DEVELOPMENT AND EVALUATION OF AN IN VITRO ISOLATION OF STREET RABIES VIRUS IN MOUSE NEUROBLASTOMA CELLS AS COMPARED TO CONVENTIONAL TESTS USED FOR DIAGNOSIS OF RABIES

*M Chhabra, V Mittal, R Jaiswal, S Malik, M Gupta, S Lal

Abstract

In vitro isolation of rabies virus using mouse neuroblastoma cells (MNA) was evaluated. 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. Concordance of Sellers stain, FAT, RTCIT with MIT was found to be 66.6, 100 and 93.93% respectively. Two samples which were positive by FAT and MIT showed gross contamination in cell lines, which is one of the drawbacks of RTCIT. However, rabies virus could be isolated in MNA cells from two of the eight human cerebrospinal fluid (CSF) samples from clinico-epidemiologically suspected cases of rabies. Both MIT and FAT showed negative results in the two CSF samples. RTCIT appears to be a fast and reliable alternative to MIT and holds promise in antemortem diagnosis of rabies, which is otherwise, a challenging task for a reference laboratory.

Key words: Mouse inoculation test, rabies diagnosis, rabies virus isolation, rapid tissue culture infection test

Role of laboratory in the diagnosis of rabies is of paramount importance in the present era. With survival of hydrophobia patients being reported, increasing need being felt for institution of rabies control measures and characterisation of street rabies virus for maintenance of molecular epidemiological surveillance, the laboratories will have to gear up for rapid and precise diagnosis of rabies. In rabies endemic countries like India, the treatment is started immediately after the animal bite on the presumption that biting animal is rabid unless proven otherwise. With cessation of production of nervous tissue vaccine in the country and a wide gap between demand and supply of tissue culture vaccines along with its prohibitive cost, laboratories in India may have to provide rapid and accurate diagnosis to guide/modify post-exposure prophylaxis (PEP), as is being practised in developed countries.

The fluorescence antibody test (FAT) has proven to be a fast and sensitive procedure of antigen detection when performed by an experienced laboratory with high quality reagents. However, isolation of virus by intracerebral inoculation of laboratory mice for confirmation of diagnosis is the recommended practice based on its ability to detect small quantities of rabies virus in very weakly positive specimens that may give false negative FAT result. This method yields delayed results and is possible only in centres where facility of continuous supply of mice is available and hence remains only of academic and epidemiological importance. On the other hand, rabies virus can be grown in cell lines in a very short time. The use of tissue culture for the growth of rabies virus was described as early as 1913. However, it was presumed that the street virus strains adapt slowly to growth in cell lines and thus this procedure was not used in routine diagnostic laboratory. Subsequently, BHK-21 and CER cell lines were identified as suitable host systems for isolation of street virus. Recent studies indicate that murine neuroblastoma cell line was found to have superior sensitivity.

Before any new technique can be accepted as a recognized diagnostic test its sensitivity must be compared with that of established tests in the laboratory. The purpose of this study was to compare the sensitivity of rabies tissue culture infection test (RTCIT) with that of mouse inoculation test (MIT), FAT and Sellers stain in an attempt to replace MIT with RTCIT for providing confirmatory diagnosis of rabies.

Materials and Methods

Samples

Thirty three brain samples of different animals viz. dog (28), cat (1) horse (1), bull (1), buffalo (1), blue bull (1) and eight human cerebrospinal fluid (CSF) samples from suspected cases of rabies were processed in the laboratory for diagnosis of rabies.

