Laboratory Diagnosis of rabies

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Rabies or hydrophobia in human beings is a neurotropic virus disease which affects all mammalian species. It is transmitted usually by bite of infected animals resulting in a rapidly fatal encephalomyelitis after a some what lengthy incubation period. The virus is maintained and disseminated mainly by:

1. Enzootic infection in stray dogs known as the canine or urban rabies - seen mainly in Asia, Africa and Latin America.

2. Wild carnivorus-known as sylvatic rabies - the animals often encountered are foxes, raccoon, wolves, skunk etc. This cycle of rabies is seen in Europe, and North America.

3. Bats- in Southern United States, Mexico and South America.

At present in over 90 countries rabies is still present in its most dangerous reservoir, the dog population. In this area about 95% of the human cases is due to dogs. In India about 70,000 persons have to undergo post exposure treatment against this disease and nearly 30,000 die from rabies annually.

Rabies virus is an RNA virus belonging to the family Rhabdoviridae. It is a bullet shaped enveloped virus in which the nucleocapsid is wound inside the envelope. The envelope comprises two virus specific proteins the M or matrix protein and the transmembrane glycoprotein G. The nucleocapsid has three proteins the N, L and NS.

Exposure to the virus does not result always in infection even when the exposure is by the bite of a rabid animal. This is because, in certain cases the saliva of the rabid animal may not contain the virus at the time of bite. The clinical behaviour of animal is not a reliable indication to say that the virus is present in the saliva or not. It has been reported that the virus may be present before the clinical symptoms or it may be absent throughout or intermittently during the clinical course. Even if the exposing animal's saliva contains rabies virus, the chances of getting the disease depends on various other factors.

The disease is probably the most fearful disease both in man and animals as there is no cure once the clinical symptoms have started and death is certain. Hence diagnosis of this disease in the exposing animals is very much important as there are effective vaccines that can be used for the prevention of the disease in the exposed animals/human beings.

Rabies cannot be diagnosed on clinical grounds especially during the early phases of the disease due to the characteristic symptoms of this disease. In addition symptoms simulating an rabies may be shown by an animal in various other conditions also.

All these suggest that laboratory techniques in detecting the disease have immense value in the prevention and control of this disease.

Specimens to be collected for laboratory diagnosis

It is essential to take special care to avoid infection of the person who collect the specimen.

1. From live animals:

1. Saliva
2. Corneal impression swabs
3. Skin biopsy of the chin or nape including hair follicles
4. Cerebrospinal fluid
5. Brain biopsy
6. Serum

2. Postmortem laboratory diagnosis in man and animals:

Brain sampling by opening the skull is hazardous when practised outside the laboratory. It can be collected by one of the following methods without opening the skull.

1. Through the occipital foramen with a drinking straw or a disposable plastic pipette.

2. Retroorbital route: Push the eyeball to one side, use a trocar to make an entry through the posterior wall of the eye socket. Introduce through this hole a straw or 2ml plastic pipette screwing it in the direction of the occipital foramen.

LABORATORY DIAGNOSTIC TECHNIQUES

1. Detection of Negri bodies:

Negri bodies are the inclusion bodies seen in the infected nerve cells. This was first described by Negri in 1902 who gave a complete description of the bodies and their relationship to the disease. These inclusions are aggregates of nucleocapsids and are specific to rabies virus infection. They are seen in the cytoplasm as round oval or oblong, varying in size from 0.25 mm to 27 mm. They are mainly localized in the pyramidal cells of Ammon's horn, purkenji cells of the cerebellum, cells in the medulla and various ganglia. They are small in the neurons of salivary glands and tongue. The number of Negri bodies varies according to the animal, the virus isolate and the passage of the virus. In cattle the inclusions are very large in size.

If fluorescent antibody technique is not available, demonstration of Negri bodies is the only means of rapid screening. The animals should not be killed as the development of Negri bodies is related to the period of illness before death. Though the Negri bodies can be demonstrated in nerve cells of various regions, Ammon's horn is the best area for finding Negri bodies, for detection of rabies antigen by FA test and the select site for histologic diagnosis and mouse inoculation.

Impression smears from Ammon's horn, cortex, cerebellum or medulla when stained with Seller's stain, the Negri bodies appear as well defined magenta red or cherry red with blue staining granules or inner bodies. The inclusions though seen in the cytoplasm frequently, seen outside the affected cell due to cell damage while preparing the smear. Hence in smear examination, the intracellular position of the Negri bodies is not required as a diagnostic criterion.

