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Review Article

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Review on Rabies with Emphasis on Recent Diagnostic Assays

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ABSTRACT

Rabies is a zoonotic and acute viral disease of the central nervous system (CNS) caused by rabies virus of the genus Lyssavirus and family Rhabdoviridae. All warm-blooded animals are susceptible for this disease. Around 2000 peoples die every year in India due to rabies. This disease is mainly transmitted by biting of rabid animals. Main reservoirs for rabies are wildlife like skunks, raccoons, bats and foxes. This virus causes neurological signs and death. Incubation period of rabies virus varies from 14 days to 6 years. Rapid diagnosis is important for taking prevention and control measures against rabies. Conventional and rapid methods for diagnosis of rabies are Seller's staining and histopathological technique. The gold standard test for diagnosis of rabies as per OIE is direct immunofluoroscent test in brain tissues of dogs. For routine diagnosis polymerase chain reaction (PCR) and Mouse inoculation test (MIT) are also commonly used. This review highlights the recent diagnostic tests which are commonly in use for diagnosis of rabies.

Key words: *Rabies, CNS, Disease, MIT, immunofluoroscent test.*

INTRODUCTION

Rabies is a zoonotic disease caused by rabies virus of genus *Lyssa*. Rabies could be prevented by vaccines and a disease of public health importance. Around 2000 peoples die every year in India due to rabies and over 60,000 in the world[34,69]. In 99% cases, the virus is transmitted by dog bite[67] whereas bat rabies predominates in United States and Canada[20]. Rabies virus belongs to the *Lyssavirus* genus of the *Rhabdoviridae* family and *Mononegavirale* order and is neurotropic, non-segmented, negative sense, single-stranded RNA virus and it primarily affects central nervous system[16].

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There are seven distinct genotypes of the *Lyssa* virus[6,13,21] namely the rabies virus itself (RABV, serotype 1, genotype 1), Lagos bat virus (LBV, serotype 2, genotype 2), Mokola virus (MOKV, serotype 3, genotype 3), and Duvenhage virus (DUVV, serotype 4, genotype 4). The European bat lyssaviruses (EBLV) is further subdivided into two biotypes (EBLV1, genotype 5 and EBLV2, genotype 6) and the Australian bat lyssavirus (ABLV, genotype 7)[27], are also members of the *Lyssavirus* genus, but are not yet classified as serotypes. Viruses of serotypes 2–4, EBLV and ABLV are known as rabies-related viruses. The genome size of rabies virus is around 12 kb and is composed of five structural proteins that are nucleoprotein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G) and RNA-dependent RNA polymerase (L)[2].Rabies virus mainly affects carnivores and bats but also cause disease in humans and wild life species. Rabies mainly causes acute encephalomyelitis. All warm-blooded animals act as reservoirs for rabies virus[53]. Rabies is fatal in nature and cannot be treated by antiviral drugs and is widespread globally[26]. This disease is more prevalent in Asian and African continent. The India and Bangladesh is the most affected region in Asia. Presence of low levels of virus in samples like saliva and multiple genotypes are the main problems in diagnosis of rabies[49]. The rabies can also be transmitted through cornea transplants, and infection of open wounds, abrasions and mucous membranes with saliva[59] Conventional and traditional method for diagnosis of rabies detection is presence of Negri bodies by Sellar's staining. According to OIE and WHO, direct immunofluoroscent test in brain tissues of dogs is the gold standard test for diagnosis of rabies. For routine diagnosis mouse inoculation test (MIT) is also commonly used due to its high sensitivity[40]. For antemortem diagnosis of rabies, polymerase chain reaction (PCR) of nucleic acid of rabies virus in the clinical samples (saliva, skin biopsy, CSF, and corneal impression smear) is a rapid, cheap and sensitive diagnostic assay[39]. The present review describes the advances in diagnostic assays for diagnosis of rabies in infected hosts.

