Detection of *Clostridium perfringens* and its toxinotypes by enzyme linked immunosorbent assay from enterotoxaemic goats in Bangladesh

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Abstract

An enzyme-linked immnnosorbent assay (ELISA) has been performed for the detection of the prevailing toxinotypes of *Clostridium perfringens* obtained from conventional culturing of intestinal contents of goats which have died of suspected enterotoxaemia. The test was found effective to detect the toxins as well as types of the organism with less time and labor. The most prevailing type of *C. perfringens* causing enterotoxaemia in goat was *C. perfringens* type D (68.75%) and followed by *C. perfringens* type B (25%) and C (6.25%). No *C. perfringens* type A was detected. This study showed an intelligible picture of prevailing toxinotypes of *C. perfringens* in goats in Bangladesh. The use of the ELISA for the detection of clostridial types and toxins allows the differential diagnosis of *C. perfringens* types A, B, C and D enterotoxaemias from samples of intestinal contents and the typing of cultures of *C. perfringens*.

Key words : Enterotoxaemia, *Clostridium perfringens*, Toxins, Toxinotypes, ELISA

INTRODUCTION

Clostridium perfringens is a Gram-positive, ubiquitous, anaerobic, spore forming bacterium which is found widely in the environment and also in the intestinal tract of humans and animals (Vaikosen and Ikhatua, 2005). Due to the production of several exotoxins *C. perfringens* is of great importance as a pathogen in humans, domestic animals and wildlife (Songer, 1996; Sipos et al, 2003). The production of four major toxins alpha (α), beta (β), epsilon (ε) and iota (ι) allows its discrimination into five types from A to E (Hatheway, 1990; Vaikosen and Ikhatua, 2005). In veterinary medicine, each of these types has been linked to specific diseases (Songer, 1996; Petit et al, 1999). *C. perfringens* enterotoxin (CPE) serves as an additional virulence factor which is believed to cause enteritis in various animal species (Werdeling et al, 1991; Songer, 1996).

C. perfringens can be a normal inhabitant of the intestines of most animal species (Nilo, 1980) including humans (Johnson & Gerding, 1997), but when the intestinal environment is altered by sudden changes in diet or other factors, *C. perfringens* proliferates in large numbers and produces several potent toxins (Uzal, 2004). These toxins can act locally; can be absorbed into the general circulation producing systemic effects or can act both locally and systemically usually with devastating effects on the host (Uzal and Kelly, 1996; Lewis, 2000).

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Little or no work has been reported on the prevalence and toxinotypes of this organism causing enterotoxemia in goat in Bangladesh. Enterotoxaemia of sheep and goats is mainly caused by *C. perfringens* type D world wide although other type were also been reported as causal agents (Guss, 1977; Delgaris, 1978; Chakrabarti et al, 1980; Uzal et al, 1994; Songer, 1996; Uzal and Kelly, 1996). The predisposing cause of the disease is often a sudden change from a relatively poor to rich diet. Those animals that thrive best and are presumably good feeders appear to be most susceptible (Bullen, 1970; Odendaal et al, 1989). Naylor et al, 1987 reported that the principal toxin of *C. perfringens* type D is epsilon (ε-toxin) formed in the intestine of animals as a non toxic precursor (protoxin).

The classical identification of the toxins is based on neutralization tests in mice or in the skin of guinea pigs (Sterne and batty, 1975). In recent years, various enzymes immunoassays have been developed (Naylor et al, 1987, 1997; Martin et al, 1988; El-Idrissi and Ward, 1992; Younan et al, 1994) and molecular methods have been adapted to identify the *C. perfringens* toxinotypes (Daube et al, 1994; Buogo et al, 1995; Yoo et al, 1997).

The ELISA was found efficient in toxinotyping of *C. perfringens* because of the stability of the reagents, ease of manipulation; high sensitivity and specificity (Jackson et al, 1985). In the present study an enzyme linked immunosorbent assay was carried out to identify the toxiontypes of *C. perfringens* causing caprine enterotoxaemia in native Black bangle goats in Bangladesh so that it can be used to keep a check on prevailing toxinotypes involved in caprine enterotoxaemia in the future. Such information can be useful in vaccine development can be carried out keeping view.

