

Evaluation of MDCK Cell Culture Assay for the Potency Testing of Enterotoxaemia Vaccines

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Abstract Control of enterotoxaemia, fatal disease of small ruminants is mainly focused on the vaccina-

tion. The disadvantages of mice neutralization test (MNT), the present gold standard assay to validate enterotoxaemia vaccines, necessitate the standardization of alternative *in-vitro* assay. Henceforth, an attempt was made to optimize MDCK cell culture assay and results were compared with indirect ELISA and MNT. There was a significant correlation found between ELISA and cell culture assay. 56% or above cell viability in the optimized assay corresponds to 100% protection in MNT. Therefore, MDCK cell culture assay is a suitable, cheap and simple alternative test for MNT.

Keywords Enterotoxaemia, MDCK cells, *Clostridium perfringens* type D.

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Introduction

Epsilon toxin one of the major lethal toxins produced by *Clostridium perfringens* type D and B, is responsible for a rapidly fatal disease in sheep and other farm animals [1]. The reality of enterotoxaemia to evolve as acute or super acute disease makes it difficult to treat, leading to sudden death in small ruminants [2]. Hence the emphasis to control enterotoxaemia is mainly placed on the prevention either by vaccination or by administration of antitoxin [3]. In order to assess the efficacy and to validate the quality of clostridial vaccines in terms of their prophylactic and therapeutic potential, official control agencies or vaccine manufacturers use toxin antitoxin neutralization assay in mice [4]. However, this test is cumbersome, time consuming, slow, expensive and can be relatively imprecise as often only ranged results are obtained [5]. In addition, the use of living

Table 1. Percent cell viability in MDCK cell culture assay.

Vaccine	Days post vaccination										
	7	14	21	28	60	90	120	150	180	210	240
TV	47.05	59.62	76.73	87.17	55.60	53.12	50.13	43.71	36.95		
SV	42.26	56.97	69.20	54.15	61.51	43.80	29.85	24.12	15.48		
AHV	52.01	56.54	69.38	79.64	71.94	57.91	55.26	44.05	41.49		
ASV	46.62	58.34	66.98	64.76	58.68	78.70	72.46	72.80	61.42	65.18	59.54
OAV	37.21	57.49	59.45	79.38	99.23	76.56	71.34	71.69	60.91	63.05	62.10

animals, this assay may suffer from the disadvantages inherent in biological tests such as variation in animal sensitivity [6, 7]. Therefore, there is a need to develop and standardize low cost *in vitro* methodologies that present quick results with good sensitivity and specificity [8]. In recent years the use of cell lines has become a viable and efficient option to replace animal models [9]. Thus, with an aim to evaluate *Clostridium perfringens* type D antibodies from Sheep sera vaccinated against different experimental vaccines MDCK cell line was used with comparison with the animal bioassay and indirect ELISA.

Materials and Methods

Bacterial strain : A highly toxigenic strain of *C. perfringens* type D procured from Division of Biological Standardization, IVRI, Izatnagar was used for toxic and vaccine development. The strain was characterized by morphological, cultural and toxigenic properties.

***Clostridium perfringens* type D epsilon toxin :** The toxin was prepared and MLD (Mouse minimum lethal dose) of the toxic was determined in mice according to the procedures described in British Pharmacopoeia [10].

Animals : Clinically normal Swiss albino mice of either sex weighing not less than 18–20 g were procured from Lab Animal Resource (LAR) section IVRI and maintained under standard conditions of nutrition and management. The animal ethics committee of Indian Veterinary Research Institute, Deemed Uni-

versity approved this study. Clinically healthy, enterotoxaemia sero-negative sheep which were maintained in the animal shed of Biological Products division, IVRI, Izatnagar were used for immunization trials. The large animal experimentation associated with present study had approval from CPCSEA.

Test sera : All the test sera were pools of sheep sera obtained from sheep immunized with five different vaccine formulations namely Toxoid vaccine (TV), Saponine vaccine (SV), Aluminium hydroxide gel vaccine (AHV), aluminium hydroxide gel saponified vaccine (ASV), aluminium hydroxide gel oil adjuvant vaccine (OAV) and control group [4].

