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Molecular detection of rabies virus in animals from Kerala, India

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Abstract
Rabies is fatal neurotropic disease caused by *Rabies virus* and other *Lyssavirus* species of the family Rhabdoviridae. The fluorescent antibody test (FAT) is a rapid, sensitive, specific method for diagnosing rabies in humans and animals and is the gold standard for diagnosis. Over the last thirty years, molecular methods for diagnosing rabies that rely on finding the genetic makeup of the virus have gained increased acceptance. The development of reverse transcription polymerase chain reaction (RT-PCR) provided an alternative method for post-mortem diagnosis and enabled ante-mortem diagnosis. In this study, we compared the application of FAT and RT-PCR for confirmatory diagnosis of rabies in suspected brain samples of animals from different parts of Kerala, India. Thirty-six brain samples from dog, cat, goat, horse, jackal and mongoose were subjected to both tests. The results support the utility of RT-PCR as a confirmatory diagnostic test. In samples of degraded brain tissue, RT-PCR is more sensitive in detecting the rabies virus.

Keywords: RT-PCR, rabies virus, nucleoprotein gene, Kerala

1. Introduction
Rabies is an important fatal neurotropic disease caused by *Rabies virus* (RABV) and other *Lyssavirus* species of the family Rhabdoviridae. It is a widespread zoonotic infectious disease, with an estimated 55,000 human deaths globally each year. All continents, excluding Antarctica, have rabies, but Asia and Africa account for more than 95% of all human deaths from the disease [1]. Rabies is usually transmitted through the bite of a rabid animal. People can also catch rabies from non-bite exposures, which might include scratches, abrasions, or open wounds exposed to saliva or other potentially infectious material from a rabid animal [2]. The disease is generally fatal once signs and symptoms appear. Although no specific treatment has been developed for rabies, there is an effective and safe prevention method called post-exposure prophylaxis (PEP) [1]. It is tragic that rabies, which can be prevented in humans and controlled in domestic animals is neglected and continues to cause a significant social and economic burden even in the 21st century [3].
Since 1985, India has reported 25,000 to 30,000 human rabies deaths per year. The mainland of India is endemic to rabies, although the islands of Andaman & Nicobar and Lakshadweep are rabies-free. The dog was the animal most responsible for human rabies deaths (96.2%) in India [4]. Underreporting of human cases is exacerbated by cultural, religious, and societal taboos, as well as lack of surveillance due to insufficient health-care facilities such as laboratory capabilities, competent employees, and medical and veterinary infrastructures [5].
Currently, rabies diagnosis relies on laboratory tests for detection of viral antigens. Among these tests, FAT is considered as gold standard test for post-mortem diagnosis [6]. However, the higher cost involved in fluorescent microscopy, the requirement for specialized training and its unsuitability for highly decomposed samples limit the wide usage of FAT [7]. Other antigen detection methods include enzyme-linked immunosorbent assay (ELISA), direct rapid immunohistochemical test (DRIT), and indirect rapid immunohistochemistry test (IRIT). Virus might have to be isolated to confirm the results of antigen detection tests and for further amplification or characterization of isolate. Confirmatory tests are virus isolation in cell culture and mouse inoculation test, which involve complex and time-consuming propagation and isolation of virus. Hence, simple-to-use tests that provide quick and economical diagnosis, with no loss of sensitivity or specificity would greatly enhance the diagnostic environment [8].

Over the last thirty years, molecular methods for diagnosing rabies that rely on finding the genetic makeup of the virus have gained increased acceptance. The development of RT-PCR provided an alternative method for post-mortem diagnosis [9] and enabled ante-mortem diagnosis [10]. If brain tissue is available, the FAT or DRIT should be used for primary diagnosis of viral antigens and molecular techniques can be used for confirmatory testing and epidemiological surveys.

2. Materials and Methods

2.1 Samples: Brain samples were collected from 36 rabies suspected cases which came for post-mortem to the Department of Veterinary Pathology, College of Veterinary and Animal Science, Pookode, Wayanad. All the samples were screened for rabies by FAT at the Department of Veterinary Pathology. The samples were stored at -80 ºC till further processing.

2.2 Extraction of total RNA from clinical samples: Total RNA present in the brain samples of suspected mammals is isolated by using TRIzol® reagent (Thermo Scientific, USA) as per manufacturer's protocol. About 10 mg of sample was taken in 750 µL of TRIzol® reagent and homogenised using a homogeniser. The suspension was transferred to a 1.5mL Eppendorf tube and incubated at room temperature for 5 min. Subsequently 0.2 mL of chloroform (Sigma Aldrich, USA) was added. The contents were thoroughly mixed by inverting Eppendorf tube a few times and incubated for 2 to 3 min at room temperature. The mixture was centrifuged at 10,000 rpm for 5 min at 4 ºC. Aqueous phase was collected in a new 1.5 mL Eppendorf tube and 0.7 volume of 100 per cent isopropanol was added. The contents were mixed and incubated for 5 min at room temperature. The mixture was centrifuged at 14,000 rpm for 10 min at 4 ºC. Supernatant was discarded leaving only the RNA pellet. RNA pellet then washed with 1 ml of 75% ethanol. After vortexing, the mixture was centrifuged at 14,000 rpm for 10 min at 4 ºC. Supernatant was discarded and pellet was air dried for 15 min in a dry block at 37 ºC. Then 40 µL Nuclease free water is added and stored at -80 ºC.