MATERIALS AND METHODS

An ELISA kit (Cypress Diagnostics, ref. VBO44, Belgium) was used to detect *C. perfringens* enterotoxins (alpha, beta and epsilon) as well as semi quantitative detection of the bacterium itself.

Bacterial strains

Organism in pure culture was isolated from goats died of suspected enterotoxaemia by conventional culture based methods and kept at $-20°C$ until use.

Principle of the test

Specific monoclonal and polyclonal antibodies produced against alpha, beta, and epsilon toxins of *C. perfringens* and a monoclonal antibody specific for a structural which protein of this bacterium have been immobilized on alternative rows of the micro titer plates. These antibodies allow specific capture of the corresponding toxins or bacteria that may be present in the samples. Rows A, C, E, and G have been sensitized with these antibodies and rows B, D, F, and H with specific antibodies.

Test procedure

The ELISA test was carried out following the manufacturer's instruction with some modifications. Briefly, the ELISA kit was brought to room temperature at least half an hour before use and the dilution buffer was diluted 1 : 5 with deinonized water. This reconstituted dilution buffer was stored at 4°C until use. Organisms isolated from the collected samples were grown on 5% sheep blood agar media until considered pure and used as the sample for ELISA. The samples were diluted volume per volume in to dilution buffer. A concentrated washing solution (containing 0.85% NaCl, 0.05% Tween 20 and 0.5% bovine serum albumin) was diluted 20 fold in deionized water to make a working solution and stored at 4°C prior to use. Then 100μl aliquots of the diluted samples were added to the wells as follows: Sample 1 in the wells of column 2, Sample 2 in the wells of column 3 and so on. After this, 100μl of control antigen was added to the wells of column 1. The plate was then incubated at room temperature for 1 hour. After the first one-hour incubation at room temperature, the kit was subjected to wash with the reconstituted washing solution. The washing procedure involved removing the contents of the wells by flipping it sharply over a sink, tapping the micro plates upside down again-

st a piece of clean absorbent paper to remove all the liquid, filling the wells with 100μl of freshly prepared washing solution per well and gently shaking the plate, then the wells were emptied once more by turning the plate over a sink. The entire washing procedure was repeated three times and care was taken to avoid the formation of bubbles in the micro wells. The conjugate was diluted 20 fold with the buffer dilution and 100μl of the diluted conjugate solution was added to each well as follows: anti-alpha toxin conjugate in rows A and B, anti-beta toxin conjugate in rows C and D, anti-epsilon toxin conjugate in rows E and F, and anti-*C. perfringens* conju-gate in rows G and H. The ELISA plate was then incubat-ed at room temperature for another one hour. After the second incubation, washing of the micro wells were carried out with the washing solution following the same procedure as described above. Then 10ml of indi-cator solution was prepared by adding 12 drops (500μl) of chromogen to 9.5ml of substrate solution just prior to use and the chromogen-substrate mixture was added to the plate in volume of 100μl per micro well and incubated for 10~15 minutes at room temperature. Lastly 50μl of stop solution was added to each well and the plate was subjected to reading. The optical densities in the micro wells were read at 450nm using a plate reader (das, Italy, Model No. AT-365; 2001). Results were read carefully soon after the stopping solution had been added since the chromogen might be crystallized in the wells with strong signals and thereby distort result.

Interpretation of the result

The net optical density was calculated for control antigens as well as each sample. To do so, the signal of the corresponding negative control wells (B, D, F, H) were subtracted from each value recorded in rows A, C, E and G. The test was considered valid only if the posi-tive control antigens yield a difference in optical density at 10 minutes that is greater or equal to the values given on the QC data sheet ($\alpha \geq 1.774$, $\beta \geq 1.988$, $\varepsilon \geq 1.128$, *C. perf* ≥ 1.161). Any sample that yielded an optical den-sity difference greater or equal to 0.150 was considered positive for the valence in question.

RESULTS AND DISCUSSION

There was development of color (bright yellow) in the wells (col-1) for control antigens as well as in the wells of rows for samples. Strong bright color was observed in the wells sensitised by specific antitoxin for which the sample was positive while light or no color was developed in the wells sensitized by nonspecific anti-body.