MDCK cells : The Madin Darbin canine kidney (MDCK) cell line was maintained in Eagle's minimal essential medium (MEM) with Earle salts and non-essential amino acids (NEAA) (Sigma, Germany) supplemented with 10% foetal calf serum (FCS), 2 mM glutamine and 50 mg/ml gentamicin at 37°C and 5% CO₂ was procured from CADRAD, IVRI, Izatnagar.

Seroneutralization with MDCK cells : The test was done following the method of Petit et al. [11] with little modification. Briefly, the monolayer was treated with different dilutions of purified toxin and 50% end point was determined. Pooled sheep sera were neutralized with equal quantity of diluted toxin having mld 300 and incubated for 1 h at RT. The cell monolayer were then layered with the respective toxin serum mixture and incubated at 37°C for 18 h. The seratoxin mixture was then replaced with the MTT dye and incubated for 3 h. Subsequently the dye was re-

Vaccine	Days post vaccination										
	7	14	21	28	60	90	120	150	180	210	240
TV	6.45	7.90	9.88	11.09	7.44	7.15	6.80	6.06	5.28		
SV	5.89	7.59	9.01	7.27	8.12	6.07	4.46	3.79	2.79		
AHV	7.02	7.54	9.03	10.22	9.33	7.70	7.40	6.10	5.80		
ASV	6.40	7.75	8.75	8.50	7.79	10.11	9.39	9.43	8.11	8.54	7.89
OAV	5.31	7.65	7.88	10.19	12.49	9.86	9.26	9.30	8.05	8.30	8.19

was received 0.1 ml of the diluted toxin containing 300 mouse minimum lethal doses per ml.

Enzyme-linked immunosorbent assay (ELISA) - For the analysis of immune response by indirect ELISA, proper dilution of the antigen, serum and conjugate was determined using checker board method [12]. Sera of sheep tested negative for enterotoxaemia was used as negative sera. After finding out the titre of pooled sera samples the results were expressed as positive/negative ratio (P/N ratio) [13].

$$\frac{\text{Absorbance of test-Absorbance of well containing control sera and toxin}}{\text{Absorbance of well containing control sera and media}} \times 100$$

Mouse neutralization test: Neutralization test was done for accessing the Antitoxic Units in the pooled sheep sera [10]. For this test one ml of the pooled sera was mixed with one ml of the epsilon toxic of *Clostridium perfringens* type D containing 300 mouse minimum lethal doses and kept at room temperature for half an hour. Two mice were each given intravenously 0.2 ml of the mixture. As controls two mice

Results and Discussion

Morphological and cultural confirmation of *Clostridium perfringens* type D : The *Clostridium perfringens* type D procured from the Division of Biological Standardization showed typical morphological, cultural and toxigenic properties. The toxin titer expressed in terms of MLD/ml of the bacterial growth

Table 3. Anti-epsilon mouse neutralization test (MNT). ND –Not died, D–Died.

[illegible]

Table 4. Anti-epsilon toxin ELISA titers (P/N ratio) of sheep immunized against different vaccines.

Vaccine	Days post vaccination										
	7	14	21	28	60	90	120	150	180	210	240
TV	1.48	1.85	1.98	1.77	1.54	1.43	1.28	1.05	1.03		
SV	1.42	1.52	1.47	1.39	1.29	1.17	1.13	0.97	0.82		
AHV	1.35	1.84	2.16	2.32	2.12	1.71	1.39	1.3	1.15		
ASV	1.51	2.1	2.33	2.5	2.44	2.16	2.07	1.73	1.68	1.62	1.56
OAV	1.37	2.32	3.59	3.89	3.68	3.46	3.41	3.46	3.25	2.42	2.22

was 1:2000 as 1:200 dilution killed both the mice within short period of time.

Effect of epsilon toxin on MDCK : In MDCK cell culture assay highest ability for toxin neutralization was shown by the 60th day sera of OAV vaccinated sheep with 99.23% cell viability (Table 1). The titres of pooled sheep sera obtained with MDCK cell culture assay were also expressed in P/N ratio and showed the varying degree of anti epsilon antibody level ranging from 2.79 to 12.49 (Table 2). The P/N value observed in MDCK cell culture assay showed almost similar trend like indirect ELISA with highest P/N value on 28th DPV with subsequent fall in antibody titer. In statistical analysis highest significant correlation was found for SV, AHV and OAV (Pearson correlation value 0.875 for SV, 0.953 for AHV and 0.750 for OAV) at $p < 0.01$. TV also showed significant correlation (0.762) at $p > 0.05$. No significant correlation was found for ASV.

