Ante-Mortem Diagnosis of Rabies in Pakistan

Shahida Afzaal, Muhammad Numan*, Zafar ul Ahsan Qureshi, Munazza Shaukat, Intiaz Ahmad Khan1, Zaheer Hussain2, Zain ul Abidin, Abdul Whab Manzoor and Mudasser Habib3

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

*Corresponding author: numan_vri@yahoo.com

Abstract

The present study was carried out to compare the conventional diagnostics for the diagnosis of rabies with advanced diagnostic technique. Ante-mortem diagnosis in food animals is of utmost importance for herd and/or flock management and to ensure proper post-exposure treatment of infected animals. Conventional methods used for the diagnosis of rabies include bite history, clinical symptoms and different tests like direct immunofluorescence (FAT), cell culture (TCIT), animal inoculation tests (MIT) etc. However, all the above conventional diagnostics are laborious and time-consuming although they are assumed to be the gold standard for the diagnosis of rabies. In the present study ante-mortem diagnosis of rabies 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 satisfactory and can be applied in replacement of routine laboratory test i.e. MIT (21 days required) for ante-mortem diagnosis of rabies virus from saliva.

Key words: 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, cattle, buffalo, sheep, goat and other 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). Though postmortem diagnosis of animal rabies using brain specimen and direct immunofluorescence in impression smears from infected brain is almost 100% specific and sensitive and considered gold standard method for rabies diagnosis, there have always been problems with accurate ante-mortem diagnosis in the laboratory (Trimarchi and Smith, 2002). The use of direct immunofluorescence and brain samples for the postmortem diagnosis of rabies give almost 100% specific and sensitive results yet there have been difficulties with exact ante-mortem diagnosis of the disease in the laboratory. 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 vary in sensitivity 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) and now discouraged in rabies diagnosis. The aforementioned tests are laborious, time consuming and are restricted to research (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 suspected animals.

**Materials and Methods**

**Samples:**

After the appearance of clinical signs and symptoms in affected cows and buffaloes, ten saliva samples were collected in wide mouth sterile containers having glycerol in it. At an interval of 4-5 hours, three saliva samples from each affected animal were collected and pooled together. Before processing, all the samples were stored at -70°C. For the negative control, five healthy animals were selected. Saliva samples form these animals 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 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:**

1. The RT-PCR was performed as described by Tordo et al (1996) 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.

**Isolation of RNA:** 1mL of Trizol-reagent was added to 0.1ml of saliva sample and kept for 5 minutes, kept on ice for 5 minutes, added 200 µL chloroform into same eppendorf tube and kept on ice for 15 minutes, centrifuged at 4°C for 15 minutes at 12000 rpm, liquid phase was taken and added to it 600 µL of isopropanol and mixed it by inverting and kept on ice for 5 minutes, centrifuged at 4°C for 10 minutes at 12000 rpm, discarded the isopropanol and pallet was observed, washed RNA pallet with 75% ethanol and centrifuged at 4°C at 9000 rpm for 5 minutes, air dried the pallet and dissolved in nuclease free 100 µL diethylpyrocarbonate (DEPC) treated water, kept at 55°C for 10 minutes in water bath (using chemicals/enzymes/kits of Fermentas ®).

**cDNA preparation:**

Step I: 2 µL of DEPC treated water and 1 µL of reverse primer were added into 5 µL of RNA. The mixture was incubated at 65°C for 5 minutes in thermocycler (Advanced primus 96 peqlab®). Chilled on ice.
Ante-Mortem Diagnosis of Rabies in Pakistan

Step II: 8 µL of dNTPs and 4 µL of 5X RT buffer were added in the mixture and incubated at 37°C for 5 minutes in thermocycler.

Step III: After incubation, 1 µL reverse transcriptase enzyme was added and incubated at 42°C for 60-90 minutes.

PCR of cDNA: Following mixture was prepared; dNTPs (4 µL), Taq buffer (5 µL), cDNA (5 µL), MgCl₂ (2 µL), Taq polymerase (1 µL), DEPC treated water (30) µL, reverse primer (1 µL) and forward primer (1 µL). Following PCR conditions were optimized for this mixture: five initial cycles of denaturation (60 seconds at 94°C), annealing (90 seconds at 45°C, followed by 20 seconds at 50°C) and elongation (90 seconds at 72°C) and 30 additional cycles reducing denaturation and elongation to 30 seconds and 60 seconds, respectively. Final elongation was obtained at 72°C for 10 minutes. Gel electrophoresis of the final product was conducted using 1% agar gel and results were visualized (Figures 1 & 2) using gel documentation system of BioRad® (Model: Universal Hood II).

