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Bacteriological and molecular studies on *Clostridium perfringens* isolated from sheep in three Egyptian provinces

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The study was intended to determine types of *Clostridium perfringens* and their toxins in diseased sheep with suspected enterotoxaemia, apparently healthy in contact sheep and soil in three Egyptian provinces (one year study). A total of 800 sheep were visited regularly over a period of one year to record cases of enterotoxaemia and collection of samples for bacteriological examination and toxin genotyping using multiplex polymerase chain reaction (PCR) by using four primers set specific for genes encoding toxin production (alpha, beta, epsilon and iota). Based on bacteriological examination, the percentage of *C. perfringens* isolated from soil, apparently healthy, and diseases sheep were 41, 12 and 59%, respectively. The results of multiplex PCR indicated that *C. perfringens* type A was the predominant followed by *C. perfringens* type D with an incidence rate of 43 and 42.7% from positive samples, respectively. While, *C. perfringens* type B was successfully recovered only from 14.61% of positive samples. Worth mentioning, the data presented collectively highlighted the role of soil and apparently healthy as a potential source of re-infection. Moreover, it is recommended that *C. perfringens* type A should be included in vaccine schedule in order to afford adequate protection and lessen the adverse economic loses of sheep clostridial diseases.

Key words: Clostridia, sheep enterotoxaemia, multiplex polymerase chain reaction (PCR), Egypt.

INTRODUCTION

Sheep and goat are considered as one of the important pillars of income in many developing countries including Egypt. Thus, sheep diseases especially that caused by *Clostridium perfringens* most notably sheep enterotoxemia

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are considered the most risk factor opposite this industry (Ahmed, 2004).

C. perfringens is a normal inhabitant of the intestine of most animal species, present in a small number and produce a little amount of toxin easily removed by the normal gut movement, but when the intestinal environment is altered by sudden changes in diet or other factors, *C. perfringens* proliferates and produces potent toxins that act locally or are absorbed into the general circulation with usually devastating effects on the host (Uzal and Songer, 2008). The diseases are generally called enterotoxemia in sheep, goats and other animals (Uzal and Songer, 2008).

C. perfringens is classified into 5 major toxin types (A, B, C, D, and E) on the basis of expression of 4 major toxins: alpha, beta, epsilon, and iota (Gokce et al., 2007). The clinical manifestation and severity of the infection in correspondence varies markedly according to toxigenic type involved and the specific toxin produced (Greco et al., 2005).

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 enterotoxaemia ranging between 24.13 and 100% (el Idrissi and Ward, 1992; Fayez et al., 2013).

Presumptive diagnosis mainly depends on clinical signs and gross post-mortem findings. In addition, identification of the toxins in intestinal content is crucial for accurate diagnosis. Traditional methods of toxins identification are mainly based on neutralization test in mice or skin of guinea pigs. In the last decades, Enzyme-linked immunosorbent assay (ELISA) kits have been used for the detection of Clostridial toxins (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 (Baums et al., 2004).

To the author knowledgement, only a few studies were conducted to estimate the incidence of *C. perfringens* toxin in camels and humans (Habashy et al., 2009; Mohamed et al., 2010). Moreover, there is a scarcity of data regarding the toxigenic type of *C. perfringens* predominantly implicated in sheep enterotoxaemia in Egypt. In addition, the role of soil and apparently healthy sheep is still unknown.

Thus, this study was undertaken to estimate the incidence of *C. perfringens* induced sheep-enterotoxaemia in three Egyptian provinces using conventional bacteriological methods and multiplex PCR for elucidating the correct toxigenic type.

