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Molecular characterization of Clostridium perfringens in small ruminants

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Abstract

A significant foodborne pathogen as *Clostridium perfringens* (C. perfringens) has been linked to many diseases in sheep and goat, included; Enterotoxaemia, pulpy kidney disease,struck, and even animal mortality. So, The objective of this study is to discuss the presence of *C. perfringens* in small ruminants with focusing on the virulence factors, antimicrobial resistance profile, and biofilm-forming capacities of the organism. A total 166 samples, (95 from sheep, 71 from goats) collected from apparently healthy, diarrheic animals and dead carcasses suspected to be infected with enterotoxaemia in Sohag, Egypt. These samples were subjected for Animal health research institute lab for microbiological examination. The phenotypic identification revealed that 25 isolates (15.1%) were *Clostridium* spp. 10 isolates were identified as *C. perfringens* by PCR using a species-specific *16S rRNA* gene. The results of multiplex PCR, revealed that the *cpa* gene, which is responsible for *C. perfringens* toxintype A, had the highest prevalence (90%) followed by the *cpe* gene, which is responsible for type F, while none of the isolates had the *cpb*, *etx*, and *iap* genes. Phenotypically, the majority of the isolates displayed multidrug resistance (MDR) patterns for, vancomycin, tetracycline , oxacillin, and piperacillin,. erythromycin and ceftazidime and whereas genetically, our isolates had the *bla*(70%), *ermB* (40%) and *tetK* (30%) genes. Our isolates demonstrated a moderate (10%) and weak (60%) ability to form biofilm.

Keywords: *C. perfringens*, small ruminants, toxigenic genes, resistance genes,biofilm.

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Introduction

The majority of enteric infections, often known as enterotoxaemia, in sheep and goats were caused by *C.perfringens*. This microbe is typically present in the intestinal flora of both humans and animals (Pawaiya et al., 2020), but under exceptional conditions where the physiological balance of the intestine is disturbed, it can proliferate rabidly and cause disorders related to a variety of toxins (Bourlioux et al., 2003).

C. perfringens is typical gram-positive, anaerobic, non-motile, rod-shaped, and endospore-forming bacteria , can be found in soil, dust, waste water, dung, feed, and sheep litter (Songer, 1996; Juneja et al., 2011). Seven serotypes of *C. perfringens* can be distinguished (A–G)), the major serotypes were (A-E) Based on the production of certain exotoxins (alpha, beta, epsilon, and iota) (Yoo et al.,1997). The (α) toxin is produced by a plasmid-mediated *cpa* gene and is linked to *C.* p*erfringens* serotype A as well as all other serotypes (Cooper et al.,2010). The serotype B also possesses the plasmid-mediated genes *cpb* and *etx* respectively, which encode for the β and ε toxins. Moreover, the β and ε toxins and are linked to serotypes C and D, respectively (Chen et al., 2011). Serotype E possesses the plasmid-mediated *iap* gene, which produces the (ι) toxin (Park et al.,2015). The genes that create *C. perfringens* enterotoxin (CPE) and *C. perfringens* beta2 toxin (CPB2) can be found in all serotypes (Uzal et al.,2010).

C. perfringens type A is the most frequently isolated type in sheep and goat enterotoxaemia, whereas *C. perfringens* type D is the principal reasons of ovine enterotoxaemia (Karthik et al., 2017). These types are responsible for secreting various toxins that act locally within the intestine to cause a necrotizing and hemorrhagic enteritis, pulpy kidney, struck, lamb dysentery in sheep and goat (Uzal et al., 2010). They also caused numerous severe enterotoxemic diseases in domestic animals and food poisoning in human (Souza et al., 2010)

According to recent investigations, most strains of *C. perfringens* were MDR strains (Ngamwongsatit et al., 2016). The incidence of Clostridial disease in ruminants is increased by high frequencies of these strains (Raymond and Hall, 2018). The two basic antimicrobial resistance mechanisms of *C. perfringens* involve mutation of intrinsic genes or acquisition of resistance gene(s) (Hall et al., 2004). Tetracycline resistance was usually provided by the *Tet*A (P) protein, which regulates tetracycline active efflux (Bannam et al., 2004). It's notable that the presence of β-lactamase (*bla* gene) caused greater minimum inhibitory concentration (MIC) values for ciprofloxacin and amoxicillin to exist (Ali et al., 2021). The *erm* gene may function similarly in the macrolide-resistant *C. perfringens*, acting as a reservoir and facilitating its conjugal transmission (Soge et al., 2009).

