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ANTE MORTEM DIAGNOSIS OF RABIES FROM BODY SECRETION/EXCRETIONS: A REVIEW

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ABSTRACT

The clinical diagnosis of rabies suggested by epidemiological and clinical manifestations is not reliable and often mistaken for other disorders. Differentiation from other neurologic diseases requires extensive investigations resulting in late confirmatory diagnosis. It leads to increased contacts and extensive exposure to rabid animals and lays unnecessary financial burden to render post exposure prophylaxis. Conventional diagnostic assays are rarely optimal and entirely dependent on the nature and quality of the sample supplied. In the course of the past few decades, the application of molecular biology has aided in the development of tests that result in a more rapid detection of rabies virus and also enable viral strain identification from clinical specimens. Rabies virus though neurotropic spread to almost all organs by centrifugal spread this has led to ante mortem diagnosis of rabies from body secretion and excretions using highly sensitive molecular approaches. The challenges in the 21st century for the development of rabies diagnostics are soon going to end up with molecular based diagnostic assays. These assays will no longer remain unaffordable for routine use considering the cost/benefit ratio and will be validated and accepted internationally. Their global acceptance and implementation where they are most needed will provide substantial improvements for rabies diagnosis and surveillance. This paper provides a review of recent publications focusing precisely on the ante mortem diagnosis of rabies from body secretion/excretions in order to highlight the sampling methods and techniques that are likely to give the most reliable results.

Key Words: *Nested RT-PCR, TaqMan Real Time PCR, Saliva, Urine, Rabies*

INTRODUCTION

Rabies virus belongs to the order *Mononegvirales*, family *Rhabdoviridae*, genus *Lyssavirus*, and is a bullet-shaped virus with an approximate length of 180 nm and diameter of 75 nm. The genome consists of a single-stranded, negative-sense, non-segmented RNA, 12 kb in length. Five genes (3'-N-P-M-G-L-5') encode for five proteins: nucleoprotein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G), and the polymerase (L) Fauquet *et al.*, (2005).

Rabies is a neurological disease, but the rabies virus spread to several organs outside the central nervous system by centrifugal spread. The rabies virus antigen or RNA has been identified from the salivary glands, the lungs, the kidneys, heart, liver and almost every organ Jogai *et al.*, (2002), Vieira *et al.*, (2011). Balachandran and Charlton (1994) reported the ultra structural lesions in rabies-infected adrenal medulla, cornea, and nasal glands.

Seroconversion in acute rabies patients is often delayed or absent. In addition, vaccine-acquired antibodies in patients who received rabies biological (full or partially completed vaccination regimens) may interfere with accurate serological laboratory confirmation of cases. Therefore, laboratory diagnosis of ante mortem rabies cases should be aimed at detecting *Lyssavirus* RNA in body fluids i.e., saliva, cerebrospinal fluid (CSF), tears, and urine Blumberg *et al.*, (2007), Dacheux *et al.*, (2008) and Wacharapluesadee *et al.*, (2010).

Here the present study aims to lead a precise focus on the ante mortem diagnosis of rabies from body secretion/excretions. This paper provides a review of recent publications relating to the evaluation of methods for rabies diagnosis from secretion/excretion in order to highlight the sampling methods and techniques that are likely to give the most reliable results. The advantages of RNA detection methods are

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in the transferability of the technology to a wide variety of other sample types that may be unsuitable for the direct fluorescent antibody test, such as saliva and cerebrospinal fluid Crepin *et al.*, (1998) PCR has been used for the confirmatory diagnosis of human rabies when other tests could not be readily applied, Centers for Disease Control and Prevention (1997).

Biological Fluid Samples for Intravital Diagnosis of Rabies

With the advent of molecular approaches, it is now possible to detect rabies ante mortem from range of biological samples e.g. nuchal skin biopsy (Dacheux *et al.*, 2008), saliva (Crepin *et al.*, 1998), CSF (Saengseesom *et al.*, 2007) and urine samples (Wacharapluesadee and Hemachudha 2010).

