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Major and minor toxins of *Clostridium perfringens* isolated from healthy and diseased sheep

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ABSTRACT

Clostridium perfringens (*C. perfringens*) is a major cause of enteric diseases in sheep, goats, and other animals, since it produces several potent toxins in the intestines of the animal, some of which have proven to be involved in the pathogenesis of the diseases. The purpose of this study was to investigate the presence of major toxin genes along with some of the minor toxins in the isolates obtained from intestinal samples in healthy (non-ENT) and diseased (enterotoxemia = ENT) sheep. Forty intestinal samples from sheep (ENT = 16, non-ENT = 24) were examined to detect *C. perfringens*. Out of 40 samples, 25 samples were *C. perfringens* positive in bacteriological investigation and the isolates were typed by multiplex PCR assay. Of the total of 16 ENT isolates, 10 (62.5%) were type A and 6 isolates (37.5%) were type C, while 6 out of 9 (66.6%) isolates taken from non-ENT samples were type A and 3 (33.3%) isolates were type C. Toxin gene *cpb2* was found in 9 of 16 (56.2%) isolates of ENT lesions and in 4 of 9 (44.4%) isolates of non-ENT sheep. However, 6 out of 12 isolates (50%), in which *cpb2* were negative in multiplex PCR in both groups, were found to be positive using single PCR technique and specific total *cpb2* oligonucleotides. The results of this study showed that type A was the dominant type which could threaten the sheep livestock in Iran.

1. Introduction

Most developing countries including Iran need to develop the efficiency and productivity of their agricultural systems. In Iran like its neighboring countries, animal rearing and production is mainly at subsistence level. Improvement of rearing small ruminants which is the most common domesticated animal in this region is limited to a variety of factors. Most likely it seemed that one of the main limiting factors in sheep rearing is infectious bacterial diseases like enterotoxemia caused by *Clostridium perfringens*. *C. perfringens* is a ubiquitous gram positive, anaerobic, non-motile, rod-shape, endospore forming bacterium which is dominant in soil, dust, waste water, feces, feed and sheep litter (Songer, 1996; Juneja et al., 2011). It is considered that this organism is a normal inhabitant of the intestinal tract of human and animals. This bacterium can be a causative agent of gastrointestinal and enterotoxemic diseases in animals which could result in considerable economical losses due to the livestock high mortality rate and efficiency decrease (de la Rosa et al., 1997; Greco et al., 2005), on the other hand it causes food poisoning, gangrene, and necrotic enteritis in humans in opportunistic conditions (Davies and Wray, 1996; Songer, 1996). Its role as an important pathogen, causing enterotoxemia in sheep, depends on the ability of producing a wide range of exotoxins (Meer and Songer, 1997), therefore, *C. perfringens* is the most prolific toxin-producing species among clostridium bacteria group (Li et al., 2013). Depending on the toxin produced by *C. perfringens*, which causes enteritis and enterotoxemia among sheep, clinical symptoms and the severity of the disease would vary because particular type of toxins are produced by this bacterium (Petit et al., 1999).

Abbreviations: C. perfringens, *Clostridium perfringens*; ENT, Enterotoxemia; PCR, polymerase chain reaction; SBA, sheep blood agar; TSC, tryptose sulfite cycloserine agar; TSN, tryptose sulfite neomycin agar; CPA, *Clostridium perfringens* alpha toxin; cpb2, *Clostridium perfringens* beta2 toxin; netB, necrotic enteritis toxin B; cpe, *Clostridium perfringens* enterotoxin; etx, epsilon Toxin; itx, iota toxin

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According to the production of one or more of the four main lethal toxins: alpha (CPA), beta (CPB), epsilon (ETX) and iota (ITXA and ITXB), each is associated with a specific enteric infection in various animals; this species is subdivided into 5 toxinotypes (A–E) (Sterne and Warrack, 1964; Niilo, 1980). Type A strain is a member of the commensal flora of warm-blooded animals and responsible for several known disorders and syndromes (Sawires and Songer, 2006). However, *C. perfringens* can produce up to 15 extracellular toxins in a variety of compounds which include lethal toxins such as perfringolysin O (PFO), enterotoxin (*cpe*), collagenase, beta2 toxin (*cpb2*) and etc (Songer, 1996; Petit et al., 1999; Sawires and Songer, 2006). Traditionally, classical characterization of toxins was based on serum neutralization tests on mice or skin of guinea pigs (Sterne and Thomson, 1963; van Asten et al., 2009). Unfortunately this method has some limitations; it is expensive, time-consuming, with low sensitivity, lack of commercial origin for antitoxic antibodies (antitoxic antibodies are rarely found) and the use of laboratory animals (Yoo et al., 1997; Petit et al., 1999; Baums et al., 2004). The main difficulty of using toxicological procedures is the diversity of the in vitro production of toxin by cultures of *C. perfringens*. Thus, Molecular genotyping methods, which are mainly based on PCR, had been established and become the criterion for toxinotyping of *C. perfringens* isolates through identifying their encoding gene (s) (Titball et al., 1989; Hunter et al., 1992; Perelle et al., 1993; Yoo et al., 1997).

