APPROACHES FOR ANTEMORTEM DIAGNOSIS OF RABIES

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ABSTRACT
Diagnosis of rabies is of paramount importance. The diagnosis of this deadly disease has gained impetus with advent of molecular approaches. The major blockade in the usefulness of rabies diagnosis was the waiting period associated with dependence of most of the authentic diagnostic approaches on the brain tissue that would be available only after the death of the suspected animal. Now, with standardization of molecular approaches, antemortem diagnosis of rabies is becoming realizable with significant authenticity. The present paper reviews various molecular approaches that offer promise for antemortem diagnosis of rabies from saliva, skin, hair follicles and urine of animals. Along with the characteristics of the symptoms, the time tested pathological alterations and the gold standard immune pathological approaches have also been highlighted.

Key Words: Antemortem, Diagnosis, Rabies

INTRODUCTION
Rabies is an infectious, fatal disease characterized by profound dysfunction of the central nervous system, caused by single stranded negative sense RNA virus belonging to the genus Lyssavirus of the family Rhabdoviridae (Tordo et al., 1986). The virus particles have a bullet shaped structure with a diameter of 75nm and a length of 100-300nm (Meslin et al., 1996). Rabies is worldwide in distribution, with the exception of a few areas and countries that have been free from it primarily due to their geographical isolation. Rabies is endemic in India except for probably the water-locked islands of Lakshadweep and Andaman and Nicobar (APCRI 2003). An estimated 55,000 people, mostly (88%) in Asian countries and particularly in the Indian subcontinent, die of rabies each year (WHO, 2005). A National Multicentric Rabies Survey conducted by the Association for Prevention and Control of Rabies in India in collaboration with WHO revealed an annual incidence of 20,565 human deaths per year due to rabies in India (Sudarshan et al., 2007). The mortality caused by rabies ranks 10th among the infectious diseases. About 2.3 million people receive anti-rabies vaccination every year (APCRI, 2003). Although the loss of livestock due to rabies is significant, there are few publications on estimates of the incidence of rabies in livestock (Knobel et al., 2005). The appearance of overt disease is usually preceded by a prodromal period in which there are a number of non-specific symptoms of malaise (Woldehiwet 2005, De Mattos et al., 2001). In infected clinical cases, the distribution of lesions in the nervous system depends on the mode of transmission. It is invariably fatal, once symptoms appear. Rabies is transmitted from animal to animal or animal to humans through bites scratches on skin or licks on the mucosal surface (De Mattos et al., 2001).

The absence of definite cure and certainty of death makes this disease more dreadful than any other known disease. Therefore, early detection of this dreaded disease is of great significance. However, until 1958, the classical Negri-body examination was used and accepted as the only diagnostic tool. Later, the specific detection of rabies viral antigen by Fluorescent Antibody Test (FAT) was developed (Dean and Abelseth, 1973) and since then this technique is one of the most accurate and reliable laboratory test available for diagnosis of rabies. But application of this approach is possible only post mortem i.e. after death; however, with the advent of molecular approaches, it is now possible to detect rabies ante-mortem i.e. before death with the reverse transcription-polymerase chain reaction (RT-PCR). PCR has become a
valuable tool being faster than MIT and more sensitive and specific than the FAT. It can also be used for rapid epidemiological analysis (Heaton et al., 1999).

Since rabies virus is secreted and excreted in all secretions and excretions of rabid animals, the molecular approaches can be employed for reliable in-vitro diagnosis. Ante-mortem detection of rabies by molecular techniques based on detecting virus or viral RNA has been reported in body fluids of live animals such as saliva (Crepin et al., 1998) and CSF (Saengseesom et al., 2007). The rabies virus is also present in nerve cells surrounding the base of hair follicles (Madhusudana and Sukumaran 2008). The ante-mortem diagnosis of rabies can be established from skin samples collected from living animals (Dacheux et al., 2008). The rapid identification of suspect rabies infection is essential to allow specific control strategies (Heaton et al., 1999).

Real-time PCR has been developed (Nagaraj et al., 2006) to increase sensitivity and to obtain results even faster by using both SYBR Green based RT-PCR (Saengseesom et al., 2007) and TaqMan based real-time RT-PCR (Hughes et al., 2004).

