APPLICATION OF HEMINESTED RT-PCR TO THE DETECTION OF EBLV1 AND CLASSICAL RABIES VIRUS INFECTIONS IN BATS AND TERRESTRIAL ANIMALS

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Abstract

The paper describes application and optimisation of hNRT-PCR in the detection of the fragment of nucleoprotein gene of Lyssaviruses (genotypes 1 and 5) as a method for laboratory diagnosis of rabies in terrestrial animals and bats. The hNRT-PCR, because of its sensitivity and rapidity, may be used as a technique for rabies diagnosis. The method can be applied both in living animals as well as in the case of post mortem collected samples. It provides not only rapid detection of rabies virus but also gives the material for sequencing of the PCR products for final identification of origin of the strain and epidemiological analysis.

Key words: Lyssavirus, rabies, laboratory diagnosis, hNRT-PCR.

Rabies is a fatal viral disease of mammalian species including humans. The infection affects the central nervous system causing acute encephalomyelitis. Rabies virus belongs to the genus Lyssavirus, the member of the order Mononegavirales, Rhabdoviridae family. Currently, basing on RNA sequence, there are seven recognised genotypes of Lyssaviruses (2), but four new viruses isolated recently from bats (Aravan virus, Khujand, West Caucasian Bat virus, and Irkut virus) (8) are awaiting for classification. They are supposed to be new members of the Lyssavirus genus. In Poland, there are two genotypes out of seven recognised (13). These are classical rabies virus defined as genotype 1 and European Bat Lyssavirus type 1 (EBLV-1) defined as genotype 5. Classical rabies virus is related to terrestrial animals and the cases were diagnosed in domestic and wildlife animals (15, 1), while the EBLV-1 virus is limited to bats. Infected bats have been reported in some European countries (6, 9). So far, it was claimed that the virus transmission between bats and terrestrial animals was impossible. However, recently a few rabies cases have been reported in terrestrial animals (sheep, marten) and humans caused by bat rabies viruses.

Material and Methods

Virus isolates. The study included terrestrial (genotype 1) and bat (genotype 5) isolates coming from different regions of Poland. The bat isolates were propagated in mice to achieve more material for examination. All samples were confirmed for the presence of rabies virus by the direct fluorescence antibody test.

Virus propagation. Mice were inoculated with 10% suspension of bat brain homogenised in water for injection. Inoculation was done intracerebrally at the dose of 0.03 ml per mouse. After inoculation, the mice were observed every day. To confirm rabies, FAT was performed in any mouse that died.

The direct fluorescence antibody test. The FAT was performed on impression smears of different parts of the brain fixed for 1 h in cold acetone. The
smears were incubated for 30 min at 37°C with polyclonal fluorescein isothiocyanate-labelled rabbit anti-rabies nucleocapsid immunoglobulin G (IgG) (Bio-Rad). Next, the slides were washed twice in phosphate-buffered saline and once in distilled water, dried, and examined for characteristic fluorescence at 400x magnification under microscope (Zeiss – Axiovert 200) equipped with an UV lamp and relevant filters.

**RNA extraction.** RNA was extracted directly from the rabid animal brain or brain of mice inoculated previously with bat isolates. Brain samples were homogenised in water for injection and RNA extraction was performed by using commercial kit QIAamp Viral RNA Mini Kit (Qiagen) according to the manufacturer’s instructions. Extracted RNA was re-suspended in RNAase free water in a final volume of 50 µl and used immediately. Remaining RNA was stored frozen at -20°C for the further investigations.

**RT-PCR assay.** Reverse transcription (RT) and first PCR was performed using OneStep RT-PCR Kit, Qiagen. Two microlitres of RNA were added to the mixture containing: 3 µl of 5x OneStep RT-PCR buffer, 0.6 µl of dNTPs each at a concentration of 10 mM, 0.5 µl of RNAse inhibitor, 0.6 µl of enzyme mix, 1 µl of each primers: JW12 (5'-ATG TAA CAC CYC TAC AAT G-3'), and JW6DPL (5'-CAA TTC GCA CAC ATT TTG TG-3') at a concentration of 10 mM, and 7.3 µl of RNAase free water to a final volume of 15 µl. Amplification was performed in Personal Cycler (Biometra) using the following programme: one cycle of RT at 50°C for 30 min, followed by denaturation at 95°C for 15 min, 35 cycles with denaturation at 94°C for 30 s, annealing at 49°C for 30 s and elongation at 72°C for 1 min, and the final extension at 72°C for 10 min.