According to severa researches, employing low doses of antibiotics results in the formation of bacterial biofilms (Kaplan, 2011). Successful biofilm formation has been shown to depend on both *C. perfringens'* Type IV pilus (TFP)-dependent entgliding motility and the catabolite control protein (CcpA), a key regulator of the response to carbohydrate limitation (Donelli et al., 2012; Charlebois et al., 2014). Biofilm formation cells have survival rate over planktonic cells after

exposure to penicillin and an increased survival to environmental stresses (Varga etal., 2008).

Owing to the increase in the emerging threat of *C. perfringens* infections in small ruminants، we explored the detection and identification of *C. perfringens* in different organs and fecal samples of sheep and goat and spotlighted the evolution hazards of biofilm formation and wide spread of MDR strains phenotypes and genotypes

Material and Methods

Ethical approval:

This work was performed in accordance with laws and ethical guidelines of the South Valley University National Ethics Committee. All procedures were carried out in conformity with all applicable rules and regulations. No. 86/19.10.2022

Sampling

Cases history

The history of the cases used in this study were collected for both diseased and apparently healthy cases prior to sample collection. Clinical signs of the disease include recumbence, mild to severe (blood-tinged to bloody) diarrhoea, stomach pain, and bloating. In other instances, intestinal inflammation, ulceration, and necrosis with watery fluid, blood, and fibrinous clots were also present alongside death. The supposedly healthy cases exhibited no clinical symptoms.

Collection of Samples

In Sohag province, a total of 166 samples included; fecal, Liver, Kidney, and intestinal samples were collected from 80 sheep and 60 goats in different localities farms. Out of the 80 sheep, 48 fecal sample was collected from apparently healthy, 22diarrheal samples from infected sheep while; 9 Liver, 6 Kidney, and 10 intestines were collected from 10 carcasses. Among 60 goats, 27fecal samples were collected from healthy cases and 25diarrheal samples from diseased cases while8 liver, 3 kidneys and 8intestinal samples were collected from 8 dead animals. These samples were taken aseptically, then placed in a buffer saline and transferred to the lab in an ice tank.

Isolation and identification of *C. perfringens*

Isolation and identification steps were done according to (Nazki1 et al.,2017). Briefly, 1gm of each sample was inoculated into 9 mL Robertson's cooked meat broth (RCMB) (Hi media Labs, Mumbai, India) (Robertson, 1916) , inoculated tubes incubated anaerobically at 37 °C for 18-24 hours. 1 ml from previously inoculated broth was streaked onto tryptose sulfite cycloserine (TSC) agar (Hi media Labs, Mumbai, India) plates, at 37˚C for 24 h in an anaerobic jar, with gas generating kits (Oxoid,). Suspected colonies were cultured onto 5% sheep blood agar with 200ug/ml neomycin sulphate under anaerobic condition at 37 °C for 24 hours. Suspected colonies were subjected for biochemical scheme according to (Macfaddin, 2000)

Molecular confirmation of *C. perfringens* using *16S rRNA* Gene species specific gene primers

DNA extraction:

DNA was extracted using QIAamp DNA Mini Kite according manufacturer's instructions the extracted DNA were stored at -20 ^{0}C.

PCR analysis

The PCR procedure was conducted in accordance with Wu et al. (2009) in a total volume of 25µl, composed of 12.5µl of Emerald Amp GT master mix (Takara,

Code No. RR310A), 5.5 µl of nuclease-free water, 5 µl of DNA template, and 1µl of primer set. The PCR amplification program was implemented with the following parameters: initial denaturation at 94°C for 5 minutes, followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 35°C for 30 seconds, and final extension at 72°C for 30 seconds. The PCR results were seen on a 1.5% agarose gel stained with ethidium bromide and seen under UV light.