**Structural Morphology**

Melnick and McCombs (1966) suggested the term Rhadbovirus after it was found that the unusual bullet-shaped morphology of vesicular stomatitis virus was shared with other viruses such as Rabies and Sigma virus. Thus, in 1970, the International Committee on Nomenclature of Viruses recommended it after the term Stomatovirus was rejected as inappropriate (Wildy 1971).

In the present universal taxonomic scheme of the International Committee on Taxonomy of Viruses, its usage has been finalized as the family Rhadboviridae (Fenner 1976) in the order Mononegavirales (Pringle 1991). Rabies virus possesses a single-stranded, nonsegmented, negative-sense RNA approximately 12 kb in length (Tordo et al., 1986). Rhadboviruses are enveloped, rod-shaped particles. Typically, mature virions appear either as bullet-shaped particles with one rounded and one flattened end, or as bacilliform particles that appear hemispherical at both ends (de Mattos et al., 2001).

The viral genome encodes five structural proteins: nucleoprotein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G), and RNA-dependent RNA polymerase (L) (Wunner 1988) and all are capable of eliciting clinical rabies in mammalian species (Tordo et al., 1998). The G protein controls major aspects of host cell infection, such as receptor binding, antigenicity, and host adaptation (Badrane and Tordo 2001). The order of the genes, namely 3'-N-P-M-G-L-5', is highly conserved (Finke and Conzelmann 2005).

The Lyssavirus genus includes seven genotypes: rabies virus (RABV, genotype 1), Lagos bat virus (genotype 2), Mokola virus (genotype 3), Duvenhage virus (genotype 4), and European bat Lyssavirus 1 (EBLV-1, genotype 5), European bat Lyssavirus 2 (EBLV-2, genotype 6), and Australian bat Lyssavirus (ABLV, genotype 7) (Arai et al., 2003). In addition to the rabies virus (RABV, genotype 1), Mokola virus (genotype 3), Duvenhage virus (genotype 4), European bat Lyssavirus 1 (genotype 5), European bat Lyssavirus 2 (genotype 6), and Australian bat Lyssavirus (genotype 7) have been associated with cases of human rabies (Jackson et al., 2003). The viruses are also called rabies related viruses.

Four new rabies-related viruses (Aravan, Khujand, Irkut, and West Caucasian bat viruses) have been isolated recently from Eurasian bats, and are described as new putative lyssavirus species. There is a reduced protection with pre-exposure vaccination and with conventional rabies post-exposure prophylaxis against all four new bat variants of rabies virus (Hanlon et al., 2005).

**Symptomatology**

Despite development of advanced diagnostic approaches, yet, the significance of keen observation of clinical signs of rabid animals cannot be ignored. And appearance of characteristic disease is preceded by prodromal period in which there are a number of non-specific symptoms of malaise (Bishop 1979). (Sinchaissri et al., 1992) suggested that clinical signs such as ataxia or depression leading to death may be due to the direct effect of the virus on the functions of neuronal cells, but not to inflammatory reactions. Woldehiwet (2005) suggested that the clinical disease can be divided into three phases: (1) Prodromal phase, (2) Excitation phase, furious rabies or acute neurologic phase, and (3) Paralytic phase, dumb rabies
or coma preceding death. However, these phases may not be distinct, as some of the early phases are not always apparent.

(Baltazar et al., 1986) observed symptoms in sheep that mainly included refusal to eat and drink, paresis, paralysis, muscular tremors and death ensuing in 2–4 days while (Rissi et al., 2008) reported the signs of abnormal gait, trembling, lateral recumbency, convulsion, opisthotonus, and fever in sheep suffering from rabies. (Soria Baltazar et al., 1988) studied the effect of the inoculation of a canine strain of rabies virus in sheep and clinical signs observed were anorexia, emaciation, nervous reactions and prostration before death.

In cattle and buffalo symptoms like anorexia, hypersalivation, constipation, conjunctivitis, corneal opacity and nervous signs (Srínunthapanth et al., 1985), ataxia, sluggish movements, recumbence and pharyngeal paralysis (Tanyi et al., 1988); ocular discharge, nasal discharge and sluggish movements(Asha Rani 2001) have been reported. Hudson et al., (1996a) observed signs of excessive salivation, behavioural changes, muzzle tremors, hyperaesthesia, aggression and pharyngeal paralysis in experimentally induced rabies. Singh and Grewal (1998b) reported the symptoms of experimental rabid buffalo calves which included bellowing, conjunctivitis, ocular and nasal discharge, arched back and sluggish movements. Aytekin and Mamak (2009) reported the symptoms of anorexia, apathy, pain, salivation, tenesmus, bellowing with tongue hanging out, leaning against objects, attempting to bite of objects, occasional postural appearance of kyphosis, hydrophobia and dysphagia in cattle suffering from rabies. (Pedroso et al., 2009) reported that paralytical form was the most common clinical picture in cattle that included in-coordination, paresis, and paralysis of the pelvic members, besides recumbence, paddling, and death.

