COMPARISON OF REAL-TIME PCR AND HEMINESTED RT-PCR METHODS IN THE DETECTION OF RABIES VIRUS INFECTION IN BATS AND TERRESTRIAL ANIMALS

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Abstract

The aim of the paper was to compare the real-time PCR with the heminested RT-PCR method, both applied for the detection of nucleoprotein gene of rabies viruses in bats and terrestrial animals. The study involved 32 rabies virus isolates collected from bats and terrestrial animals coming from different regions of Poland. For both methods, the sensitivities related to TCID₅₀/mL of CVS virus were estimated. The comparison of the methods revealed that the TaqMan PCR was 10-fold more sensitive than the heminested RT-PCR and the detection of rabies virus by this method was possible from 0.1 TCID₅₀/mL on up. The use of the heminested RT-PCR allowed for detection of rabies virus from 1 TCID₅₀/mL on up. Next, the examination of 32 archive samples using both methods revealed 23 positive samples and nine negative samples. The agreement between the results obtained by the methods was 100%. It confirms using the real-time PCR and heminested RT-PCR in laboratory diagnosis of rabies in terrestrial animals and bats. However, the real-time PCR does not require visualisation of the amplification product in agarose gel and the results are observed during the run of the test, which makes the method more rapid than the heminested RT-PCR. Additionally, it is done in a single closed tube and does not require multiple transfer of materials like at the heminested RT-PCR, thus making the virus detection faster and minimising the opportunity for cross-contamination.

Key words: rabies, rabies virus, heminested RT-PCR, real-time PCR.

The rabies virus (RV) and the rabies-related viruses belong to the genus of *Lyssavirus*, the member of the *Mononegavirales* order, *Rhabdoviridae* family. *Lyssaviruses* have an unsegmented negative-stranded RNA genome, coding five structural proteins: N (nucleoprotein), G (glycoprotein), M1 (phosphoprotein), M2 (matrix protein), and L (polymerase). Based on the nucleotide sequences of N protein, the *Lyssavirus* genus has been divided into seven genotypes (2, 3, 6, 8): classical rabies virus (genotype 1), Lagos virus (genotype 2), Mokola virus (genotype 3), Duvenhage virus (genotype 4), European bat lyssavirus (EBLV) type 1 and 2 (genotype 5 and 6, respectively), and Australian bat *Lyssavirus* (ABLV) (genotype 7). Another four new viruses isolated recently from bats (Aravan virus, Khujand, West Caucasian Bat virus and Irkut virus) (7) are awaiting the classification. The first and the fifth genotypes of rabies virus have been diagnosed in Poland and there were 82 cases in total in 2006 (15). Rabies was present in terrestrial animals (domestic and wildlife) and four cases were diagnosed in bats.

Rabies viruses cause acute encephalomyelitis. The disease affects all mammalian species including humans. Due to the fatal course, the infection with rabies viruses needs accurate and rapid virus detection. Three methods are used for routine diagnosis (12, 16): the direct fluorescent antibody test (FAT), mouse inoculation test (MIT), and rabies tissue culture inoculation test (RTCIT). However, the sensitivity of these methods can be reduced, especially when the brain tissues submitted for testing are in a decomposed state (5). Thus, traditional methods of rabies virus detection need to be confirmed by the alternative methods.

Recently, a great improvement in molecular biology methods has been achieved. Thus, traditional methods can be replaced by e.g. polymerase chain reaction (PCR). Heminested RT-PCR (hnRT-PCR) and real-time PCR seem to be more reliable and more sensitive than traditional methods used for rabies diagnosis. They are widely used as confirmatory methods in many microbiological and virological laboratories including those for rabies diagnosis as well (1, 9, 13, 17). They allow for the detection of genetic material of viruses in a relatively short time. The real-time PCR is especially useful for that purpose; it does not require visualisation of the amplification product in agarose gel and the results are observed during the run of the test. This makes the method more rapid than the heminested RT-PCR. Beside of the pathogen detection, real-time PCR allows also for its quantification. It is especially important in biomedical and molecular
research related to pathogen quantitation or relative and absolute quantitation of gene expression. Real-time PCR seems to become a standard technique in many diagnostic laboratories.

The aim of the study was to compare two molecular techniques (heminested RT-PCR and real-time PCR) for the detection of nucleoprotein gene of Polish rabies virus isolates.

Material and Methods

Virus isolates. The study included terrestrial (genotype 1) and bat (genotype 5) isolates coming from different regions of Poland. Bat isolates were propagated in mice to get more material for the examination.

Virus propagation. Mice were inoculated with 10% suspension of bat brain homogenised in the water for injection. Inoculation was done intracerebrally in the dose of 0.03 ml per mouse. After inoculation, the mice were observed every day. Each brain of any died mouse was checked for rabies virus by FAT and those with positive results were subjected for RNA extraction.

