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Comparative Evaluation Of Reverse Transcriptase Polymerase Chain Reaction (Rt-Pcr) With Direct Fluorescent Antibody Test For Diagnosis Of Rabies In Fresh And Decomposed Brain Samples

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Abstract

A total of 85 one human & 84 animal brain samples(76 fresh and nine decomposed brain samples) were tested by three methods namely Seller's Staining, direct Fluorescent Antibody Test (dFAT) and Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) to evaluate the role of RT-PCR in comparison with conventional methods. Out of 76 fresh brain samples, 42(55.26%) samples were found positive by Seller's staining, 46 (60.52%) were found positive by both RT-PCR & dFAT which is a gold standard. Sensitivity, Specificity and Concordance of RT-PCR with dFAT was 100% in fresh brain samples. Of the nine decomposed brain samples, eight (88.88%) samples were positive by RT-PCR whereas dFAT confirmed positivity in four (44.44%) samples and one sample was found negative by both RT-PCR and dFAT. Further Gene sequencing of 26 fresh and 8 decomposed RT-PCR positive brain samples was done for confirmation .As per this study RT-PCR appears to be a useful diagnostic tool for detection of rabies virus as this test is sensitive for both fresh as well as decomposed brain samples.

Key words: dFAT, RT-PCR, diagnosis, rabies, animal, brain.

Introduction

In India, rabies is enzootic in nature where dog is the principal reservoir of virus for transmission in the country (Bhatia et al., 2007; Chhabra et al., 2003). Approx. 20,000 human rabies deaths are estimated per year in the country (Sudarshan et al., 2007). The description of inclusion bodies in the cytoplasm of nerve cells was the true beginning of rabies diagnosis by Adelchi Negri in 1903. Gold Wasser and Kissling in 1958 described immunofluorescent antibody test which was further modified (Kissling., 1975). Since then Direct Fluorescent Antibody Test (dFAT) has been the most widely used test for diagnosis of rabies and is considered as gold standard due to its high sensitivity and specificity (WHO., 2005).

Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) as an alternative method for routine diagnosis of rabies has been reported earlier by (Sacramento et al., 1991) as it is sensitive in fresh as well as in highly degraded samples. Decomposition of samples in the tropical country like India is a common problem. Reasons may be breakage in cold chain or shipment of samples taking longer time to reach laboratories for diagnosis. The aim of this study was to compare RT-PCR with dFAT for the diagnosis of rabies in fresh and decomposed samples.

Materials & Methods

Study Samples

A total of 85 brain samples of different species viz dog(25), Cat(2), Buffalo (4),Monkey(1), Human(1), Cheetal (51) outbreak in Delhi Zoo,2016 and squirrel (1) received from different states of India (Uttar Pradesh, Delhi, Haryana ,Himachal Pradesh, Punjab and Manipur) from 2012 to 2016 were processed in the Rabies laboratory at NCDC, Delhi for diagnosis of rabies . Of these 76 were fresh brain samples and nine were decomposed brain samples.

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Processing of samples

Each brain sample was processed as soon as it was received in the laboratory. Impression smears were made from hippocampus whenever available or other areas for Seller's Staining and dFAT. 10% suspension from brain sample was prepared in 0.01mol/l phosphate buffer saline (PBS) pH7.4. This was centrifuged at 4000rpm for 10 minutes and supernatant was collected for RT-PCR.

Seller' staining

Smears (moist) immediately after preparing were stained with Basic fuchsin with methylene blue as their base (1:2 ratio). The presence of intracytoplasmic (Pink to purplish-pink in color) small inclusion bodies known as Negri bodies examined under oil immersion in light microscope at 100X indicated the presence of rabies infection.