In equines, muzzle tremors were the most common and initial sign besides pharyngeal spasm, ataxia, lethargy and furious form in experimentally induced rabies in horses (Hudson et al., 1996b). (Url et al., 2004) described clinical and diagnostic peculiarities concerning equine rabies on the basis of a rabid horse originating from the area of Austria and reported apathy, colic and unpecific CNS symptoms in an 18 year old warm blooded gelding. (Luciano et al., 2009) reported decrease or absence of sensitivity testing of the anus, tail and varying degrees of paresis and paralysis in horses.

In dogs, recumbency, dropped jaw, dyspnoea, ataxia, salivation, conjunctival congestion and glazed eyes were observed (Okolo 1986, Fekadu et al., 1988) while in cats, (Frymus et al., 2009) reported exaggerated emotional responses viz. irritability, rage, photophobia, attacking inanimate objects, seizures, muscular twitching or tremors and aimless pacing.

In camels, (Afzal et al., 1993) reported hyper excitability, attacking inanimate objects, self-biting of forelimbs, colic, drooling of saliva, internal recumbency and paralysis of hind limbs.

In wild animals, Delpietro et al., (2009) reported signs of paralysis, difficulty in walking, opisthotonos, pedaling of posterior limbs and aggressiveness, (Oertli et al., 2009) reported typical behaviors of rabid skunks were entering a dog pen, appearing outside during daytime, and attacking pets. (Delpietro et al., 1990) conducted seven trials, in which infected Calomys Musculinus had evident nervous symptomatology consisting of excitability aggressiveness and paralysis.

**Pathological Studies**

Negri (1903) described inclusions in the neurons of brains from rabid animal, that later became the pathognomonic lesions of the disease. These Negri bodies consist of masses of ribonucleoprotein of rabies virus but not seen in all cases (Derakshan et al., 1978). (Zimmer et al., 1990) found no Negri bodies in brains of animal dying of rabies. However, Salem et al., (1995) reported Negri bodies in purkinje cells of cerebellum of fetus of the pregnant dam that died of rabies. (Sinchaisri et al., 1992) reported absence of Negri body in CNS of mice experimentally infected with CVS strain of fixed rabies virus although antigen was detected in these mice by avidin-biotin peroxidase technique. (Kat et al., 1995) revealed eosinophilic intracytoplasmic inclusions upon histological examination of 2 brain samples of African wild dogs supporting a diagnosis of rabies viral encephalitis. Kaplan and Koprowski (1973) reported that Negri body detection gradually was superseded by very reliable and rapid Fluorescent Antibody Technique. (Lahaye et al., 2009) reported that Negri bodies are composed of the viral N and P proteins.
Gross lesions in rabies have never been very characteristic (Sullivan, 1985). (Baltazar et al., 1986) also found that gross lesions even in experimentally produced rabies in sheep were non-specific. Similarly, (Dutta et al., 1992) reported no gross lesions in experimentally inoculated virus in four groups except edema of meninges in the mice. Some workers have nevertheless reported some non-specific gross lesions in experimental studies on rabies viz., Singh (1999) and Archana (2001) revealed congestion and edema in the brain of experimentally infected buffalo calves.

Histopathologically, various workers reported encephalitis or myelitis with (Gonzalez and Stephano, 1984; Stoltenow et al., 2000; Jamadagni et al., 2008) or without (Macruz et al., 1977) intra-cytoplasmic inclusions. Non-purulent encephalitis has been reported either confined to medulla oblongata (Schulz, 1986) or extended up to pons, spinal cord, cerebrum, cerebellum and medulla oblongata (Singh and Grewal, 1998).