Enterotoxemia is one of the most frequent diseases among sheep and goats worldwide (Uzal and Songer, 2008). Various global studies have reported a prevalence rate for Enterotoxemia ranging between 24.13% and 100% (El Idrissi and Ward, 1992; Greco et al., 2005). Sudden changes in diet or heavy grain-feeding enable the bacteria to multiply rapidly, so food movement along digestive tract decreases and the produced toxin (s) cannot be removed or neutralized (Songer, 1996). Enterotoxemia in sheep is caused by different toxin types of *C. perfringens* (Niilo, 1980; Petit et al., 1999) and results in considerable economic losses to the livestock industry. Pathogenicity and lesions are correlated with the major produced toxins, thus typing of the bacterium has a diagnostic and epidemiological significance.

Detecting different types of *C. perfringens* in an area is important for the improvement of the most appropriate vaccines (Kalender et al., 2005). From 2012 to 2016, more than 21,515,866 sheep were vaccinated against enterotoxemia in Khorasan-e-Razavi province in northeastern of Iran. The aim of this study was to genotype *C. perfringens* isolates obtained from healthy and diseased sheep by PCR method. In this study, we collected *C. perfringens* isolates from both ENT and non-ENT sheep, and then analyzed them using a multiplex PCR assay in order to determine the presence of alpha (*cpa*), beta (*cpb*), epsilon (*etx*), iota and enterotoxin (*cpe*) toxin genes. In addition, single PCR was used to determine *tpeL*, *netB* and *cpb2* toxin genes and to verify their relationship in the outbreaks of enterotoxemia among sheep and their possible role in the development of lesions and pathogenicity of enterotoxemia.

2. Materials and methods

2.1. Sampling and culture

Out of 40 collected samples, 25 samples were *C. perfringens* positive in microbiological investigations. The *C. perfringens* isolates used in this study had been isolated from sheep with clinical signs of enterotoxemia (ENT = 16) and those died of other diseases rather than enterotoxemia (non-ENT = 9). Scraping of affected intestinal mucosa of both ENT and non-ENT samples were subjected to the necropsy and isolation of *C. perfringens*. All 16 cases of enterotoxemia had remarkable clinical signs and necropsy lesions include abdominal pain, mild to severe diarrhea, sudden death and severe intestinal hyperemia. Subsequently, the intestinal mucosa samples were smeared onto blood agar plates, containing 7% defibrinated sheep blood, the plates were then incubated in anaerobic condition at 37 °C for 48 h. In the first cultures on blood agar plates, colonies of *C. perfringens* were recognized by their morphology, characteristic dual beta hemolytic zones and with the use of Gram staining. Following, one positive colony was re-cultured on blood agar plates and incubated at 37 °C for 48 h; the same colony was sub-cultured on *C. perfringens* selective medium include Tryptose Sulfite Cycloserine agar (TSC) and Tryptose Sulfite Neomycin agar (TSN) incubated for 24–48 h at 37 °C under anaerobic conditions for purification and confirmation of diagnosis (Ahsani et al., 2011; Greco et al., 2005; Gkiourtzidis et al., 2001; Tooloei and Masodei, 2008). Identification of the *C. perfringens* isolates was confirmed by their colonial and microscopical morphology and also by hemolytic pattern, Gram staining and selective culture medium. Reference strains of *C. perfringens:* ATCC 13124 (*cpa*), CIP 106157 (*cpa*, *cpe*), CIP 60.61 (*cpa*, *cpb*, *etx*, *cpb2*) and also JRMTK01 (*netB*) and JRTK01 (*tpeL*) were used as positive controls.

2.2. DNA extraction and multiplex PCR

C. perfringens reference and isolated strains were cultured on blood agar and the extraction of total DNA was performed. In order to extract bacterial DNA, a few colonies of each *C. perfringens* isolate were grown on blood agar plate at 37 °C, then they were suspended in 100 µl distilled water, boiled for 10 min and then centrifuged at 10,000 × *g* for 10 min. The supernatants were collected carefully and used as template DNA for PCR (Ghavidel et al., 2016). In this study six pairs of primers which are shown in Table 1 were used to determine the presence of *cpa*, *cpb*, *cpb2*, *cpe, iA* and *etx* genes using a multiplex PCR technique for all isolates. PCR materials used in the reactions were provided by Ampliqon (Odense, Denmark). The main PCR master mix for the six mentioned genes was provided according to Songer and Bueschle, (1999) study. The amplification process was programmed in a thermocycler (Techne TC-3000, England) as follows: 95 °C for 3 min followed by 35 cycles at 95 °C for 1 min, 55 °C for 1 min (annealing), 72 °C for 1 min (extension) and a final extension step at 72 °C for 10 min (Meer and Songer, 1997).

Table 1
Sequence of primers used in multiplex PCR technique for amplification of genes encoding *C. perfringens* toxins.

Target gene	Primer sequences (5`-3`)	Product size (bp)	Annealing temp.(X °C)	References
Cpa	GCTAATGTTACTGCCGTTGA CCTCTGATACATCGTGTAAG	324	55	Meer and Songer, (1997)
Cpb	GCGAATATGCTGAATCATCTA GCAGGAACATTAGTATATCTTC	196	55	Meer and Songer, (1997)
Etx	GCGGTGATATCCATCTATTC CCACTTACTTGTCCTACTAAC	656	55	Meer and Songer, (1997)
Ia	ACTACTCTCAGACAAGACAG CTTTCCTTCTATTACTATACG	446	55	Meer and Songer, (1997)
Cpe	GGAGATGGTTGGATATTAGG GGACCAGCAGTTGTAGATA	233	55	Meer and Songer, (1997)
Cpb2	AGATTTTAAATATGATCCTAACC CAATACCCTTCACCAAATACTC	567	55	Songer and Bueschel (1999)