Communication

[*Comunicação*]

Reverse transcription-polymerase chain reaction assay for rabies virus detection

[*Protocolo de reação em cadeia pela polimerase precedida de transcrição reversa para a detecção de vírus da raiva*]

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Rabies has been reported since antiquity and its economical importance is related to its occurrence in domestic animals, which are source of food. Rabies in herbivorous animals, mainly in cattle, transmitted by bats, represents an important limitation to the development of animal husbandry in Latin America (Constantine, 2003; Belloto, 2001).

The comparison between sequences and segments of different lyssaviruses and among samples of the Pasteur Virus strain (PVS) enabled the identification of preserved regions inside of the *Lyssavirus* genome, allowing the design of primers able to amplify the viral nucleoprotein, glycoprotein and the pseudogene (Sacramento et al., 1991; Botvinkin et al., 2003).

Based on those acquired knowledge, protocols for lyssaviruses identification in clinical specimens were developed (Sacramento et al., 1991; Botvinkin et al., 2003). Furthermore, phylogenetic analysis of those viruses was performed after amplicons sequencing (Ito et al., 2001; Botvinkin et al., 2003).

The goal of this study was to establish a reverse transcription-polymerase chain reaction (RT-PCR) assay to identify *Lyssavirus* genus in clinical specimens from different origins, by using primers designed in the nucleoprotein region.

A total of 50 samples of encephalic tissue from animals infected with the rabies virus were used: 44 bovines, 5 equines and 1 chiroptera. Those samples were received from several municipalities of the State of Rio de Janeiro, between January 1999 and June 2001.

The fluorescent-antibody test (FAT) was carried out according to Meslin et al. (1996). Briefly, the imprint of the fragments were incubated with polyclonal anti-rabies fluorescein-labeled immune globulins (Instituto Nacional de Controle de Qualidade em Saúde – INCQS / Fiocruz) and visualized under an immunofluorescent microscope (Zeiss) afterwards.

The intra-cerebral mouse inoculation test (MIT) was conducted according to Meslin et al. (1996). The encephalic tissues were triturated in a mortar, diluted to 10% in phosphate buffered saline (PBS) pH 7.2, containing penicillin 50IU – streptomycin 0.02mg/ml and clarified by centrifugation at 900x g for five minutes. The supernatant was collected and 0.03ml of each sample was used to inoculate 21 day-old albino mice by intra-cerebral route. PBS pH 7.2 was used for inoculation as negative control. The

inoculated animals were observed daily up to 30 days.

The viral RNA was extracted by using TRIzol[TM1] according to the manufacture's recommendations in 10% of clarified supernatant. The RT-PCR assay was performed as described by Heaton et al. (1997) with modifications. Briefly, 10μl of the extracted RNA was denatured at 75ºC for 10 minutes, cooled on ice, reverse-transcribed in 40μl of the RT mix: 2μl of 10μM primer JW12 (5' ATGTAACACCC/TCTACAATTG 3' [nt 55-73]), 200U of SuperScript II Reverse Transcriptase, 1mM of each dATP, dCTP, dTTP, dGTP, 10U of RNAsin, 5μl of 5X PCR buffer, 1mM dithiothreitol and 1,5μl of 50mM magnesium chloride[1]. The RT mix was incubated for 60 minutes in water bath at 42ºC, boiled for five minutes, and then chilled on ice. Fifty microliters of the amplification mix (PCR) containing 1mM of each dATP, dCTP, dTTP, dGTP, 2,5U of Taq-Platinum polymerase, 5μl of 10X PCR buffer, 1,5μl of 50mM magnesium chloride and 4μl of a cocktail of 10μM of the following primers: JW12, JW6 (nt 660-641)(5' CAATTCGCACACATTTTGTG 3' / 5' CAGTTAGCGACATCTTATG 3'/ 5' CAGTTGGCACACATCTTGTG 3')[2], corresponding to the sequencing of Duvenhage virus, EBLs 1 and 2, and Mokola virus, respectively. After denaturation at 94ºC for two minutes, the PCR assay consisted of: 40 cycles (94ºC/30 seconds, 45ºC/30seconds, 72ºC/60seconds), linked by a final extension cycle at 72ºC/7minutes. The challenger virus strain (CVS), kindly provided by the INCQS / Fiocruz, Brazil and distilled milli-Q water were used as positive and negative controls, respectively, during all procedures. In order to avoid false-positive results, precautions of manipulation were carried out during the amplification step, as recommended by Kwok and Higuchi (1989).