Characteristic perivascular cuffing, neuronal degeneration and necrosis of neuron in pons, hippocampus and medulla oblongata has been reported by various workers (Singh, 1999; Hudson et al., 1996b) Congestion and hemorrhages were also major alterations in the nervous tissues of infected buffalo calves. Bundza and Charlton (1988) revealed moderately extensive spongiform lesions that rarely affected basal ganglia or hippocampus in skunk’s inoculated intra muscularily with street rabies virus. Spongiform lesions were characterized by less number of small vacuoles. Green et al., (1992) reported major histopathological findings in rabid horses, comprising diffuse lymphocytic perivascular cuffing with lymphocytes in the meninges, neuronal degeneration, neuronophagia, gliosis and malacia of gray matter of spinal cord. Peixoto et al., (2000) compared the sensitivity of three diagnosis techniques, microscopic examination of Negri bodies, fluorescent-antibody test (FAT) and mouse inoculation test (MIT) in 3,713 samples and observed that in equine rabid samples, only in few opportunities the Negri bodies could be observed. The absence of inclusion bodies and the longer incubation period for equine samples suggested that rabies pathogenesis studies for equine species are different.


Impression Smears
The greatest strength of impression smear detection is its rapidity. An attempt was reported to further decrease the time by Davis et al., (1997) wherein micro-wave fixed specimens were reported to be in complete agreement with conventional acetone fixed smears for detection of rabies.

Fluorescent Antibody Technique (FAT)

FAT was first developed by Goldwasser and Kissling (1958) for the diagnosis of rabies. Kaplan and Koprowski (1973) reported that the "direct" FAT took the lead over all others for speed and accuracy. However, all fluorescent - negative specimens have been recommended to be tested in mice to maintain a constant check on FAT. Dean and Abelseth (1973) observed that combined Fluorescent - positive and mouse - negative specimens could be expected since the FA test detected inactivated as well as live antigen. Howard (1977) described the fluorescent antibody test for the detection of rabies virus antigen in brain, salivary gland, cornea, lip, tactile follicle, and skin and the study indicated that these tissues were 100% effective for the diagnosis of rabies in naturally infected skunks. Umoh et al., (1985) found that FAT could detect 90 per cent of cases which would have been missed by Negri body staining and mouse inoculation. Kulonen (1989) described that routine diagnosis of rabies in Finland is performed by FAT on cerebral cortex, hippocampus, cerebellum and occasionally spinal cord tissues. Virus isolation in murine neuroblastoma cells and newborn mice are used as confirmatory tests. Aubert (1982), Roehe et al., (2002), Bingham and Merwe (2002) and Barrat and Aubert (1995) suggested that FAT gives reliable
results on fresh specimens within a few hours in more than 95–99% of cases. However, the sensitivity of the FAT depends on the specimen, the degree of autolysis, how comprehensively the brain is sampled and on the proficiency of the diagnostic staff. Different workers employed FAT to determine viral antigen in different body parts e.g. in optic nerve (Shashenko et al., 1985), hind limb peripheral nerve (Meyer et al., 1986) and in various body organs (Singh and Grewal 1998, Singh 1999).

Meslin et al., (1996) and Whitfield et al., (2001) observed that fresh, frozen and glycerolated material might be examined with FAT and found that this test was fast, comparatively inexpensive and more accurate than either the examination of films or sections by recommended procedures or mouse inoculation test.

FAT has been used to conduct epidemiological studies of rabies in Sudan (Ali et al., 2006) and Brazil (Teixeira et al., 2008). Carriere et al., (2006) reported that in equine, greater amount of rabies viral antigen is present in the brainstem and cervical medullar tissues than in the hippocampus, cortical and cerebellar tissues using FAT.


Polymerase Chain Reaction (PCR)

PCR has made the most significant impact on the rabies diagnosis. Various types of PCR have been employed on various clinical samples of various animal species.

Brain Samples

Ermine et al., (1989) attempted to improve the sensitivity of the rabies genome hybridization test, so PCR amplification was used following reverse transcription of rabies RNA extracted from infected brain. Sacramento et al., (1991) investigated the PCR amplification technique of viral nucleic acids as an alternative protocol for diagnosis and epidemiological studies of rabies virus. Kamolvarin et al., (1993) described a simple, sensitive, and specific PCR protocol for detection of rabies virus. Rabies nucleocapsid sequence was amplified from all brain samples from 95 dogs and 3 humans with rabies confirmed by fluorescent antibody (FAT) and mouse inoculation tests (MIT).

PCRs were conducted on a 10% suspension of a post-mortem sample from the patient's brain, and comparison with equivalent regions of known rabies viruses, confirmed that the fragments originated from a virus belonging to the rabies virus serotype. This case demonstrated the advantage of using a range of laboratory techniques to obtain a definitive diagnosis.