2.3 Reverse Transcription Polymerase Chain Reaction (RT-PCR): Complementary DNA (cDNA) was synthesized from total RNA using the RevertAid First Strand cDNA synthesis kit (Thermo Scientific, USA) as per the manufacturer's protocol. To the PCR tube kept on ice, 8 µL of total RNA and 1 µL (0.2 µg/µL) of random hexamer are added. Then make up the volume to 12 µL by adding nuclease-free water (NFW). The mixture was incubated at 65 ºC for 5 min. The above mixture was cooled down to 25 ºC and 4 µL of 5X reaction buffer, 1 µL (20 U/µL) Ribolock RNase inhibitor, 2 µL of 10 mM dNTP mix and 1 µL (200 U/µL) RevertAid H minus M-MuLV reverse transcriptase enzyme were added. Then the mixture was incubated at 25 ºC for 5 min followed by 60 min at 42 ºC. The reaction was terminated by heating at 70 ºC for 5 min. The cDNA was stored at -20 ºC for further use. A fragment of N gene was amplified by RT-PCR, as described previously [11], using primers RABNF (5'ACTGATGTAGAAGGGAATTG3') and RABNR (5'GAACGGAAGTGGATGAAATA3') yielding amplicons of 533 bp size. The PCR was carried out in a PCR tube containing 12.5 µL of EmeraldAmp GT PCR master mix (2x), 1 µL of each primer set (10 pmol each), 2 µL of cDNA (500 ng) and NFW to make up the volume to 25 µL. The PCR condition was standardized as 95 ºC for 3 min (initial denaturation), 35 cycles of 95 ºC for 30 sec (denaturation), 50 ºC for 40 sec (annealing) and 72 ºC for 30 sec (extension). After 35 cycles PCR final extension was carried out at 72 ºC for 5 min. The PCR products were analysed by 1.25% agarose gel electrophoresis in comparison with a 100 bp DNA ladder, and visualized using a gel documentation system (Syngene, Cambridge, England).

3. Results and Discussion

Despite the fact that nearly 60% of the worldwide human rabies deaths occur in the country [4], it is not a notifiable disease in India [12]. The lack of simple, sensitive and cost-effective laboratory methods for rabies diagnosis underestimates the disease burden and public health impact due to rabies [13].

FAT is rapid, sensitive and specific method for diagnosing rabies in animals and humans. It is the golden standard test approved by WHO and WOAH for rabies diagnosis [6]. In the current study, 24 out of 36 samples collected were positive. Of the 12 negative samples, four were unfit for testing by FAT and the results produced were ambiguous due to decomposition of carcass by the time of necropsy.

RT-PCR to identify the RNA of RABV is more accurate for the diagnosis of partially degraded samples compared with FAT and mouse inoculation test (MIT) [14]. Of the 36 samples tested by RT-PCR targeting partial N gene, 28 were positive and 8 were negative for RABV. Positive samples yielded amplicons of size 533 bp and were observed in agarose gel electrophoresis as shown in Figure 1. A pictorial representation of the comparative results of RT-PCR and FAT is shown in Figure 2.

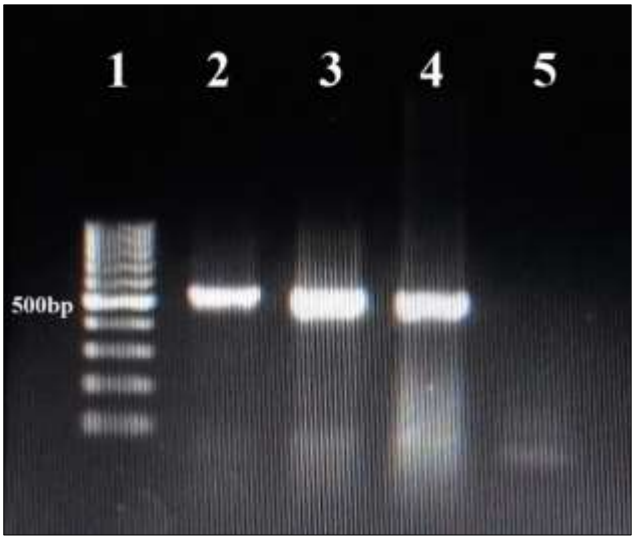

Fig 1: A pictorial representation of the comparative results of RT-PCR and FAT is shown in Figure 2.

Figure 1, PCR amplification of N gene having product size of 533 bp (clinical samples in lanes 2 to 4, 100 bp DNA ladder in lane 1 and negative control in lane 5).

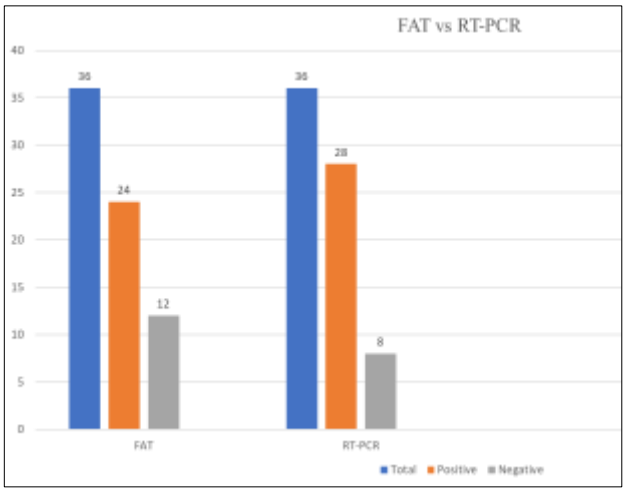

Fig 2: Comparative results of RT-PCR and FAT

4. Conclusion

In fresh specimens, there is 100% agreement between the two tests. However, there is a considerable loss of sensitivity of FAT when decomposed tissues are subjected to testing. RT-PCR shows excellent performance qualities in regard to reliability of the result. Hence the results of this study support the utility of RT-PCR as a confirmatory diagnostic test.

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