Lab ID	Virus	Material	Yr.	Host	Country of origin
1	11164	RABV	bs	2005	fox	Germany
2	11329	RABV	bs	1988	dog	Nigeria
3	11332	RABV	bs	1982	dog	India
4	11333	RABV	bs	1981	dog	Mexico
5	12858	MOKV	bs	1993	-	Ethiopia
6	L-23	EBLV-1	bs	1968	bat	Germany
7	L-24	EBLV-2	bs	1985	human	Finland

yr.: isolation year; bs: brain suspension; -: not applicable.

ferent Rabies virus positive samples from different host species, various isolation years, and from all over the world could be detected (Tab. 1B). Recently, a two-step SYBR®Green-based universal real-time PCR assay for the detection of lyssaviruses was described that detects not only RABV, EBLV-1 and -2 but also between five and 50 copies of LBV, MOKV, DUVV, ARAV, KHUV, IRKV, WCBV and SBV (Hayman et al., 2011) (Tab. 1B).

Interestingly, most of the published assays use the nucleoprotein (N) gene as a target sequence. However, the polymerase (L) gene bearing highly conserved regions was in some cases also used as a target region (Dacheux et al., 2010). A conventional RT-PCR assay based on the L-gene also exists for the detection of RABV, LBV, MOKV, DUVV, EBLV-1 and ABLV (Dacheux et al., 2008) (Tab. 1A).

Microarray analysis

A drawback for PCR-methods is the genetic variance of the lyssavirus genome, which could led to an impaired primer annealing. Therefore, a broader range of variability of the detection probes could be necessary which is e. g. solved by the microarray or DNA-chip technology. Furthermore, microarray systems use sets of immobilized oligonucleotide probes with a length between 60 and 70 nucleotides; this allows hybridization also in the absence of complete homology. Thus microarray analyses offer the chance of rapid and simultaneous detection and identification of defined or unclassified lyssaviruses (Fooks et al., 2009; Gurrala et al., 2009).

For the identification and characterisation of lyssavirus species Gurrala et al. (2009) used specific probes based on the N-gene. The authors developed a microarray system capable to identify seven lyssavirus species by using glass slides spotted with 624 probes. Unfortunately, because of the lower sensitivity with clinical samples and the high costs of the system it is not particularly suitable for routine diagnostic purposes at the moment.

Sequencing

Distinct identification of lyssavirus species is classically determined by Sanger-sequencing (Sanger et al., 1977). This technique is based on an enzymatic synthesis of labeled single stranded DNA molecules, which is aborted base specifically (Piotrowski, 2003). Sanger-sequencing is both time- and money consuming; however, the output is viral sequence information of up to 1200 base pairs per run allowing to identify genetic variations. The application of novel sequencing technologies, like the recently presented pyro-sequencing protocol by de Benedictis et al. (2011) and the next generation sequencing techniques (Höper et al., 2012) may enhance available sequence information even on whole genome level.

Experimental layout

Molecular diagnosis of rabies and lyssaviruses has received some attention in recent times and various protocols have been published. However, such assays only evaluated the sensitivity and specificity using a single specific PCR kit. There is evidence that the test performance of different commercially available PCR kits can influence the results obtained, but this has not yet been considered for rabies diagnosis using RT-qPCR.

Therefore, the objective of this study was to test two published primer systems for the identification of different lyssaviruses by RT-qPCR. Furthermore the performance of the selected primer combinations was analysed using several one-step and two-step RT-PCR kits.

Material and Methods

Virus strains and RNA isolation

A panel of seven lyssavirus samples, consisting of four RABV strains from different countries, different host

TABLE 3A: *Analytical sensitivity of two-step and one-step RT-PCR protocols using primer set JW12/ N165-146 as mean quantification cycle (C_q) values*

Sample no.	Lab ID	Virus	Two-step RT-PCR					One-step RT-PCR			
			RNA-to-cDNA (C_q)*	VILO™ – (C_q)	VILO™ + (C_q)	DyNAmo™ (C_q)	M-MuLV (C_q)	AgPath-ID (C_q)	QuantiTect (C_q)	SuperScript III (C_q)	IC
1	11164	RABV	31.3	30.6	30.2	33.4	34.9	23.6	23.0	28.5	26.8
2	11329	RABV	28.7	27.7	26.8	30.2	30.8	20.0	19.8	24.9	26.8
3	11332	RABV	27.2	27.3	26.2	28.1	29.3	19.2	18.0	21.7	26.6
4	11333	RABV	28.0	28.0	27.2	29.6	32.4	21.8	20.7	26.4	26.5
5	12858	MOKV	36.1	31.9	32.7	34.0	no C_q	34.5	no Cq	no C_q	26.4
6	L-23	EBLV-1	33.2	34.9	33.2	34.8	36.2	31.7	34.0	no C_q	26.6
7	L-24	EBLV-2	34.8	37.7	35.3	37.6	39.4	29.3	28.2	37.3	26.5

no C_q: C_q not available; * baseline correction necessary; IC: internal control.