MATERIALS AND METHODS

Clinical and necropsy finding

A total of 20 sheep flocks located at Desert Road, Waedielnatron, Merkz bader and Sadat city were regularly visited for one year to inspect the animals for clinical signs of enterotoxaemia. Each flock was examined clinically according to Radostitis et al. (2007). Clinical symptoms of clinically diseased sheep were recorded. Necropsy findings of the dead animals were recorded according to Griffiths (2005) within 6 h of death. Samples for bacteriological examination were taken from rectum and intestinal contents of sheep which displayed a variety of clinical signs such as abdominal pain, bloat, mild to severe (blood-tinged to bloody) diarrhea and recumbence. Small intestine specimens (duodenum) were rapidly taken from recently dead or sacrificed animals with clinical symptoms of dysentery, in order to avoid non-specific *C. perfringens* (Osman, 1993; Ahmed, 2004). In some animals, nervous symptoms (dullness, muscular tremors, opisthotonos and convulsions) were observed, while in other cases death was the only clinical sign observed.

Histopathological examination

Specimens from brains, kidneys, livers and small intestines were collected after necropsy and fixed in 10% buffered neutral formalin solution. Five-micron thick paraffin sections were prepared, stained with Hemotoxylin & Eosin stain (H&E), and then examined microscopically for histopathology (Bancroft and Gamble, 2002).

Samples for bacteriological examination

Four hundred (400) samples were collected from the soil of sheep farms, clinically healthy and sheep with enterotoxemia for isolation of *C. perfringens* (Table 1). Regarding soil samples, about 10 g of soil were collected in sterile plastic bags after removal of the superficial layer of the soil at a depth of 20 cm and sent directly to a laboratory for bacteriological isolation followed by toxin identification using multiplex PCR. Briefly, the fecal samples were collected from the rectum of diseased sheep and healthy in contact sheep and directly sent to laboratory for bacteriological examination. While, in case of intestinal samples, portions of small intestine (congested and/or ulcerated) from diseased and healthy in contact sheep were ligated from both ends, cut and put in sterile plastic bags and directly sent to laboratory in cold ice (Osman, 1993; Ahmed, 2004).

Isolation and identification of *C. perfringens* (Willis, 1977)

C. perfringens was isolated on cooked meat medium (Oxoid), 10% sheep blood agar (Oxoid), Perfringens agar medium LAB 194 (Lab M): Typical colonies were identified as described by Murray et al. (2003), and full identification of the isolates mainly depending on their characteristic colonial morphology, hemolysis activity, Gram staining and biochemical tests according to Cruickshank et al. (1975), Koneman et al. (1988) and Quinn et al. (2002).

Multiplex PCR

The whole genomic DNA was extracted according to Sambrook and Russell (2001). Briefly, 1 ml of overnight culture in fluid thioglycollate broth (FTB) supplemented with D–cycloserine was centrifuged for 20 min at 13,000 rpm. The pellets were then re-suspended in 600 µl of ice cold cell lysis buffer. Then 3 µl of proteinase K solution was added and incubated at 55°C for 3 to 16 h to increase the yield of genomic DNA. Finally, the extracted DNA pellet was stored in 100 µl of tris-EDTA buffer at -20°C for further analysis. The PCR reactions conditions were carried out using four

Table 1. Types and numbers of samples collected for bacteriological examination.

Types of samples collected		Number
Soil	Villages area	50
	Newly reclaimed area	50
Examined suspected sheep	Faecal samples	70
	Intestinal contents	130
Apparently healthy sheep	Faecal samples	50
	Intestinal contents	50
Total		400

Table 2. The nucleotide sequences of primers used for multiplex PCR amplification (Greco *et al*., 2005).

Toxin/Gene	Oligonucleotide sequences	Fragment length (bp)
α/cpa	5-TGC TAA TGTTAC TGC CGT TGA TAG-3 5-TGC TAA TGTTAC TGC CGT TGA TAG-3	247
β/cpb	5-AAC TTA ACT GGA TTT ATG TCT TCA-3 5-ATA GTA GAA AAA TCA GGT TGG ACA-3	317
ε/etx	5-ATT AAA ATC ACA ATC ATT CAC TTG-3 5-CTT GTG AAG GGA CAT TAT GAG TAA-3	206
í/iap	5-TTT TAA CTA GTT CAT TTC CTA GTT A-3 5-TTT TTG TAT TCT TTT TCT CTA GGA TT-3	298