The calculated net optical density values for each
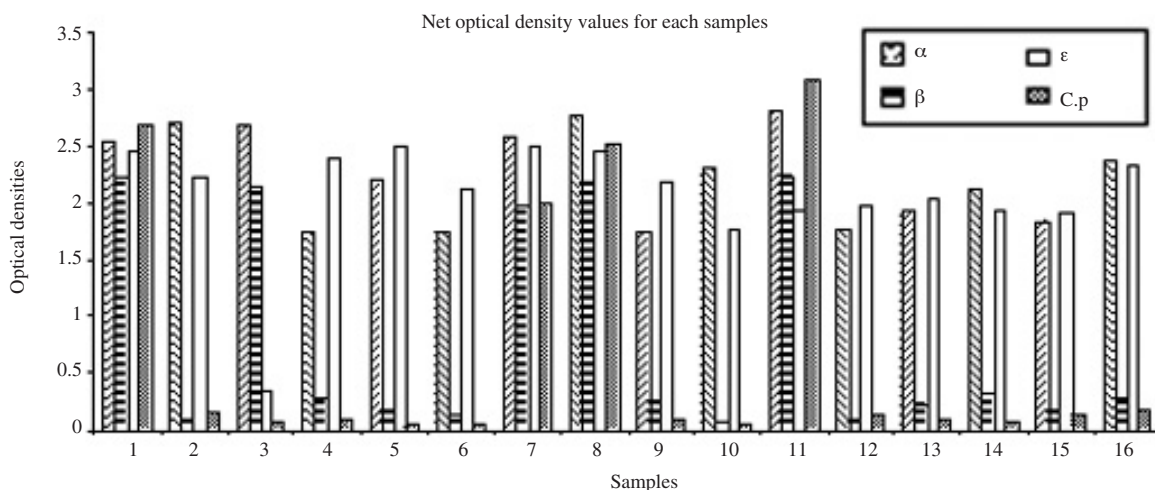
Fig. 1. Calculated net optical density values for each samples for the detection of *C. perfringens* enterotoxin. The OD at 450nm plotted for each enterotoxin and sample was determined by subtraction of the OD 450 of the corresponding negative control wells (rows B, D, F, H; wells sensitized by aspecific antibodies) from that of test samples containing enterotoxin (rows A, C, E & G; wells sensitized by specific antibodies for α, β, ε and *C. perfringens,* respectively).

Table 1. Summary of ELISA test for the detection of *Clostridium perfringens* and toxinotype identification. Rows A, C, E and G were sensitized by anti-alpha toxin, anti-beta toxin, anti-epsilon toxin and anti *C. perfringens* respectively and rows B, D, F, and H were sensitised by aspecific antibodies. Column-1 indicates the result of control while Column-2 to 17 show the results of different samples

Rows	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
A α+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
C β+	+	+	−	+	−	−	−	+	+	−	−	+	−	−	−	−	−
E ε+	+	+	+	−	+	+	+	+	+	+	+	+	+	+	+	+	+
G Cp+	+	+	−	−	−	−	−	+	+	−	−	+	−	−	−	−	−

Table 2. Prevailing toxinotypes of *C. perfringens* at BLRI goat farm determined by ELISA

Type	Toxins	No. positive (n=16)	% Positive
A	α	Nil	Nil
B	α, β & ε	4	25
C	α & β	1	6.25
D	α & ε	11	68.75
E	α & ι	Nil	Nil

sample and control antigens based on which toxinotypes of each sample were determined are given in Fig. 1. The differences in net optical density values for positive control antigens at 10 minutes were with in the accepted limit as described in materials and methods. The result of 16 samples tested is summarized in Table 1.

Among the 16 samples tested 11 samples produced bright color both in the rows sensitised for alpha and epsilon toxins (i.e. rows A and E). Besides these, four samples were found producing bright color both in the rows sensitive for alpha, beta and epsilon toxins (i.e. rows A, C and E). The remaining one sample was found positive to row A and C. Four samples developed color in the wells of row which was specially sensitised for *C. perfringens* type B. Other types of *C. perfringens* were also determined from the result, type A producing only alpha toxin, type B alpha, beta and epsilon toxins, type C alpha and beta toxins and type D alpha and epsilon toxins (Table 2).

