Prevalence and Toxinotyping of the Toxigenic Clostridium perfringens in Sheep with suspected Enterotoxemia

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Abstract: The study was designed to determine the types of Clostridium perfringens and their toxins in sheep with suspected enterotoxemia in Al- Ahsa province, KSA. Out of 240 intestinal content samples collected from dead sheep with suspected enterotoxemia, 34 Clostridial strains were isolated. Diagnosis was based on classical diagnostic tests, enzyme-linked immunosorbent assay (ELISA) and multiplex polymerase chain reaction (PCR) to determine the types and toxins of C. perfringens. All isolates exhibited characteristics of Clostridium perfringens. Subsequently, molecular typing of the bacterial isolates was performed by multiplex PCR. Genotyping of isolated strains revealed that, 18 (52.94%) were type A, 2 (5.8 %), were type B, 6 (17.64 %), were type C and 8 (23.52%) were type D. Based on the ELISA results, 73 (30.41%) intestinal content samples were positive for the toxins produced by C. perfringens. Clostridium perfringens type A and D were the dominant types in cases of enterotoxemia in sheep in Al- Ahsa province detected by ELISA and multiplex PCR tests. The enterotoxaemia causes considerable economic loss to the sheep industry. Therefore, it is recommended that a proper vaccination schedule against enterotoxemia should be implemented for sheep flocks in Ahsa province. These vaccines should provide adequate protective immunity against all C. perfringens types specially types A and D.
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1. Introduction

Clostridium perfringens is a gram-positive, anaerobic bacterium that causes a wide range of diseases in humans and animals. It is widely spread in the environment and is commonly found in the gastrointestinal tract of animals. The pathogenicity of this organism is associated with exotoxins **(Songer, 1996; Uzal, 1996; Uzal, and Song, 2008)**. C. perfringens is classified into five types, A, B, C, D and E, based on the synthesis of four major lethal toxins including, alpha, beta, epsilon and iota.The enterotoxin and β2-toxin are further important toxins produced by C. perfringens, and both have been reported in the last decade **(McDonel, 1986; Yamagashi et al., 1997; Gkiourtzidis et al., 2001)**. 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. **(Songer, 1996).**

Enterotoxemia caused by C. perfringens is fatal enteric disease that affects all species of domestic animals and humans. It is one of the most frequently occurring diseases of sheep and goats worldwide with reported prevalence rates ranging between 24.13% and 100% (**El Idrissi and Ward, 1992; Greco et al., 2005**).

The diagnosis of enterotoxemia is usually based on clinical signs and pathological findings, but identification of toxins in intestinal contents is necessary to confirm the diagnosis. The classical identification of the toxins is based on neutralization test in mice or in skin of guinea pigs , however, this method is tedious, time-consuming, expensive and monovalent. Furthermore, it is improper and unethical to apply it at the expense of laboratory animals (**Yoo et al., 1997**). In recent years, enzyme-linked immunosorbent assay (ELISA) kits have been used for the detection of clostridial toxins (**El Idrissi and Ward, 1992; Naylor et al., 1997 Uzal et al., 2003**).

DNA-based techniques, such as polymerase chain reaction (PCR), have been developed for C. perfringens genotyping and are a reliable alternative method to testing in laboratory animals. Various PCR protocols have been established to genotype C. perfringens isolates with respect to the genes cpa, cpb, etx, iap, cpe, and cpb2, which encode the alpha, beta, epsilon, iota, entero- and β2-toxins, respectively (**Petit et al., 1999; Baums et al., 2004**).

In Saudi Arabia, indigenous sheep and goats are estimated at 7.7 and <3.5 million heads, respectively. They are economically the most important farm animals in the Kingdom, serving as major sources of meat, milk and income for a large sector of the population. More than 5 million sheep and goats are also imported annually during the Hajj season and other religious events (**Anonymous, 2001**).

Vaccination against C. perfringens is very important for preventing enterotoxemia. Detection of C. perfringens types in an area is important for the development of the most appropriate vaccines (**De la Rosa et al., 1997; Bernáth et al., 2004**).

The study was designed to determine the types of Clostridium perfringens and their toxins in sheep with suspected enterotoxemia in Al- Ahsa region in 26° 35' 00" N, 48° 10' 00" E Saudi Arabia using the conventional diagnostic methods, enzyme-linked immunosorbent assay (ELISA) technique and polymerase chain reaction (PCR).