Sensitivity of testing saliva was higher than testing extracted hair follicles CSF and urine. While the sensitivity from liquid saliva samples was greater than in saliva swab samples Dacheux *et al.*, (2008). RT-PCR assay of saliva allowed a positive intravital diagnosis in all of the nine laboratory-confirmed cases of human rabies presented Crepin *et al.*, (1998).

Saengseesom *et al.*, (2007) conducted study in order to look for evidence of rabies virus in saliva and cerebrospinal fluid (CSF) of suspected live rabid dogs at the time of quarantine by using a SYBR Green real-time RT-PCR based assay for the detection of rabies virus RNA. Hemachudha and Wilde (2009), Wacharapluesadee and Hemachudha (2010) conducted ante mortem rabies diagnosis from CSF samples.

Debbie & Trimarchi (1970) showed the presence of rabies virus within tubular cells of the kidneys. The same authors reported that mice inoculated with urine from a rabid fox died of rabies.

Wacharapluesadee and Hemachudha (2002), Dacheux *et al.*, (2008) and A Kaw *et al.*, (2011) used urine as an ante mortem specimen for confirmatory rabies diagnosis using molecular techniques.

Tears can also be a source of the virus, which was demonstrated in at least one patient using RT-nPCR, where corneal impressions and CSF were negative by DFA and RT-nPCR, respectively (Elmgren *et al.*, 2002)

In our study also for ante mortem diagnosis of rabies (unpublished data) saliva, milk and urine was used with resultant higher sensitivity. Milk may not be feasible tool for intravital diagnosis of rabies in lactating animals as it offers a lowest sensitivity.

Collection, Transport and Preservation of Samples

Results obtained with molecular assays have close relationship with timing of sample collection related to the clinical onset and the type of sample. Also the transport and preservation of intravital biological fluid samples is of utmost importance. Various biological fluid samples which can be used for intravital rabies diagnosis should be stored at -20°C or below WHO expert consultation (2005).

Cotton swabs were used for collection of saliva samples directly from oral cavity of rabies suspected animal by Larghi *et al.*, (1975), Crepin *et al.*, (1998), Shankar *et al.*, (2004) and Johnson *et al.*, (2008).

Sponge tipped applicators were immersed into 2ml of phosphate buffered saline prior to swabbing the anterior surface of tongue and cheek mucosa (of the dog) for 15-20 sec. by Kasempimolporn *et al.*, (2000) and Saengseesom *et al.*, (2007).

Aspiration technique was reported by Crepin *et al.*, (1998) for daily collection of saliva samples (from human patients) for intravital diagnosis of rabies.

Liquid saliva sample (200 µl) was preferred than saliva swab by Nagaraj *et al.*, (2006), Dacheux *et al.*, (2008), Hemachudha *et al.*, (2009), Wacharapluesadee *et al.*, (2010) and Brito *et al.*, (2011).

Use of sterile eye dropper pipette and syringe for collection of saliva sample directly from the mouth of animal was suggested by centers for disease control and prevention (CDC) and Kaw *et al.*, (2011).

An indwelling urinary catheter was inserted in all patients suspected of having rabies therefore; urine samples can be obtained throughout the illness (Wacharapluesadee and Hemachudha 2002).

As per Dacheux *et al.*, (2008) at least 1 ml and minimum three urine samples should be collected in an interval of 3–6 hours and stored at -20°C/-80°C.

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At least 2 ml of urine should be collected once daily and repeat testing is to be done if result is negative. Fresh urine is preferable to stored samples Hemachudha and Wacharapluesadee (2004), Wacharapluesadee and Hemachudha (2001) and Wacharapluesadee *et al.*, (2008).

Kaw *et al.*, (2011) collected urine samples from the rabies suspected animals directly while animal was urinating.

Because the viral shedding in saliva, CSF, and urine is suggested to be intermittent, multiple and different samples (e.g. saliva, urine) must be collected and analyzed by molecular methods for an intra-vitam rabies diagnosis (Dacheux *et al.*, 2008, Wacharaplusadee and Hemachudha 2001).

