Measurement of Epsilon Antitoxin in Sheep Sera by ELISA

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Summary

For measuring the quantity of epsilon antitoxin in the sera of sheep vaccinated with polyvalent enterotoxaemia vaccine, an enzyme linked immunosorbent assay (ELISA) has been developed, standardised and compared with toxin neutralisation test in mice. A solution of epsilon toxoid, as antigen, was prepared at concentrations of 1, 0.75, 0.5, and 0.2 mg/ml. From each concentration, 0.15 ml was used for coating wells of 96-well microplates. To optimise the test, each antigen dilution was tested, separately, against standard antitoxin and unknown vaccinated sheep sera. In every trial, standard antitoxin and test-sera were tested in duplicate wells. A standard curve was built upon the results of OD values obtained from the known concentrations of the standard antitoxin. Correlation studies between ELISA and SN values were carried out. The most significant correlation ($r=0.998$, $p<0.001$) was observed when sheep sera, diluted 1/50, 1/100 and 1/200, were tested against 0.2 mg/ml of the antigen and rabbit antisheep peroxidase conjugate was diluted 1/50. The test was considered to be specific, rapid, economical and an alternative to the serum neutralisation test. It reduced the demand for laboratory animals in testing the potency of clostridial vaccines by SNT.

Introduction

Epsilon is the major toxin of *Clostridium perfringens* Type D. It is also produced by Type B of the microorganism. This polypeptide chain with 311 amino acids and 37 kD molecular weight is released in the yield as an

inactive prototoxin that can be activated by treatment with proteolytic enzymes such as trypsin (Habeeb, 1975). Epsilon toxin is the causal agent of enterotoxaemia (pulpy kidney) in sheep, and the usual cause of necrotic enteritis in very young lambs (lamb dysentery). Occasionally, it causes a similar condition in foals. Humoral immunity that is mainly produced by vaccination with epsilon toxoid, alone or in combination with 6ther clostridial antigens, plays an important role in protection against enterotoxaemia. Conventionally, epsilon antitoxin against epsilon toxoid is measured by serum neutralisation test (SNT). According to *British pharmacopoeia*, there must be at least 5 international units of epsilon antitoxin per ml of pooled sera of 12 rabbits bled two weeks after two injections, four weeks apart, of the toxoid. SNT is difficult to standardise and a cumbersome method. Also, there is little information about relationship between the serological responses of the sheep and those of the rabbit. Consequently, for evaluating potency of enterotoxaemia vaccine and seroepidemiological studies it is necessary to have access to a method that is not only rapid but reduces the dependence on laboratory animals. Accordingly, the present study was undertaken to measure epsilon antitoxin in sheep sera by ELISA and compare the results with those of SNT .

Materials and methods

Sheep sera: 35 sheep were divided into 7 groups, five each, and injected with polyvalent enterotoxaemia vaccine that comprised inactivated cultures of Types D, B and C of *Clostridium perfringens*. The animals received two injections four weeks apart. They were bled before and after first injection and two weeks after the second injection. For each group sera were pooled. Pre-injection sera were used as controls.

Serum neutralisation test: 10 mixtures were prepared for each pooled sera. Into each of 10 tubes 0.4 mg of standard epsilon toxin was put. To 5 tubes 0.1, 0.3, 0.5, 0.7 and 1 ml of standard epsilon antitoxin and to the other tubes 0.1, 0.3, 0.5, 0.7 and 1 ml of unknown sheep sera were added. Physiological saline was added to the final volume of 2 ml. The mixtures stood at room temperature, protected from the light, for 30 minutes. Then, 2 mice were injected intravenously with 0.5 ml of each mixture. The mice were observed for 72 hours and, based on comparison between the mortality in mice that had received unknown sera mixtures and the mice received standard mixtures, the amount of epsilon antitoxin per ml of sheep sera were calculated.