Processing of samples

Each brain sample was processed in the laboratory as soon as it was received. Impression smears were made from...
hippocampus and/or other areas, depending on availability of the material. A set of impression smears was stained with Sellers stain for detection of Negri bodies and another set was used to perform FAT for detection of rabies antigen using rabies conjugate anti-nucleocapsid (BIORAD). CSF was centrifuged at 3000 rpm for 10 min, smear was made with the deposit, air dried and fixed in chilled acetone for FAT. The supernatant was used for isolation of virus. Mouse inoculation test (MIT) was performed by inoculating the brain suspension and CSF intracerebrally in 3-4 week-old Swiss albino mice. Eight mice were inoculated per sample, each received 0.03 mL of the sample. The mice were observed for the development of signs and symptoms of rabies. The sick or dead mice brain was harvested and were observed for the development of signs and symptoms of rabies. The sick or dead mice brain was harvested and confirmed by Sellers stain and FAT.11

**RTCIT**

The *in vitro* isolation of virus in cell culture was performed in culture tubes (corning 16 x 125 mm). Brain suspension (10%) was prepared in 0.01 mol/L phosphate buffer saline (PBS), pH 7.4, treated with antibiotics and was allowed to settle for at least an hour at 4°C. This was then diluted with minimum essential medium supplemented with 10% foetal calf serum (MEM-10) to form 1% brain suspension. Murine neuroblastoma (MNA) cell cultures (Indian Immunologicals Ltd., Hyderabad) were grown in MEM-10 and used in concentration of approximately 5 x 10⁶ cells/mL. Each culture tube received 2 mL of cell suspension and 4 mL of 1% brain suspension or 0.5-1 mL of CSF (depending upon the available quantity). Known positive street control and a negative sample were inoculated along with the cell control tube with each batch of test to maintain internal quality control. The tubes were incubated at 37°C for four days. The infected cells were coated on multi-spot slide, fixed in chilled acetone, stained with commercially available rabies anti-nucleocapsid antibodies tagged with FITC dye (Rabies conjugate antinucleocapsid, BIORAD) and examined under the fluorescence microscope.

**Results**

Of the 33 brain samples tested, 24 (72.72%) were positive by MIT (Table). Of these, 13 (54.16%) showed the presence of Negri bodies and the rabies antigen was detected in all (24, 100%) by FAT. Concordance of Sellers stain and FAT with MIT was found to be 66.6 and 100% respectively. All the brain samples were also inoculated in MNA cell lines. Twenty two (91.6%) samples out of 24 positive by MIT showed positive results in RTCIT. Cell lines were grossly contaminated on inoculation of two brain samples, which were partially decomposed (2-3 days old). FAT and MIT showed positive results in both these samples where as Sellers was positive in one of them. Attempts to remove the contamination as described in literature were unsuccessful. Concordance of RTCIT with MIT was found to be 93.93%.

Eight human CSF samples from clinico-epidemiologically suspected cases of rabies were inoculated intracerebrally in mice and MNA cell lines. The virus was isolated in two CSF samples in cell lines whereas none could be isolated by MIT. Rabies antigen was not detected in any of the CSF samples by FAT.

**Discussion**

A battery of tests are being performed in our laboratory for diagnosis of rabies, each having its own merits and de-merits. A positive test by any one of the standardized procedures overrides negative reaction in others. Whereas a doubtful result obtained in any single test, necessitates recourse to other tests available in order to arrive at definite conclusion.

A quick, easy and economical procedure for diagnosis of rabies is the use of Sellers stain for detection of intracytoplasmic inclusion bodies i.e., Negri bodies. In the present study, sensitivity and concordance of Sellers stain to MIT was found to be 54.2 and 66.6% respectively. These results are comparable with our earlier reports and with that of other authors, who report sensitivity of Sellers stain ranging from 53 to 75%. The low sensitivity of Sellers Stain is attributed to number of factors as has been discussed.

