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Full Length Research Paper

Typing of toxigenic isolates of *Clostridium perfringens* by ELISA in ostrich

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Toxigenic types of *Clostridium perfringens* are significant causative agents of enteric disease in domestic animals, although type E is presumably rare, appearing as an uncommon cause of enterotoxemia of lambs, calves and rabbits. In 100 feces samples collected from ostrich farm, 40 strains of *C. Perfringens* were isolated by biochemical test, of these, 4 strains were toxigenic strains type A and any toxigenic strains of type B and type C and type D are not fond. Enzyme-linked immunosorbent assay (ELISA) kit for detection of type E not exist and isolation of type E not purpose. The technique of isolation and ELISA test are described in this paper.

Key words: Toxigenic strains, *Clostridium perfringens*, Enzyme-linked immunosorbent assay (ELISA).

INTRODUCTION

Enterotoxaemia is a fatal enteric disease that affects all species of domestic animals (Gibert et al., 1997). This disease attribute to a toxigenic type of *Clostridium perfringens*. *C. perfringens* is a gram-positive, spore-forming, anaerobic bacterium (Rood, 1998) that produces at least 15 different protein toxins (Hatheway, 1990), is found in environment and domestic animals intestine (Mcdonel, 1986).

Since *C. perfringens* was first described as *Bacillus acrogens capsulatus* in 1892 (Welch and Nuttall, 1892), the bacterium has been identified as an anaerobe responsible for a wide range of diseases in humans and animals (Niilo, 1993).

The pathogenicity of the organism is associated with several toxins. The alpha, beta, epsilon, and iota toxins are the major lethal toxins produced by the organism and are closely related to its virulence, even though they produce several minor extracellular toxins (Hatheway, 1990).

Usually, *C. perfringens* has been classified into five toxigenic types (A through E) on the basis of its ability to produce the major lethal toxins (Katayama et al., 1993). Alpha toxin is commonly produced by all five types and is a phospholipase C that can hydrolyze lecithin into phosphorylcholine and diglyceride and is believed to be a major factor responsible for the organism's tissue pathology (Jolivert-Reynaud et al., 1988).

This is the predominant product of *C. perfringens* type A (Awad et al., 1995). Therefore, type A exhibits several powerful toxicities, and infection with type A may result in myonecrosis, hemolysis, an increase in vascular permeability (Ohsaka et al., 1978), and platelet aggregation (Sugahara et al., 1977).

The major lethal effects associated with this toxin are gas gangrene in humans and necrotic enteritis and enterotoxaemia in animals (Daube et al., 1994). Beta toxin is a major lethal toxin produced by type B and C strains of *C. perfringens* and is a single-chain polypeptide of approximately 40 KDa which is highly sensitive to trypsin (Hunter et al., 1993).

The beta toxin is known to play a major role in the pathogenesis of necrotic enteritis in humans and animals (Mcdonel, 1996).

In humans, the disease has been termed pig-bel which is caused by *C. perfringens* type C infection and which

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shows clinical signs of vomiting, abdominal pain, and bloody diarrhea (Lawrence et al., 1984).

Also, necrotic enteritis associated with toxigenic *C. perfringens* has been reported in calves, lambs, and piglets (Niilo, 1986).

In experimental infection and clinical studies (Murrell et al., 1966), the presence of trypsin or protease inhibitors in the gut has been shown to be the most important cofactor in beta toxin-induced necrotic enteritis. However, it remains unclear whether the presence of dietary trypsin inhibitors is a prerequisite for the disease.

Also, type B of this organism was identified as a causative agent of enterotoxaemia or necrotic enteritis in foals, lambs, sheep, and goats (Stubbings, 1990).

Epsilon toxin is produced by types B and D of *C. perfringens* and is responsible for a rapid fatal enterotoxaemia in economically important livestock (Itodo et al., 1986).

This toxin is secreted as a relatively inactive protoxin and is activated to a potent heat labile toxin with the loss of an N-terminal peptide by a proteolytic enzyme produced by the organism (Haagsma 1991).

The activated protein is highly toxic and can have lethal, dermonecrotic and edematic activities .The effect on the brain is the most critical since the effect can induce death by cerebral edema and necrosis of brain tissue. Iota toxin is produced only by type E of this bacterium and is also produced as a protoxin .

The protoxin can permeate the vascular wall as a result of proteolytic activation. This toxin consists of two independent polypeptides: Ia, which is an ADP transferase, and Ib, which is involved in the binding and internalization of this toxin into the cell.

Although they can be distinguished immunologically and biochemically, their activities assist each other to produce toxicity such as dermonecrosis in mice.

This toxin has also been implicated in calf and lamb enterotoxaemia. The isolation and identification of *C. perfringens* from intestinal contents of animals and man had been reported by many workers. *C. perfringens* type A food poisoning currently ranks as the third most commonly identified food borne illness in the United States (Mcclane, 2001).

The Centers for Disease Control and Prevention conservatively estimates that this food poisoning sickens approximately 250,000 Americans annually (Adak et al., 2002).

Deaths from *C. perfringens* type A food poisoning are not common but do occur in the elderly and debilitated. Per year this food poisoning is estimated to kill approximately 7 people in the United States and 50 to 100 people in the United Kingdom (Mead et al., 1999).

The diarrheic and cramping symptoms of *C. perfringens* type A food poisoning result from *C. perfringens* enterotoxin. This toxin is both necessary and sufficient for the enteric virulence of *C. perfringens* type A food poisoning isolates.

The object of this study was isolation and identification of the toxigenic strains of *C. perfringens* from the feces of ostrich in Iran.

