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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.



## 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™ 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 ($2 \times 10^6$ to $2 \times 10^{-1}$ 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 $2 \times 10^6$ to $2 \times 10^{-1}$ 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