Detection of toxigenic genes in C. *perfringens* isolates

Multiplex PCR

By using certain sets of primers, the multiplex PCR was carried out with the goal of amplifying four toxigenic genes α (*cpa*), β (*cpb*), ε (*etx*), and ι (*iap*) (Table 1) The reaction was carried out in a 50µl total volume utilizing 25µl of master mix Amp GT (Takara, Code No. RR310A Emerald), 12 µl of nuclease-free water, 1 µl of an oligonucleotide primer set (Table 1), and 5 µl of DNA. The following 35 cycles of amplification were performed, starting with a denaturation stage at 94°C for 5 minutes. Each cycle consists of the following steps: 94°C for 1 min of denaturation, 55°C for 1 min of annealing, 72°C for 1 min of synthesis, and 72°C for 10 min of the final extension phase (Yoo et al., 1997). Amplicons were separated in 1.5% agarose gel and stained with ethidium bromide under UV light.

Table (1): Oligonucleotide primers sequences

gene	Sequence	Amplified product	Reference
16S rRNA	AAAGATGGCATCATCATTCAAC TACCGTCATTATCTTCCCCAAA	279 bp	Wu *et al.*, 2009
Cpa (Alpha toxin)	GTTGATAGCGCAGGACATGTTAAG CATGTAGTCATCTGTTCCAGCATC	402 bp	YOO *et al.*, 1997
cpb (Beta toxin)	ACTATACAGACAGATCATTCAACC TTAGGAGCAGTTAGAACTACAGAC	236 bp	
etx (Epsilon toxin)	ACTGCAACTACTACTCATACTGTG CTGGTGCCTTAATAGAAAGACTCC	541 bp	
Iap (Iota toxin)	GCGATGAAAAGCCTACACCACTAC GGTATATCCTCCACGCATATAGTC	317 bp	
Cpe (entero toxin)	ACATCTGCAGATAGCTTAGGAAAT CCAGTAGCTGTAATTGTTAAGTGT	247 bp	Kaneko *et al.*, 2011
ermB	GAA AAG GTA CTC AAC CAA ATA AGT AAC GGT ACT TAA ATT GTT TAC	638 bp	Soge *et al.*, 2009
bla	ATGAAAGAAGTTCAAAAATATTTAGAG TTAGTGCCAATTGTTCATGATGG	780 bp	Catalán *et al.*, 2010
tetK	TTATGGTGGTTGTAGCTAGAAA AAAGGGTTAGAAACTCTTGAAA	382 bp	Gholamiandehkordi *et al.*, 2009

Uniplex PCR

According to Kaneko et al. (2011), *cpe* enterotoxin gene Table (1) was detected by uniplex PCR in a total volume of 25 µl, which contained 12.5 µl of Emerald Amp GT master mix ((Takara) Code No. RR310A), 5.5 l of nuclease-free water, 5 µl of DNA template, and 1µl of primer set. This was carried out under particular cycling circumstances, including 35 cycles

at 94°C for 30 seconds, 55°C for 45 seconds, 72°C for 45 seconds, and a final extension step at 72°C for 10 minutes. The 1.5% agarose gel used to segregate the PCR product was stained with ethidium bromide and exposed to ultraviolet light to see the results.

Detection of resistance genes in C. *perfringens* isolates

bla (Penicillin), *erm*B (erythromycin), and *tet*K (tetracycline) resistance genes were detected using a uniplex PCR assay utilizing specified primers (Table 1). A 25 µl combination containing 5.5 µl of grade water, 1 µl of each set of primer (forward and reverse), 12.5 µl of master mix (Emerald Amp GT), and 5µl of DNA template was used to prepare the three genes. The amplification protocols were adjusted to denaturation at 94°C for five minutes, 35 cycles at 94°C for 30 seconds, and final extension at 72°C for ten minutes. Each primer's annealing temperature was varied, including; (*bla*) at 50°C for 45sec and 72°C for 45 sec for extension (Catalán et al., 2010), erythromycin (*erm*B) at 57°C for 45 sec 72°C for 45 sec(Soge et al., 2009) while tetracycline (*tet*K), at 50°C for 50 sec and 72°C for 40sec (Gholamiandehkordi et al., 2009). PCR product was run on agarose gel (1.5%) and bands were visualized under UV light (Sambrook et al., 1989).