Nadin-Davis et al., (1994) reported a protocol applying reverse transcription-polymerase chain reaction (RT-PCR) and restriction endonuclease analysis (REA) to the rabies virus nucleoprotein gene that was useful for discrimination of rabies virus variants in Ontario. Four main types, which showed no host species specificity but which did exhibit different geographical distributions, were identified.

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). 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.

Luo-Ting Rong et al., (2000) employed polymerase chain reaction (PCR) for detecting rabies virus and revealed that PCR could detect 3TCID_{50} of rabies virus and gave a positive result with 0.8 pg of RNA.
Nested PCR could identify RNA of rabies virus in the liver, heart, lung, and spleen of mice 5 days after inoculation. 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. Heaton et al., (1999) reported a comparison of the sensitivity of the standard fluorescent antibody test (FAT) for rabies antigen and that of hnRT-PCR for rabies viral RNA with degraded tissue infected with a genotype 1 virus.

Various workers combined the immunofluorescent technique with molecular approach wherein Kulonen et al., (1998) compared direct immunofluorescence and PCR detection methods for sensitivity in evaluating the rabies status of archival specimens of Carnoy-fixed, paraffin-embedded brain tissue. Similarly, Smreczak et al., (2009) described the first case of the isolation of the European bat Lyssavirus Type 1b in the Serotine bat in Poland by combination of immunofluorescent as well as molecular techniques wherein rabies was diagnosed by FAT as well as heminested RT-PCR.

The biggest advantage of PCR over FAT was that rabies diagnosis was possible even in decomposed samples by PCR which is not feasible with FAT. Accordingly, David et al., (2002) used RT-PCR wherein 10 decomposed brain samples that were diagnosed as negative by direct fluorescent antibody test (FAT), were found positive. Three of the ten decomposed brains were confirmed as positive even by isolation of rabies virus in tissue culture and by mouse inoculation (MIT), whereas the other seven decomposed samples were found positive only by RT-PCR.

Romijn et al., (2003) carried out an epidemic-geographic rabies study in which 72 animal and human brain samples were analyzed for Lyssaviruses by a direct immunofluorescent technique (DIFT) and a reverse transcriptase-polymerase chain reaction (RT-PCR) assay. Similarly, Paez et al., (2003) derived phylogenetic relationships between rabies viruses isolated in three regions in Colombia (Caribbean Region (northern Colombia), Arauca (eastern Colombia) and in the Central Region). Nadin-Davis (1998) also reported molecular methods for rabies virus typing wherein restriction fragment length polymorphism (RFLP) of PCR products and strain-specific PCR (SS-PCR), in which sequences of specific viral strains were amplified differentially using strain-specific primers. In India, Nagarajan et al., (2006) studied the molecular epidemiology of RV isolates in India based on nucleotide sequence analysis of 29 RV isolates originating from different species of animals in four states. Phylogenetic analysis using RT-PCR (one step RT-PCR) revealed that the RV isolates belong to genotype 1 and that they were related geographically but were not related according to host species. Analysis of the data indicated that the dog rabies virus variants are the major circulating viruses in India that transmit the disease to other domestic animals and humans as well. Wacharapluessadee et al., (2008) developed a TaqMan real-time RT-PCR assay as a rabies surveillance tool. Thirteen rabies proven samples from Myanmar, Cambodia, Indonesia and India; 3 of which had up to 7 mismatches at primer/probe binding sites, could be detected.

Molecular approaches have also been employed for strain identification. Picard Meyer et al., (2004) developed a simplified hemi-nested reverse transcriptase polymerase chain reaction (hnRT-PCR) to determine specifically the European Bat Lyssa virus nucleoprotein gene (EBLV-1) and observed that compared to the rabies diagnostic methods. Likewise, Gupta et al., (2005) used a method based on RT-PCR and restriction endonuclease digestion of amplified PCR product to differentiate rabies laboratory fixed and street viruses. Further, utilizing PCR and real time PCR, Wakeley et al., (2005) described a single, closed-tube, non nested RT-PCR with TaqMan technology that distinguishes between classical rabies virus (genotype 1) and European bat lyssaviruses 1 and 2 (genotypes 5 and 6) in real time. The TaqMan assay is rapid, sensitive, and specific and allows for the genotyping of unknown isolates concomitant with the RT-PCR.