primers sets as described previously (Greco et al., 2005) (Table 2). The reactions were performed in 50 µl volumes in PCR tubes. The reaction mixture consists of 10 µl DNA template, 5 µl of 10 × Dream Taq green buffer, 3 µl of dNTPS (10 mM of each), 0.5 µl of Dream Taq polymerase (2.5 u), 1 µl of each primer (50 pmoles/µl) and 23.5 µl of DNA-free water. The thermal conditions were 95°C for 5 min followed by 35 cycles consisting of 1 min of denaturation at 94°C, 1 min of annealing at 50°C and 1 min of extension at 72°C. The final extension was performed at 72°C for 10 min. The PCR products were analyzed using 1.5% agarose gel and stained with ethidium bromide. Amplified bands were visualized and photographed under UV illumination.

RESULTS

Clinical symptoms

Two hundred sheep out of the 800 (25%) examined sheep showed signs of clostridial enterotoxemia, with a mortality rate of 16.25% (130/800). In addition, 80 diseased sheep out of 200 (40%) showed peracute and acute forms of illness (sudden death to death within 24 h), while 120 (60%) of the diseased sheep showed sub-acute illness (death occurs within 72 h from the beginning of illness). Three clinical forms had been identified; the first form is characterized by acute onset of depression, pale mucus membrane, collapse, dyspnea and severe abdominal pain. This form ended by the death of diseased animals within the same day of onset of illness while few animals may survive for several days. The second form is characterized by sudden death. In some cases, depression, with abdominal pain and severe bloody to greenish diarrhea were noticed. The last observed form is characterized by diarrhea, inappetence, depression ended by collapse and death. In some cases, progressive weakness with the development of nervous signs such as dullness, ataxia, incoordination and convulsive movement of the head with the neck rest laterally on the shoulder were observed.

Necropsy finding

The necropsy findings of the examined cases were summarized (Figure 1A, B, C and D). The post-mortem