Antibiotic susceptibility of *C. perfringens*

Using the Kirby-Bauer disc diffusion method, the antimicrobial susceptibility of the *C. perfringens* isolates was assessed based on interpretive criteria and previously developed standards by the Clinical and Laboratory Standards Institute [2018].

Nine classes of antibiotics (Oxoid, UK) were used in this test, including; Tetracycline (30µg) and oxytetracycline (30 µg), Penicillin (oxacillin (5µg), amoxicillin clavulanic(30µg) and piperacillin (100µg)) Glycopeptide (Vancomycine (30µg)) Quinolones (Ciprofloxacin(5µg) Levofloxine (5µg)), Macrolids (Erythromycin (15µg) and Clarithromycin (15µg)), Lincosamides (Lincomycin (2µg)), Cephalosporine (ceftazidime (30µg) and cefoperazone/sulbactm (105µg)), Aminoglycoside (Gentamiycin (10µg)), Polypeptides(colistin sulphate(10µg) and Metronidazole (5µg)(Bioanalyses).

The CLSI guidelines [2018] were used to interpret the diameter of the inhibitory zone. Since there are no break points for *C. perfringens*, the *Staph. aureus* ATCC 25923 strains were employed as the positive control.

The number of antibiotics an isolate is resistant to (a) divided by the total number of antibiotics used in the study (b) [31] is used to construct the Multi-Antibiotic Resistant (MAR) index. Equation displayed below:

MAR Index is equal to a/b.

Biofilm Formation

According to Charlebois et al. (2014), *C. perfringens* blood agar cultures were used to refreshment *C. perfringens* colonies. The revived colonies were injected in 1% glucose, tryptic soy broth and allowed to develop for 24 hours at 37 °C. 96-well polystyrene tissue culture plates were filled with 100µL of cultures after they had been standardized to 0.5 MacFarland. Each isolate was tested in three wells in triplicate. These plates were then incubated for 6 days at 44^{o}C in anaerobic jars.

The formation of the biofilm was evaluated using the crystal violet technique

(Varga et al., 2008). *C. perfringens* ATCC13124 (Animal health research institute, Dokki-Giza),was used as a positive control because it has been proven to generate biofilm in the past. The media only was used to generate negative control. According to the Stepanovicet al. (2007) biofilm formation was classified by following criteria: Negative biofilm formation if OD ≤ ODc, weak if ODc < OD < 2 ×ODc and moderate if 2 × ODc < OD < 4 ×ODc. Strong at 4 × ODc < OD., isolates were divided into four categories based on the calculated optical density (OD) values measured at 570 nm: non-biofilm producers, weak biofilm producers, moderate biofilm producers, and strong biofilm producers.

Statistical analysis

Graphpad(version 9.5.2) data processing was used to compare the values. The T-test with a p value ≤0.05 indicating a significant difference.

Result

The obtained results in this study showed that, out of 80 sheep and 60 goat, 16 (17.8%) and 9 (15%) of them were suspected to be infected with *C. perfringens* respectively (Fig.1). The microbiological analysis of collected samples cleared that 16.8% and 12.7% of sheep and goat samples (fecal, diarrheal and organs) showed a suspected colony for *C perfringens on* Tryptose_ sulfite_ cycloserine media (TSC) respectively (Table 2). The intestinal content was the most infected samples in sheep (70%) and goat (50%). The suspected isolates were characterized with black colors on TSC , double hemolytic zones on blood agar and production of opaque halo zone on egg yolk agar (Fig.2a, b and c). Twenty- five isolates (15.1%) suspected to be *Clostridium* spp were subjected for biochemical and PCR analysis. The biochemical profile revealed that all isolates were positive for lecithinase production, gelatin hydrolysis, negative for catalase, indole and lipase test and variable results in sugar fermentation tests (supplementary table 1). PCR results, using species specific *16SrRNA* primers confirmed that out of 25 isolates from both sheep and goat samples, 10 were *C. perfringens*. (6 isolates from sheep and 4 from goat) (Fig. 3).