**hnRT-PCR assay.** Heminested RT-PCR was carried out with Enhanced Avian HS RT-PCR Kit, Sigma. One microlitre of 10-fold diluted RT-PCR product was added to the mixture containing: 2.6 µl of accuTaq buffer, 1.5 µl of dNTPs each at the concentration 2 mM, 1 µl of each primers JW 12 and for genotype 1: JW10P (5'- GTC CCG AGT GAG ATC TTG A – 3') or for genotype 5: Jeb1l (5'- GTC CCG AGT GAG ATC TTG A – 3') at the concentration of 10 µM, 0.25 µl of JumpStart polymerase, and 13.65 µl of PCR water to the final volume of 20 µl. The amplification was done in the same thermocycler as the RT-PCR was performed. The following programme was set: one cycle of polymerase activation 95°C for 5 min, 30 cycles with denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and elongation at 72°C for 1 min, and the final extension at 72°C for 10 min.

**Optimisation of RT-PCR and heminested RT-PCR assays.** Optimisation was performed for RT-PCR and hnRT-PCR assays. It included the selection of optimal annealing temperature for both pairs of primers as well as estimation of a proper concentration of primers and Mg$^{2+}$ ions in the reaction mixture. Optimal annealing temperature was evaluated using gradient thermocycler (Tgradient, Biometra) within 12-degree temperature gradient 44°C–56°C.

Proper primers concentration was estimated in the reaction mixture containing 1.25-50 pmol/volume of reaction mixture. Optimum Mg$^{2+}$ ions concentration was evaluated in the range 0.25–5 mM. Selection of primers and Mg$^{2+}$ ions concentrations were performed in Personal Cycler (Biometra).

**Assessment of the RT-PCR and heminested RT-PCR sensitivities.** The sensitivities of RT-PCR and heminested RT-PCR methods were evaluated by using 10–fold dilutions of CVS strain at an initial concentration of 10 000 TCID$_{50}$/mL. Subsequently, RNA isolation as well as RT-PCR and heminested RT-PCR were conducted according to the procedures described above.

**Electrophoretical analysis.** Nine microlitres of RT-PCR and heminested RT-PCR products were analysed in 2% agarose gel stained with ethidium bromide at the concentration 10µg/mL. Electrophoretic separation was performed in 1x TAE buffer for 45 min at 90V. The samples were analysed under UV light by comparison with the molecular mass marker.

**Results**

All of the samples from rabid animals, diagnosed as positive in FAT test, gave a positive signal in the heminested RT-PCR assay. Some isolates were not detected in the first amplification when the external primers were used (RT-PCR); however, the second PCR (hnRT-PCR) using internal primers revealed all of positive samples. In the first step, amplification with primers JW12 and JW6DPL produced single band of 606 bp, while the second amplification of the primary PCR products with the primer pairs JW12 – Jeb1l and JW12 – JW10P gave products of 410 bp and 586 bp, respectively (Fig. 1). To confirm the specificity of both methods, one step RT-PCR as well as the second PCR (hnRT-PCR) was performed with additional genotype 1 prototype laboratory strain CVS-11, used as a positive control.

RT-PCR and heminested RT-PCR optimisation revealed that optimal annealing temperature for RT-PCR was in the range: 44°C–52.1°C and was lower than optimal annealing temperature of heminested RT-PCR: 54.8°C-56°C. Proper concentration of primer pairs was 5–10 pmol/15 µl of RT-PCR reaction mixture and 5–50 pmol/20 µl of heminested RT-PCR reaction mixture. Significant role during PCR amplification plays Mg$^{2+}$ concentration. The optimal concentration of Mg$^{2+}$ was presented as 2.5–3 mM/volume for both RT-PCR and heminested RT-PCR reaction mixtures. Optimisation of both methods allowed for virus detection.