MATERIALS AND METHODS

The feces samples were collected from farms in Tabriz. Sample portion, from feces of ostrich, was transferred into a plastic tube.

Bacterial isolation

A small portion, about the size of a pea, of feces was transferred into a sterile PBS solution and blended in PBS solution. After awhile we was separated supernatant, this supernatant contained spores of *C. perfringens*, then we was centrifuged this supernatant in 4000 rpm. Fresh culture was made by streaking the material onto blood agar plates. Also we were added two antibiotics to this medium (Neomycin and polymixin). These antibiotics only allow gram positive bacillus to growing. The plates were incubating, aerobically, in Gas-pak jar at 37°C for 24 h.

Characterization of isolated strain

The colonies of *C. Perfringens*, after an overnight incubation, were low convex semi opaque with an entire margin. The colonies were surrounded by narrow zone of complete haemolysis of the theta toxin. A wider zone of incomplete haemolysis, due to alpha toxin, engulfed the narrow zone of complete haemolysis. After biochemical tests for these sample (lactose, glucose, galactose, nitrate, gelatin, starch, stormy milk) strains of *C. perfringens* are isolated.

The isolated strains of *C. perfringens*, was typed by ELISA kit made in biox corporation in belgium and 100% sensasive for toxins. For determination of toxins, alpha, beta, epsilon, the culture inoculate into a freshly cooked meat broth and incubate for 5 h. each culture centrifuged at 3000 rpm and supernatant was used for typing of *C. Perfringens* by ELISA kit. The test uses 96-well micro titration plates sensitized by specific monoclonal antibodies for the alpha beta and epsilon toxins. These antibodies allow a specific capture of the corresponding antigen which is present in the samples. These control rows allow the differentiation between specific immunological reaction and non specific bindings.

Biological samples (for example: contents of the small intestine, peritoneal fluid....) are diluted in dilution buffer and incubated on the micro plate for 60 min at room temperature. Culture supernatants are used without any dilution. After this first incubation step, the plate is washed and incubated for 60 minutes with the conjugate – Peroxides labeled anti-alpha anti-beta-toxin and anti-epsilon specific polyclonal antibody. After this second incubation, the plate is washed again and the enzyme substrate (hydrogen peroxide) and the chromogen tetramethyl benzidine (TMB) are added.

This chromogen has the advantages of being more sensitive than the other peroxides chromogens and not being carcinogenic. If alpha beta or epsilon-toxin is present in the tested samples, the conjugate remains bound to the corresponding micro wells and the enzyme catalyses the transformation of the colorless chromogen into a pigmented compound.

The intensity of the resulting blue color is proportionate to the titer of beta-toxin in the sample. Enzymatic reaction can be stopped by acidification and resulting optical density at 450 nm can be recorded using a photometer. The signals recorded for the negative control micro wells are subtracted from the corresponding positive micro wells.

RESULTS AND DISCUSSION

From 100 feces samples collected from ostrich farm only 40(40%) strains of *C. Perfringens* were isolated regard to biochemical tests. In their strains only 4(4%) strains were toxigenic and ELISA test of their sample for alpha toxin is positive and ELISA test for epsilon and beta toxin were negative. The kits for detection of iota toxin were not available and therefore detection of type E was not purpose.

Toxins of *C. perfringens* are responsible for enterotoxaemia in domestic animals. *C. perfringens* is a member of normal intestinal flora that reproduces at high rates and produces toxins in some conditions such as overeating and when diet changed suddenly (Popoff, 1984).

In this study, 40 strains isolated from ostrich, 4 (4%) were toxigenic type A. In accordance with our results, several studies conducted in other countries reported that the most predominant type in domestic animals is type A (Deligaris, 1978; Popoff, 1984; Itodo et al., 1986; Efuntoye and Adetosoye, 2003). *C. perfringens* type A, B, C and D types were isolated from sheep with enterotoxaemia in Turkey (Kurtkaya and Alver, 1969; Ozcan and Gurcay, 2000).

In sheep, enterotoxaemia has been reported to be produced by all five types of *C. perfringens* (Songer, 1996), although the role of type A in disease production is considered doubtful by some researchers (Niilo, 1980).

In the study in Korea in 1997 only type A was isolated from calves and chickens, while type C (2 of 14 isolates), in addition to type A, was isolated from piglets (Yoo et al., 1997).

In same study in Turkey, *C. perfringens* strains isolated from healthy and diseased sheep were analysed by multiplex PCR in order to detect the presence of the alpha, beta, epsilon, iota and enterotoxin genes. *C. perfringens* was isolated from 52 of 104 sheep with enterotoxemia signs and from 61 of 194 clinically healthy sheep. Genotyping of 52 strains from diseased sheep indicated that 33 (64%) were type A, 11 (21%) type D and 8 (15%) type C. Of 61 strains from healthy sheep, 58 (95%) were type A and 3 (5%) type D (Kalender and Ertas, 2005).

The results of our study show that the most prevalent type of *C. perfringens* in the intestinal flora of ostrich in Iran is type A and thus the detection of toxins rather than isolation, in intestinal content of ostrich in enterotoxemia cases is more convenient tool for the diagnosis. However, the zoonotic characteristic of *C. perfringens* type A should be taken in account. Some strains of *C. perfringens* may not be able to produce toxin in measurable amounts under laboratory conditions and this causes an obstacle for typing by classical methods.

ELISA kits use in this study was more sensitive for detection of toxins of *C. pefringens*.

Further studies should be carried out to detect the virulence factors of *C. perfringens* isolates from animals.

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