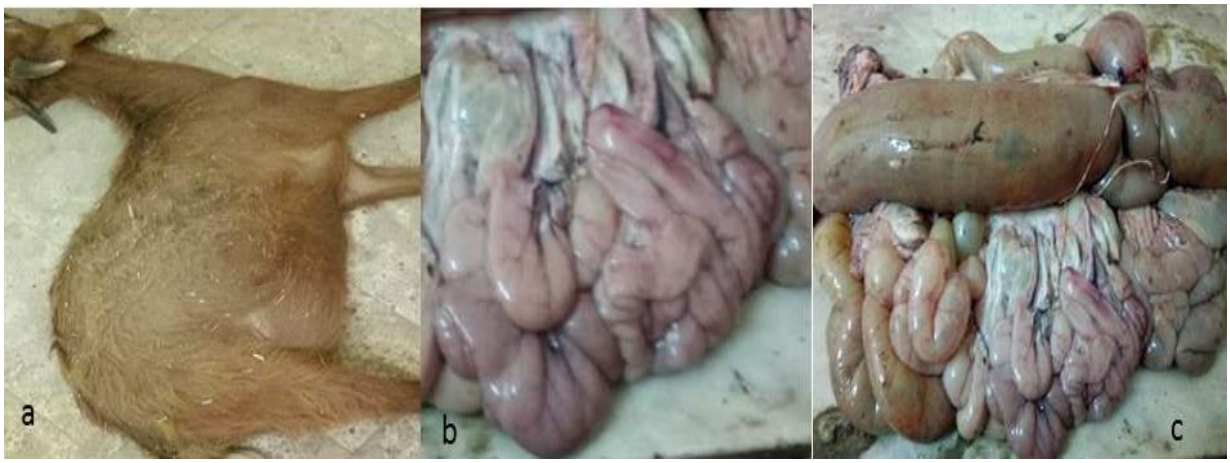

Fig. (1): Postmortem view of animals infected with C. perfringens (a) Dead animal with bloating symptoms, (b and c) intestinal hemorrhage and bloating

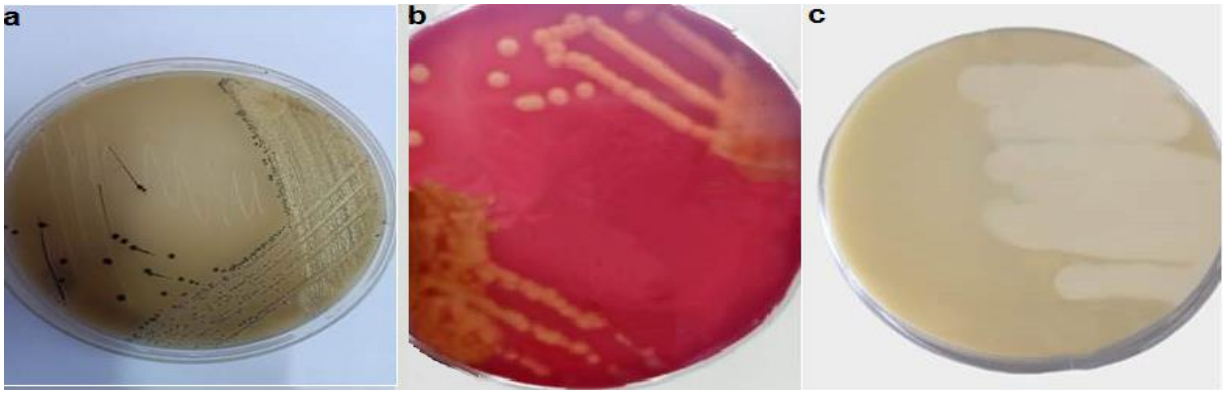

Fig.(2): Colonies of *C. perfringens* on different media. (a) *C. perfringens* colonies in the colour black on tryptose sulphite cycloserine TSC agar.(b) *C. perfringens'* beta hemolytic activity on 5% sheep blood agar. (c) On egg yolk agar, *C. perfringens* showed good lecithinase activity.