Direct Fluorescent Antibody Test

The dFAT was performed according to the procedure described by the Office International des Epizooties (OIE) and World Health Organization (WHO). Briefly, the impression smears were air dried for 25-35 minutes and fixed in chilled acetone for 2-4 hrs. The slides were encircled around the smears. Brain samples from mice infected with challenge virus standard (CVS) and normal mouse brain were used as positive and negative controls, respectively. The smears were covered with FITC conjugate anti-rabies antibody (Bio-Rad, USA) and incubated for 30 min in humidified dark chamber at 37°C. The slides were washed twice with PBS (0.01M, pH 7.5) and twice with distill water for 5 minutes each in slide holding glass coplin jar. After washing, slides were mounted (90% buffered glycerol, pH 8.5) examined under fluorescent microscope at 400 nm (Olympus, USA). Apple green fluorescence confirmed the presence of rabies infection in brain sample.

Isolation of RNA

Total RNA from supernatant of brain suspension was extracted by TRIZOL method using a kit (QIAGEN QI Amp Viral RNA Mini Kit). In brief 1000µl of Trizol was added to 200µl of supernatant of brain suspension. This was mixed, incubated at room temperature for 10 minutes, 200µl of chloroform (Amresco, USA) was added and mixed by vortexing and incubated on ice for 5-15minutes with frequent vortexing. The mixture was then centrifuged at 14,000rpm for 15 minutes. The upper layer was collected carefully in sterile micro centrifuge tube and equal volume of 70% ethanol was added and mixed .The whole volume was passed through the spin column twice (700µl each time) by centrifuging at 10,000rpm for 1 minute. Column was washed with AW1 and AW2 wash buffers thrice as per the kit protocol and RNA was eluted in 60 µl elution buffer. Concentration of RNA was estimated by spectrophotometer (Nanodrop 2000cc).

One step Reverse Transcriptase Polymerase Chain Reaction

RT–PCR was performed using Quigen One-step RT–PCR kit. The 443 bp of the nucleocapsid (N) gene of Rabies Virus was amplified with Forward Primer N1 (+) sense: (587)5'-TTT GAG ACT GCT CCT TTT G-3'-(605) and Reverse Primer N2 (-) sense :(1029)5'-CC CAT ATA GCA TCC TAC -3'(1013) .The RT-PCR cycling conditions were: five initial cycles of denaturation (D: 60 seconds at 94°C), annealing (A: 90 seconds at 45°C, then 20 seconds at 50°C) and elongation (E:90 seconds at 72°C)and 30 additional cycles where D and E were reduced to 30 seconds and 60 seconds ,respectively .The final elongation was carried out at72°C for 10 minutes (Tordo et al.,1996).

Amplified products were visualized on 1.2% agarose gel with 0·5 µg/ml ethidium bromide. The PCR products were purified using the QIquick PCR purification kit according to the manufacturer's instructions (Qiagen).

Automated Nucleotide Sequencing

N gene region positive samples were subjected to automated nucleotide sequencing using Big dye terminator cycle sequencing ready reaction kit V3.1 (Applied Biosystems, USA). For each sequencing reaction 25 ng(1µl) of purified PCR product was mixed with 3.2 pmol(1 µl) of respective primer (Forward and Reverse of N gene) and 1 µl of Big dye with 2 µl of 5X sequencing buffer and 5 µl of nuclease free water. The reaction mixture was placed onto a pre-heated ABI 9700 Thermal cycler (Applied Biosystems, USA). Cycle sequencing parameters consisted of 25 cycles at 96°C for 10 seconds, 50°C for 5 seconds and 60°C for 4 minutes. The reaction mixture was purified by precipitation with 3M sodium acetate (pH 4.6) and 100 % ethanol. The purified product was lyophilized, reconstituted in 10µl Hi-Di formamide, incubated at 95°C for 2 minutes and immediately chilled on ice. The chilled sample was mixed and briefly centrifuged before loading onto an ABI 3130XL automated capillary DNA

sequencer (Applied Biosystems, USA). Basic Local Alignment Search Tool was used for confirmation of sequences obtained. N gene sequences were submitted to GenBank, NCBI (National Centre for Biotechnology Information) and accession numbers were acquired.