2. Material and Methods

2.1. Intestinal contents samples:

Intestinal contents were collected at necropsy from 240 sheep (aged between two weeks to 3years) with suspected enterotoxemia or mortality due to sudden death during the period from 2011 to 2012. Other 20 intestinal content samples were collected from slaughtered healthy sheep. The animals had belonged to herds raised in Al- Ahsa region. All samples were taken aseptically from ileum and jejunum. Samples were divided into two parts the first was used for bacterial isolation and the other part was diluted (1:5) in phosphate buffer saline (PBS) and centrifuged at 2000 ×g for 20 min at 4 °C. After centrifugation, supernatants were filtrated through 0.45-µm membrane filters and kept at –70 °C until used for detection of toxins**.**

2.2. Bacterial isolation:

intestinal contents and intestinal scrapings were inoculated directly into cooked meat broth medium (Oxoid) and incubated anaerobically in an anaerobic Gas pack jar for 24 h at 37 Co. Loopfuls of growth were then streaked onto 5% sheep blood agar (Oxoid), supplemented with neomycin sulphate. The plates were incubated anaerobically for 48 h at 37 C^{o}. The suspected colonies were identified by characteristic colony morphology, Gram staining. Biochemical identifications were performed using a commercial kit (API 20A, bioMérieux SA, Marcy-l'Etoile, France).

2.3. Biological assay for C. perfringens toxin:

Following culture of the C. perfringens isolated strains in cooked meat broth medium, the cells were harvested by centrifugation at 3 000 rpm for 15 min and the cell-free culture supernatants were recovered; one part of the supernatant fluid was treated with trypsin (**Sterne and Batty, 1975**). White mice (25 - 40 g) were injected intraperitonealy with 0.3 ml of the culture supernatant and then observed over a period of 3 days for either death or disease symptoms. In the same way, mice were injected with 0.3 ml of intestinal content filtrate. Control group of mice were injected with PBS.

2.4. ELISA:

Broth cultures supernatants from isolated strains and intestinal content filtrates were examined by an indirect ELISA commercial kit (Bio-X Diagnostics, Belgium), according to the manufacturer's instructions, for detection and identification of toxins

2.5. Isolation of C. perfringens DNA:

Colonies from 24 hours old cultures on blood agar plates were picked and suspended in 1.5 ml Eppindorf tubes containing 1 ml of distilled water. Following 3 cycles of freeze-thawing, the tubes were incubated at 95°C for 20 min and finally centrifuged for 15 min at 5 000 rpm. The supernatants were collected and kept frozen until use as templates in the PCR. The DNA samples were analyzed spectrophotometrically at 260 and 280 nm to check the presence of DNA and its purity. Samples were also subjected to electrophoresis on a 1.5% (w/v) agarose gel in 1 × TBE buffer to ensure the presence of intact DNA (**Maniatis et al., 1982**).

2.6. Identification of toxigenic strains by Polymerase Chain Reaction (PCR):

All C. perfringens isolates were also PCR-screened for the detection of alfa (cpa), beta (cpb), epsilon (etx), and iota (iap), toxin encoding genes, as described by (**Baums et al., 2004**). The multiplex PCR was performed on a 25 µl mixture containing 2.5 mM MgCl2, 250 µM each deoxyribonucleotide triphosphate (dNTP), 0.5 U Platinum Taq DNA polymerase (Invitrogen),and 0.1 µM of each primers listed in **Table** (1). The thermal cycling was performed as follows: initial denaturation for 2 min 30 sec at 95°C followed by 35 cycles of 1 min at 95°C, 1 min at 60°C, 1 min 20 sec at 72°C and a final extension for 2 min at 72°C. The PCR reaction mixtures were analyzed by electrophoresis on a 1% (w/v) agarose gel in the presence of 100-bp DNA ladder (Fermentas Life Science, EU). The agarose gel was supplemented with ethidium bromide in order to visualize the DNA on an UV transilliminator.

3. Results and Discussion:

C. perfringens causes numerous gastrointestinal infections in most mammalian species. These infections are often generically called enterotoxemia because toxins produced in the intestine may be absorbed into the general circulation. However, while this is true for many C. perfringens toxins, some toxins produced in the intestine act only locally, while others act both locally and systemically (**Songer and Uzal, 2005**). It is well known that enterotoxemia causes considerable economic loss to the sheep industry due to a high fatality rate, decreased productivity, and increased treatment costs (**Özcan and Gürçay, 2000; Greco et al., 2005**).

