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Original Research

Ante-Mortem Diagnosis of Rabies in Cows and Buffaloes

Shahida Afzaal[1], Muhammad Numan[1]*, Zafar ul Ahsan Qureshi[1], Munazza Shaukat[1], Imtiaz Ahmad Khan[2] , Zaheer Hussain[3], Zain ul Abidin[1], Abdul Whab Manzoor[1], Mudasser Habib[4]

[1]*Veterinary Research Institute, Zarrar Shaheed Road Lahore Cantt; Pakistan;*
[2]*Department of Pathobiology, PMAS-Arid Agriculture University, Rawalpindi, Pakistan*
[3]*School of Biological Sciences, University of the Punjab, Lahore, Pakistan;*
[4]*Nuclear Institute for Agriculture and Biology, Faisalabad, Pakistan.*

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Abstract

The current study was carried out to compare the conventional diagnostics for the diagnosis of rabies with advanced diagnostic technique. Ante-mortem diagnosis of rabies in cows and buffaloes is described using newer molecular technique and conventional methods for the detection of rabies virus RNA from saliva samples. Saliva samples from animals at different time intervals were collected and tested for rabies virus using reverse transcriptase polymerase chain reaction (RT-PCR) and mouse inoculation test (MIT). Results obtained by RT-PCR (1-2 days required) were easy, non-laborious, satisfactory and can be applied in replacement of routine laboratory test i.e. Mouse inoculation test (21 days required) for ante-mortem diagnosis of rabies virus from saliva.

Keywords: Animal rabies; saliva; RT-PCR; MIT; N gene

Introduction

Rabies is an acute and fatal viral encephalopathy caused by Lyssa virus of family Rhabdoviridae. The virus is bullet shaped non segmented single stranded negative sense RNA virus (Wilkinson, 2002). Carnivores along with bats are the primary hosts. Human and domesticated animals can harbor the infection through bite of infected animals through the contamination of wounds with saliva of infected ones (Singh and Sandhu, 2008). Post-mortem diagnosis of animal-rabies using direct immunofluorescence on brain-tissue impression smears from the dead infected animal is almost 100% specific and sensitive, and considered gold standard method for diagnosis of rabies. There have always been problems with accurate ante-mortem diagnosis in the laboratories (Trimarchi and Smith, 2002). Different laboratory techniques including frozen section skin biopsy, isolation of virus from saliva or cerebrospinal fluid (CSF), detection of antibodies in serum and CSF and fluorescent antibody test (FAT) on corneal impression smears (in wild carnivorous animals) vary in sensitivity and specificity depending on the stage of disease and the skill levels of a laboratory (Trimarchi and Smith, 2002). On the other hand detection of Negri bodies in stained tissue section is also less sensitive (Hanif *et al*., 2009). The aforementioned tests are laborious and time consuming (Dean *et al*., 1996). The most recent molecular techniques such as PCR and its modifications are proved to be more specific and sensitive for the ante-mortem diagnosis of rabies. In developing countries like Pakistan, no data is available for the use of such techniques for diagnosis of rabies in animals. The aim of the present study was to detect rabies virus using RT-PCR from saliva samples of clinically suspected diseased cases of cows and buffaloes.

*Corresponding author: Muhammad Numan

E-mail address: numan_vri@yahoo.com

Materials and methods

Samples

After the appearance of clinical signs of rabies of affected cows and buffaloes, ten saliva samples were collected in wide mouth sterile containers containing glycerol. At an interval of 4-5 hours, three saliva samples from each affected animal were collected and pooled together. Before processing, the collected samples were stored at -70°C. For the negative control, five healthy animals were selected and their saliva were also obtained, processed and stored in the same way as for the affected animals.

Controls for the assays

For this purpose, two rabbits were selected. One rabbit was infected with the Pasteur strain of rabies virus (FAO donation) and its brain was collected. A 10% homogenate of the brain was prepared to use as positive control. The second rabbit's brain homogenate was used as negative control.

Test procedures

The RT-PCR was performed using most highly conserved region of "N" protein gene (443 bp). Primer sequences used were:
N1 (+) sense: (587) 5'- TTT GAG ACT GCT CCT TTT G-3' (605)
N2 (-) sense: (1029) 5'- CCC ATA TAG CAT CCT AC-3' (1013)

The N1 primer was used to prime cDNA which was thereafter amplified by the N1-N2 set. All steps of RT-PCR including isolation of RNA and preparation of cDNA preparation were carried-out using chemicals, enzymes, kits of Fermentas® as described previously by Tordo *et al*. (1996) using thermocycler (Advanced primus 96 peqlab®). Gel electrophoresis of the final product was conducted using 1% agar gel and results were visualized (Figures 1 and 2) using gel documentation system of BioRad® (Model: Universal Hood II).

The Mouse Inoculation Test (MIT) was performed as described by Koprowski (1996).

Results

Results of the MIT and RT-PCR tests indicated that 6 samples were found positive for rabies (Table 1). Amplification of "N" ' region gene through RT-PCR (Fig. 1, 2) resulted into specific bands of 443 bp. Out of 10 saliva samples, 6 were found positive by RT-PCR. Saliva samples collected from negative controls (five healthy animals and a normal rabbit brain sample) were found negative indicating the specificity of the primers used.

Table 1. Saliva samples indicating the results by RT-PCR and MIT.