Calculation of Sensitivity, Specificity, Concordance, Positive Predictive Value & Negative Predictive Value

Sensitivity was calculated using the formula [(TP/TP+FN)] × 100 where TP was the number of samples with true positive results as determined by the reference assay dFAT and FN was the number of samples with false negative results, Specificity and Concordance were calculated using formula [TN/(TN +FP)] × 100 and [(TP+ TN) / (TP+ FP+ FN+ TN)] × 100 respectively where TN was the number of samples with true negative results and FP was the number of samples with false positive results. Positive and Negative predictive values were calculated using formula [TP / (TP + FP)] × 100 and [TN / (TN +FN)] ×100 respectively.

Results

In the present study three diagnostics tests were compared viz Seller' Staining, dFAT and RT-PCR considering dFAT as a gold standard. A total of 85(76 fresh and 9 decomposed brain samples) suspected rabies brain samples of dog (25) ,Cat (2), Buffalo(4),Monkey (1),Human(1) , Cheetal (51) and squirrel (1) from different states of the country were tested(Table 1). Out of 76 fresh brain samples 42(55.26%) samples were found positive by Seller's stain, 46 (60.52%) were found positive by both RT-PCR & dFAT. All the three assays gave negative results for 30 samples. Sensitivity of Seller's staining and RT-PCR was 91.30% and 100% respectively in fresh brain samples whereas Specificity of both Seller's and RT-PCR was 100% with dFAT. The Negative Predictive Values of Seller's and RT-PCR was 88.23% and 100% and Positive Predictive Values for both Seller's and RT-PCR was found to be 100% . Concordance of Seller's and RT-PCR with dFAT was found to be 94.73% & 100% respectively (Table 2). Out of nine decomposed samples tested by dFAT and RT-PCR, four samples (2 dog & 2 cheetal brain samples) were found positive by both dFAT & RT-PCR and four samples (1 cat & 3 cheetal brains) were found positive only by RT-PCR and one sample (1 cheetal brain) was found negative by both dFAT & RT-PCR. Seller's staining was not done in decomposed brain samples. Further sequencing of 26 positive samples and 8 decomposed samples was done for confirmation of diagnosis and accession numbers (KU050052-KU050075 and KY595092-KY595101) were obtained by submitting in Gen Bank (NCBI).

Table 1: Details of brain samples with results:

Host/ Species	Area	Total No.	Seller's		FAT		RT-PCR	
			+	-	+	-	+	-
Human	UP	1	0	1	0	1	0	1
Dog	Delhi, Haryana, HP, Manipur, Punjab	25	16	9	17	8	17	8
Cat	Delhi	2	0	2	0	2	1	1
Monkey	Delhi	1	0	1	0	1	0	1
Cheetal	Delhi	51	22	29	29	22	32	19
Squirrel	Delhi	1	0	1	0	1	0	1
Bovine	HP	4	4	0	4	0	4	0
		85	42	43	50	35	54	31

Table 2: Comparison of tests in fresh brain samples.

		dFAT						
		+	**-**	**Sensitivity**	**Specificity**	**Concordance**	**Negative predictive value**	**Positive predictive value**
Seller's	**+**	30	12	91.30%	100%	94.73%	88.23%	100%
	-	16	18					
RT-PCR	**+**	30	16	100%	100%	100%	100%	100%
	-	16	14					

Discussion

Diagnosis of human rabies is mainly clinic-epidemiological however laboratory holds a prominent place for making a reliable and confirmed diagnosis. Clinically rabies can be confused with Guillian-Barre syndrome, Poliomyelitis and other viral encephalitis (Plotkin. 2000).

Rabies, an acute progressive, fatal encephalomyelitis, transmitted most commonly through the bite of a rabid animal, is responsible for an estimated 61,000 human deaths worldwide (Laboratory Diagnosis of Human Rabie:RecentAdvances.,2013) .In India Association of the Prevention and Control of Rabies (APCRI) estimated 20,000 human deaths due to rabies per year (Sudarshan et al., 2007).