Thus 11 samples were found producing alpha and epsilon toxins and considered as *C. perfringens* type D. On the other hand, 4 samples were considered as *C. perfringens* type B as they were found to produce alpha, beta and epsilon toxins while the remaining one was

identified as *C. perfringens* type C as it produced alpha and beta toxins. Thus 68.75% of goat enterotoxaemia at BLRI goat farm were considered due to *C. perfringens* type D where as *C. perfringens* type B was responsible for 25% caprine enterotoxaemia. Only 6.25% cases of goat enterotoxaemia was due to *C. perfringens* type C among the 16 samples tested (Table 2).

The ELISA test used in this study for the detection of the toxinotypes of *C. perfringens* causing enterotoxaemia in goats is the first time of its kind in Bangladesh. Although other investigators have described ELISA procedures for the detection of *C. perfringens* and its toxins from enterotoxaemia suspected animals abroad (Narayan et al, 1983; Bartholomew et al, 1985; Jackson et al, 1985; Naylor et al, 1987; Martin et al, 1988; Nagahama et al, 1991; El-Idrissi and Ward, 1992; Holdsworth and Parratt 1994; Hale and Stiles, 1999; Uzal et al, 2003) no report has being available regarding this issue in Bangladesh.

Routinely, diagnosis of enterotoxaemia is based on the presence of toxins of *C. perfringens* in intestinal contents (Sterne and Batty, 1975). The key components of the diagnosis system of enteritis caused by *C. perfringens* in domestic animals are the evaluation of clinical signs and gross and microscopic lesions, bacteriologic cultures of appropriate specimen and detection of toxins in pathologic specimens and in supernatant fluids of pure cultures (Smith, 1980; Hoefling, 1989). Selective media may be employed (Dafwang et al, 1987). However, the isolation of *C. perfringens* type D from intestinal contents is not necessarily confirmatory diagnosis as this organism can be a normal inhabitant of the

Table 3. Types of *Clostridium perfringens* responsible for diseases in different animal species

Type	Toxin (s) produced	Disease
A	α	Gas gangrene in man and animals, avian necrotic enteritis, food poisoning in man, fatal enterotoxaemia in lambs, kid, goats, cattle, calves and foals
B	α, β & ε	Lamb dysentery, enterotoxaemia of foals, enterotoxaemia in sheep and goats, enterotoxaemia of calves and feedlot cattle
C	α & β	Enterotoxaemia of sheep (struck), neonatal haemorrhagic enterotoxaemia of calves and foals, lambs, enterotoxaemia of piglets, necrotic enteritis of man and fowls
D	α & ε	Enterotoxaemia of sheep, goats and possibly cattle
E	α & ι	Sheep and cattle enteritis, enteritis of rabbits, calves etc

bowel of healthy sheep and goat (Warren et al, 1999). Demonstrations of major toxins in the gut, the bloodstream or serous exudates (Carter, 1984; Timoney et al, 1988) by *in vivo* assays were also used. Cytotoxicity assays for enterotoxins have also been reported (Berry et al, 1988; Estrada-Correa and Taylor, 1989). Most other *in vitro* methods for the detection of toxigenic *C. perfringens* are based on enzyme immunoassays, gene probes or PCR for detection of toxin genes (Songer, 1996). Enterotoxin has been detected by immunoassays including enzyme linked immunoassay (ELISA) and reverse passive latex agglutination (Narayan et al, 1983; McClane and Strouse 1984; Bartholomew et al, 1985; Jackson et al, 1985; Harmon and Kautter, 1986; Jackson et al, 1986; McClane and Synder, 1987; Berry et al, 1988; Metha et al, 1989; Germany et al, 1990; Hunter et al, 1993).