Saliva samples	Rabies positive or negative result by RT-PCR	Rabies positive or negative result by MIT
1	+	+
2	+	+
3	+	+
4	+	+
5	+	+
6	+	+
7	-	-
8	-	-
9	-	-
10	-	-

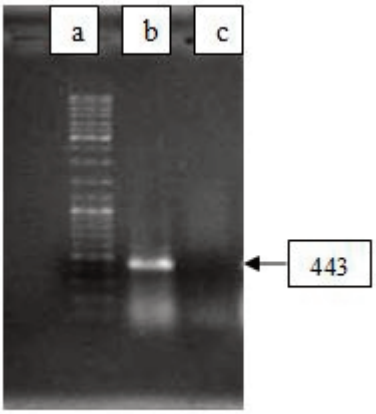

Fig. 1. Reference bands
a = marker, b = saliva sample positive for rabies virus, c = saliva sample negative for rabies virus

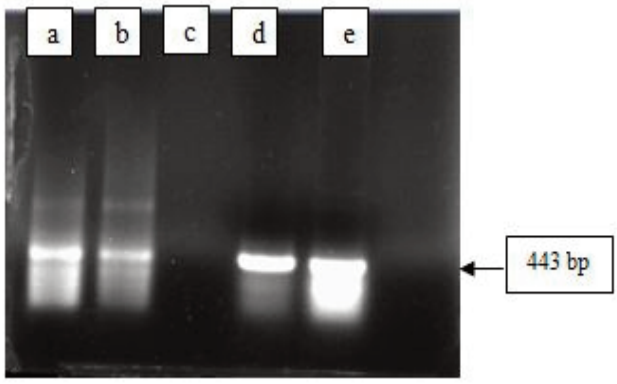

Fig. 2. Test samples with positive and negative controls
a = saliva sample positive for rabies virus, b = saliva sample positive for rabies virus, c = negative control, d = saliva sample positive for rabies virus, e = positive rabies (band of 443bp) sample as reference

Discussion

Rabies is a neglected disease for the most part in developing countries including Pakistan. In one study, 24 human saliva samples of suspected rabies patients were molecularly tested and found that 21 samples were positive (Nagarajan *et al*., 2006). Nadin-Davis (1998) and Hanif *et al*. (2009) reported a single band of 762 bp by RT-PCR in rabies positive cases. Crepin *et al*. (1998) used RT-PCR to test twenty-eight saliva samples and confirmed the occurrence of rabies virus nucleic acid in five cases. In this study, from 6 of ten, rabies virus samples, RNA was isolated and resulted into specific bands of 443 bp.

Unadventurous techniques used for postmortem diagnosis of rabies are of limited value to support the ante-mortem diagnosis of the disease (Hemachudha *et al*., 1988; Warrel and Warrel, 1995). Additionally, methods like FAT can not be applied with clinical samples such as saliva and CSF for laboratory confirmation. When saliva is preferred to be used as clinical sample then the only conventional method for diagnosis of rabies is the isolation of virus. However, the classic MIT (Koprowski, 1996) used for virus isolation can lead to a substantial delay in the evaluation of an end point. Moreover, it is labour-intensive as it needs facilities for the use of experimental animals. Other techniques of viral isolation like cell culture isolation methods are demanding due to the failure of certain rabies virus variants to multiply easily in cell lines (Hughes *et al*., 2004). Many tests have been used for the ante-mortem diagnosis of the rabies including the frozen section skin biopsy (Blenden *et al*., 1986) and corneal smear examination (Schneider, 1969). The corneal smear method is too insensitive for exact clinical diagnosis. The merely test considered to be trustworthy is the immunofluorescence test on skin biopsy samples. However, Crepin *et al.* (1998) noticed that at least 20 sections were required to make sure the observation of hair follicles. This might grounds discomfort to the animal due to collection of skin at biopsy in addition to being tedious and difficult to be done in the laboratory. It was also observed that sensitivity of finding rabies antibodies in serum and CSF by ELISA and serum neutralisation test on cell culture was very low. Incidence of seroconversion in the course of disease makes serological testing of limited value (Schuller *et al.*, 1979).

The use of newer techniques such as the dot blot enzyme immunoassay has shown unsatisfactory results for the detection of rabies antigen in saliva and CSF samples (Madhusudana *et al*., 2004). Fooks *et al.* (2003) corroborated that salivary secretions of the virus are intermittent so at least three samples should be taken at different time intervals. The duration of virus shedding in the saliva and quantum of virus are of principal importance for rabies diagnosis. Smith *et al*. (2003) conducted MIT and rapid tissue culture infective test (RTCIT) alongside the detection of rabies specific antigen in skin biopsies from nape of the neck and hand, and observed unconvincing result whilst RT-PCR yielded a positive result. The FAT is considered the basic method that is used for routine diagnosis of rabies infection, however this technique was found of no value for decomposed samples (Kamolvarin *et al*.,1993) and as most detection reagents are specific for rabies virus, one could face lowered sensitivity for the detection of other Lyssaviruses (Echevarria *et al*., 2001). Moreover, brain samples found negative for rabies virus by FAT were observed as positive when tested by RT-PCR (Echvarria *et al*., 2001; Serra-Cobo *et al*., 2002; Muller *et al.*, 2004).

Conclusion

RT-PCR is an efficient and sufficient procedure used for rapid ante-mortem diagnosis of rabies in cows and buffaloes. Although the MIT have a similar sensitivity and specificity rate, but requires 3 weeks to give final results. Comparatively, RT-PCR results can be achieved within 1-2 days and can be applied as routine laboratory technique in replacement of MIT for ante-mortem animal rabies virus diagnosis. However, RT-PCR is economically precious procedure, in contrast to MIT.

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