Abstract

Rabies is a fatal zoonotic infection of the central nervous system transmitted by the bite of rabid animal and capable of infecting all mammalian species (OIE, 2009). Rabies is still a cause of global concern, affecting over 55,000 people worldwide, particularly in the developing countries, especially Asia where over 30,000 people succumb annually, 40% of whom are children less than 15 years of age (Sugiyama and Ito, 2007., Wilde et al. 2008).

Rabies can be controlled by pre exposure prophylaxis or post exposure prophylaxis. Post exposure prophylaxis is a combination of administration of vaccine and specific antirabies serum or the rabies immunoglobulin (RIG), and is advised in severe bite cases. Rabies immunoglobulin is available in two forms, the Human rabies immunoglobulin (HRIG) and the Equine rabies immunoglobulin (ERIG). HRIG is a homologous purified immunoglobulin preparation, but is costly as well as available in limited quantities. On the contrary, ERIG a heterologous purified immunoglobulin preparation is manufactured on a large scale and hence available in large quantities. Moreover its safety has been proved beyond doubt (Wilde et al. 2009).

Currently, there are only two reference testing methods approved by World Health Organization, to test the antirabies antibodies titer in antirabies serum. They are the ‘Virus Neutralization Test using mice (VNT) and the Rapid Focus Fluorescent Inhibition Test (RFFIT). Although ELISA is used to test the antirabies antibodies titer, it is still a technique under development as per the World Health Organization (Meslin et al. 1996). Both VNT and the RFFIT require the use of live rabies virus to determine the antirabies antibody titer in the antirabies serum. As per the Indian Pharmacopeial requirements, all the manufacturers of antirabies serum have to determine the antirabies antibody titer of antirabies serum by the VNT using mice.

During the manufacture of antirabies serum, a large number of samples have to be tested for their antirabies antibody titer. **VNT is time consuming, utilizes a large number of mice, results are prone to errors and costly for routine testing. Hence, there is an need to develop an alternative test in place of the VNT using mice.**
The present study was undertaken to standardize indirect ELISA for quantification of antirabies antibodies in equines used for antirabies serum production and to explore the possibility of using indirect ELISA for routine use in place of Virus Neutralization Test (VNT) as an alternative test. During production of antirabies serum, broadly, four types of samples have to be tested for antirabies antibody titer. They were designated as horse sera, Plasma, Purified sera and batch.

The salient findings of the study were:

1. **During standardization of indirect ELISA for horse sera, plasma, purified sera and batch sera, the optimum dilutions determined for antigen, primary antibody and the secondary antibody conjugate.**

   The optimum dilution for antigen and primary antibody was 1:1,000, whereas for secondary antibody conjugate it was 1:20,000 (Fig. 3.3 to 3.8, Results).

2. **The correlation between the values of VNT and indirect ELISA test was excellent.**

   The Spearman’s ‘r’ value was 1.0 for horse sera, whereas for plasma, purified sera and batch sera, the values were 0.82, 0.923 and 0.874 respectively at p < 0.005 (Table 3.2, Results).

3. **The sensitivity and specificity amongst VNT and ELISA was highest.**

   The sensitivity and specificity values as determined by Receiver-Operator Characteristic curve analysis were 100% (Fig. 3.15, Results) as the area under the curve for all the samples tested was 1.0. Similarly, the sensitivity and the specificity values were nearing 100% when manual calculations were performed (Table 3.3, Results).

4. **The inter-rater reliability index between VNT and ELISA as determined by the Cohen’s Kappa index was near to 1.0, for all the samples tested.**

   The results of inter-rater reliability index as computed using the Cohen’s Kappa index formula for VNT and ELISA tests on horse sera and plasma samples was 1.0, while for purified sera and batch sera, it was 0.931 and 0.933 respectively (Table 3.3, Results).

5. **The consistency and the repeatability of the ELISA test were ascertained.**

6. **Based on the ELISA results for horse sera samples, the horses were segregated as High Responders, Medium Responders and Low Responders.**
Those samples with ELISA O.D. values higher than 1.0 were termed as High Responders, while those having O.D. values between 0.5 to 1.0 were Medium Responders and those having O.D. values less than 1.0 were termed as Low Responders (Fig. 3.11, Results). Moreover, based on the change in the responding status of the horses, they were identified as Late Medium Responders and Late High Responders (Fig. 3.12, Results).

In conclusion, indirect ELISA test was standardized and validated for quantification of antirabies antibodies in equines used for antirabies serum production. At Haffkine Biopharmaceutical Corporation limited, Pune, India; this indirect ELISA test has been applied as a routine test for evaluation of antirabies antibody titer in antirabies serum except for the batch serum samples which requires testing by VNT using mice as a Pharmacopeial requirement. Based on the application of ELISA test, Haffkine Biopharmaceutical has saved 99.5% in terms of cost of mice. On presenting the results of our study to Indian regulatory authorities, ELISA test can replace VNT using mice, even for batch sera samples.

Thus, the present study successfully standardized and validated indirect ELISA test for quantification of antirabies antibodies in equines used for antirabies serum production. The indirect ELISA eliminates the use of a large number of mice, gives fast, yet reliable, reproducible results and proves to be an optimum and ethical method for determining antirabies antibody titers of samples during manufacturing of antirabies serum.