RNA-to-cDNA: High Capacity RNA-to-cDNA Master Mix Kit with primer JW12.

VILO™-: SuperScript® VILO™ cDNA Synthesis Kit without primer JW12.

VILO™+: SuperScript® VILO™ cDNA Synthesis Kit with primer JW12

DyNAmo™: DyNAmo™ cDNA Synthesis Kit for qRT-PCR.

M-MuLV: Roche M-MuLV.

AgPath-ID: AgPath-ID One-Step RT-PCR Kit.

QuantiTect: QuantiTect SYBRGreen RT-PCR Kit.

SuperScript III: SuperScript III Platinum SYBRGreen One-Step qRT-PCR Kit.

TABLE 3B: *Analytical sensitivity of two-step and one-step RT-PCR protocols using the pyro-primer set as well as species specific PCRs as mean quantification cycle (C_q) values*

Sample no.	Lab ID	Virus	Two-step RT-PCR		One-step RT-PCR		
			RNA-to-cDNA (C_q)*	M-MuLV (C_q)	AgPath-ID (C_q)	QuantiTect (C_q)	species specific (C_q)
1	11164	RABV	37.5	39.7	27.7	37.7	21.2
2	11329	RABV	35.1	no C_q	22.1	32.8	19.5
3	11332	RABV	33.2	39.0	22.5	33.3	19.2
4	11333	RABV	35.5	38.6	25.9	36.5	20.4
5	12858	MOKV	39.3	37.0	38.0	no C_q	-
6	L-23	EBLV-1	38.8	37.8	36.4	no C_q	21.2
7	L-24	EBLV-2	39.5	36.5	33.1	no C_q	24.2

no C_q: C_q not available; *baseline correction necessary.

RNA-to-cDNA: High Capacity RNA-to-cDNA Master Mix Kit with primer JW12.

M-MuLV: Roche M-MuLV with primer JW12.

AgPath-ID: AgPath-ID One-Step RT-PCR Kit.

QuantiTect: QuantiTect SYBRGreen RT-PCR Kit.

species specific: sample 1–4 (Hoffmann et al., 2010), samples 6 and 7 (Hoffmann et al., unpublished).

-: no species specific PCR performed.

species as well as one MOKV, EBLV-1 and EBLV-2 sample each (Tab. 2) was used. Viral RNA was extracted from field samples (brain suspension) using the RNeasy kit (Qiagen, Germany) according to the manufacturer's instructions. After the column was washed twice with the appropriate buffer, RNA was eluted using 50 µl of RNase free water and stored at –70°C until use. All analyses were performed in duplicates and the C_q values listed in the tables represent the mean.

Two-step RT-PCR

cDNA synthesis

The extracted RNA was reverse transcribed using four different commercial systems:

The High Capacity RNA-to-cDNA Master Mix Kit (Applied Biosystems, USA) applying the included random primers with or without the JW12 primer (7.5 pmol);

SuperScript® VILO™ cDNA Synthesis Kit (Invitrogen, USA) using the included random primers with or without the JW12 primer (7.5 pmol);

DyNAmo™ cDNA Synthesis Kit for qRT-PCR with random primers (Finnzymes Oy, Finland);

1 µl reverse transcriptase M-MuLV (Roche, Germany) and 2 µl incubation buffer (Roche, Germany) in combination with 1 µl dNTP mix (20 nmol for each nucleotide; Finnzymes Oy, Finland) and 1 µl JW12 primer (7.5 pmol) filled up with RNase free water to a final reaction volume of 10 µl.

All kits were used according to the manufacturer's recommendations and in all experiments a qualified number of controls (no template controls [NTC], positive controls [PC] and internal process controls [IC]) were co-amplified.

