Comparative Analysis of Routine Laboratory Diagnostic Tests for Rabies

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Abstract Present study was undertaken to compare various routine laboratory diagnostic tests for rabies detection. Seller's staining, mouse inoculation test (MIT), Dot-ELISA, Agar gel precipitation test (AGPT) and counter immunoelectrophoresis test (CIET) were the main basic tests performed in the laboratory for the rabies diagnosis. Out of 200 brain specimens, Negri bodies were observed in 52 brain samples by Seller's staining. Rabies virus was isolated in 56 samples by intra-cerebral inoculation in newborn Swiss-albino mice. Dot-ELISA and AGPT could detect rabies antigen in 55 and 57 samples respectively. Comparative analysis revealed that the CIET is the most sensitive and rapid test among performed diagnostic tests.

Keywords Rabies · Seller's stain · MIT · Dot-ELISA · CIET

Rabies is one of those endemic diseases, which is known for centuries. Though all warm-blooded animals fall prey to rabies, dogs (canines and wild canids) are mainly responsible for maintaining the disease and transmitting it to human and other animals [1, 27]. Every year 50,000 to

55,000 people die of hydrophobia all over the world; half of which are from India alone [5, 35, 36]. In India, the majority of rabies cases are from rural areas, due to lack of awareness about proper post-exposure immunization [32]. The majority of people who die of rabies are of poor or low-income socioeconomic status [26].

As per World Health Organization standards, rapid viral diagnostic techniques fall into four main categories: electron microscopy, immuno-fluorescence, enzyme techniques, and radioimmunoassay [2]. However, availability of cutting-edge instruments like electron microscope etc. in the developing countries is uncertain. Radioimmunoassay also perchance falls into the category of techniques, such as electron microscopy, that may not be suitable for all countries, and it may need to be replaced when other methods become available for the diagnosis of specific viral infections [2].

Available literature shows that researchers have compared routine diagnostic tests like Seller's staining and/or MIT with recent technologies like PCR [22]. Although, application of molecular biology has supported in a rapid and accurate detection of rabies, still majority of rabies detection laboratories, especially in rabies-endemic countries use routinely based clinical and epidemiological information for rabies diagnosis due to economic benefits [13]. Therefore, in present study we have selected the diagnostic tools keeping the easy availability of the equipment and chemical or reagents in mind. These studied diagnostic tests were also compared with Seller's staining and mouse inoculation test (MIT) to find out the most effective tool for detection of the viability of the virus with respect to rapidity, cost effectiveness and sensitivity.

Total 200 brains samples of different species like dogs, cats, goats, bulls etc. suspected of rabies infection were screened for the presence of rabies virus at the Microbiology

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department, Bombay Veterinary College, Parel, Mumbai. All animal experimental protocols were approved by Bombay Veterinary College ethical committee and Control and Supervision of Experiments on Animals (CPCSEA). Appropriate controls were maintained in all the performed tests except Seller's staining.

Brain tissues from the area of Hippocampus were used to make impression smears. These smears were then stained with Sellers stain as described earlier by Tierkel and Atanasiu [33]. The stained impression smears were scrutinized for the presence of intracytoplasmic, eosinophilic inclusion bodies called 'Negri bodies'.

Weanling (2–3 days old) Swiss albino mice of any sex were selected for intracranial inoculation. These animals were housed under controlled conditions of light (12 h light and 12 h darkness), temperature (24°C) and humidity (50%) and maintained on normal chow and water. Ammon's horn (carnivores) or cerebellum (cattle/buffaloes) or cerebral cortex (sheep/goats/pigs) part of the brain were used to prepare 20% suspension, with the help of sterile phosphate buffer saline (PBS) containing 0.2% streptomycin sulfate (Sigma Aldrich, MO, USA) as a diluent. Prepared brain suspensions were aliquoted for further tests after centrifuging at 200 g for 5 min. Mice inoculation protocol was adapted from Koprowski [20]. 0.1 ml of brain suspension was inoculated intra cerebrally in pre-anesthetized mice. Total 6 mice were used per group. These experimental animals were observed for 30 days for rabies symptoms.