ELISA can be used to determine the presence of toxins and *C. perfringens* in suspected samples, pure cultures, culture supernatant fluids etc. The present study result demonstrated that ELISA could be used to determine the presence of toxins and as well as to determine the toxinotypes of the organism and indicated that this assay might be useful in examining the production of toxins by various types of *C. perfringens* to identify toxinotypes within a few hours which much faster than conventional assays like mouse protection test, dermonecrotic test etc. In the present study the toxigenic strains of *C. perfringens* identified by ELISA produced alpha (α), beta (β) and epsilon (ε) toxins. No strain of *C. perfringens* was found to produce only one of the toxins. Based on the observations, it was possible to estimate the toxinotypes of the organism as divided in five types; A through E based on the toxin produced by them (Cato et al, 1986; Hatheway, 1990; Vaikosen and Ikhatua,

2005) and each type is associated with specific enteric infections of various animal species (Table 3) (McDonel, 1986). All types of *C. perfringens* produce alpha toxin (Warren et al, 1999). In addition, type B produces beta and epsilon toxins, type C produces beta toxins, type D produces epsilon toxins and type E produces iota toxin (Niilo, 1980).

In the present study, 68.25% of the samples were identified as *C. perfringens* type D as they produced α and ε toxins, 25% of cases were reported to be *C. perfringens* type B (produced α, β and ε toxins) and only 6.25% cases were found to be *C. perfringens* type C (produced α and β toxins). Previous investigators reported type D as the principal type of *C. perfringens* associated with caprine enterotoxaemia (Chakrabarti et al, 1980; Uzal et al, 1994; Songer, 1996; Uzal and Kelly, 1996) although *C. perfringens* type A, B and C have also been reported as the causal agents of enterotoxaemia in goat (Guss, 1977; Delgaris, 1978). Thus present study was in accordance with the previous reports although no *C. perfringens* type A strain was detected to cause enterotoxaemia in goat in the study area. This may be due to the variation of location and type A seldom causes disease. It was reported that epsilon toxin played the most important role in the pathogenesis of the disease in goats (Uzal and Kelly, 1996). The epsilon toxin is produced as the minimally toxic ε prototoxin (McDonel, 1986), which is converted to a $> 1,000$ fold more toxic form by proteolytic removal of 14 N-terminal amino acids (Bhown and Habeeb, 1977). Although its precise biological activity has not been identified, epsilon toxin is necrotizing and lethal (Batty and Glenny, 1947; Buxton, 1978).

The α toxin produced in various amounts by all iso-

lates of *C. perfringens* and the only toxin produced by type A, which is hemolytic (Songer, 1996), necrotizing (Timoney et al, 1988) and potentially lethal (Rood and Cole, 1991) also reported to be responsible for goat enterotoxaemia (Guss, 1977; Delgaris, 1978). But in the present study, no strain was found to produce alpha toxin alone and hence could not be placed in *C. perfringens* type A.

The beta toxin is highly trypsin sensitive protein (Sakurai and Duncan, 1977; Jolivet-Reynaud et al, 1986), which is responsible for mucosal necrosis and possibly for central nervous system signs in *C. per-fringens* induced diseases in domestic animals (Jolivet-Reynaud et al, 1986; McDonel, 1986).

Type C producing alpha and beta toxins and reported to be responsible for goat enterotoxaemia in some extent (Guss, 1977; Delgaris, 1978). In the present study, one sample was found to produce alpha and beta toxins and considered as type C. It appears that *C. perfringens* type C is less frequent in enterotoxaemic goats. This finding is also correlated with previous reports.

The prevalence of *C. perfringens* type E (major toxins α & ι) could not be calculated due to the lack of investigation facilities. But it could be assumed that no *C. perfingens* type E was responsible for goat enterotoxaemia in the study area as all the samples could be placed to different types. If any sample that was found as *C. perfringens* by culturing could not be placed to any type, there might be a question of being type E. But there was none. In addition, type E has not yet been reported to cause goat enterotoxaemia (Songer, 1998; Uzal, 2004). So, the possibility of occurrence of *C. perfringens* type E was omitted for the study samples. But for definite result, further study should be carried out.

CONCLUSIVELY

For the first time, ELISA was employed to identify the toxinotyping of *C. perfringens* which causes enterotoxaemia in goats in Bangladeshand the prevalence of *C. perfringens* type B, C and D was determined. Among the types, type D was found most prevalent and followed by type B and C.

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