The sensitivity of RT-PCR assay was established at 100 TCID$_{50}$/mL. Positive signals were obtained in the range of 10 000-100 TCID$_{50}$/mL. The heminested RT-PCR assay showed 100–fold increase in the sensitivity in comparison with RT-PCR by detecting the expected fragment of 586 bp at the dilution corresponding to 1 TCID$_{50}$/mL. Positive signals during heminested RT-PCR were noticed within 10000–1 TCID$_{50}$/mL (Fig. 2).
**Fig. 1.** Results of hnRT-PCR amplifications.

Row A presents products of hnRT-PCR amplifications of genotype 5 (EBLV-1) isolates. Lines 1, 3, 5, 7, 9: products of RT-PCR, lines 2, 4, 6, 8, 10: products of hnRT-PCR, lines 11, 12: positive controls of RT-PCR and hnRT-PCR, respectively, and 13 line: negative control.

Row B presents products of hnRT-PCR amplifications of genotype 1 isolates. Lines 1, 3, 5, 7, 9: products of RT-PCR, lines 2, 4, 6, 8, 10: products of hnRT-PCR and lines 11, 12: positive and negative controls of RT-PCR, respectively.

PCR products in 2% agarose together with 100 bp DNA ladder (Invitrogen) – electrophoretic pattern.

**Fig. 2.** Assessment of sensitivities of RT-PCR (A) and hnRT-PCR (B) assays. Lanes 1 – 9 correspond to 10-fold serial dilutions of CVS virus stock containing 10000 TCID<sub>50</sub>/mL (line 1). PCR products were electrophoresed through 2% agarose together with 100 bp DNA ladder (Invitrogen).
**Discussion**

The paper describes the application of a molecular biology method - hnRT-PCR - for rabies virus diagnosis in brain samples collected from bats and terrestrial animals. In routine diagnosis of rabies, FAT and MIT are mainly used for the detection of rabies virus in the brain of suspected animals. However, a significant improvement in the rabies diagnosis has been reported recently. The molecular methods have replaced classical diagnostic techniques. They are not only rapid and specific but also sensitive. It is important, especially, when the brain samples are in a decomposed state. Very often animal brain tissues received by diagnostic laboratories for rabies diagnosis are rooted and not fitting for FAT or MIT.

The reduction of a sensitivity of FAT can also occur with bat rabies virus isolates. It is because conjugates available on the market use the antibody against proteins of rabies virus genotype 1. Thus, alternative techniques, which would detect both genotypes (1 and 5) with the same sensitivity, are necessary. Additionally, there is a need of methods, which allow for virus detection in live animals in micro-samples, such as saliva or cerebrospinal fluids. Crepin et al. (4) reported that hnRT-PCR was an excellent tool for rabies diagnosis in living animals. They have diagnosed rabies in saliva and cerebrospinal fluid in humans.

This paper, however, describes the use of hnRT-PCR method for rabies diagnosis in samples collected post mortem. In the first step (RT-PCR), using specific oligonucleotides dedicated for both genotypes, there was possibility of virus detection in samples collected from bats and terrestrial animals as well. Rabies was diagnosed in all samples recognised as positive in FAT. In the second step (hnRT-PCR) when two primer pairs specific for the first and the fifth genotypes, respectively, were used, rabies virus in each group of samples collected from bats and terrestrial animals was detected. The use of separate primer pairs for both of genotypes could give additional information about the origin of virus isolates. It could be also an indicator of virus transmission from bats to terrestrial animals including humans. Brookes et al. (3) reported that sheep following intracranial and intramuscular inoculations with EBLV-1 and EBLV-2 developed clinical infection. Fooks et al. (7) described also the human rabies transmitted by bites of the rabid bats.

The hnRT-PCR described in this paper demonstrated high sensitivity in rabies virus detection. Much higher sensitivity was obtained in the second step when nested primers were used in comparison to the first one. Our results correspond to other studies, which demonstrate higher sensitivity of hnRT-PCR compared to the sensitivity obtained in MIT (5, 10, 12).

In conclusion, hnRT-PCR because of its high sensitivity and rapidity can potentially constitute a suitable technique for rabies diagnosis. The method can be applied for living animals as well as for post mortem collected samples. It could provide not only rapid detection of viruses but also give the material for sequencing of the PCR products for final identification of origin of the strain.

**References**