The FAT for detection of rabies antigen is quick and one of the most reliable methods for diagnosis of rabies. It has been perfected to such an extent that many laboratories have opted to abandon the MIT. In the present study also the sensitivity of FAT was 100% and it gave cent percent concordant results with MIT. False negative FAT results are not common but can occur. Experience and proficiency tests have shown a few failures with FAT and have demonstrated that it is not totally infallible. Errors have usually been traced to inadequate sampling, faulty equipment, unsatisfactory conjugate, lack of control slide or lack of experience in reading the slide and hence it has been suggested that in a standard diagnostic centre for rabies, confirmation of FAT results may be performed by MIT particularly in the samples which are negative by FAT. Intracerebral inoculation of mice has served as a confirmatory and ideal test for rabies diagnosis as virus isolation provides unambiguous identification of the causative agent. MIT has ability to detect small quantities of virus in the sample. Apart from that it has its applicability to partially decomposed

| Table: Results of all tests performed for diagnosis of rabies |
|-------------|-------|-------|-------|-------|-------|-------|
| Sample      | MIT   | Sellers stain | FAT   | RTCIT |
| Animal brain| +ve   | +ve           | -ve   | +ve   |
| Human CSF   | 0     | N/A           | N/A   | 8     | 2     | 5     |

*Contaminated, N/A Not applicable, RTCIT - Rapid tissue culture infection test, CSF - Cerebrospinal fluid, FAT - Fluorescence antibody test, MIT - Mouse inoculation test"
specimens. It is practical, sensitive, reliable and technically non-demanding. Its main drawback, besides the inherent environmental and ethical issues with the use of live animals in the laboratory, is the typical 7 to 21 days interval before the result is obtained. MIT also requires a well-maintained animal house for continuous supply of mice and larger number of mice per sample and is labour intensive.

The unpredictable and problematic delay associated with in vitro isolation has been reduced by RTCIT. The in vitro growth of rabies virus has been described since 1913. Fixed rabies virus can grow in wide variety of cells.\textsuperscript{13} Initially RTCIT was performed to study the virus and produce large quantities of virus for preparation of vaccine.\textsuperscript{13} It had been widely assumed that street rabies virus strains adapted slowly to growth in cell lines. Comparative studies have indicated that neuroblastoma cell lines are superior over baby hamster kidney cells (BHK-21) and chick embryo related (CER) cell lines because of its neural origin for isolation of neurotropic rabies virus.\textsuperscript{10} Mouse neuroblastoma (NA-C1300) cells (hypoxanthine guanine phosphoribosyl transferase deficient) are useful as they share number of characteristics with human neurons, including gross microscopic and fine structural neuron-like morphology and the presence of microtubular protein, neurotransmitter synthetic enzymes and electrically excitable cell membranes with acetyl choline receptors.\textsuperscript{13}

In the present study, MNA cell lines were used. The results were obtained in four days as compared to three weeks in MIT. Of the 33 brain samples tested, 22 were positive by FAT, MIT and RTCIT. However, two brain samples that were positive by FAT and MIT showed gross contamination in RTCIT. This is one of the major drawbacks of RTCIT. These samples were partially decomposed (two to three days old). Other authors\textsuperscript{5} have also reported that the tissue culture procedure is less tolerant to decomposed tissue than either FAT or MIT.

On the other hand, of the eight CSF samples collected from clinico-epidemilogically suspected cases of hydrophobia, none showed positive results with FAT or MIT. However, virus was grown in MNA cells in two samples. The CSF contains very small amount of virus and hence larger inoculum used in this system (0.5-1 mL as compared to 0.03mL per mouse) could be the reason for RTCIT being more sensitive than MIT.\textsuperscript{14} Other studies have also indicated that with inocula containing very small amount of field virus, MIT in 21-day old mice demonstrated virus in 50% where as RTCIT detected virus in 99% with the same inocula.\textsuperscript{13} Isolation of virus from CSF samples provides definitive antemortem diagnosis of rabies, which is otherwise a challenging task for a reference laboratory as no single test is positive in all cases of human rabies.

Virus isolation not only provides unambiguous diagnosis of the disease, it is also necessary for characterisation of the street strains to maintain molecular surveillance in comparison to vaccine strains, especially when vaccine failures are reported.\textsuperscript{3} RTCIT proved to be reliable method for isolation of street rabies virus for further characterization. For laboratories that are equipped for tissue culture work, RTCIT offers a fast and sensitive alternative to MIT.

References


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