All specimens from suspected rabies patients should be handled with caution and considered infectious. Sample containers must be sealed securely. Samples should be taken under sterile techniques in a sterile container and transferred on ice to the laboratory within 24 hour after collection. If not, they should be stored frozen at -20°C or below and then shipped frozen on dry ice.

When it is not possible to send refrigerated samples, other preservation techniques may be used. Whenever possible samples should be kept in glycerol/saline and refrigerated. As the virus is not inactivated by glycerol/PBS, all laboratory tests can be used on these samples Shankar (2009)

Oropharyngeal swabs were collected and stored in tubes containing 1.5 ml lysis buffer at -80°C by Echevarria *et al.*, (2001), Jackson *et al.*, (2008) and Reyens *et al.*, (2011).

In our study of ante mortem diagnosis from secretion/excretion saliva (collected directly or by syringe aspiration), milk (strip milking of teats of lactating animals), urine samples (direct while urinating or urethral catheterization) were collected in sterilized vials from the animals suspected for rabies.

Choice of the Most Appropriate Molecular Techniques for Viral RNA Detection

Molecular tools based on the detection and manipulation of the genetic information of the virus is becoming more widely accepted and accessible for the diagnosis of rabies. The advent of molecular biology is changing the face of diagnostic virology generally enabling high throughput and short turn around-time analysis of samples. In the 21st century, it is expected that diagnostic virology techniques for high throughput rabies virus detection will progress rapidly towards the use of molecular diagnostics replacing more conventional testing techniques such as virus isolation and histopathology.

New technological advances will undoubtedly be faster, more accurate and may, in time, offer a cost-effective alternative to traditional rabies diagnostic tests. These paradigm shifts including modern advances in technology will lead to the effective control of rabies in animals and wildlife Rupprecht *et al.*, (2006).

RNA Extraction and Purification

RNA extraction is the most critical phase during the virus studies as RNA is highly degradable. All surfaces where RNA extraction is being carried out should be free from RNase and all other inhibitors. Various commercial preparations available in market like RNase Zap (Applied Biosystem) can be used. Always gloves should be put on and barrier pipette tip should be used in all nucleic extraction steps, since as little as 20 ng of contaminating material can produce a false-positive reaction Trimachi and Smith (2002).

The area for sample preparation should be separate from amplification and post-amplification areas to prevent contamination. A known rabies-negative control sample should be included through all steps of extraction and amplification.

RNA extraction with TRIzol®, a commercial reagent, is the most common method for total rabies RNA purification. TRIzol reagent is a monophasic solution of phenol and guanidine isothiocyanate.

Total RNA was extracted from saliva or CSF specimens using TRIzol® (GIBCO-BRL, Gaithersburg, Md, USA) by Elmgren *et al.*, (2002) and Saengseesom *et al.*, (2007). Precipitated RNA was dissolved in 20 to 40 µl of diethyl polycarbonate water and stored at -80°C for further use.

TRIzol® (Invitrogen, Canada) reagent protocol was used for extraction of RNA by Shankar *et al.*, (2004) and Brito *et al.*, (2011). Former also supported addition of 100 µl lysis buffer (10 mMTris-HCL, pH 7.5,

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150 mM NaCl, 1.5mM MgCl₂ and 0.65% NP-40) with ~100 µl of the saliva sample prior to addition of Trizol[®].

TRIzol LS reagent (Invitrogen, Canada) was used for RNA extraction with a Roche MagNA Pure LC (model JE 379, Roche Diagnostics, Indianapolis, Indiana, USA) system by Jackson *et al.*, (2008).

TRIzol LS reagent (Invitrogen, Canada) was used for recovery of RNA from human and bat saliva samples by Nadin Davis *et al.*, (2009) and Reynes *et al.*, (2011) respectively.

The QIAamp UltraSens Virus kit (Qiagen) was used to extract RNA from a 1-mL urine sample, according to the manufacturer's instructions. QIAamp viral RNA mini kit (Qiagen, Germany) was used for extraction of RNA from 200 µl saliva samples by Johnson *et al.*, (2008), Coertse *et al.*, (2010) and Panning *et al.*, (2010).