ELISA method: Freeze dried epsilon prototoxin was used for coating polyvinyl microplates. Four concentrations of epsilon prototoxin, namely, 1, 0.75, 0.5 and 0.2 mg/ml in PBS (pH=7.2) were prepared and 96 wells of

each microplates were coated with 0.15 ml of each concentration. After incubation for 24 h at 4°C the plates were washed five times with PB (pH=7.2, 0.01 M) and Tween 20 (0.05%). The sera of vaccinated sheep and standard epsilon antitoxin were diluted to 50 , 100 and 200 folds in PBS (pH=7.2, 0.01 M and 0.1% gelatine). All dilutions of unknown and standard sera were tested, in duplicate wells, against all concentration of the antigen. Blanks were included in each assay and microplates were incubated for 2 h at 24°C. After similar washings, 0.15 ml of rabbit antisheep IgG peroxidase conjugate (prepared by Razi Vaccine and Serum Research Institute), diluted at 1/50, was added to each well and incubated for 1 hour at 24°C. Following another washing, 0.1 ml substrate consisting of hydrogen peroxide and O-phenylenediamine was added and incubated for 20 min at 24°C. Then, absorbencies of the yellow coloured product was read at 490 nm by a Merck ELISA reader. A standard curve was made using absorbencies of standard antitoxin dilutions. This curve was used for calculating epsilon antitoxin in sera of the vaccinated sheep.

Statistical analyses: The results of epsilon antitoxin titrations by ELISA and serum neutralisation tests were compared using the correlation coefficient method.

Results and discussion

The data shown in Table 1 indicate that SNT failed to measure the amount of antitoxin less than 0.1 iu/ml. Also, the titres in Group 4 demonstrated the presence of antitoxin in some of the pre-vaccinated sheep sera, this corroborates the views of Howard (1989). The values of ELISA tests for epsilon antitoxin using four concentrations of the antigen are shown in Table 2. The results indicate that amounts of antitoxin less than 0.1 iu/ml could be measured by ELISA. The titres by ELISA and SNT were compared, by correlation coefficient method, and the data are shown in Table 3. The correlation coefficients were found significant, at $p<0.001$ level, for 0.75 mg/ml , 0.2 mg/ml and, at $p<0.02$, for 1 mg/ml dilution of the antigen. These data demonstrated that ELISA could be an alternative technique to SNT for the measurement of epsilon antitoxin in sheep sera. There are a few works on ELISA and its correlation, 0.92 and 0.93, with SNT (Wood, 1991; Rood and Stewart, 1991). Different concentration of epsilon prototoxin, 0.04 mg/ml, 0.02 mg/ml, 0.01 mg/ml and 0.1 mg/ml had previously been tried as the antigen. The most significant correlation was found when 0.04 mg/ml concentration was used as the antigen (Sojka *et al.*, 1989). However, in this study the best correlation was found to be at 0.2 mg/ml concentration of the antigen and the dilution of sera and the conjugate at 1:100 and 1:50, respectively. This might have been due to the

fact that the epsilon prototoxin used in this work was prepared from culture filtrate of *C. perfringens* Type D and wasn't completely purified. Nonetheless, this prototoxin was also used in SNT, therefore, correlation are significant and valid. As 0.15-0.2 iu/ml of antitoxin in sheep serum is protective it seems that ELISA could be a useful tool for detecting protective amounts of epsilon antitoxin in epidemiological studies.

Table 1. *The results of epsilon antitoxin titration by SNT*

No. of Sera	Bleeding time	Titre (iu/ml)
1	Before 1st injection	0.2
2	After 1st injection	0.5
3	After 1st injection	1.0
4	After 2nd injection	3.0
5	After 2nd injection	1.0
6	After 2nd injection	1.0
7	After 2nd injection	3.0

Table 2. *The results of epsilon antitoxin titration by ELISA (iu/ml)*

No of sera	ELISA 1	ELISA 2	ELISA 3	ELISA 4
1	4.3	3.9	3.4	2.9
2	2	1.75	1.3	1
3	0.7	1.5	1.25	0.8
4	1.35	0.8	0.6	0.15
5	1.6	1.3	1.1	0.9
6	2.35	2.8	3.4	2.8
7	1.3	1	1.4	0.45

ELISA 1, ELISA 2 , ELISA 3 and ELISA 4 were conducted with 1 , 0.75 , 0.5 and 0.2 mg/ml concentrations of antigen respectively.

Table 3. *Correlation coefficient for the results of ELISA and SNT*

Test	r	p
ELISA 1	0.790	<0.02
ELISA 2	0.950	<0.001
ELISA 3	0.976	<0.001
ELISA 4	0.998	<0.001

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