Junior et al., (2006) produced a digoxigenin-labeled probe from the Pasteur virus strain for the detection of the rabies virus N gene. The probe hybridization was performed from amplified N gene obtained by reverse transcription polymerase chain reaction and the results by RT-PCR and hybridization showed 100% agreement.
Rojas et al., (2006) used reverse transcription-polymerase chain reaction (RT-PCR) to determine the stability of rabies virus genomic RNA in brain samples. Reverse-transcriptase PCR experiments were performed in 3 different inoculated brains, in which the direct fluorescent antibody (DFA) test was previously conducted to detect rabies viral antigen in the brains kept at room temperature and in the frozen brains. These results indicate that brain samples kept at ambient temperature (up to 27°C) may reach a reference laboratory in an adequate state for rabies diagnosis by RT-PCR.

Nadin-Davis et al., (2007) compared selected Indian viruses with representative rabies viruses recovered worldwide by using nested PCR to amplify a portion of the viral N gene and showed a close association of all Indian isolates with the circumpolar Arctic rabies lineage distributed throughout northern latitudes of North America and Europe and other viruses recovered from several Asian countries. Picard-Meyer et al., (2007) evaluated the feasibility of the use of the FTA Gene Guard System (a commercial product consisting of filter paper impregnated with patented chemicals supplied by the Whatman Company) for the shipment, storage and detection of RNA rabies viruses by a simplified hemi-nested reverse transcriptase polymerase chain reaction (hnRT-PCR).

Biswal et al., (2007) found examination of archival samples by molecular techniques as a valuable tool in providing retrospective and epidemiological data. Study was carried to evaluate the usefulness of RT-PCR in unfixed archival samples to assess whether a retrospective diagnosis of human rabies could be made from archival brain samples from patients suspected to have died of rabies. These results demonstrate the importance of RT-PCR in the detection of rabies virus RNA in 5-6 year old preserved samples without substantial loss. Likewise, 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.

Orlowska et al., (2008) aimed at the comparison of the real-time PCR with the heminested RT-PCR method, both applied for the detection of nucleoprotein gene of rabies viruses in bats and terrestrial animals. The comparison of the methods revealed that the TaqMan PCR was 10-fold more sensitive than the heminested RT-PCR. Likewise, Panning et al., (2010) found quantitative real-time RT-PCR to be more sensitive than virus isolation.

Saliva

Crepin et al., (1998) reported an optimized reverse transcription (RT)-PCR protocol for the intravitam detection of rabies virus genomic RNA in clinical samples obtained from 28 patients suspected of having rabies. 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. Isolation of rabies from saliva was attempted in 15 of the 20 cases of rabies diagnosed before death, and in 9 cases virus was found in 1 or more samples. 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. 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 by using a SYBR Green real-time RT-PCR based assay for the detection of rabies virus RNA.

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. 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 (Madhusudan and Sukumaran 2008). Wacharapluesadee and Hemachudha (2010) obtained a sensitivity of 75.8% (47/62 samples) by applying nucleic acid-amplification test methods with saliva samples for ante-mortem detection in human patients.

Rabies RNA may be found in saliva, CSF, skin biopsy tissue and urine. Nested PCR techniques enhance the sensitivity. Real time PCR methods are being evaluated (Principles and Practices of Clinical Virology...
Molecular techniques can improve clinical diagnosis. Although molecular diagnosis facilities of rabies are limited in developing countries, these do exist in parts of India, the Philippines, Latin America, Sri Lanka and Thailand. The best specimens include saliva, tear secretions, nuchal skin biopsy specimens, CSF and urine. Secretions of virus are intermittent in saliva, urine and even CSF ( Principles of Neurologic Infectious Diseases 2005).

**Skin Samples**

Various workers (Noah et al., 1998; Strauss et al., 2005; Macedo et al., 2006) have reported detection of rabies from nuchal skin biopsy specimen by RT-PCR. Real time PCR methods improve clinical diagnosis ( Principles and Practices of Clinical Virology 2009; Principles of Neurologic Infectious Diseases 2005).

**Hair Follicles**

Detection of rabies from hair follicle s is a fairly non-invasive approach of detection of rabies. Wacharapluesadee and Hemachudha (2010) reported a sensitivity of 50% (13/26) with the use of nucleic acid-amplification tests on extracted hair follicles from human patients for ante-mortem detection of rabies.