Amplification of cDNA

For amplification of the cDNA regardless which reverse transcription protocol was performed the Power SYBR®Green Hot Start Kit (Applied Biosystems, USA) was applied using reverse transcribed DNA (1:10 diluted in nuclease-free water) as template. The PCR was performed in a 40 µl total volume consisting of 20 µl SYBR®Green Hot Start, 13 µl nuclease-free water, 1 µl JW12 primer (20 pmol/µl) and 1µl N165-146 primer (20 pmol/µl) or 1 µl RabForPyro (20 pmol/µl) and 1µl RabRevPyro-biot 1–3 (20 pmol/µl) (de Benedictis et al., 2011) respectively and 5 µl cDNA-template or RNase free water for the no template control (NTC). The pyro-primers were only tested with cDNA generated with the High Capacity RNA-to-cDNA Master Mix Kit in combination with JW12 primer and cDNA generated with Roche M-MuLV reverse transcriptase. The size of the amplified product is 110 bp for JW12 (position: 55–73; [Heaton et al., 1997]) combined with primer N165-146 (position: 146–165; [Wakeley et al., 2005]) and 603 bp for the pyro-primers RabForPyro (position: 59–75) and RabRevPyro-biot 1–3 (position: 641–662). The following thermal profile was used: PCR initial activation step at 94°C for 2 min; 40 cycles of three-step cycling consisting of denaturation at 94°C for 1 min, annealing at 54°C for 30 s, and extension at 72°C for 30 s.

One-step real-time RT-PCR

Different one step RT-PCR kits were tested: AgPath-ID™ One-Step RT-PCR Kit (Applied Biosystems, USA) in combination with ResoLight Dye (Roche, Germany), QuantiTect® SYBR®Green RT-PCR Kit (Qiagen, Germany) and SuperScript® III Platinum® SYBR®Green One-Step qRT-PCR Kit (Invitrogen, USA). All assays were optimized by using a total reaction volume of 25 µl. For one reaction with the AgPath-ID™ One-Step RT-PCR Kit (Applied Biosystems, USA) 4.5 µl RNase-free water, 12.5 µl 2x RT-PCR buffer, 1 µl 25x RT-PCR enzyme mix, 1 µl ResoLight Dye, 0.5 µl of each primer (JW12/ N162-146 or RabForPyro/ RabRevPyro-biot 1–3, 20 pmol/µl each) and 5 µl of prediluted RNA (1:100 dilution in RSB buffer; Hoffmann et al., 2006) or RNase free water for the no template control (NTC) was used. The following thermal program was applied 1 cycle of 45°C for 10 min and 95°C for 10 min, followed by 44 cycles of 95°C for 15 s, 56°C for 20 s, and 72°C for 30 s. Functionality of amplification was tested exemplarily using the AgPath-ID™ One-Step RT-PCR Kit and the heterologous internal control (IC) system according to Hoffmann et al. (2006; Tab. 3A).

For one reaction with the QuantiTect®SYBR® Green RT-PCR Kit (Qiagen, Germany) 6.25 µl RNase-free water, 12.5 µl 2x QuantiTect SYBR Green RT-PCR Master Mix, 0.25 µl QuantiTect RT-Mix, 0.5 µl of each primer (JW12/ N162-146 or RabForPyro/RabRevPyro-biot 1–3, 20 pmol/µl respectively) and 5 µl of prediluted RNA or RNase free water for the no template control (NTC) were used. The thermal program was as follows: 1 cycle of 50°C for 30 min and 95°C for 15 min, followed by 40 cycles of 94°C for 15 s, 55°C for 30 s, and 72°C for 30 s.

For one reaction applying the SuperScript® III Platinum® SYBR®Green One-Step qRT-PCR Kit (Invitrogen, CA) we used 6 µl RNase-free water, 12.5 µl 2x SYBR®Green Reaction Mix, 0.5 µl SuperScript® III RT/ Platinum® Taq Mix, 0.5 µl of each primer (JW12 and N162-146, 20 pmol/µl respectively) and 5 µl of prediluted RNA or RNase free water for the no template control (NTC). The following heating and cooling program for reverse transcription and amplification was performed: 1 cycle of 50°C for 3 min and 95°C for 5 min, followed by 40 cycles of 95°C for 15 s, 60°C for 30 s.

All reactions (two-step and one-step real-time RT-PCR) were carried out as technical duplicates in Bio-Rad 96-well PCR plates using a CFX96 quantitative PCR system (Bio-Rad Laboratories Inc., USA). For each RT-PCR, a critical threshold 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.

Results

Two-step RT-PCR

In order to optimize the two-step strategy, reverse transcription kit protocols from different suppliers (High Capacity RNA-to-cDNA Master Mix Kit, SuperScript® VILO™ cDNA Synthesis Kit, DyNAmo™ cDNA Synthesis Kit for qRT-PCR) as well as the enzyme Roche M-MuLV for cDNA synthesis were tested. The reverse transcription step is crucial for cDNA production and the subsequent success of the SYBRGreen PCR. All generated cDNAs were amplified with the Power SYBR®Green Hot Start Kit. For these assays a panel of four RABV strains from different regions or different host species, and also one MOKV, EBLV-1 and EBLV-2 sample each (Tab. 2) was tested.