Glycoblue (Ambion) and Glycogen (Ambion) were used as co-precipitant along with Trizol LS reagent for extraction of RNA by Hughes *et al.*, (2004) and Dacheux *et al.*, (2008) respectively.

Use of Guanidinium thiocyanate together with silica particles for purification of RNA was reported by Crepin *et al.*, (1998) and Echevarria *et al.*, (2001).

Cationic surfactants (Catrimox-14; Iowa Biotechnology Corporation) and Chelating resin (Chelex 100; Bio-Rad) techniques explored by Crepin *et al.*, (1998) for production of RNA yielded negative results.

Proteinase K method [200 µl of fluid sample was incubated for 2 hrs at 37°C with 400 µl of Proteinase K buffer containing 40 µg of Proteinase K (Gibco BRL)] prior to purification by phenol-chloroform extraction was reported by Crepin *et al.*, (1998).

Nucleic Acid Amplification Target

The N gene is mostly conserved and strongly expressed in lyssaviruses. Its sequences have been the most exhaustively reviewed Bourhy *et al.*, (1993), Kissi *et al.*, (1995) Delmas *et al.*, (2008) and it has become the most common amplification target. It has been extensively used as a target for real-time PCR assay.

N gene was used as an amplification target in saliva samples Hemachudha and Wilde (2009), Wacharapluesadee and Hemachudha (2010), Nagaraj *et al.*, (2006), Crepin *et al.*, (1998), Dacheux *et al.*, (2008), Kaw *et al.*, (2011), Saengseesom *et al.*, (2007).

Dacheux *et al.*, (2008) used L gene as an amplification target for saliva samples.

Hemachudha and Wilde (2009), Wacharapluesadee and Hemachudha (2010) used N gene amplification target for ante mortem diagnosis of rabies from urine as well as CSF samples.

Reverse-transcription PCR

Nested RT-PCR

Whitby *et al.*, (1997) concluded that reverse transcriptase-polymerase chain reaction (RT-PCR) was a useful additional tool for the detection of rabies and rabies related viruses, which was easy to perform and was rapid and highly sensitive.

Luo-Ting Rong *et al.*, (2000) developed a nested polymerase chain reaction (PCR) for detecting rabies virus and revealed that PCR could detect 3TCID₅₀ of rabies virus and gave a positive result with 0.8pg of RNA. Nested PCR could identify RNA of rabies virus in the liver, heart, lung, and spleen of mice 5 days after inoculation.

In our study sensitivity obtained with nested RT-PCR using saliva, milk and urine samples was 68%, 54.54% and 62.5% respectively (unpublished data).

Hemi Nested RT-PCR

Crepin *et al.*, (1998) tested an optimized reverse transcription (RT)-PCR protocol for the intravital detection of rabies virus genomic RNA in clinical samples obtained from 28 patients suspected of having rabies, 9 of whom were confirmed to have had rabies by postmortem examination.

Noah *et al.*, (1998) observed a higher sensitivity of >98% was obtained by using RT-PCR for ante-mortem diagnosis of rabies in human saliva samples.

RT-PCR on saliva for viral nucleic acid detection yielded a sensitivity of 50-70% and a specificity of 100% Madhusudana and Sukumaran (2008).

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Dacheux *et al.*, (2008) standardized a new reverse-transcription; heminested polymerase chain reaction (hnRT-PCR) protocol at 3 participating centers in Cambodia, Madagascar, and France. In this study, saliva samples provided the second-best results for sensitivity testing (63.2% [57 samples in group 1] and 70.2% [84 samples in group 2]). A sensitivity of 100% was obtained with the saliva sample when analyzed at least 3 successive samples per patient.