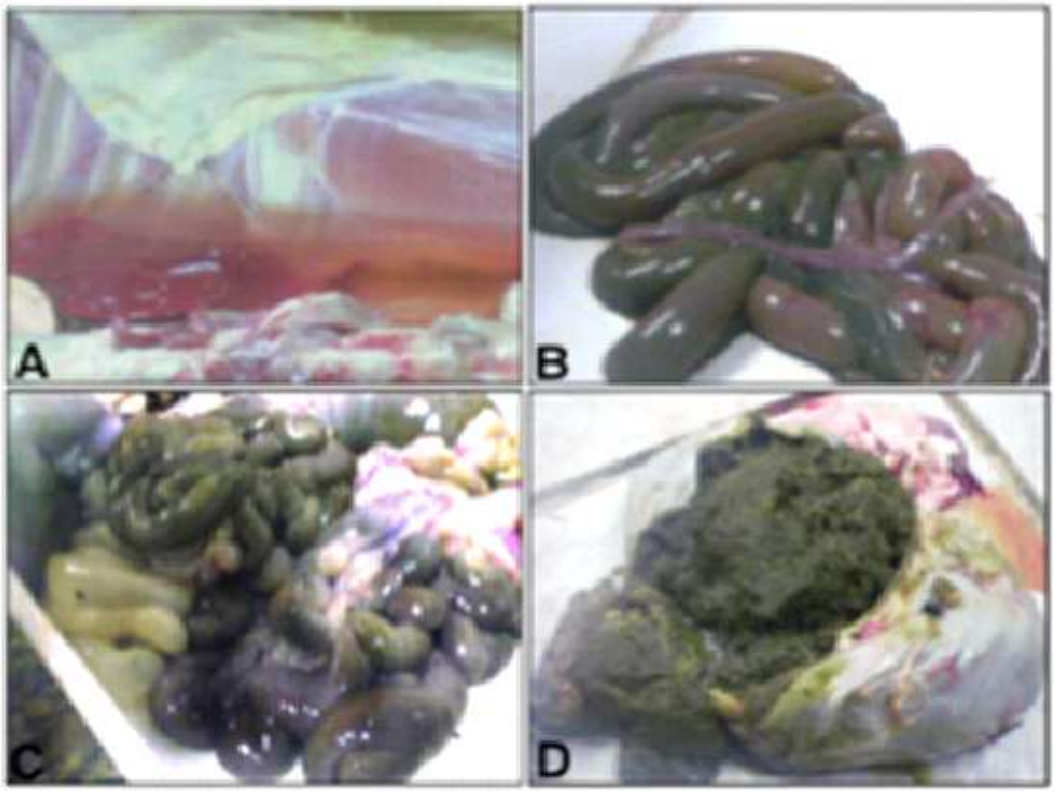

Figure 1. Necropsy findings of the diseased sheep. A) Bloody fluids in body cavities. B) Severely congested part of sheep small intestine. C) Severely congested sheep small intestine with gases. D) Rumen of sheep overloaded with ingesta.

examination of dead cases of the three forms showed bloody fluid in body cavities (Figure 1A) with flappy heart, severely congested intestine (Figure 1B), the presence of gasses in the small intestine with the overloaded rumen (Figure 1C to D), congestion of brain and meninges with friable congested liver and kidneys. Of note, there is no post-mortem changes that could be reported in some cases.

Histopathology

The histopathological changes showed severe necrosis and destruction of intestinal villi, hemorrhages, edema in the intestinal lamina propria with severely congested blood vessels (Figure 2A and B). The liver showed dissociation of hepatic cords, hemorrhages, congestion of central vein, edema under the endothelial lining of a hepatic sinusoid with portal edema and coagulative necrosis (Figure 2C and D). The kidneys showed hyper-cellularity with red blood cells infiltration in Bowman's space, medullary hemorrhages and nephrosis with necrosis of proximal and distal convoluted tubules (Figure 2E, F and G). Malacia, demyelination, perivascular hemorrhages, edema and congestion of both cerebrum and cerebellum were also observed in brain samples.

Isolation and identification of *C. perfringens*

C. perfringens was isolated and characterized based on typical colony morphology on sheep blood agar with a characteristic double zone of hemolysis, while on perfringens agar medium supplemented with D-cycloserine and egg yolk emulsion, the micro-organism appeared as small black colonies surrounded by halo area due to lecithinase activity. Gram-stained smear from the colonies revealed the typical appearance of Gram-positive straight sided rods arranged singly or in pairs. The percentage of *C. perfringens* isolation from the soil, apparently healthy and diseased sheep were 41% (41/100), 12% (12/100) and 59% (118/200), respectively.

Multiplex PCR for clostridia toxin type

Depending on the results of PCR and toxin genotyping, the data presented in Table 3 showed that the percentage