The RT-PCR amplicons were resolved by electrophoresis on 1.5% agarose gels[1], stained with ethidium bromide (0.5μg/ml)[2], visualized and photographed by using a polaroid system gel Doc 1000[2]. After electrophoresis, a specific 606bp amplicon was observed in all 50 samples

analyzed, showing a 100% agreement with the MIT, including one false-negative sample by the FAT from an equine.

The present RT-PCR assay can provide a same day laboratory diagnosis for rabies virus by using different animal species. The results obtained highlighted the potential of the RT-PCR assay as rapid, specific and sensitive tool for the diagnostic investigation of rabies viruses. Despite the excellent results obtained by the MIT and FAT, the RT-PCR has the ability to make a more rapid and definitive diagnosis allowing more directed intervention strategy. Furthermore, RT-PCR assay is useful for the laboratorial diagnosis of rabies viruses from brain tissues from different animal species from different geographic regions. The use of specific primers for the nucleoprotein region of rabies virus and rabies-related viruses increases substantially the possibility of success to detect rabies infection (Heaton et al., 1997).

The future of the amplification techniques lays in their capacity to surpass the sensitivity of the *post-mortem* diagnostic techniques or in their suitability in diagnosis the infection from live animal secretions. However, diagnostic laboratories are still performing time-consuming and less sensitive techniques (Sacramento et al., 1991). For Kamolvarin et al. (1993) the RT-PCR technique for rabies diagnosis shows important additional advantages compared to the FAT and the MIT: a) it should be used in clinical specimens in bad conditions of conservation (material in decomposition) that often occurs in localities with a tropical climate such as Brazil; b) it is faster than the MIT which often requires an interval of 10 to 30 days for a conclusive result. Our data corroborate with those findings when one clinical specimen (equine 22/06/01) was false-negative by the FAT, but it was identified as positive by the RT-PCR and by the MIT as well, however only 21 days after animal inoculation.

Another importance in routinely using molecular techniques for rabies virus detection is that they allow the implementation of prophylactic and control measures. It also plays an important role in the disease epidemiology once it helps to identify the reservoir in the routine diagnosis (Ito et al., 2001, 2003).

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[1] All reagents furnished by Invitrogen, Carlsbad, CA, USA
[2] Bio-Rad, Laboratories, USA

Recent findings of new bat lyssaviruses throughout the world are showing the importance of molecular methodologies used for diagnosis and for characterization of *Lyssavirus* genus as well (Botvinkin et al., 2003). Our study, in agreement with previous studies performed in Brazil, shows valuable contribution of molecular analysis methods for rabies virus diagnosis, justifying the implementation of a RT-PCR as a methodology in routine rabies diagnosis in Brazil. It is concluded that the methodology implemented in this study could be successfully used in the routine diagnosis because it was rapid, specific and showed a high sensitivity, contributing positively for the laboratory diagnosis of rabies virus in tropical countries.

Keywords: rabies virus, reverse transcription-polymerase chain reaction, diagnosis

RESUMO

Este estudo teve por objetivo implantar um protocolo de amplificação genômica, precedida de transcrição reversa (RT-PCR) para o gene da nucleoproteína do vírus da raiva, para a utilização dessa metodologia em laboratórios onde são realizadas investigações para a detecção do vírus rábico. Foram utilizadas 50 amostras de tecido encefálico de animais (44 bovinos, 5 eqüinos e 1 quiróptero) oriundos do Estado do Rio de Janeiro, positivos por imunofluorescência direta e/ou prova biológica para o vírus rábico. A extração do RNA foi feita a partir da suspensão a 10% em PBS pH7,2 do tecido encefálico utilizando-se a metodologia de TRIzolTM (Life Technologies) e o protocolo de RT-PCR descrito por Heaton et al. (1997), incluindo algumas modificações. Dentre as 50 amostras analisadas, 50 foram positivas pela prova biológica e pela RT-PCR e destas, 49 foram positivas pela imunofluorescência direta. Estes resultados demonstram ser este protocolo de RT-PCR uma metodologia sensível, específica, rápida e extremamente valiosa, podendo ser utilizada como rotina em laboratórios que trabalham no diagnóstico de vírus rábico.

Palavras-chave: vírus da raiva, transcrição reversa/reação em cadeia pela polimerase, diagnóstico

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