In first validation tests, the High Capacity RNA-to-cDNA assay using random primers only revealed no satisfying results. The application of the specific primer JW12 for the reverse transcription step resulted in raw data, which required a manual correction (Tab. 3A, B). In summary, the two-step SYBRGreen PCRs with the primer set JW12/ N165-146 and the cDNA produced with I) the High Capacity RNA-to-cDNA Master Mix Kit in combination with JW12, II) the SuperScript® VILO™ cDNA Synthesis Kit with or without JW12, III) the DyNAmo™ cDNA Synthesis Kit for qRT-PCR with JW12 and IV) Roche M-MuLV with JW12 allowed the detection of all tested species with exception of MOKV in the case of Roche M-MuLV (Tab. 3A). The range of detected mean C_q value differences reached a maximum of 5.2 cycles for sample 4 (RABV) (~50 times less sensitive). Clearly, cDNA produced by the Roche M-MuLV enzyme scored with the highest C_q values (Tab. 3A). The SuperScript® VILO™ cDNA assay in combination with the specific primer JW12 generated the lowest C_q values in four out of seven samples (Tab. 3A), and three out of seven samples resulted in relatively low mean C_q values. Therefore, the SuperScript® VILO™ cDNA assay in combination with the specific primer JW12 represented the most sensitive assay of the two-step RT-PCR protocols tested.

Two-step SYBRGreen PCR using the pyro-primer set RabForPyro/RabRevPyro-biot 1–3 was performed with cDNA generated with the High Capacity RNA-to-cDNA assay in combination with JW12 and generated with Roche M-MuLV reverse transcriptase together with JW12. Compared to the assays using the JW12/ N165-146 primers the application of the pyro-primers resulted in a lower sensitivity in most cases (Tab. 3A, B). Thus, the High Capacity RNA-to-cDNA/pyro-primer assay recognized all seven samples with high mean C_q values. In contrast to all other two-step assays the assay using cDNA generated via Roche M-MuLV and amplified with the JW12/ N165-146 primer set failed to detect the MOKV sample. Furthermore, the assay using cDNA generated via Roche M-MuLV and amplified using pyro primers scored negative for the RABV sample from Nigeria (sample 2).

One-step RT-PCR

Regarding the three one-step RT-PCR kits (AgPath-ID™ One-Step RT-PCR Kit + ResoLight; QuantiTect® SYBR®Green RT-PCR Kit; SuperScript® III Platinum® SYBR®Green One-Step qRT-PCR Kit), which were used with the JW12/N165-146 primer set, only the AgPath-ID™ One-Step RT-PCR Kit in combination with ResoLight Dye was able to detect all seven samples. The QuantiTect® SYBR®Green RT-PCR Kit detected all isolates with exception of MOKV. Using the SuperScript® III system (Tab. 3A) this assay scored negative for MOKV as well as EBLV-1.

The one-step assays yielded in most of the validation experiments markedly lower C_q values than the optimized two-step assay (Tab. 3A). The best performance was achieved with the AgPath-ID™ One-Step RT-PCR Kit in combination with ResoLight Dye. The application of a heterologous internal control (IC) ensured the functionality of the amplification. The IC2 RNA was detected with around 26 C_q values, as described by Hoffmann et al. (2006), which indicates that there were no PCR inhibitors present.

The application of the pyro-primer set RabForPyro/ RabRevPyro-biot 1–3 in combination with the one-step RT-PCRs AgPath-ID™ One-Step RT-PCR Kit + ResoLight and QuantiTect® SYBR®Green RT-PCR Kit did not improve the sensitivity of the assays compared to the JW12/ N165-146 primer set (Tab. 3A, B). The AgPath-ID™ One-Step RT-PCR Kit tested with the pyro-primer set detected all seven samples, however, resulting in generally higher mean C_q values. The QuantiTect® SYBR®Green RT-PCR system in combination with the pyro-primer set detected only four out of seven lyssavirus samples, while MOKV as well as EBLV-1 and -2 (Tab. 3B) scored negative.