Table (1): Primers for the four toxins genes of C. perfringens used in multiplex PCR

Toxin gene	Toxin	Sequence 5′→3′	Amplicon (bp)
cpa	**alpha**	5'- AGT CTA CGC TTG GGA TGG AA -3' 5'- TTT CCT GGG TTG TCC ATT TC -3'	900
cpb	**beta**	5'-TCC TTT CTT GAG GGA GGA TAA A -3' 5'- TGA ACC TCC TAT TTT GTA TCC CA -3'	612
etx	**epsilon**	5'- GGG GAA CCC TCA GTA GTT TCA -3' 5'- ACC AGC TGG ATT TGA GTT TAA TG -3'	396
iap	**Iota**	5'- AAA CGC ATT AAA GCT CAC ACC -3' 5- CTG CAT ACC CTG GAA TGG CT -3'	293

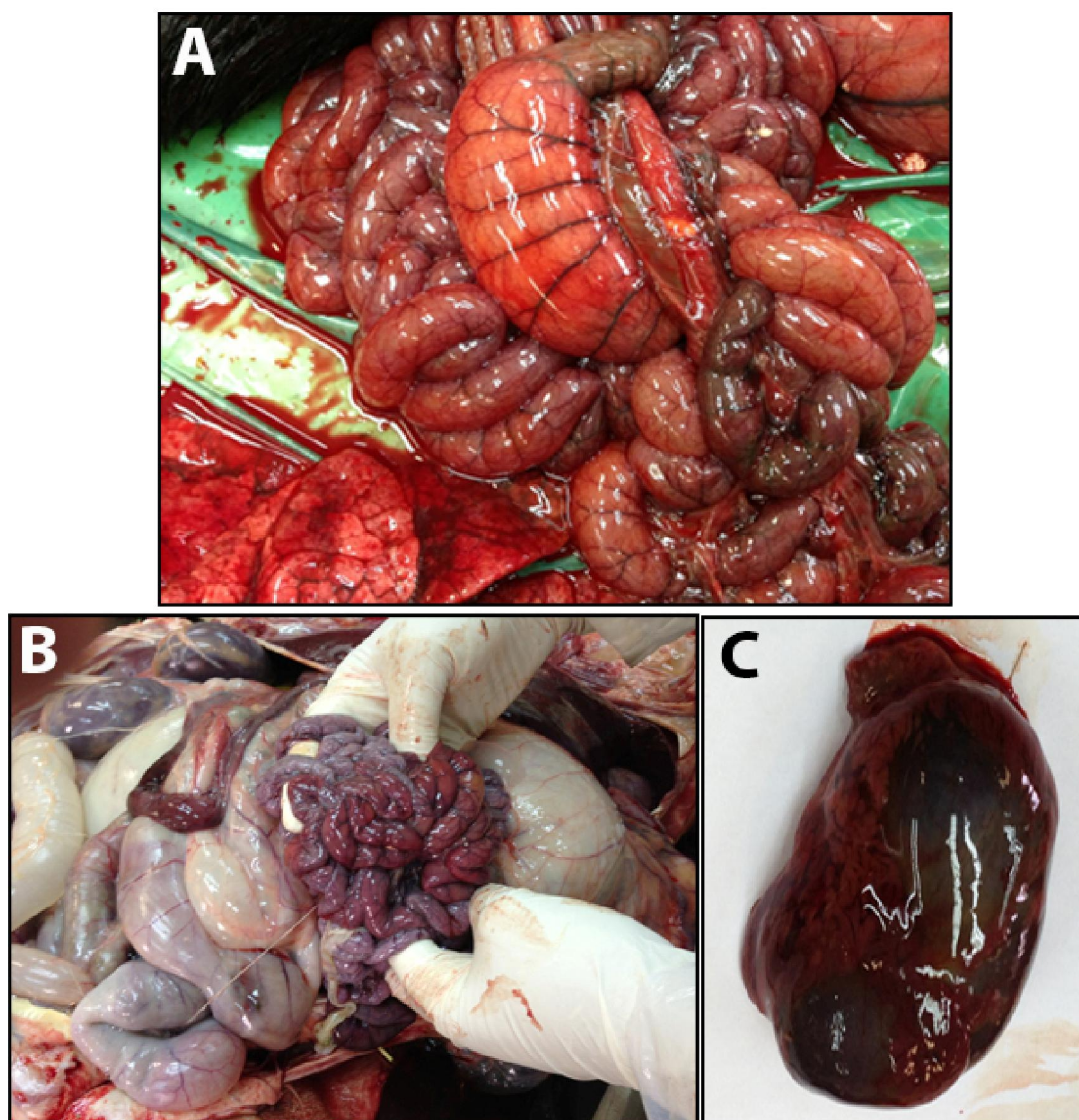

Fig (1) Postmortem findings: The large and small intestines showed severe congestion and edema with a high collection of watery blood and fibrinous clots and distension of intestine with gases (A, B). Congestion and enlargement were also observed in the, kidney (C).

At postmortem examination as illustrated in **Fig** (1) the large and small intestines showed a high collection of watery blood and fibrinous clots with gas distension. Congestion, small ulcers, and edema were observed in the mucous membranes of the abomasum, and small and large intestines. Congestion and enlargement were also observed in the liver, kidney, lung, and spleen. In addition to these sings , many petechiae were observed in the small intestine, kidney, epicardium, and bladder. Distension of intestine with gases as well as with thin watery, mucoid or bloody content observed through postmortem examination come in accordance with (**Mansour, 2004**). C. perfringens is one of the best-known toxin-producing bacteria. It can produce both major (mouse lethal) and minor (non-lethal) toxins (**Cavalcanti, 2004**). The pathogenesis of C. perfringens diseases is directly associated with the many toxins and enzymes. Our postmortem findings are documented by the reports of (**Songer, 1996; Songer and Uzal, 2005)**

Of 240 sheep intestinal samples, 34 (14.1 %) were culture positives, where 29 strains were isolated from 210 adult sheep and 5 strains from 30 kid sheep. The intestinal contents collected from slaughtered healthy sheep not revealed any isolates.