Rabies diagnosis on the basis of history and clinical signs

Clinically signs of rabies may only lead to a suspicion of rabies because signs of the disease are not characteristic and may vary from animal to animal[70]. Incubation period of rabies virus varies from 2 weeks to 6 years [26,29]. The early signs (2– 5 day's duration) progress to paralytic or dumb forms in 75% of dogs which is risky while attending cases of the dumb form[50]. After the appearance of clinical signs at 4 to 8 days, death or Paralysis occurred in both clinical forms. Tentative diagnosis can be made based on typical clinical signs in dogs[11]. In the prodromal phase of rabies, changes in behaviour are seen like more alert, restless and friendly, aggressive (more in cat), irritable, increased sensitivity to noise and light, and attack without provocation, hiding in dark places, or become depressed, slight pyrexia, self-mutilation at the site of the bite and impaired corneal reflex where as in excitative phase, nervous signs like vicious bite and attack, flaccidity or in-coordination, irritability, muscle tremors, pica, paralysis of deglutination and spasm, change in voice, difficulty in swallowing, drooling as well as frothing of saliva, paralysis, dropping of jaw, coma and death[50]. Grossly no specific lesions are observed in the brain. Bite wound and presence of foreign bodies in stomach due to pica may be suspected for rabies. The disease should be differentiated from canine, equine and bovine encephalitis, canine distemper, thiamine deficiency (cats), poisoning due to lead, strychnine poisoning, pseudorabies, spongiform encephalopathy and listeriosis[58]. Rabies occurred due to bats can happen with different clinical manifestations than dog acquired rabies which can help in improving diagnosis of rabies[61]. In human, rabies occured in two forms encephalitic (furious) or paralytic (dumb) forms[25].Prodromal symptoms of rabies are itching, pain at the site of bite and GIT upset[46]. In furious form it is characterized by agitation, irritability, hyperaesthesia, hydrophobia, fear

of swallowing, painful laryngospasm, generalised flaccid paralysis and aerophobia[43]. Furious form of rabies should be differentiated from diphtheria, botulism, drugs like phenothiazines and amphetamines, and plant poisoning like *Datura fastuosa*, whereas paralytic rabies should be distinguished from polio virus and Guillain-Barre syndrome[36]. The Magnetic resonance imaging (MRI) technique gives same image for both paralytic and furious forms of rabies; but is less pronounced in furious form than paralytic forms of rabies. Other techniques like diffusion- weighted imaging and diffusion tensor imaging are used in the infected dogs to compare with the non infected dogs to create maps of mean diffusivity and fractional anisotropy[35]. Quantitative proteomics using various biomarkers for rabies have been identified[62]. The Seller's staining and HP methods are not sensitive methods as they don't detect positive rabies cases when negri bodies are not present.

Samples required for diagnosis

Rabies virus is present in high level in the medulla, pons and thalamus. In 3.9–11.1% of the positive brains, rabies was found to be negative when hippocampus (Ammon's horn), cerebellum and different parts of the cerebrum have been used for diagnosis. Rabies was positive in all the infected brains when thalamus was collected and tested, so thalamus is the structure of choice for rabies detection. A pool of brain tissues (including brain stem) is recommended for collection and testing of rabies[8]. During large epidemiological studies in the field, sampling can also be done through the occipital foramen[7]or through the orbital cavity[45]. The central nervous system tissues from suspected rabies cases should be handled with high preacutions. To prevent aerosols entry gloves should always be worn. To prevent injury and contamination, use of scissors, cutting tools and scalpels, should be used with care. For antemortem diagnosis clinical samples like saliva, CSF, eye and throat, swabs from the nasal mucosa, impression smears from eyeball and cornea, biopsies from facial and nuchal skin and in dead animals tissues from the brain[64].The first indicated diagnostic test for rabies is intra-vitam test which is applicable to either dead or living individuals. For the intra-vitam diagnosis, cornea test is a new method for rabies. Transportation of rabies samples for diagnosis should be done in glycerol or saline at low temperature[55]. For postmortem diagnosis of rabies, viral antigen can also be found in the nerves surrounding the hair follicles[12].