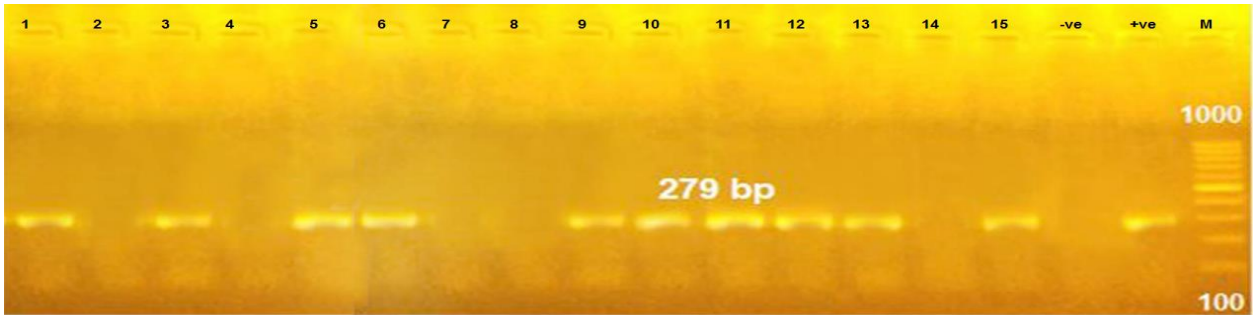

Fig.(3) a. Detection of *C. perfringens'* species-specific *16S rRNA* gene amplified by PCR, lanes (L): 100base pair DNA ladder); Lane (P): effective regulation; Lane (N): Negative regulation Isolates showing 279bp of the *16srRNA* encoding gene in lanes (1-15)

Table (2): Incidence of *Clostridium* spp on TSC media

animals	No of samples	No of positive – samples
Sheep	Healthy cases Fecal swap (48) Infected cases Diarrheal swaps (22) Dead carcasses Liver(9) Kidney (6) Intestinal content(10)	Healthy cases Fecal swap 4(8.3%) Infected cases Diarrheal swaps 3(13.6%) Dead carcasses Liver 3(33.3%) Kidney 0 Intestinal content 7(70%)
Total	95	16(16.8%)
Goat	Healthy cases Fecal swap (27) Infected cases Diarrheal swaps (25) Dead carcasses Liver(8) Kidney (3) Intestinal content(8)	Healthy cases Fecal swap 1(3.7%) Infected cases Diarrheal swaps 3(12%) Dead carcasses Liver 1(12.5%) Kidney 0 Intestinal content 4(50%)
Total	71	9(12.6%)
Total for all samples	166	25 (15.1%)

Out of total 10 isolates 9 isolates were found to carry *cpa* gene alone (Fig.4a) as a major toxin gene , thus were designated as toxin type A . While the remaining one isolate from intestine of dead cases harbored both *cpa* and *cpe* genes, thus were designated as toxin type F(Fig.4a,b) , None of the isolates possessed *cpb* , *etx* and *iap* genes indicating the absence of *C. perfringens* toxin type B, C , D or E in sheep and goat samples.

The assay of antimicrobial resistance demonstrated that a large section of *C. perfringens* isolates showed highly resistance (100%) to six antibiotics such as Tetracycline, oxytetracycline, oxacillin, piperacillin, vancomycin, erythromycin, ceftazidime and metronidazole. Intermediate resistance was showed against clarithromycin (40%) and Lincomycin (10%). Amoxicillin clavulanic (20%),colistin/sulphat (40%). cefoperazone/sulbactm and gentamiycin (30%), interestingly, maximum sensitivity to ciprofloxacin and levofloxacin (100%) were observed in all isolates (Table.3).all *C.perfringens* isolates showed multidrug resistance patterns.one isolate showed resistance to eight antibiotics with resistance index (0.5) Three isolates showed resistance for 9 antibiotics with resistance index (0.56),two isolates resist to ten antibiotics with resistance index (0.62) ,four isolates resist for eleven antibiotics with resistance index (0.68)

Table 3: Antibiotic susceptibility profile of *C. perfringens* isolated from sheep and goat