2. The MIT was performed as described by Koprowski (1996). Briefly, 10% brain tissue and saliva suspensions were inoculated intracerebrally to individual mouse. Five mice were inoculated with each sample and one mouse was kept as a control in each set. Mice were checked daily for 21 days. Deaths occurring 24-28 hours after intracerebral inoculation were attributed to causes other than rabies virus, such as trauma, bacterial contamination or other viruses. Positive mice for rabies showed ruffled fur, tremors, lack of coordination of hind legs, ascending paralysis, prostration and finally death.

Results and Discussion

Rabies is a neglected disease in many parts of the world particularly in developing countries including Pakistan. A considerable number of rabies cases go unnoticed inflicting serious losses to the economy of the country in the form of mortality (Seimenis, 2008) and resulted in losses due to lack of laboratory facilities in remote areas and laborious diagnostic procedures. The MIT and RT-PCR tests were conducted to test the saliva samples collected from suspected rabies cases. Six samples were found positive for rabies by these tests (table). Amplification of “N” region gene through RT-PCR (Figures 1 & 2) resulted into specific bands of 443 bp. Out of ten saliva samples, six 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. A similar study on human saliva samples was conducted by Nagaraj et al. (2005). Twenty-four saliva samples of suspected rabies patients were tested and twenty-one samples out of twenty-four were positive. The findings of the present study were contrary with the findings of Hanif et al. (2009) and Nadin-Davis (1998), who reported a single band of 762 bp by RT-PCR in rabies positive cases. Conventional 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). Conventional diagnostic 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. Furthermore, it is labour-intensive as it requires facilities for the use of experimental animals. Other techniques of viral isolation like cell culture isolation methods are challenging due to the failure of certain rabies virus variants to proliferate easily in specific cell lines (Hughes et al., 2004). Many tests have been

Numan et al., IJAVMS, Vol. 7, Issue 1, 2013: 39-45
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).

Figure 1:
Title: Reference bands
a = marker, b = saliva sample positive for rabies virus, c = saliva sample negative for rabies virus

Figure 2:
Title: 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

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

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<th>Saliva samples</th>
<th>Rabies positive or negative result by RT-PCR</th>
<th>Rabies positive or negative result by MIT</th>
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The corneal smear method is too insensitive for exact clinical diagnosis. The only test considered to be dependable is the immunofluorescence test on skin biopsy samples. However, it was noticed by Crepin et al., (1998) that a minimum of twenty sections were required to ensure the observation of hair follicles. This might cause discomfort to the animal due to collection of skin at biopsy besides being tedious and challenging to be done in the laboratory. It was also observed that sensitivity of detection of rabies antibodies in serum and CSF by ELISA and serum neutralisation test on cell culture was very low. Occurrence 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). Crepin et al. (1998) used RT-PCR to test twenty-eight saliva samples and confirmed the presence of rabies virus nucleic acid in five cases. In this study, from six out of ten, rabies virus samples, RNA was isolated. Fooks et al., (2003) observed 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 chief importance for rabies diagnosis. Smith et al., (2003) conducted MIT and rapid tissue culture infective test (RTCTI) along with detection of rabies specific antigen in skin biopsies from nape of the neck and hand and observed inconclusive 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 which were found negative for Rabies virus by FAT test were observed as positive when tested by RT-PCR (Echvarria et al., 2001; Muller et al., 2004; Serra-Cobo et al., 2002). Conventional techniques used for postmortem diagnosis of rabies are of limited value to support the ante-mortem diagnosis of the disease but RT-PCR described in this study is an effective tool for the rapid ante-mortem diagnosis of rabies. The RT-PCR is a good tool for molecular epidemiological studies in many of countries like India (Nagarajan et al., 2006). Conventional methods which are used for the postmortem diagnosis of rabies are less supportive for the ante-mortem diagnosis of the disease but RT-PCR described here is a valuable tool for the rapid ante-mortem diagnosis of rabies. Conventional method used in this study i.e. MIT requires 21 days to give results either positive or negative, however, by RT-PCR results can be obtained within 1-2 days and can be applied as routine laboratory technique in replacement of MIT for ante-mortem animal rabies virus diagnosis.

**Conclusion**

The RT-PCR test using saliva samples is quick and reliable and the results can be obtained within 1-2 days and can be applied as routine laboratory technique in replacement of MIT for ante-mortem animal rabies virus diagnosis.

**References**


