Typing of isolates of *Clostridium perfringens* from healthy and diseased sheep by multiplex PCR

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ABSTRACT: In this study, *C. perfringens* strains isolated from healthy and diseased sheep were analysed by multiplex PCR in order to 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. The other types of *C. perfringens* were not detected, and none of the isolates contained the enterotoxin gene. This result indicates that the enterotoxin of *C. perfringens* does not play the important role in the occurence of enterotoxemia in sheep.

Keywords: *C. perfringens*; sheep; toxin genes; PCR

Clostridium perfringens is a Gram positive, sporulated and anaerobic bacteria that causes disease in animals and humans. *C. perfringens* is widely distributed in the environment and intestinal tracts of animals. The patogenicity of this organism is associated with exotoxins (Meer and Songer, 1997). *C. perfringens* is classified into five types, A, B, C, D and E, based on the synthesis of four major lethal toxins, alpha, beta, epsilon and iota (Yamagashi et al., 1997). Type A produces only alpha toxin, type B produces alpha, beta and epsilon toxin, type C produces alpha and beta toxin, type D produces alpha and epsilon toxin and type E produces alpha and iota toxin. Each of these five types has been associated with enterotoxaemia in sheep (Niilo, 1980). Enterotoxin is also one of the important toxins of *C. perfringens* (Gkiourtzidis et al., 2001; Engstrom et al., 2003). Enteroroxin is considered by many to be a virulence atribute in animal strains of *C. perfringens* (Meer and Songer, 1997*)*. The enteroroxigenic strains of *C. perfringens* was found in the cattle and horse isolates (Tschirdewahn et al., 1991). Enterotoxin is most often produced by type A, but it may be produced by all of the toxin types. Enterotoxigenic *C. perfringens* type A strains cause outbreaks of food poisoning in humans (Fach and Popoff, 1997).

Classically, typing of *C. perfringens* is performed with toxin neutralization test in mice. This procedure consumes a lot of antisera and experimental animals. Additionally it is time consuming. In recent years, molecular techniques such as polymerase chain reaction (PCR) have been used to type *C. perfringens* (Uzal et al., 1997; Yoo et al., 1997; Gkiourtzidis et al., 2001; Baums et al., 2004). Detection of *C. perfringens* types in an area is important for the development of the most appropriate vaccines. The aim of this study was to type *C. perfringens* strains isolated from healthy and diseased sheep by multiplex PCR and to investigate the presence of the enterotoxin gene in the isolates.

MATERIAL AND METHODS

Samples

Intestinal samples used in the study were taken from 104 Akkaraman breed sheep, a local breed of

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Turkey, aged 1 year or up which had enterotoxemia signs such as diarrhea, sudden death and enteritis in the east of Turkey. In addition, intestinal samples were collected from 194 slaughtered apparently healthy sheep from the same area with same age and breed at an abattoir in Elazig province. The samples were taken asseptically into sterile plastic bags and transferred to the laboratory under cold conditions in 1–2 hours and were accordingly processed. All samples were taken from jejunum.

Reference strains

The following reference strains of *C. perfringens* were used as controls in this study: strain CCUG 2035 for alpha toxin gene *cpa,* beta toxin gene *cpb* and epsilon toxin gene *etx,* strain CCUG 44727 for *cpa* and iota toxin gene *iap*, strain NCTC 10239 for *cpa* and enterotoxin gene *cpe*.

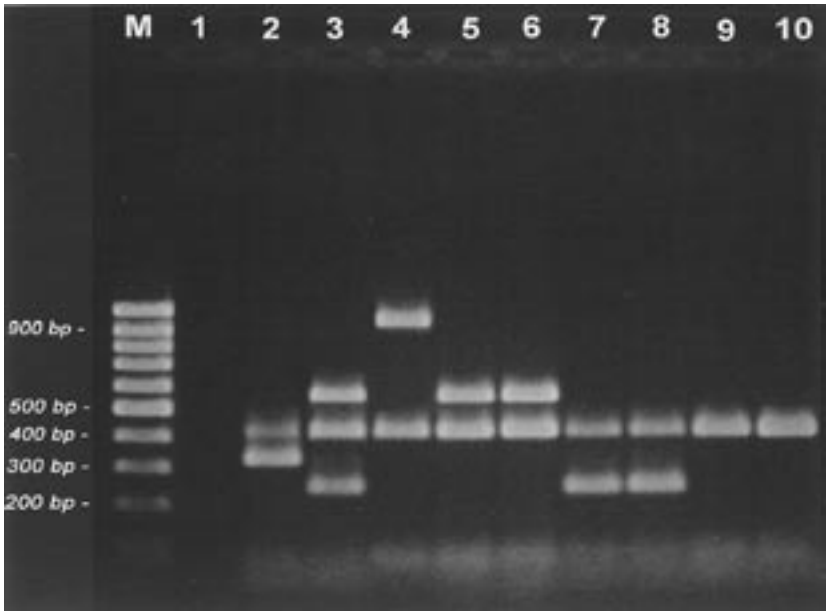
Culture

Intestinal contents were cultured onto Perfringens Agar Base (TSC Agar; Oxoid) containing egg yolk emulsion (SR47; Oxoid) and selective supplement (SR88; Oxoid) and incubated in an anaerobic chamber at 37°C for 24 hours. The suspected colonies were identified by characteristic colony morphology, Gram staining and biochemical tests.