Class	antibiotics	abbreviation/ugs	Antibiotic sensitivity		
			Resistant	Intermediate	sensitive
Tetracycline	Tetracycline	TE30	10(100%)	-	-
	Oxytetracycline	T30	10(100%)	-	-
Penicillin	Oxacillin	OX1	10(100%)	-	-
	Amoxicillin clavulanic	AMC30	5(50%)	2(20%)	3(30%)
	Pipracillin	Prl100	10(100%)	-	-
Glycopeptide	Vancomycin	VA30	10(100%)	-	-
Quinolones	Ciprofloxacin	CIP5	-	-	10(100%)
	levofloxine	LEV5	-	-	10(100%)
Macrolids	Erythromycin	E15	10(100%)	-	-
	Clarithromycin	CLR15	4(40%)	4(40%)	2(20%)
Lincosamides	Lincomycin	L2	4(40%)	1(10%)	5(50%)
Cephalosporine	Ceftazidime	CAZ 30	10(100%)	-	-
	Cefoperazone/sulbactm	CES105	3(30%)	3(30%)	4(40%)
Aminoglycoside	Gentamycin	CN10	2(20%)	3(30%)	5(50%)
Polypeptides	Colistine sulphate	CT10	3(30%)	4(40%)	3(30%)
Flagyl	Metronidazole	MTZ 5	10(100%)	-	-

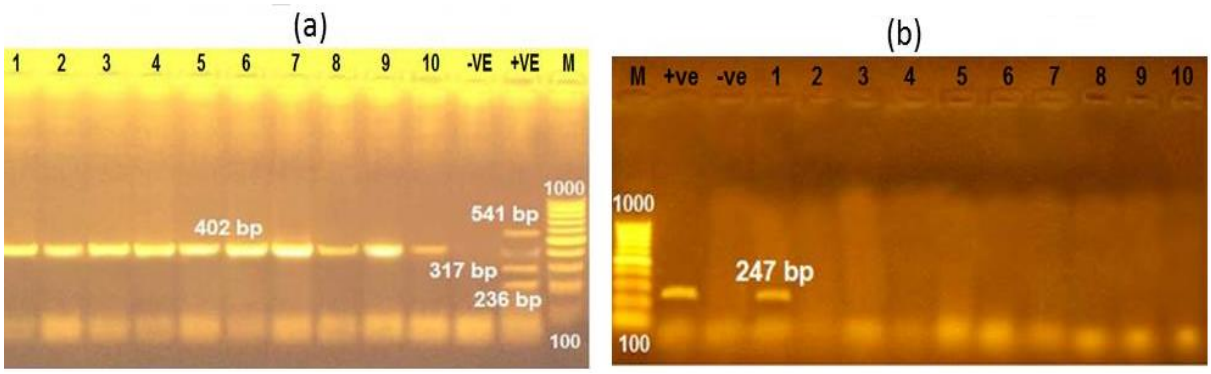

Fig. (4): a. Detection of *C. perfringens* toxin genes amplified by multiplex PCR (*cpa, cpb,etx,iap*). DNA ladder (lane L), positive control (lane P), negative control (lane N), and Isolates containing amplicon at 402 bp positive for *cpa* gene are found in lanes 1-10. No evidence of amplification was seen for other genes. (b) *C. perfringens cpe* genes was amplified by uniplex PCR lane: L (DNA ladder, 100bp), Lane (P) for the positive control, Lane (N) for the negative control, and Lane 1: Positive for *cpe* toxin-encoding genes in the isolate 274bp

In this study, PCR results showed that antimicrobial resistance genes(*bla*, *tetK* and *ermB*) were distributed in most of *C.perfringens* isolates with the following percentage; β-lactams *bla* gene was detected in 7 (70%) ,tetracycline *tetK* in 3 (30%),while erythromycin *ermB* were 4 (40 %) (Table 4& Fig 5.a,b,and c).

The measurement of biofilm ability with crystal violet assay showed that biofilm formation can vary among tested isolates of *C. perfringens*, after 6 day incubation of those intermediate 1 (10%) ,weak 3 (30 %) while 6 (60%) of isolate unable to form biofilm (Fig 6). **The statistical analysis showed significant relationship (P≤0.01) between** antibiotic resistance and biofilm formation **(supplementary table 2)**.