**Urine**

Wacharapluesadee and Hemachudha (2002) yielded highest proportion of positive results in small series test, where 2 out of 4 patients had urine sample positive for rabies where as the test sensitivity for urine was 33.3% for rabies virus RNA in another study (Hemachudha and Wacharapluesadee 2004). Dacheux et al., (2008) standardized a new reverse-transcription; heminested polymerase chain reaction (hnRT-PCR) protocol with use of biological fluid specimens (saliva and urine) and skin biopsy specimens. The study revealed sensitivity of 14.6% on human urine samples. Wacharapluesadee and Hemachudha (2010) used nucleic acid-amplification tests for ante-mortem diagnosis of rabies in urine samples of human patients and revealed a sensitivity of 39% (16/41). Rabies RNA may be found in saliva, CSF, skin biopsy tissue and urine. Nested PCR techniques enhance the sensitivity. Real time PCR methods are being evaluated ( Principles and Practices of Clinical Virology 2009).

**Comparison of Different Techniques**

Rajamanickam et al., (1994) compared the dipstick dot-ELISA with direct FAT for detection of rabies antigen and found that the dipstick dot ELISA test did not produce non-specific false positive results and was therefore specific and reliable. Jayakumar et al., (1995a) and Jayakumar et al., (1995b) tested various specimens (400) with the dot ELISA technique and FAT and it was found that dot ELISA was a simple, inexpensive, rapid and highly sensitive method for rabies diagnosis.

Singh and Grewal (1998b) compared different diagnostic techniques to detect rabies virus antigen/antibody and reported that detection of neutralizing antibodies by using modified counter immuno-electrophoresis was the most sensitive technique followed by direct immunofluorescence; Seller’s staining of Negri body, pleocytosis in cerebrospinal fluid, detection of rabies virus in nasal, salivary and rectal secretions and mice inoculation test. The least sensitive of all the diagnostic technique was the histopathological detection by Negri-body.

Silva et al., (1999) tested central nervous system samples obtained from dogs with suspected rabies or distemper by Seller’s and immunofluorescence techniques. Ratho et al., (2001) compared diagnosis of rabies among suspected human rabies encephalitis cases by Seller’s stain, fluorescent stain as well as mouse inoculation test. Out of 71 postmortem brain specimens, 26 were diagnosed as rabies positive. Negri bodies were demonstrated in 18 (25.4%) brain samples by Seller stain. Fluorescent antibody technique could detect rabies antigen in 21 (29.6%) samples. Archana et al., (2003) compared Fluorescent antibody technique (FAT), Seller's staining, mice inoculation test (MIT) and double-antibody sandwich ELISA (DAS-ELISA) to detect the rabies virus antigen in the calves.

Qureshi et al., (2003) attempted to demonstrate the rabies virus/antigen in the saliva/brain tissue of affected/suspected/healthy animals belonging to different species, using agar gel precipitation (AGPT),
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fluorescent antibody (FAT) and mice inoculation (MIT). It was shown that out of the total 321 animals, 18 (5.0%) were positive for AGPT, 69 (21.49%) with FAT and 71 (22.11%) with MIT. FAT in combination with MIT was the most sensitive, reliable and quick method for diagnosis.

Chhabra et al., (2007) evaluated in vitro isolation of rabies virus using mouse neuroblastoma cells (MNA). The sensitivity and reliability of in vitro procedure was performed in comparison with mouse inoculation test (MIT), the in vivo method of virus isolation, direct fluorescent antibody test (FAT) and Sellers staining. Of the 33 animal brain samples tested, 24 (72.72%) were positive by MIT. Sensitivity of Sellers stain, FAT and rapid tissue culture infection test (RTCIT) was found to be 54.16, 100 and 91.6% respectively.

Durr et al., (2008) evaluated the direct rapid immunohistochemical test (dRIT) by testing 35 fresh samples parallel with both the direct immunofluorescent antibody (DFA) test and dRIT. They found a 100% agreement of the dRIT and DFA in fresh samples.

CONCLUSIONS

It may be concluded that advent of molecular approaches have strengthened the diagnostic preparedness of scientific fraternity. While, close observation of symptoms, have carried their importance throughout, the interpretation of the clinical samples viz. saliva, urine, skin and hair follicles largely owing to the molecular approaches have transformed the possibility of the authentic detection of rabies antemortem that was unthinkable not very long ago.

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