Virus isolation

The isolation of rabies virus (RABV) in 2 days or 3–4-weeks-old mice and in cell lines such as BHK 21/C13, Vero cell, mouse neuroblastoma cell line – Neuro2a/CCL 131, are the most reliable methods of diagnosis of rabies[50]. The direct fluorescent antibody test (dFAT) is used to detect replication of virus in both mice and cell lines. Most susceptible cell line to street RABV is CCL 131 (ATCC) neuroblastoma cell line which doesn't require prior adaptation and used for isolation routinely. After 18 hours of inoculation of RABV results can be seen[50]. Supplements like diethyl aminoethyl cellulose (DEAE)- dextran to the cell line can increases the invasiveness of the virus strains[52]. The McCoy cells act as a useful tool based on cytopathic effect and produce more virus and have higher interferon (IFN) sensitivity[22].

Antigen and virus Detection

There are various diagnostic tests for detecting RABV antigen among reservoir hosts. By seller's staining in impression smears, Negri bodies (0.2–0.5 mm) can be demonstrated as acidophilic, magenta color, oval or round and, with small and dark blue interior basophilic granules whereas as red color to Mann's staining in nervous tissue sections[50]. Seller's staining is not a test of choice now a days in histopathological because of its low sensitivity. Electron microscopy can be used to detect viral inclusions and virus particles. To detect rabies virus variants, an indirect rapid immunohistochemistry test (IRIT) has been developed which reduces costs of maintaining laboratory equipments. In this test impressions of frozen brain or monolayer cell culture fixed

in buffered formalin and a set of anti-nucleoprotein monoclonal antibodies of murine origin and goat anti-mouse antibody is required[50]. This test helps in antigenic characterization and localization of RABV in humans and animals[18]. The immunoperoxidase technique (IPT), immunofluorescence and enzyme immunoassays are the assays used for direct detection of RABV antigen which are quicker and reliable also[51] In tissue sections fixed with formalin and paraffin- embedded, immunoperoxidase tests are used. The counterimmunoelectrophoresis (CIEP), IPT, dFAT, and rapid rabies enzyme immuno-diagnosis (RREID) tests were performed by Jayakumar and Ramadas[32] for detection of rabies in suspected cases and found CIEP and IPT had 78.6% and 92.9% specificity, respectively, while RREID and had 100% dFAT sensitivity. Zimmer *et al.*[33] also compared specificity of these tests using 187 suspected samples. In FAT and PAP 98% sample were positive, MIT 95%, cell culture 81% and HP only 53% cases. The PAP was found to be reliable when formalin-fixed and paraffin-embedded material was used for detection. Direct immunofluoroscent test (dFAT) is most commonly and routinely test which is performed by the laboratories working on diagnosis of rabies. It is also most widely recommended test for diagnosis of rabies by both OIE and WHO[48,68]. The dFAT is the gold standard test for detection of rabies virus in fresh brain samples[56]. The dFAT was used to detect RABV antigens in touch impressions of corneal epithelial cells, skin biopsy specimens, or fresh brain tissue as described. The sensitivity of the dFAT is 100% and gives results within 2 h. The dFAT is not suitable when the brain samples are autolysed due to higher temperature[5,41]. RABV can also be detected in the brain by rapid immunochromatographic test using monoclonal antibody[1]. For rapid detection of rabies a direct rapid immunohistochemical test (dRIT) has also been developed recently[38]. RT-PCR–ELISA is a recently developed technique for the diagnosis of RABV[4].

Detection of anti-rabies antibodies

Serological tests are not commonly used for the diagnosis of rabies as the disease is fatal and acute in nature and animal does not survive longer period to produce sufficient antibodies. But serolological tests can be used for evaluation of potency of rabies vaccines[66]. Serological tests usually target outer transmembrane G-protein epitopes which is serotype specific and N-protein of RABV capsid which is group specific. Mouse neuroblastoma cells and G-protein reactive monoclonal antibodies (mAb-Gs) are required for performing serum neutralizing tests. Jayakumar *et al.*[30,31] in his study observed that avidin-biotin dot ELISA for detection of rabies antigen was more specific and sensitive than conventional ELISA. Seventeen monoclonal antibodies (mAbs) were developed against RABV, out of which 9 mAbs (IgG 2b) were against G protein and 8 were against N protein and these mAbs reacted only with conformational epitopes of G or N protein[57]. In another study, Jayakumar *et al.*[30,31]found dipstick ELISA is more specific and reliable than dFAT. Esterhuysen *et al.*[19] developed liquid phase blocking ELISA for diagnosis of rabies virus antibody against N-protein in serum of any species and could be used for epidemiological studies. Sandwich ELISA was developed by Xu[71]. Both sandwich ELISA and MIT detected 17 samples however two samples tested negative initially for MIT were found positive for SEIA. For identification of RABV neutralizing antibodies, rapid neutralizing antibody detection test has been developed[47]. Tao and Li[60] developed an immunochromatographic test strip for detection of RABV antibodies in the serum. This test was 93.1% and 92.2%, specific and sensitive respectively. The fluorescent antibody virus neutralization test (FAVN) and rapid fluorescent focus inhibition test (RFFIT) is the WHO recommended tests for estimation of RABV neutralizing antibodies[14]. RFFIT is used for estimating the prophylactic vaccine efficacy, and also used in ante-mortem diagnosis of rabies. As RFFIT is costly, Madhusudana *et al.*[37] developed a cheaper immunohistochemistry- based neutralization