Heaton *et al.*, (1997) described a heminested reverse transcriptase PCR (hnRT-PCR) protocol which is rapid and sensitive for the detection of rabies virus and rabies related viruses. Sixty isolates from six of the seven genotypes of rabies and rabies-related viruses were screened successfully by hnRT-PCR and Southern blot hybridization. Of the 60 isolates, 93% (56 of 60) were positive by external PCR, while all isolates were detected by heminested PCR and Southern blot hybridization.

Soares *et al.*, (2002) evaluated heminested-PCR (hnRT-PCR) using primers to the nucleoprotein-coding gene in a nested set in the detection of Brazilian strains of rabies virus (RV). A representative number of RV nucleoprotein sequences belonging to genotype 1 were aligned. Based on such alignment, primers were directed to highly conserved regions. All 42 clinical samples positive by both fluorescent antibody and mouse inoculation tests were also positive by the hnRT-PCR.

Picard Meyer *et al.*, (2004) developed a simplified hemi-nested reverse transcriptase polymerase chain reaction (hnRT-PCR) to determine specifically the European Bat Lyssavirus nucleoprotein gene (EBLV-1) and observed that compared to the rabies diagnostic methods, the hnRT-PCR showed a higher sensitivity for the detection of small amount of EBLV-1 virus.

Lima *et al.*, (2005) evaluated the heminested RT-PCR for the study of rabies virus distribution in mice inoculated experimentally. Inoculation was by the intramuscular route in 150 mice, using the dog street rabies virus. HnRT-PCR was shown to be more efficient for the study of rabies virus distribution in different tissues and organs viz. brain, spinal cord, salivary gland, limbs, lungs, liver, spleen, urinary bladder, tongue and right kidney.

Araujo *et al.*, (2008) evaluated the RT-PCR and hnRT-PCR for rabies virus detection in original tissues stored at -20°C for different periods considering their use for rabies virus detection in stored and decomposed samples. The RT-PCR and hnRT-PCR results were compared with previous results from Direct Fluorescent Antibody Test and Mouse Inoculation Test. From the 50 positive fresh samples, 26 (52%) were positive for RT-PCR and 45 (90%) for hnRT-PCR. From the 48 positive decomposed samples, 17 (34.3%) were positive for RT-PCR and 36 (75%) for hnRT-PCR.

Real-time PCR

In molecular biology, real-time polymerase chain reaction, also called quantitative real time polymerase chain reaction (Q-PCR/qRT-PCR) or kinetic polymerase chain reaction (KPCR), is a laboratory technique based on the PCR, which is used to amplify and simultaneously quantify a targeted DNA molecule. For one or more specific sequences in a DNA sample, Real Time-PCR enables both detection and quantification. The quantity can be either an absolute number of copies or a relative amount when normalized to DNA input or additional normalizing genes.

The procedure follows the general principle of polymerase chain reaction; its key feature is that the amplified DNA is detected as the reaction progresses in real time. This is a new approach compared to standard PCR, where the product of the reaction is detected at its end. Two common methods for detection of products in real-time PCR are: (1) non-specific fluorescent dyes that intercalate with any double-stranded DNA, and (2) sequence-specific DNA probes consisting of oligonucleotides that are labeled with a fluorescent reporter which permits detection only after hybridization of the probe with its complementary DNA target.

SYBR Green assay

The products of SYBR Green-based real-time PCR can be detected by a DNA-intercalating dye (SYBR Green), which detects all double stranded DNA products within a reaction. PCR product confirmation can be achieved with nonspecific fluorescent dyes by performing melting (denaturation) curve analysis at the

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completion of the amplification. SYBR Green detection chemistry is preferable when more variability of probe-target binding sites is presented Wacharapulsadee and Hemachudha (2010).

Nagaraj *et al.*, (2006) evaluated the utility of conventional RT-PCR and SYBR Green I Real time PCR in the ante mortem diagnosis of rabies using saliva samples. Saliva samples collected from twenty-four patients presenting with typical clinical manifestations of rabies were tested in the two assays. Real time PCR assay was more sensitive than conventional RT-PCR assay (sensitivity 75% versus 37%, $p = 0.0189$).