One aliquot of brain suspension prepared during MIT was used to detect the presence of rabies virus by immunodiagnostic methods. The experimental protocol for Dot-ELISA was adapted from Jayakumar et al. [18]. In brief 1 µl of brain suspension was dotted on to 0.2 µm nitrocellulose paper and dried at room temperature for 30 min. The endogenous peroxidase from dotted samples was blocked using 30% hydrogen peroxide followed by thorough washing with PBS containing Tween 20. Non-specific binding sites were blocked with 3% bovine serum albumin and incubated in Polyclonal antirabies antibodies raised in a rabbit [8] for 30 min at 37°C. After horse radish peroxidase treatment membrane was treated with 3,3'-diaminobenzidine substrate for colour development. Enzyme reaction was stopped by washing membrane under tap water.

AGPT was performed as per the methods described by Lèpine [21] employing a seven well Ouchterlony's system. Test samples (brain suspension) were placed in the peripheral wells while prepared anti-rabies antiserum was placed in a central well punched on 1% agar containing glass slide. After incubation at room temperature under humid conditions for 48 h slides were observed for precipitation line between the test wells and central serum

containing well. The precipitation was also confirmed after 0.2% amido black staining.

The counter immunoelectrophoresis test (CIET) was carried out on a glass slide measuring 75 mm × 50 mm. 1% agarose was layered on top of these slides and wells were etched out according to template described by Diaz [12]. Initial electrophoresis was carried out for 45 min at potential difference (PD) 150 V between both the ends of slide after filling the antibody well with anti-rabies antibodies. Later antigen wells were filled with prepared test samples (brain suspension from MIT) and electrophoresed for 20 min at PD 50 V. The results were read immediately and after 12 h incubation in a humid chamber at room temperature. At the end of incubation slides were treated with 5% sodium citrate solution to remove nonspecific precipitation. Slides were stained with 0.2% amido black stain and observed for precipitation bands.

MIT was used as a gold standard test and proportion agreement beyond chance was calculated using kappa values. Concordance percentage of all the performed tests with respect to CIET was calculated. In conclusion the kappa value over 0.75 was considered as excellent while 0.40 to 0.75 as fair to good, and below 0.40 as poor.

The rabies diagnosis in animals depends upon appropriate specimens collection and their testing [15, 17]. Hence, collection of proper brain part according to species for rabies diagnosis was strictly followed in this study. A total of 200 brain samples were screened for the presence of rabies virus using multiple diagnostic tests viz. Seller's stain, MIT, Dot-ELISA, AGPT and CIET. The results of these diagnostic tests are summarized in Table 1.

Round, oval, sharply demarcated magenta colour Negri bodies were observed in 52 brain smears stained with Seller's stain suggesting rabies infection. Negri bodies were seen only in 86% of rabies positive cases. This result is in accordance with the observations made by most of the laboratories where Seller's method is used as a preliminary diagnostic test [25, 28, 30].

MIT, which is considered as the most effective tool for detection of the viable rabies virus [14] showed around 93% sensitivity similar to previously observed results [14, 28]. After inoculation of brain suspension into weanling mice 56 test samples inoculated mice showed clinical symptoms of rabies which were mainly encephalitic in nature. Four samples which were diagnosed positive for rabies by CIET showed no rabies symptoms in mice after MIT. The possibility of survival of mice throughout the observation period could be the development of neutralizing antibodies by mice due to the prolonged incubation period which leads to decrease in virus titer [3, 16]. When compared to Seller's staining, MIT showed additional four positive cases proving that the MIT is more sensitive test

Table 1 Comparative evaluation of tests performed for the rabies diagnosis with CIET as the concordance comparative and calculation of kappa value with MIT as a gold standard test