Drawbacks

1. can identify only 80-85% of the rabies infected brain.
2. unsuitable for the identification of infected salivary gland tissues.
3. Negri bodies are to be differentiated from other vital inclusions such as canine distemper, infectious canine hepatitis and nonviral inclusions as in the case of cats and rodents.
4. Autolysed brain tissues are unsuitable for detection of Negri bodies.

The above facts show that absence of Negri bodies is not a confirmatory negative diagnosis and confirmatory diagnosis must be obtained by other tests such as fluorescent antibody test or mouse inoculation.

2. Fluorescent antibody test

The usefulness of immunofluorescence for identifying rabies antigen in tissues of naturally infected animals was demonstrated by Goldwasser and Kissling in 1958. By 1969, this technique was adopted as the routine method for diagnosis of rabies in public health laboratories. This is preferred over microscopical examination of Negri bodies because it is highly sensitive. This test can be used both for detection of rabies antigen and specific antibodies.

Fresh unfixed tissue is the best material for making smears or impressions. But satisfactory preparation can be made from frozen or glycerinated tissues or even from the sediment of tissue suspension when adequate tissue is not available. Impression smears from various parts of brain-Ammons horn, cerebellum, brain stem, corneal impressions, frozen sections of the skin biopsies, sediments of saliva or cerebrospinal fluid are the usual specimens used for FA test.

When salivary glands are to be examined, it is better to blot the tissue to remove excess of mucoid material, then crush the fragments on to the slide with the side of a scalpel blade. In most species submaxillary salivary glands are more likely sources of rabies virus than the parotid and sublingual glands.

Fixations of sections/smears with acetone at 20°C to 50°C for 1-4 hours gives good results. Fixing with ethyl alcohol results in excessive non specific staining and gives results inferior to those obtained with cold acetone. After treating with the conjugate has to be prepared from either polyclonal or monoclonal antibodies raised against nucleocapsids the preparation is mounted in 90% glycerine buffered to pH 8.6. The pH of the mounting medium must be above 8.0 to permit maximum fluorescence. Appropriate controls should always be kept to avoid nonspecific fluorescence.

Advantages:

1. Putrefied rabies specimens can be examined.

2. Highly sensitive and reliable. Any animal whose brain when submitted fresh is FA negative, does not present any danger of infection for man.

Submission of putrefied specimens also causes discrepancies between the FA and mouse inoculation results. In such cases, negative FA result has to be judged with epidemiological findings surrounding the exposure and treatment be initiated. In rare cases, animals with long morbidity period, especially in wild life develop high levels of antibody to rabies virus that interfere with virus isolation by mouse inoculation. This should be taken into account when a specimen is found positive by FA test, but no virus could be isolated by mouse inoculation.

Errors in diagnosis of rabies by FA staining can be reduced by:

1. Including adequate number of controls
2. Adequate sampling of each specimen
3. Avoiding undue exposure of the conjugated antiserums to light
4. Avoiding exposure of stained preparations to light especially UV light before examination begins
5. Maintaining the pH of the mounting medium 8.0 to 8.6
6. Use morphologic criteria and fluorescence to arrive at a diagnosis.
7. Examination should be made promptly without any preconceived ideas.

However, it is a fact that antigenic mass smaller than that could be resolved by light microscopy cannot be detected by FA test.

3. Mouse Inoculation:

Regarded as a sensitive supplementary test for confirming the other tests.

This gives a precise diagnosis but the animals do not die until 5-10 days after inoculation and thus does not permit a rapid decision for human treatment. Suckling mice are more susceptible than weanling or adult mouse. Mouse inoculation is slightly more sensitive than FA test. When this and FA tests are performed by competent and experienced workers agreement between them is usually 99% or higher, with few specimens giving negative FA but positive mouse inoculation. The observation period can be shortened by FA examination of brains of inoculated mouse killed 5-6 days after inoculation.