Saengseesom *et al.*, (2007) conducted a study in order to look for evidence of rabies virus in saliva and cerebrospinal fluid (CSF) of suspected live rabid dogs at the time of quarantine. In this study, 13 out of 15 dogs (87%) had positive saliva and four out of 15 had positive CSF by SYBR Green assay when primary PCR product was used as the template

In a study, sensitivity of 62.5% and specificity of 100% from saliva samples was observed with SYBR green real time PCR Kaw *et al.*, (2011).

TaqMan assay

TaqMan assay is one of the most popular chemistries employed by real-time PCR. It uses dual-labeled oligonucleotide probes that are labeled at the 5' end by a reporter dye and at the 3' end by a quencher that prevents emission of reporter dye until the two are in close proximity. The probe detection system represents an advantage on specificity over the conventional RT-PCR.

The TaqMan assay is rapid, sensitive, and specific and allows for the genotyping of unknown isolates concomitant with the RT-PCR. The assay can be applied quantitatively and the use of an internal control enables the quality of the isolated template to be assessed. Despite sequence heterogeneity in the N gene between the different genotypes, a universal forward and reverse primer set has been designed, allowing for the simplification of previously described assays (Wakeley *et al.*, 2005).

The sensitivity of the real-time assays, either with TaqMan or SYBR Green, is often comparable with or even better than conventional RT-PCR or nested PCR (Wakeley *et al.*, 2005, Foord *et al.*, 2006, Smith *et al.*, 2002, Nagaraj *et al.*, 2006).

The TaqMan-based assay was 1000-fold more sensitive than traditional RT-nested PCR in raccoon rabies viral standard stock Nadin-Davis *et al.*, (2009). One-step TaqMan RT-PCR was ten-fold more sensitive than the RT-hnPCR and the limit of detection of rabies virus was 0.1 and 10 TCID₅₀/ml, respectively Orłowska *et al.*, (2008).

TaqMan real time PCR could be advantageous in lot ways over that of other conventional techniques and also other molecular approaches such as Nested and Heminested PCR. In this assay multiple transfers of materials is not required and it is done in a single closed tube that makes the virus detection fast and minimizes the opportunity for cross-contamination observing the results under UV lamp in the presence of ethidium bromide commonly known as a carcinogen has to be used. The incorporation of TaqMan technology into a real-time assay not only enables the *Lyssavirus* template to be detected but also allows it to be genotyped Orłowska *et al.*, (2008).

The real-time RT-PCR assay is less than half as expensive as the NASBA-ECL assay and twice as rapid. For rabies amplification and detection, TaqMan real-time RT-PCR requires the shortest assay time of only 1.5 h, while NASBAECL, real-time NASBA and RT-hnPCR require 3.0 h, 2.0 h and 5.0 h respectively. The NASBA assay includes an extensive time course between pre-amplification steps, and the number of handling steps per sample for RT-hnPCR was higher than the other four methods. Although this advantage may increase risk of PCR product carry over contamination (Vander Meide *et al.*, 2008).

Ante mortem diagnosis of human rabies using TaqMan real-time PCR targeting the highly conserved N gene Hughes *et al.*, (2004) Nadin-Davis *et al.*, (2009) Wacharapulsadee *et al.*, (2008) in conjunction with RT-hnPCR which targets conserved blocks among the L gene Dacheux *et al.*, (2008), would likely ensure an accurate diagnosis Wacharapulsadee *et al.*, (2011).

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The TaqMan PCR assay to detect RV RNA was as sensitive as the primary PCR assay but had a considerably reduced detection limit compared to the detection limit of a heminested PCR. Methods adopted to increase sensitivity, such as increasing the template concentration and the number of thermocycles, failed to increase the detection limit of the assay Hughes *et al.*, (2004).

The single-tube TaqMan RT-PCR assay performed well compared with the gel-based assay with respect to its sensitivity. A direct comparison of the heminested assay and the TaqMan assay revealed that a positive *Ct* value was still obtained when only 1 pg of total RNA was added to the assay, whereas 100 times more RNA was required to generate a visible band on an ethidium bromide-stained agarose gel. In addition, with the cloned classical rabies virus N gene sequence, only one molecule was required in order to produce a positive result in the single-tube TaqMan assay, which is the theoretical minimum amount of template required to produce a positive *Ct* value Wakeley *et al.*, (2005).