Discussion

Various protocols for molecular diagnosis of rabies and lyssaviruses have been published (Fooks et al., 2009; Dacheux et al., 2010). Usually in published assays the sensitivity and specificity was determined with a specific commercially available PCR kit. Here we present for the first time a study in which two published primer systems (Wakeley et al., 2005; de Benedictis et al., 2011) for the identification of a wide range of lyssaviruses were used for real-time RT-PCR with different one-step and two-step PCR kits using RNA from lyssavirus positive animal brain suspensions as sample materials.

Varying the RT-chemistry revealed that the SuperScript® VILO™ cDNA Synthesis Kit in combination with the Power SYBR®Green Hot Start Kit for cDNA amplification applying the JW12/N165-146 primer set showed the highest sensitivity. However, all tested one-step systems provided an improved performance compared to the optimized two-step assay. Especially the AgPath-ID™ One-Step RT-PCR Kit in combination with the ResoLight Dye delivered more positive results and at

lower C_q values indicating the amplification power of the system and its accordingly higher test sensitivity. Furthermore, the AgPath-ID™ One-Step RT-PCR assay produced similar results for all tested RABV RNAs as could be observed with highly sensitive species-specific real-time RT-PCR (Tab. 3A, B).

The pyro-sequencing method developed by de Benedictis et al. (2011; Tab. 1A) facilitates the differentiation of lyssavirus species and can be used for further classification of lyssavirus genome positive samples. The one-step AgPath-ID/ResoLight assay in combination with the pyro-primers resulted in lower C_q values compared to the two-step protocols. However, the set of pyro-primers achieved no further improvement of sensitivity neither in the two-step nor in the one-step assays (Tab. 3B). Notably, the QuantiTect® SYBR®Green RT-PCR system failed to detect three out of seven lyssavirus samples, MOKV, EBLV-1 and -2 (Tab. 3B). The reason for this observation could be a reduced capability of the kit to amplify longer PCR fragments.

All species specific lyssavirus detection assays published so far do have certain limitations. Nagaraj et al. (2006) published a SYBRGreen-based two-step assay for the detection of RABV (Tab. 1B). A one-step TaqMan PCR system developed by Coertse et al. (2010) concentrated on the detection of African lyssaviruses namely RABV, LBV, MOKV and DUVV. Wakeley et al. (2005) and Hoffmann et al. (2010) used TaqMan technology for a species specific detection, but mismatches between probe- and target sequence according to the high genome variability of lyssaviruses might impair binding of the probe followed by detection failures (Wacharapluesadee et al., 2008; Coertse et al., 2010; Nadin-Davis and Real, 2011). To avoid this difficulty the pan-lyssavirus PCR systems were performed using the non-specific dyes SYBRGreen or ResoLight to allow the detection of a preferably wide range of lyssaviruses.

Although molecular detection by polymerase chain reaction and nucleic acid sequence-based amplification techniques have the highest level of sensitivity they still require standardization and very stringent quality control (WHO, 2005). That is one of the reasons why a recommended standard protocol for rabies RT-PCR from OIE or WHO has not been published yet. In our approach reliability of results was achieved by testing seven different isolates in duplicates. Furthermore, the generation of false negative results caused by inhibition of PCR is ruled out by using a heterologous internal control developed by Hoffmann et al. (2006).

Although the recently developed universal two-step real-time PCR assay for the detection of lyssaviruses was able to detect single representatives of all lyssavirus species (Hayman et al., 2011) it remains to be proven if this could serve as a universal molecular approach for the detection of lyssaviruses as its specificity and sensitivity still needs to be confirmed on a broader panel of lyssavirus isolates. Furthermore the results of this study give hints to hypothesize that the switch to the one-step RT-PCR strategy can increase the sensitivity of the assay.

Similar to other viral diseases a cascade-type diagnostic procedure would be preferable for the identification and characterization of lyssaviruses (Hoffmann et al., 2007). Such a cascade-protocol would probably start with a pan-lyssavirus assay, followed by species specific lyssavirus approaches. With the protected status of bats, lyssavirus surveillance in this host requires highly sensitive intra vitam diagnostics. Given the wide variety of lyssaviruses in one geographic area, e. g. in Africa, and Eurasia a pan-lyssavirus real-time RT-PCR assay would be of utmost importance. In fact, when discovering the novel BBLV from a Natterer's bat, species specific real-time RT-PCRs gave no C_q values and only a pan-RT-PCR revealed the presence of a lyssavirus (Freuling et al., 2011).

In conclusion, the described systems have yet to be validated. We intend to use a representative panel of samples, comprising the widest range of lyssavirus species available. Furthermore, the development of a multiplex targeting the N-gene together with a system based on the L-gene recognition might improve the diagnostic sensitivity of lyssavirus detection.

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