4. Enzyme immuno Technique:

In this group of tests, the antigen antibody complex is visualized by treating with antibody, enzyme (horse radish peroxidase), conjugate and a substrate. The colour developed will be proportional to the concentration of antigen/ antibody.

a) **Indirect Immunoperoxidase test:**

Has the same sensitivity as FA test. Here the enzyme horse radish peroxidase is used for labelling the antispecies antibody. It has got the advantage that ordinary light microscope is sufficient for examination of the preparation unlike in a FA test. However, it can give rise to errors of interpretation in the hands of inexperienced persons.

b) **Peroxidase Antiperoxide test:**

This technique is preferred when only formalin fixed tissues are available.

c) **Avidin-Biotin Peroxidase Complex test:**

Simple and sensitive method to localize antigens in formalin fixed tissues. This method involves the use of biotin labelled secondary antibody followed by the addition of avidin-biotin complex labelled with HRPO. This method gives a more sensitive result when compared to the previous peroxidase antiperoxidase test.

d) **Rapid rabies enzyme immunodiagnosis (RREID)**

This test is based on the immunocapture of the rabies nucleocapsid antigen by an antinucleocapsid polycloned globulin coated on the ELISA plates, followed by antigen detection with the same globulin conjugated to peroxidase and addition of a chomogenic substrate with hydrogen peroxidase. Positive reaction is indicated by development of yellow colour and the results can be visualized by naked eye reading. In this case the results can be obtained in three hour time and the correlation with FAT is 96-99%. This is quite suitable for epidemiological surveys.

The above test can be done by using nitrocellulose strips as solid support in place of ELISA plates. Here antigen in very small quantities (1µl) is placed on to nitrocellulose strips, followed by use of precipitable chromogenic substrate as above resulting in the formation of an easy to read coloured dot on white nitrocellulose. The sensitivity and specificity are equivalent in dipstic, dot ELISA and direct FAT.

5. Virus Isolation:

Not a routine practice, but can be done in murine neuroblastoma cells. The cell line usually used in Neuro-2A; which is seeded on cover slips or microtitre plates. The test can be performed within 48 hours. This test can detect 96-97% positive samples. Here the antigen detection in the cells is done by mixing the cells with acetone and subjecting it to FAT

6. Detection of Viral RNA

a) **Dot hybridization technique:**

By this technique, the rabies viral nucleic acid (RNA) can be detected in brain sample even when it is as little as 80mg. Here the viral RNA is extracted from the brain specimen and separated by agarose electrophoresis. After filtration onto nilon membranes, hybridi-

zation is done with 32 P DNA probes, complimentary to rabies genome-RNA and rabies RNAs. Hybridized labelled probes are detected by autoradiography. The results are obtained within 48 hours. Non radioactive probes can also be used.

b) **Polymerase chain reaction technique:**

After phenol extraction of RNAs from brain specimen antigenomic primers are annealed to the RNA which is reverse transcripted to DNA. This DNA is amplified through 30 cycles of PCR. Its specific detection is achieved with 32 P labelled probes, complimentary to the rabies N gene region, by Southern blot. The PCR technique is rapid, sensitive and specific and can be used as an alternative for the routine diagnosis. It is efficient even in highly degraded samples.

Intravitam Diagnosis of Rabies in Humans

The choice of the technique for intravitum diagnosis varies greatly according to the stage of the disease. Antigen detection is generally sensitive during the first few days while virus neutralizing antibodies in cerebrospinal fluid and serum usually tend to appear after 7-10 days of illness.

Viral antigen may be detected by FA test in corneal impressions or skin biopsies from patients with rabies. Skin biopsies are taken from the nuchal region with hair follicles containing peripheral nerves. Corneal impressions are taken from patients with enecephalitis by likely touching the central part of the cornea with a microscopic slide. Both corneal and skin biopsies should be refrigerated immediately after collection until the test is carried out.

The sensitivity of intravitam diagnosis is limited. Though a positive result is indicative of rabies, a negative result does not rule out the possibility of infection. Overall, sensitivity of FA is higher with skin biopsies than with corneal impressions.

Rabies virus may be isolated in cell culture from certain body tissues and fluids particularly saliva and cerebrospinal fluid.

Antibody Titration

Neutralizing antibodies in the serum of CSF of non-vaccinated patients may be measured either by mouse neutralization (MNT) or rapid fluorescent focus inhibition test (RFFIT). The latter is more rapid and atleast as sensitive as MNT

ELISA test using purified rabies glycoprotein as the antigen has been used to determine VN antibody levels in the serum of several species including man. The test can be carried out in the field with a special kit and it provides results within a few hours. The results are quite reproducible also.

Whatever may be the test employed, the result obtained will assist in determining whether the post exposure treatment need to be continued, modified or terminated. This is very much important with the traditional nervous tissue vaccines whose use is complicated by severe side effects.