RNA extraction. RNA was extracted directly from either the rabid animal brains or mouse brains inoculated previously with bat isolates. The brain samples were homogenised in the water for injection and RNA extraction was performed by using commercial kit QIAGen Viral RNA Mini Kit (Qiagen) according to the manufacturer’s instructions. Extracted RNA was re-suspended in RNAse free water in a final volume 50 µl and part of that RNA was used for injection. The remaining part was frozen and stored at -20°C for further study.

RT-PCR assay. Reverse transcription (RT) and the first PCR were 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 of primers: JW12 and JW6DPL (9) at a concentration of 10 µM, and 7.3 µl of RNase free water to a final volume of 15 µl. The amplification was performed in Personal Cycler (Bioterm) 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 55°C for 30 s, elongation at 72°C for 1 min, and the final extension at 72°C for 10 min.

Heminested RT-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 of primers JW12 and for genotype 1: JW10P (9) or for genotype 5: Jbel1 (13) at the concentration of 10 µM, 0.25 µl of JumpStart polymerase and 13.65 µl of PCR water to a final volume of 20 µl. The amplification was done in the same thermocycler as for RT-PCR. 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, elongation at 72°C for 1 min, and the final extension at 72°C for 10 min.

Design of TaqMan primers and probes. All TaqMan primers and probes were designed by the Primer 3 (v. 0.4.0) computer programme available on the Internet and are summarised in Table 1. The primers gt1P, gt1L and the probe AWgt1 for the detection of genotype 1 of rabies virus were designed based on the Pasteur virus (PV) nucleoprotein sequence available in GenBank database (accession number M13215). For genotype 5 detection, consensus sequence was generated for the sets of bat (EBLV type 1) nucleoprotein sequences from a sequence alignment generated with the BioEdit computer programme. From this alignment, the area of relative conservation was selected as an input to the Primer 3 computer programme to generate primers and probe (resulting in generation of gt5L, gt5P, and AWgt5).

All TaqMan probes were labelled at the 5’ end with a fluorescent reporter dye and at a 3’ end with quencher. AWgt1 was conjugated with the FAM as a fluorophore dye and TAMRA as a quencher molecule. AWgt5 was joined with HEX (fluorophore) and TAMRA, respectively.

Real-time PCR assay. Real-time PCR was performed using Quantitect Probe RT-PCR kit (Qiagen). 24 µl of reaction mixture contained 8.025 µl of RNase-free water; 12.5 µl of 2x Quantitect probe RT-PCR Master Mix; 0.25 µl of Quantitect RT Mix; 0.5 µl each of the PCR primers (a 10 µM concentrated each): gt1L, gt1P, gt5P, gt5L, and 0.5 µl each of 10 µM concentrated TaqMan probes AWgt1 and AWgt5. One microlitre of total RNA was added to 24 µl of the reaction mixture. The reactions were carried out in Mx3005P thermocycler (Stratagene) according to the following programme: 1 cycle of reverse transcription at 50°C for 30 min, 1 cycle of 95°C for 15 min followed by 45 cycles of 95°C for 15 s and 60°C for 1 min.

For each real-time PCR reaction, the software associated with Mx3005P system determined a threshold of cycle number (Ct). It corresponded to the number of PCR cycle at which the fluorescence of reaction exceeded the value determined higher than the background.

Assessment of the RT-PCR and heminested RT-PCR sensitivities. The sensitivity of RT-PCR and heminested RT-PCR methods was evaluated by using 10-fold dilutions of CVS strain at an initial concentration of 10 000 TCID50/mL. Subsequently, RNA isolation as well as RT-PCR, heminested RT-PCR, and real-time PCR were conducted according to the procedures described above.

Electrophoretic analysis. Nine microlitres of RT-PCR and heminested PCR products were analysed in 2% agarose gel stained with ethidium bromide at a 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.
Table 1
Primers and TaqMan probes

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<th>Name</th>
<th>Role</th>
<th>Sequence 5’ – 3’</th>
<th>Target</th>
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<td>GTC CCG AGT GAG ATC TTG A</td>
<td>genotype5</td>
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<td>real-time PCR primer</td>
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<td>genotype1</td>
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<td>GGGATGAATGCTGCTAAATTAGACCCA</td>
<td>genotype5</td>
</tr>
</tbody>
</table>

All TaqMan primers and probes were designed by the Primer 3 (v. 0.4.0) computer programme available on the Internet.

Fig. 1. The sensitivity of RT-PCR (A) and heminested RT-PCR (B) assays. Lanes 1-9 correspond to 10-fold serial dilutions of CVS virus stock starting from 10000 TCID<sub>50</sub>/mL (line 1).
Fig. 2. Real-time PCR sensitivity related to CVS titre (TCID<sub>50</sub>/mL).