All bacterial isolates exhibited the characteristic features of C. perfringens. The colonial characters on blood agar showed dew drops smooth greyish convex colonies with a double zone of haemolysis. Microscopic characters revealed Gram positive non motile rods. Biochemical identification of the isolates showed catalase, lecithinase positive and a haemolytic activity on sheep blood agar showing double zone of haemolysis. Gas and acid from glucose, fructose, lactose sucrose and mannitol were seen, urease negative and gelatinase positive. Based on biochemical tests, and in consultation with Bergey's Manual of determinative bacteriology (**Holt et al., 1994**), the isolates were identified as C. perfringens.

The prevalence of C. perfringens in the intestinal contents come in agree with (**Moussa, and Ashgan 2011, Al-Humiany, 2012**) where they reported the prevalence of toxigenic clostridia in the central and north regions of KSA. Enterotoxemia is one of the most frequently occurring diseases of sheep and goats worldwide. Reports from countries around the world have reported prevalence rates of enterotoxemia ranging between 24.13% and 100% ((**El Idrissi and Ward, 1992; Greco et al., 2005**).

Regarding to the biological assay for C. perfringens toxin, all culture supernatants from the 34 isolates were toxigenic to mice, whereas only 73 intestinal content filtrates were toxigenic for mice. Samples collected from apparently healthy sheep at slaughtering time were not toxigenic for mice. Control mice injected with PBS were still alive. C. perfringens is a prolific bacterial toxin producer, reportedly producing >17 different toxins. Each C. perfringens type is associated with particular diseases suggesting that certain toxins, or combinations of toxins, are important contributors to those diseases (McClane, 2004). However, since each C. perfringens isolate produces multiple toxins, it can only be conclusively determined whether a specific toxin is important for virulence by fulfilling Molecular Koch's postulates (**Falkow, 1988**).

The use of ELISA for the detection of C perfringens toxins allows the differential diagnosis of enterotoxemia cases caused by C. perfringens types A, B, C, and D from samples of intestinal content as well as the typing of C perfringens cultures (**Naylor, et al 1997).**It has been reported that ELISA is 95% reliable for the detection of C. perfringens toxins in the intestinal contents of sheep suspected of having enterotoxemia (**El Idrissi, and Ward, 1992**).