test and compared it with RFFIT and found it as valuable diagnostic tool in developing countries with poor resource. The BioPro ELISA rabies Ab kit was proved to be a good diagnostic tool for detecting the antibody level against rabies in wildlife which was also confirmed by 25 international laboratories[65]. A novel neutralization test was developed by Moeschler *et al.*[44] using vesicular stomatitis virus. They deleted a segment of glycoprotein G gene and were substituted with RABV glycoprotein which allowed the fast, safe and sensitive detection of RABV antibodies.

Nucleic acid detection

There are several diagnostic tests which are based on nucleic acid of RABV and are used by reference laboratories of OIE and WHO like PCR, in situ hybridization, genomic sequencing, etc.[42]. The NA hybridization technique is more sensitive and specific than any other nucleic acid based tests. In situ hybridization (ISH) can also be used on formalin fixed tissues and paraffin-embedded to detect genomic RNA and mRNA. The ISH requires interaction of rabies specific probes with complementary RNA of virus to form hybrids. These specific probes are either non-radioactive label (digoxigenin) or radioactive label (3H). Jackson and Reimer[28] used 35S and 3H probes specific to N-protein of RABV in paraffin embedded sections of mice brain. In rabies diagnosis use of non-radioactive label probes has increased due to safe, cheap and easy in use[63]. Ganesh and Jayakumar[23], labelled PCR product of rabies positive brain with digoxigenin and it could detect 63 out of 64 brain samples that were also positive by FAT. The PCR based tests could also used for studying viral pathogenesis and epidemiological work apart from diagnosis[50]. PCR can detect few numbers of viral particles also in samples which are autolysed and could not be diagnosed by FAT. The sequencing of amplified PCR product can provide accurate genotyping of rabies virus. Heaton *et al*[24]. in his study developed a hemi-nested PCR assay and detected 6 genotypes of RABV and RRVs. Arai *et al.*[3] did single step RT-PCR using N-gene of 11 RABVs by amplifying the most variable region. In one study Sacramento *et al.*[54] designed a primer for mapping N-protein cistron of infected brains. The RT-PCR was developed by Black *et al*[10]. for detection of RABV (genotype 1) and RRV. The real time PCR using TaqMan probes has been developed to differentiate all the six genotypes of RABV and RRVs[9]. For the diagnosis of RABV, RT-PCR–ELISA has been developed[4]. The RVG gene was sequenced by pyrosequencing to know the vaccine associated rabies cases[15]. Quantitative RTPCR (qRT-PCR) technique was compared with the dFAT by Dupuis *et al.*[17]and results were found comparable.

CONCLUSION

Diagnosis of rabies depends upon the available diagnostic tests and clinical signs and symptoms. Passive surveillance for various genotypes and serotypes of rabies virus circulating in rabid animals and bat is required. Development of rabies specific RT-PCR technology has made the diagnosis of rabies easy. Rapid molecular diagnostic techniques should be developed for early confirmatory diagnosis and differentiation of various serotypes and genotypes of RABV. There are countries in the world which are now free from rabies due to extensive preventive measures taken by the governmental authority. So, rabies can also be eliminated from other developing countries like India through awareness of public regarding vaccination and preventive measures.

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