Fig. 3. Real-time PCR results of randomly selected samples. All samples that crossed a threshold line are positive. The remaining samples placed below a threshold line are negative.
Results

The sensitivity of real-time PCR was assessed versus the results of RT-PCR and heminested RT-PCR assays (Figs 1 and 2). The study revealed that the TaqMan PCR was more sensitive than both the heminested RT-PCR and the RT-PCR and the detection of rabies virus by this method was possible from 0.1 TCID<sub>50</sub>/mL on up (Fig. 2). Standard curve drawn after the reaction established 98% efficiency of the reaction. R square corresponding to curve fitting was 1.0 thus indicating proper optimisation of the reaction. The sensitivity of heminested RT-PCR assay was 10-fold lower and it allowed for virus detection from 1 TCID<sub>50</sub>/mL on up (Fig. 1A).

When real-time PCR and heminested RT-PCR were compared to RT-PCR (Fig. 1B), the increase in the sensitivity of both methods was obtained. The study revealed that using RT-PCR, the detection of rabies virus was possible from 100 TCID<sub>50</sub>/mL on up.

Next, 32 of archive samples (16 designed genotype 1 and 16 designed genotype 5) were examined using the heminested RT-PCR and real-time PCR assays. Heminested RT-PCR using respective primer pairs JW12-JW10P and JW12-Jeb1 revealed 23 positive assays. Heminested RT-PCR with MIT and RTCTIC. The study demonstrated higher sensitivity of the heminested RT-PCR than that of MIT and RCTIC. The heminested RT-PCR was 5x10<sup>5</sup> more sensitive than RTCTIC and 3x10<sup>5</sup> than MIT confirming the usefulness of the molecular methods in rabies diagnosis. Heminested RT-PCR for the detection of EBLV1 and classical rabies virus infections in bats and terrestrial animals was also applied by Smreczak et al. (14). The use of nested or heminested techniques following reverse transcription and the first amplification increases the sensitivity of the methods in rabies virus detection. However, it needs two-step manipulations of genetic material. It involves multiple transfers of nucleic acids between different tubes with the associated risk of contamination and production of false-positive results. Besides, another inconvenience of RT-PCR and hRT-PCR is that the electrophoretic separation of the amplification products in the agarose gel followed by observing the results under UV lamp in the presence of ethidium bromide, commonly known as a carcinogen, has to be used.

One tube real-time PCR however, was addressed to solve all of the heminested RT-PCR inconveniences. The method does not require multiple transfer of materials and it is done in a single closed tube that makes the virus detection fast and minimises the opportunity for cross-contamination. The test takes 2 h and 16 min and it is surely shorter than the run of heminested RT-PCR. The method does not require electrophoretic separation of the amplification products taking away the exposure of the operator to the ethidium bromide and additionally decreases the risk of contamination. The incorporation of TaqMan technology into the real-time PCR method offers another advantage. The use of two primer pairs and two probes labelled with different fluorophores in one tube in a single reaction enables differentiating bat isolates from isolates collected from terrestrial animals, allowing for rabies virus detection and its genotyping at the same time.

The study confirms results of the others, who have applied the real-time PCR for the detection of rabies virus genotypes in saliva and brain samples (1, 10, 11, 17, 18). Black et al. (1) used TaqMan technology for the detection of seven genotypes of rabies virus. Wakeley et al. (17, 18) developed the TaqMan technology for the detection and differentiation of lyssavirus genotypes 1, 5, and 6. They reported the sensitivity of the assay at approximately 0.1 TCID<sub>50</sub>/mL. Thus, the assay described in our study seems to be a sensitive tool for the detection and distinguishing
decomposed state. Thus, methods applying PCR technology such as RT-PCR, heminested RT-PCR, and real-time PCR due to their rapidity, sensitivity, and reliability are recently more and more frequently used for rabies diagnosis.

Detection of rabies viruses with PCR has been described by a number of authors (4, 9, 13, 19). Picard-Meyer et al. (13) and Heaton et al. (9) investigated heminested RT-PCR methods for the detection of seven rabies virus genotypes. Additionally, Picard-Meyer et al. (13) estimated the sensitivity of the method comparing heminested RT-PCR with MIT and RTCTIC. The study demonstrated higher sensitivity of the heminested RT-PCR than that of MIT and RCTIC. The heminested RT-PCR was 5x10<sup>5</sup> more sensitive than RTCTIC and 3x10<sup>5</sup> than MIT confirming the usefulness of the molecular methods in rabies diagnosis. Heminested RT-PCR for the detection of EBLV1 and classical rabies virus infections in bats and terrestrial animals was also applied by Smreczak et al. (14). The use of nested or heminested techniques following reverse transcription and the first amplification increases the sensitivity of the methods in rabies virus detection. However, it needs two-step manipulations of genetic material. It involves multiple transfers of nucleic acids between different tubes with the associated risk of contamination and production of false-positive results. Besides, another inconvenience of RT-PCR and hRT-PCR is that the electrophoretic separation of the amplification products in the agarose gel followed by observing the results under UV lamp in the presence of ethidium bromide, commonly known as a carcinogen, has to be used.

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between different genotypes of rabies virus. Real-time PCR is more rapid, and it has the potential to be used for both diagnostic and research purposes for the identification and classification of rabies virus isolates.

References