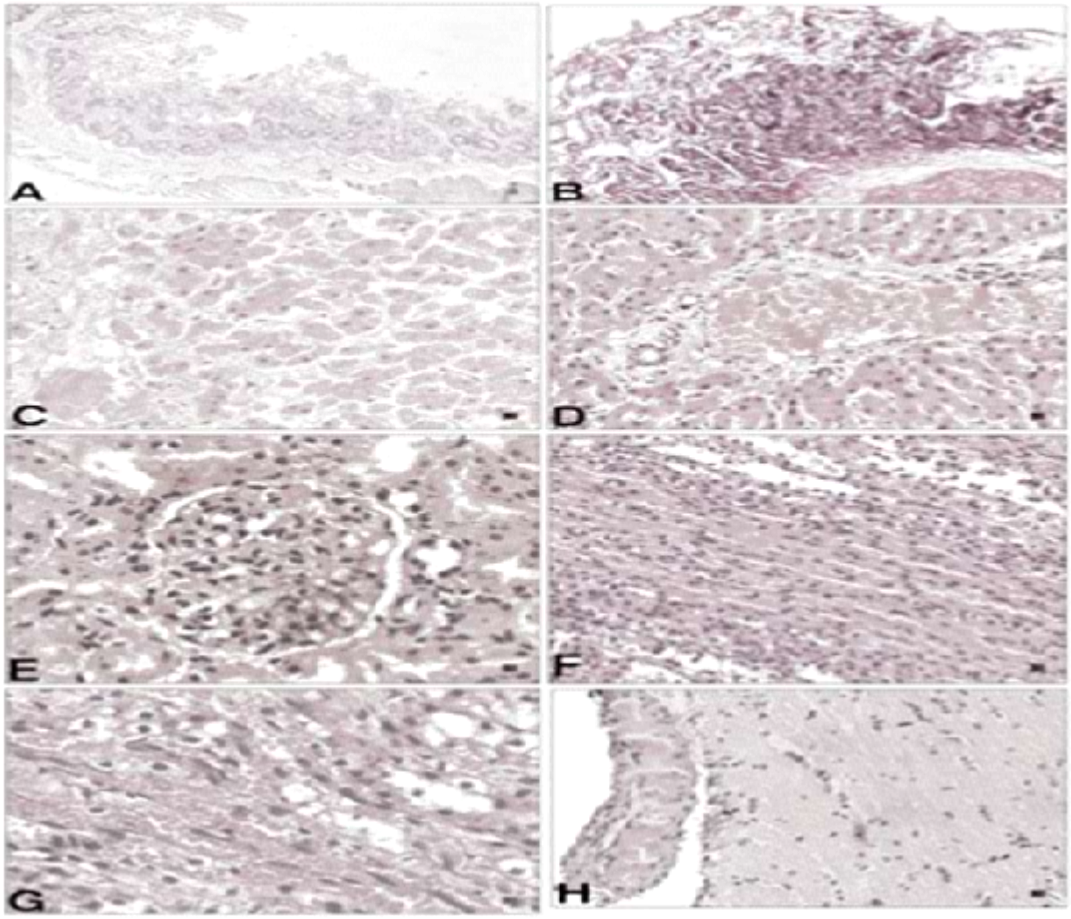

Figure 2. Histopathological examination of enterotoxemic sheep. (A) Sever necrosis and destruction of intestinal villi haemorrhages, oedema in lamina propria with severely congested blood vessels H&E (x4). (B) Sever necrosis and destruction of intestinal villi with haemorrhages H&E (x10). (C) Dissociation of hepatic cord with coagulative necrosis H&E (x20). (D) Congestion in central vein, hepatic hemorrhages, dilation in hepatic sinusoid with edema under the endothelial lining H&E (x20). (E) Dilatation of Bowman's space with red blood cells infiltration and necrosis in convoluted tubules H&E (x40). (F) Medullary haemorrhages with necronephrosis H&E (x20). (G) Haemorrhages, vacuolation and destruction in distal convoluted tubules H&E (x40). (H) Capillary congestion with edema in cerebellum H&E (x20).

of *C. perfringens* type A (cpa gene only), *C. perfringens* type B (cpa, cpb and etx genes) and *C. perfringens* type D (cpa and etx genes) in positive soil samples was 70.37% (29/41), 7.31% (3/41), and 21.95% (9/41), respectively. While in case of the apparently healthy sheep, the incidence rate of *C. perfringens* type B (cpa, cpb and etx genes) and *C. perfringens* type D (cpa and etx genes) in positive samples was 50% (6/12) and 50% (6/12) correspondingly, with no positive samples for *C. perfringens* type B. Finally, a higher incidence was observed in diseased sheep, *C. perfringens* type A (cpa gene only), *C. perfringens* type B (cpa, cpb and etx genes) and *C. perfringens* type D (cpa and etx genes) were recovered from 35.59% (42/118), 21.18% (25/118), and 45.76% (54/118) of positive samples in that order. The overall incidence showed that, *C. perfringens* type A (cpa gene only), *C. perfringens* type B (cpa, cpb and etx genes) and *C. perfringens* type D (cpa and etx genes) were recovered from 45.03% (77/171), 14.61% (25/171), and 40.35% (69/171) of the positive clostridial perfringens (Table 3).