Test	Positive samples	Negative samples	Concordance with CIET (%)	Kappa value (with MIT as gold standard)	Total time required (h)
Seller's method	52	148	88	1.0004	0.5
Mouse inoculation test (MIT)	56	144	84	–	720
Dot-ELISA	55	145	85	1.0004	3
Agar gel precipitation test (AGPT)	57	143	83	0.9915	48
Counter immuno-electrophoresis test (CIET)	60	140	–	1.0003	1
Seller's method	52	148	88	1.0004	0.5
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than Seller's staining [29, 34, 37] and hence always consider as a the confirmatory backup procedure [23, 35].

Out of the 200 test brain suspensions 55 developed red/pink colour spots on nitrocellulose paper when the bound antigen reacted with antibody followed by peroxidase conjugated immunoglobulin. Positive reactions were easily visualized as red dots after enzyme degradation of the substrate. Dot-ELISA test for rabies detection was found to be specific, faster and reliable with almost 92% sensitive which is similar to the earlier reports [18, 24]. Although, Dot ELISA test did not produce non-specific false-positive results [19], development of colour spot depends on the concentration of virus in antigen used in the test. Brain suspension with relatively less viral load could result in false negative results without colour spot.

AGPT is based on an antigen antibody precipitation reaction. In case of positive samples precipitation band was observed in between antigen and antibody well. Out of the 200 test samples 57 were found positive by AGPT showing 28.5% positivity and 95% sensitivity. However, three brain samples which were diagnosed positive for rabies by MIT were found negative in AGPT. This could be due to non-proportion of antigen and antibody.

Like AGPT, CIET is also based on an antigen–antibody precipitation reaction but under the influence of electric charge. A CIET was developed by Diaz and Diaz [12] as a diagnostic procedure for the detection of serum-neutralizing antibodies to rabies virus. Here, we have employed CIET for detection of antigen/rabies virus in test samples. Intense precipitation band was observed between antigen and antibody well for positive sample. Sixty brain samples of the 200 were found to be positive by CIET. As maximum test specimens were found positive by CIET, concordance study was carried out keeping CIET as a constant tool for comparative study. The Dot-ELISA method was showed 85% concordance to CIET while MIT, AGPT and

Seller's staining showed 85, 84 and 83% concordance with CIET respectively (Table 1).

The techniques selected for diagnostic purposes should be suitable for use in all countries; especially those in which sophisticated techniques, expensive instrumentation and virus isolation procedures may not be practicable. Most of the rabies diagnosis laboratories perform Seller's stain as a primary diagnosis test. However results obtained in this study and previous observations showed that Seller's stain is a diagnostic test for field purpose [4]. Although the viral isolation by MIT may be positive in the first week after inoculation, nevertheless the test procedure is cumbersome and results may take up to 3–4 weeks. MIT being time-intensive test is of academic value only in decision making as regards initiation of post-exposure treatment [7]. Rabies cannot be excluded by negative MIT results. On the other hand, Dot-ELISA and AGPT, both tests were found specific, less time consuming and reliable for the diagnosis of rabies.

A high degree of specificity and reasonably adequate sensitivity, availability of basic facilities in the laboratory to perform this test, seems to favor the CIET's application as screening technique to examine large number of samples [6, 9, 12, 31]. This method also provided results that correlated well with those obtained by the standard tests like MIT [10, 11]. The CIET is not just qualitative but can also be used as a quantitative test to determine rabies virus or anti-rabies antibody titer [6]. Another advantage of CIET is that the incubation period does not affect the neutralization of the virus [6]. Owing to its high specificity, rapid diagnosis and more reliability, CIET can be used to diagnose samples suspected for rabies. It can be concluded that the CIET described here can be used as confirmatory test for post mortem as well as ante mortem diagnosis of rabies in animals and humans which is otherwise, a challenging task for a reference laboratory.

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