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Genotyping of isolates of *Clostridium perfringens* from vaccinated and unvaccinated sheep

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ABSTRACT

Enterotoxaemia vaccine is a polyvalence vaccine from different types of *Clostridium perfringens*, and *C. septicum* toxins that prevents various diseases, the most important one being enterotoxaemia in sheep. The aim of the present study was to evaluate the enterotoxaemia vaccine effects in reducing isolates of intestinal clostridia genus specifically *C. perfringens*. Sheep dung samples were randomly collected from 10 places in Kerman, Iran. The samples were taken from 90 vaccinated Kermani sheep against enterotoxaemia and 50 unvaccinated sheep of same age from flocks with similar management. Following processing and culture of the samples, colonies were identified applying morphological, gram stain and biochemical tests. Using these tests *C. perfringens* were isolated from 27 out of 50 unvaccinated sheep (54.0%) and from 2 out of 90 vaccinated sheep (2.2%). All of the clostridia isolates were analyzed by multiplex PCR. Genotyping of 2 strains isolated from the vaccinated sheep indicated that these strains were type D, while the strains isolated from the unvaccinated sheep were types A, B, C and D; 14.8% (4 out of 27), 22.2% (6 out of 27), 40.7% (11 out of 27) and 22.2% (6 out of 27), respectively. However, no isolate containing the iota gene (type E) was detected. Vaccination against enterotoxaemia had a significant effect ($P < 0.01$) on reducing *C. perfringens* isolates. Occurrence of the disease in the vaccinated and unvaccinated groups was 3.3% and 64.0% ($P < 0.01$), respectively.

1. Introduction

Clostridium perfringens, one of the most widely distributed pathogenic bacteria in the environment, is an anaerobic, gram-positive and spore-forming bacillus (Songer, 1996; Vijay et al., 2008). This bacterium is the most rapidly growing foodborne pathogen and is found in soil, water, air, food, and intestinal tract of human and animals (Vijay et al., 2008). Enterotoxaemia in sheep is caused by different toxin types of *C. perfringens* (Songer, 2006) and causes considerable economic loss to the sheep industry due to high fatality rates, increased treatment costs, and decreased productivity (Greco et al., 2005). On the other hand, domestic animals are known to be sources of human food poisoning. In order to reduce or eliminate this risk, strategies must be developed to prevent entering infected animals to the food chain (Piatti et al., 2004). *C. perfringens* is classified into five types (A–E) based on the synthesis of four major lethal toxins, alpha, beta, epsilon and iota (McCourt et al., 2005; Gurjar et al., 2008). Type A produces only alpha toxin, type B produces alpha, beta and epsilon toxins, type C produces alpha and beta toxins, type D produces alpha and epsilon toxins while type E produces alpha and iota toxins (Kalender et al., 2005). Toxin typing of *C. perfringens* is important since particular toxin types are

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associated with specific enteric diseases in animals (Van Asten et al., 2009).

Traditionally, for classification of *C. perfringens*, serum neutralization tests on mice or guinea pigs are performed (Yoo et al., 1997; Van Asten et al., 2009). However, as it was pointed by Van Asten et al. (2009) these tests are laborious, expensive, and no longer considered ethically acceptable. In some diagnostic laboratories, this differentiation has been replaced by rapid and easy to use enzyme-linked immunosorbent assays (ELISAs). Although the ELISAs allow reliable typing of *C. perfringens* isolates, the options for subtyping are limited. For example, using ELISA is not possible to detect the beta toxin. In addition, high levels of enterotoxin are present only during sporulation (Baums et al., 2004). Furthermore, traditional methods are limited for subtyping. Thus, various PCR protocols including multiplex PCR assays have been established to genotype *C. perfringens* isolates (Settanni and Corsetti, 2007). PCR typing is achieved by demonstrating the presence of the encoding gene(s) in the bacterial genome (Van Asten et al., 2009) or in the plasmid since the epsilon toxin gene is thought to reside on a large plasmid (Songer, 1996; Johansson, 2006). A number of the molecular tools allowing an easier *in vitro* test and PCR method of typing *C. perfringens* have been developed (Yoo et al., 1997).