DISCUSSION

C. perfringens is spore-forming Gram-positive cocci that produce more than 17 toxins (Li et al., 2013; Popoff and Bouvet, 2013). *C. perfringens* is classified into five main groups A to E in relation to the production of four lethal toxins alpha, beta, epsilon and iota toxins (McCourt et al.,

Table 3. Prevalence of *C. perfringens* recovered from diseased, apparently healthy sheep and soil and toxin genotyping using multiplex PCR.

Type of isolate	*C. perfringens* Type A		*C. perfringens* Type B		*C. perfringens* Type D		Total
Source of samples	No	%	No	%	No	%	
Village soil	11	84.62	0	0	2	15.38	13
Newly reclaimed soil	18	64.28	3	10.72	7	25	28
Clinically healthy sheep	6	50	0	0	6	50	12
Diseased sheep	42	35.59	25	21.18	54	45.76	118
Total	77	45.03	25	14.61	69	40.35	171

2005; Gurjar et al., 2008). *C. perfringens* induced enterotoxemia in sheep and goat represents a major economic obstacle facing developing countries attributable to the high fatality rate, decreased productivity, and increased treatment costs (Özcan and Gürçay, 2000; Uzal et al., 2003; Greco et al., 2005). A conclusive diagnosis was not only based exclusively on toxin detection but also accompanied by pathological as well as microbiological findings (Uzal and Songer, 2008). Three clinical forms had been identified; the first form associated with *C. perfringens* type A infection (yellow lamb disease or hemolytic disease of sheep) and characterized by acute onset of depression, pale mucus membrane, dyspnea, and severe abdominal pain, then death on the same of onset of illness or some animals may die within few days later. The second form associated with *C. perfringens* type B infection (lamb dysentery and/or hemorrhagic enteritis) sudden death is the main feature of this form. Sometimes, symptoms of depression, abdominal pain, and severe bloody to greenish diarrhea were noticed. The last observed form was associated with *C. perfringens* type D infection (pulpy kidney disease) which begins with the sudden death of few cases, and the number of died animal markedly increased within a week or more from the first record of death. In addition, many signs were observed such as diarrhea, inappetence, depression ended by collapse and death. Noteworthy, some animals suffered from progressive weakness with the development of nervous signs such as dullness, ataxia, incoordination and convulsive movement of the head with the neck rest laterally on the shoulder. Regarding the necropsy finding, the data obtained showed that the rumen is full of ingesta, severely congested intestine and bloody fluids in the body cavities. While, histopathological examination of tissue samples revealed that, severe necrosis and destruction of intestinal villi, hemorrhages, edema in the intestinal lamina propria with severely congested blood vessels (Figure 2A and B). The liver showed dissociation of hepatic cords, hemorrhages, congestion of central vein, edema under the endothelial lining of hepatic sinusoid with portal edema and coagulative necrosis (Figure 2C and D). The kidneys showed changes in the form of hypercellularity with red blood cells infiltration in Bowman's space, medullary hemorrhages and nephrosis with necrosis of proximal and distal convoluted tubules (Figure 2E, F and G). Malacia, demyelination, perivascular hemorrhages, edema and congestion of both cerebrum and cerebellum (Figure 2H). Previous studies showed variable histopathological signs are quite similar to those presented herein (Greco et al., 2005; Van Immerseel et al., 2010). Complete identification of the isolates had revealed that all the isolates were Gram-positive, spore-forming bacilli, produce double zone of hemolysis on sheep blood agar with an incidence rate of 41, 12 and 59% of collected soil, apparently healthy and diseased sheep samples, respectively. Conversely, Habashy el al. (2009) failed to recover any *C. perfringens* isolates from apparently healthy sheep with no data about the role of soil as a potential source of infection. This can be explained as, Habashy et al. (2009) mainly focused on bacteriological isolation and characterization of clostridia with no further molecular identification of toxin type. In addition, their study was conducted in another province; and this strongly recommended further studies to evaluate the regional distribution of clostridial species in Egypt. Conversely, this result is lowered in comparison with that obtained by Mahmoud (1991) and Osman (1993), who successfully isolated *C. perfringens* from 66.5%, 17.74% of samples collected from healthy sheep. While, in case of diseased sheep, the data presented in this study is lower in comparison with that obtained by Abd El-Moez et al. (2014), who showed that, *C. perfringens* was recovered from 77.8% of diseased sheep with no data regarding apparently healthy and soil. The existence of clostridia spores in soils plus in apparently healthy sheep can produce sporadic diseases episodes that are accountable for massive economic losses in animal production through ingestion of the organism and then toxin release (Gamboa et al., 2005; Diego et al., 2012). Therefore, the data presented in this study is considered significant to emphasize the role of soil and apparently healthy sheep in the incidence of *C. perfringens*–induced sheep enterotoxaemia. Hence, further studies are requisite to authenticate the molecular association among *C. perfringens* isolated from soil and