The intestinal contents collected from 20 slaughtered healthy sheep were found to be negative for alpha, beta, and epsilon toxins. A total of 73 (30.41%) intestinal content samples were positive for the toxins produced by C. perfringens, while 167 samples (69.58%) were negative. The number of samples determined to be positive for different C. perfringens types by ELISA was 35 for C. perfringens type A (47.94%); 5 for C. perfringens type B (6.84%); 13 for C. perfringens type C (17.8%), and 19 for C. perfringens type D (26.02%) **Table 3**. According to the toxins produced by the isolated strains, Out of 32 C. perfringens isolates, 18 (52.94%) were type A, 2 (5.8 %), were type B, 6 (17.64 %), were type C and 8 (23.52%) were type D, with type A as the dominant one. **Table 2**.

Regarding to the toxin type detected in the intestinal contests, types A and D were the dominant types detected by ELISA in sheep with suspected enterotoxemia, while type E was not encountered. Our results were in compliance with those reported in previous studies, **(Moussa and Ashgan, 2011; Al-Humiany, 2012**) where they showed the high prevalence of C. perfringens type A and D among sheep enterotoxemia in central and north regions of KSA.

It is a common view that C. perfringens types B, C, and D are mostly responsible form enterotoxemia, while C. perfringens type A alone is not involved or has very limited influence. Recently, several researchers have reported on the importance of C. perfringens type A (**Manteca et al., 2001; Greco et al.; 2005)**.

Table (2): Toxigenic types of C. perfringens isolated from sheep by ELISA and PCR.

Animal	No of Intestinal Content Samples	No o f C.perfringens Isolates	No of isolates following toxin typing					No of isolates following genotyping				
			A	B	C	D	E	cpa	cpa cpb etx	cpa cpb	cpa etx	cpa iap
Sheep	210	29	17	1	3	8	-	17	1	3	8	-
Lambs	30	5	1	1	3	-	-	1	1	3	-	-
Total	240	34 (14.1%)	18	2	6	8		18	2	6	8	-

Table (3): Results of ELISA for detection of toxin types in the intestinal contents.

Animal	No of Intestinal Content Samples	No of Positive Samples	C. perfringens types				
			A	B	C	D	E
Sheep	210	64	30	4	11	19	-
Lambs	30	9	5	1	3	-	-
Total	240	73	35	5	14	19	-

Fig (2) Detection of a toxin gene in C. perfringens by multiplex PCR. Lane 1**:** 100 bp molecular marker; lanes 2, 7: cpa, etx; lanes 3, 6: cpa, cpb, etx, lanes 4, 8: cpa, lane 5: cpa, cpb and Lane: 9, 10 negative control.

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.

A multiplex PCR using primers for five toxin genes was performed in this study and all of C. perfringens isolates were successfully typed by the multiplex PCR.

In the present study, A total of 34 C. perfringens isolates from sheep were genotyped by PCR. Of these isolates, 18 (52.94%) were type A, 2 (5.8 %), were type B, 6 (17.64 %), were type C and 8 (23.52%) were type D. PCR products of alpha toxin (900 bp), beta toxin (612 bp), epsilon toxin (396 bp) were shown in (**Figure 2**).

Compared to conventional techniques, the PCR method has been shown to be much more rapid,

giving results in a few hours, and it is much more reliable

The multiplex PCR presented here includes all toxin genes considered to be important virulence factors in C. perfringens mediated enteritis or enterotoxaemia. It offers a reliable differentiation of C. perfringens isolates in a sing le reaction, even when performed with bacterial lysates.(**Baums et al., 2004**).

Enterotoxemia causes more economic loss among feedlot- and pasture-reared lambs than all other diseases combined, if vaccination is not applied (**Quinn et al., 1994**). Vaccination against C. perfringens is very important for preventing enterotoxemia; however, C. perfringens biotypes causing enterotoxemia in a particular region should be identified so as to formulate a proper vaccine.

In conclusion, the multiplex PCR can be used to type C. perfringens isolates in epidemiological studies as an alternative to conventional procedures. The majority of toxins were identified as C. perfringens type A and D. The results of the study indicated that C. perfringens type A, B, C, and D may cause enterotoxemia, but type A and D were the predominant causative agents of enterotoxemia in the sheep in Al Ahsa. Therefore, it is strongly recommended that a vaccination schedule should be implemented to reduce the incidence of enterotoxemia in Al Ahsa province. This vaccine should provide adequate protective immunity, especially against C. perfringens type A and D.

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