Detecting different types of *C. perfringens* in an area is important for the improvement of the most appropriate vaccines (Kalender et al., 2005). In addition, the effects of the developed vaccines on prevention of the disease need to be investigated. De la Rosa et al. (1997) compared vaccination schedules for ewes and their lambs to raise antibody concentrations against epsilon toxin of *C. perfringens*, the causative agent of enterotoxaemia. They observed that vaccination of lambs did not increase sera antibody concentration but prepartum vaccination of the dams increased antibody concentrations in the lambs when compared with the lambs reared by unvaccinated ewes. El Idrissi et al. (1992) reported that *C. sordellii* alone or with *C. perfringens* may be an important pathogen in sudden mortality in sheep (El Idrissi et al., 1992). They found that as there was no significant difference between vaccinated and unvaccinated sheep with regard to clostridial infections, vaccines or vaccination programs need to be improved (El Idrissi et al., 1992).

The aim of this study was to genotype *C. perfringens* isolates from vaccinated and unvaccinated sheep by multiplex PCR and evaluation of enterotoxaemia vaccine effects on reduction of *C. perfringens* isolations and prevention of enterotoxaemia in Kermani sheep.

2. Materials and methods

2.1. Samples

Sheep dung samples were randomly collected from 140 Kermani sheep, a local breed in southeast of Iran, related to 10 places in Kerman, Iran. The sheep (1–2.5 years of age) were from both sexes. All of the sheep were healthy apparently and did not have prehistory of enterotoxaemia. In 6 out of 10 farms, enterotoxaemia vaccine was injected and from each flock 15 samples (total of 90 samples) were collected. In addition, in 4 farms, no enterotoxaemia vaccine was used and from each flock 10–14 samples (total of 50 samples) were collected. All of the vaccinated and

Table 1
Biochemical tests for identification of the clostridia species from vaccinated and unvaccinated sheep dung samples.

Catalase test	Egg yolk agar		Gelatin hydrolyzed	Indole produced	Carbohydrates fermentation				Motility	Milk reaction	The identified clostridia species
	Lecithinase produced	Lipase produced			Glucose	Lactose	Sucrose	Maltose			
Unvaccinated sheep											
−	+	−	+	−	+	+	+	+	−	dc	*C. perfringens* (27 case)
−	+	−	−	−	+	w	+	w	−	c	*C. baratii* (1 case)
−	−	−	−	−	+	−	−	−	+	−	*C. scatologenes* (1 case)
Vaccinated sheep											
−	+	−	+	−	+	+	+	+	−	dc	*C. perfringens* (2 case)
−	+	−	+	+	+	−	−	w	+	d	*C. bifermentans* (2 case)
−	−	+	+	−	+	−	−	−	+	d	*C. sporogenes* (3 case)
−	−	−	−	−	−	−	+	+	−	−	*C. leptum* (1 case)
−	−	+	+	−	+	+	+	+	+	c	*C. aurantibutyricum* (1 case)
−	−	−	−	−	−	−	−	−	−	−	*C. sporosphaeroides* (1 case)
−	−	+	+	−	+	−	−	−	+	d	*C. botulinum proteolytic* (1 case)
−	+	−	+	−	+	+	+	+	−	c	*C. absonum* (1 case)
−	−	−	−	−	+	+	+	+	−	c	*C. ramosum* (1 case)
−	−	−	−	−	+	−	+	−	−	−	*C. innocuum* (1 case)
−	−	−	+	+	+	+	+	+	+	c	*C. indolis* (1 case)

c: curd; d: digestion; cd: first curd then digestion; dc: first digestion then curd; w: weak.