DNA extraction and multiplex PCR

Two or three colonies from isolates and reference strains grown on blood agar were suspended in 300 µl distilled water, and the mixture was then incubated at 56°C for 30 minutes. The samples were treated with 300 µl of TNES buffer (20 mM Tris pH 8.0, 150mM NaCl, 10mM EDTA, 0.2% SDS) and proteinase K (20 mg/ml). After incubation at 37°C for 2 h the mixture was boiled for 10 minutes. To that suspension, same volume of phenol (saturated with Tris-HCL) was added, the suspension was shaken vigorously by hand and centrifuged at 11 600 g for 10 minutes. The upper phase was transferred into another tube and sodium acetate (0.1 volume) and ethanol (2.5 volume) were added. The suspension was kept at –20°C for 1.5 h and than centrifuged at 11 600 g for 10 minutes. The pellet was washed with 95 and 70% ethanol, each step followed by 5 min centrifugation. Finally the pellet was dried and resuspended in 50 µl distilled water.

The PCR was performed in a touchdown thermocycler (Hybaid) in a total reaction volume of 50 µl containing 5 µl of 10 × PCR buffer (10mM Tris-HCL, pH 9.0, 50mM KCL, 0.1 % Trition X-100), 5 µl 25mM $MgCl_2$, 200 µM of each deoxynucleotide triphosphate, 5 U of Taq DNA polymerase, 1µM each of primers and 5 µl of template DNA. alpha, beta, epsilon, and iota toxin primers from Yoo et al. (1997) and enterotoxin primers from Gkiortzidis et al. (2001) were used in the multiplex PCR.



M: marker (DNA ladder, 100 bp); Lane 1: negative control; Lane 2: strain CCUG 44727 for *cpa* and *iap*; Lane 3: strain CCUG 2035 for *cpa, cpb* and *etx*; Lane 4: strain NCTC 10239 for *cpa* and *cpe*; Lane 5–6: *C. perfringens* type D isolates; Lane 7–8: *C. perfringens* type C isolates; Lane 9–10: *C. perfringens* type A isolates

Figure 1. Agarose gel electrophoresis of PCR products of *C. perfringens* isolates

Amplification was obtained with 30 cycles following an initial denaturating step at 94°C for 5 minutes. Each cycle involved denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and synthesis at 72°C for 1 minute. 10 µl of the amplified products were electrophoresed in a 1.5% agarose gel and stained with ethidium bromide. Amplified bands were visualised and photographed under UV illumination.

RESULTS

Of 298 samples, 113 (38%) were culture positive. *C. perfringens* was isolated from 52 of 104 diseased sheep and from 61 of 194 healthy sheep. PCR products of alpha toxin (402 bp), beta toxin (236 bp), epsilon toxin (541 bp), iotatoxin (317 bp) and enterotoxin (933 bp) were shown in Figure 1. Of 52 isolates from diseased sheep, 33 (64%) were type A, 11 (21%) type D, and 8 (15%) type C. Of 61 isolates from healthy sheep, 58 (95%) were type A and 3 (5%) type D. Type B and type E were not identified. None of the isolates were *cpe* positive.

DISCUSSION

Toxins of *C.perfringens* are responsible for enterotoxaemia in sheep. *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, of 113 strains isolated from sheep, 91 (80.5%) were type A. The majority of type C and type D isolates belonged to diseased sheep. In accordance with our results, several studies conducted in other coutries reported that the most predominant type in sheep 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). The results of our study show that the most prevalent type of *C. perfringens* in the intestinal flora of sheep is type A and thus the detection of toxins rather than isolation, in intestinal content of sheep in enterotoxemia cases is more convenient tool for the diagnosis. However, the zoonotic characteristic of *C. perfringens* type A should be taken in account.

In this study, the enterotoxin gene was not detected in any isolate. This result indicates that the enterotoxin is not involved in disease in sheep investigated in the present study. In accordance with our results, the enterotoxin gene was not detected in the *C. perfringens* isolates from lambs (Gkiortzidis et al., 2001), poultry (Engstrom et al., 2003) and pigs (Kanakaraj et al., 1998). However, enterotoxigenic strains of *C. perfringens* have been isolated from horses and cattle (Tschirdewahn et al., 1991).

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. PCR techniques have been used to determine *C. perfringens* toxin types. In PCR assay by Yamagashi et al. (1997) and Gkiourtzidis et al. (2003), individual reactions for each toxin gene were carried out. Multiplex PCR has been developed by some researchers (Meer and Songer, 1997; Yoo et al., 1997; Baums et al., 2004). A multiplex PCR using primers for five toxin genes was performed in this study and all of *C. perfringens* isolates were succesfully typed by the multiplex PCR.

In conclusion, the multiplex PCR can be used to type *C. perfringens* isolates in epidemiological studies as an alternative to conventional procedures. Further studies should be carried out to detect the virulence factors of *C. perfringens* isolates from animals.

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