The Laboratory occupies a central place in efforts to monitor control program and surveillance activities. A laboratory report should be as clear and unequivocal, as possible.

A quick and easy procedure for diagnosis of rabies is the detection of Negri bodies by Seller's staining first recognized by Adelchi Negri, 1903 and remained the mainstay of diagnosis for more than half a century before FAT was introduced in 1958. However, the sensitivity of histological techniques is much less than that of immunological methods, especially if there has been some autolysis of the specimen. Consequently, histological techniques can no longer be recommended (OIE, 2008). In this study 42 brain samples showed Negri bodies.

The most widely used test for postmortem rabies diagnosis is the dFAT, which is recommended by both World Health Organization (WHO) and World Organization for Animal Health (OIE).First described by Gold wasser and Kissling in 1958 which was subsequently modified (Dena et al,.1973; Kissling., 1975), dFAT is considered as a gold standard for rabies diagnosis (WHO., 2005) the specificity and sensitivity of the test almost approach 99% in an experienced laboratory and results are available within a few hours. Reliable results are obtained only when fresh brain tissue is used; Partially decomposed brains are not suitable for this test as it is very difficult to differentiate specific antigen from nonspecific fluorescence which may result from bacterial contamination in case of inconclusive DFA results Virus isolation is recommended by OIE (Laboratory diagnosis. 2013). Confirmatory tests for DFA are the tissue culture infection test (RTCIT) and mouse inoculation test(MIT) previous studies also describes the importance of virus isolation (Chhabra et al.,2005; Chhabra et al.,2007). In this study, of the 76 fresh brain samples 46 samples were positive and out of nine decomposed brain samples four samples were found positive by dFAT. Low sensitivity of dFAT might be due to condition of samples (decomposed), improper storage of samples, load of virus, stage of diseases and preservatives used (Trimarchi, 2000).

In our study RT-PCR detected rabies virus in 54 brain samples (46 fresh brain samples and eight decomposed brain samples). RT-PCR showed 100% Sensitivity, Specificity and Concordance with dFAT in fresh brain samples. A previous study also showed 100% Sensitivity and Specificity of RT-PCR as compared to 83.3% sensitivity of dFAT (Biswal. et al, 2012). Eight out of nine

decomposed brain samples were found positive by RT-PCR whereas only four tested positive by dFAT. Higher sensitivity and specificity of RT-PCR compared to dFAT has been reported earlier. Previous studies (Heaton., et al, 1993; Kamolvarin., 1997) in which the brain samples left at room temperature for 72 and 360 hours reported the detection of rabies virus from decomposed samples. In decomposed tissue the RNAases degraded the genome into smaller segments (David. 2008; Rojas. 2006) so results of our study demonstrated the importance of RT-PCR in detection of rabies virus in decomposed brains. Earlier studies (David.,2002 ;Biswal.,2007.,Beltran.,et al,2014) also reported the detection of rabies virus from decomposed brain and even in formalin- fixed tissues (Warner et al.,1997). Decomposed brain samples detected positive for rabies virus by RT-PCR were subjected to the sequencing for confirmation- Sequencing also plays an important role in strain identification and disease epidemiology and may help to identify the source of infection (Biswal. et al, 2012).

However RT-PCR and other amplification techniques is not recommended for routine diagnosis of rabies in humans and in animals ,but is recommended for epidemiological survey with standardization and very stringent quality control and expertise and experience (WHO.,2005; OIE.,2008)

Negative RT-PCR result in brain sample does not rule out rabies as there may be possibility that the sample may be badly deteriorated and not suitable for RNA extraction in such cases the epidemiological information for administrating appropriate treatment must be considered.

Conclusion

RT -PCR has comparable results with dFAT for diagnosis of rabies when the tests are performed in fresh brain samples. However, RT-PCR was more sensitive and specific for diagnosis of rabies in partially decomposed samples as compared with dFAT.

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