apparently healthy sheep in one hand and diseased sheep in the other to trace the source of infection. Based on the result of multiplex PCR, *C. perfringens* type A was the predominant followed by *C. perfringens* type D in either isolate of diseased sheep, apparently healthy sheep, and soil. This is consistent with the findings of Gerco et al. (2015), who showed that *C. perfringens* type A and D are the predominant causes of predominant causes of enterotoxaemia in very young lambs and kids in southern Italy. Similarly, Abd El-Moez et al. (2014) showed that *C. perfringens* type A is the predominated type isolated from humans and animals. In contrast, previous studies showed that the main cause of sheep dysentery in UK, South Africa, and Greece was *C. perfringens* type B (Gkiourtzidis et al., 2001; Bueschel et al., 2003). Noteworthy, the finding that no *C. perfringens* type E strains were identified strongly advocate that *C. perfringens* type E is rare in lambs and kids (Greco et al., 2005). *C. perfringens* in general are associated with several forms of enteric diseases including fatal enterotoxemia in animals. *C. perfringens* type A is the main causative agent of gas gangrene (myonecrosis) and diarrhea (Hatheway, 1990), while type B and type D are the predominant causes of fatal enterotoxemia in domestic animals (Yamagishi et al., 1997). *C. perfringens* type A is one of the major toxin producers among clostridia species; however, alpha is representing the main toxin type for this species (Popoff and Bouvet, 2013). This species is commonly isolated from environment and intestine of both healthy and diseased animals while other toxin types are less frequently isolated from intestinal cultures, and sometimes can be isolated from the environment in areas where the clostridial disease is present (Songer, 1996). The precise pathogenesis of enteritis induced via clostridial toxin production still needs more investigation (Timbermont et al., 2011) and most probably the production of the alpha toxin is the key element. *C. perfringens* type B and type D produce are the main producers of epsilon toxin, the infection with this subspecies is manifested via the production of epsilon toxin as a result of overfeeding, the toxin abundantly synthesized, then absorbed into circulation as a result of increased intestinal permeability, toxin then spread to various internal organs including brain, kidney, and lung causing edema, neurological disorders, and ultimately mortal effect often ends with sudden death (Finnie, 2003). Similarly, the data provided showed that the main clinical feature of the sheep infected with this subspecies is sudden death. Collectively, the data obtained herein accentuated the significance of multiplex PCR in combination with necropsy, histopathological findings and bacteriological isolation and identification to verify the infection with *C. Perfringens*. Furthermore, the data emphasize the role of soil and apparently healthy sheep in the pathogenesis of clostridial infection as a potential source of infection for the second episode, however, the genetic relationship between the various strains isolated need further investigation. Finally, the high positivity rate to *C. perfringens* type A toxins remarkably counsel counting of this strain in vaccine schedule in order to validate the ample guard to avert the disease in animals.

Conflict of Interests

The authors have not declared any conflict of interests.

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