The single-tube TaqMan assay is considerably faster to perform than the heminested RT-PCR assay, with the process taking 2 h 11 min, including a 30-min reverse transcription step. This can be reduced to a 2-min reverse transcription step for experimental samples with known levels of template RNA, reducing the overall time to perform the assay to 1 h 43 minutes Wakeley *et al.*, (2005).

Heminested PCR was more sensitive than the primary TaqMan assay. However, after performing a primary PCR with the cDNAs from salivary glands and oral swab specimens, the primary PCR amplicons, when used as templates in the TaqMan assay, produced positive *Ct* values Shankar *et al.*, (2004).

Twenty-one ante mortem samples collected over a 19-month period were evaluated for the presence of *Lyssavirus* RNA. Real-time PCR and hnRT-PCR were performed on original RNA (extracted on the collection date) stored at -70°C. Ten saliva samples tested *Lyssavirus* positive with both hnRT-PCR assays and real-time PCR and were subsequently quantified with the external standard curve. Copy numbers ranged from 149 to 37,600 RNA copies per reaction Coertse *et al.*, (2010.)

A percent positivity obtained with TaqMan real time PCR was 30% and 8% when 200 µl volume of saliva and CSF samples respectively were used for analysis and proteinase K lysis step followed Crepin *et al.*, (1998).

In our study sensitivity of 77.27%, 60% and 78.94% was obtained by TaqMan real time PCR from saliva, milk and urine samples respectively when tested with newly designed TaqMan primer/probes (unpublished data).

Mismatches between TaqMan primer and probe sequences and the target sequence may occur and are clearly detrimental to amplification. The number of mismatches reduces the efficiency of the reaction such that more than four differences can result in a weakly positive or negative result. A point mutation at the center of the probe (the site used for single nucleotide polymorphism detection) can prevent generation of a fluorescent signal although the PCR itself proceeds normally Hughes *et al.*, (2004).

Nucleic Acid Sequence-Based Amplification (NASBA)

The percent positivity obtained with Nucleic Acid Sequence Based Amplification (NASBA) on saliva, urine and CSF samples was 75.8% (n = 62), 39% (n=41), 43.3% (n=30) respectively. Hemachudha and Wilde (2009), Wacharapluessadee and Hemachudha (2010).

CONCLUSION

An earlier confirmation of diagnosis leads to lessen the contacts with affected animals and also helps in making timely decisions about PEP administration. The appropriate ante-mortem diagnostic methods should have high sensitivity that is sufficient for early detection of a very low load of rabies viral RNA in biological samples. Nucleic acid amplification techniques can be performed within hours and have been emerged with promising results and as an adjunct to other tests or even as a sole procedure in experienced laboratory facilities for ante-mortem diagnosis. Good sample collection and preparation can increase success of molecular diagnosis. Factors that may influence the quantity and quality of viral RNA are the stage of rabies infection, type of sample available for testing or proper handling of samples. RNA

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extraction control systems have been used for control of the RNA integrity to avoid false-negative results in each individual tube. These may be due to handling errors, the presence of enzyme inhibitors or viral RNA degradation. The quality and quantity of viral RNA can affect the efficiency of primer binding and amplification.

Currently high-throughput rabies virus molecular detection methods augment standard diagnostic tests or are in the process of development and refinement for use alone. As we progress through the 21st century, it is possible that these techniques will replace conventional tests while surpassing the obstacles of cost, complexity and local acceptance of their use.

It is also concluded that the various biological fluid samples including saliva, urine, CSF offers a great sensitivity and specificity for earliest ante mortem diagnosis of rabies if performed with optimized molecular approaches. It is need of hour to explore these protocols to the widest extent and make them as routine laboratory diagnostic practices of efficient surveillance and prevention of rabies in the rabies endemic countries.

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