Mice neutralization test (MNT) : Neutralization test was done for accessing the Antitoxic Units in the pooled sheep sera. Generally I international unit of epsilon anti- toxin will be able to neutralize 150 MLD/ml of epsilon toxin and 2 IU (International Unit) is considered as protective antibody titer for prevention of clinical disease in sheep. The MNT values obtained with different vaccines are presented in (Table 3). The highest MNT value was showed by OAV and ASV vaccine. In both cases mice did not die even after 240th DPV indicating higher antibody titer giving protection for longer period of time. AHV showed death of mice on 90th days. Toxoid vaccine

and SV both showed death of mice on 60th day. In addition, 56% or above cell viability in the optimized assay corresponds to 100% protection in MNT.

Indirect ELISA : An indirect ELISA was standardized to detect antibody to *Clostridium perfringens* type D epsilon toxin using concentrated cell free extract. The optimal sera and conjugate dilution determined by checker board method was 1 : 10000 and 1:400 respectively. The ELISA titres were expressed in positive negative ratio (P/N) [14] (Table 4).

Epsilon toxin is the potent clostridial toxin which causes fatal enterotoxemia in sheep, goats and occasionally in calves and other animals, resulting in heavy economic losses [15]. The Madin-Darby canine kidney (MDCK) cell line, of epithelial origin from the distal convoluted tubule, is susceptible to epsilon-toxin produced by *Clostridium perfringens* types B and D [16]. The toxin showed a cytotoxic effect on the MDCK cells at a very low concentration (LD_{50} : 2 mg/ml) [15, 17] causing both cell cycle alterations [18] and morphological changes, including swelling and large bleb formation independent of the actin cytoskeleton and endocytosis [11, 18]. The MTT colorimetric assay determines the ability of viable cells to convert the yellow tetrazolium salt into the dark blue formazane precipitate resulting in a high optical density by metabolically active cells where as dead cells show a low optical density which can be correlated with the lethal activity of epsilon-toxin [18]. In present study, antisera with high antibody titres were able to neutralize the cytotoxic effect of epsilon toxin and cells appeared as intact and confluent under the mi-

croscope. By measuring the alterations in cells using the MTT assay, characteristic P/N ratio and percent cell viability were calculated.

The MTT test, based on the concept that tetrazolium salts are readily reduced to formazan by metabolically active cells indicates metabolic, cytotoxic and proliferative reaction in cells [19]. However, the *in vitro* methods do not reflect the rather complex pathogenetic mechanism which cause the death of mice in MNT, making the comparison of *in vitro* results and results obtained in animal experiments is the most important point in validation of *in vitro* methods. In this aspect, the animal experiment is considered to be the “golden standard” and the alternative method should lead to comparable results [20, 21]. Therefore, in present study results of MDCK cell culture assay were compared with toxin antitoxin neutralization assay in mice showing 56% or above cell viability in the optimized assay correspond to 100% protection in MNT. Moreover, the antibody titre of each pooled sera tested by cell culture assay was compared with the corresponding result of indirect ELISA and correlation coefficients were calculated. The correlation was significant for all test sera ($p < 0.01$) according to the criteria given for the degree of correlation in the standard literature [22].

The use of permanently growing cells as *in-vitro* assay represents an intermediate place between the complex nature of the animal experiment and the specific detection of the antibodies using immunological/chemical methods [23]. Additionally, the cell assay requires neither the use of highly purified toxins, in order to exclude nonspecific reactions, nor the use of monoclonal antibodies, which is a prerequisite for several ELISA systems [24, 25]. The study also demonstrated that antibodies against *C. perfringens* epsilon toxin can be detected in a precise, sensitive, practical, and relatively quick way, with a significant reduction in the number of animals used. Therefore, the cell culture assay developed might be a suitable method for the determination of the antibodies against *C. perfringens* epsilon toxin and can be used by vaccine manufacturers and regulatory agencies as an alternative to the serum neutralization test for assessing vaccine potency. However, further intensive validation studies are necessary.

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