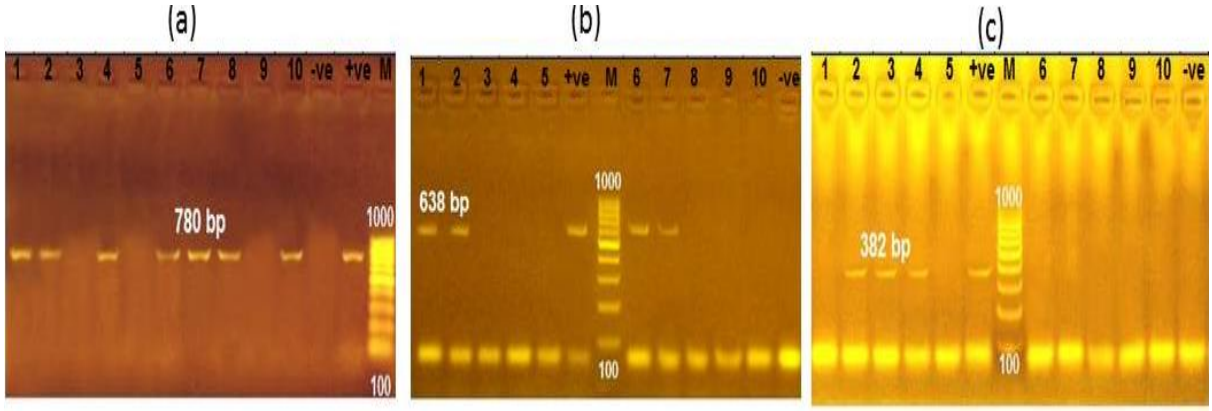

Fig. (5): (a) PCR-based agarose gel electrophoresis of the *C. perfringens bla* resistance gene amplicon. 100 bp ladderr in lane 1, Lane(1,3,4,5,7,8,9): positive *bla* resistance gene at 780bp, Lane(2,): positive control, Lane(3,): negative control. (b) the *ermB* resistance gene amplicon of C. perfringens was electrophoresed on agarose gel. 100bp Ladder Lane Lane(p) indicates a positive control, Lane(n) indicates a negative control, and Lane(4,5,9.10) indicates a positive ermB gene 638bp. (c) the tetk resistance gene amplicon of *C. perfringens* was electrophoresed on an agarose gel at 382 bp. lane l. 100 bp ladder, Lane(p) a positive control, Lane(N) a negative control, and Lane (7,8,9) positive *tetk* gene.

Fig. (6): Heat map style, Distribution of toxinotypes , toxin genes , antimicrobial resistance genes and biofilm formation among *C. perfringens* The cpa, *cpb, etx, i*ap, and *cpe* are *C. perfringens* alpha, beta, epsilon, iota, and enterotoxin genes, respectively. The *bla* , *erm*(B); *tet*(K), are genes associated with, β-lactams , erythromycin, tetracycline , resistances, respectively., M: moderate, W: weak biofilm formation.

Table 4: Toxinotype, Phenotypic resistance pattern and antibiotic resistance genes of *C.perfringenes*

No. of *c. perfringes* samples	Phenotypic resistance pattern	Toxin genes	Species type	Antimicrobial resistance genes
1	CLR15,TE30, AMC30, CAZ30, T30,OX1,CN10, MTZ5, Prl100,VA30,E15,	*cpa*	TypeA	*bla*
2	TE30,Prl100, OX1 E15,MTZ5 ,T3,CAZ30 ,VA3,L2,CES105	*cpa*	TypeA	-
3	AMC30,OX1, E15,CT10,CAZ30 ,VA30,T30, Prl100 MTZ5, T3	*cpa*	TypeA	*bla*
4	TE30, E15, AMC30, CES105, CLR15,MTZ5 ,OX1 Prl100, VA30, CAZ30, T30	*cpa*	TypeA	*bla, ermB,*
5	Prl100, CT10, CAZ30,T30, L2,E15,VA30, OX1 MTZ5, T3, CLR15	*cpa*	TypeA	*bla ermB,*
6	TE30,E15, VA30 ,OX1 ,Prl100 ,MTZ5, T3,CAZ30	*cpa*	TypeA	-
7	E15, L2, AMC30,Prl100, CAZ30,T3 OX1 MTZ5,TE30, VA30	*cpa*	TypeA	*bla, tetK*
8	CLR15,MTZ5,VA30,E15 ,OX1 TE30, Prl100, T3 ,CAZ30, CT10	*cpa*	TypeA	*bla ,tetK*
9	E15,CN10, Prl100, AMC30 TE30, MTZ5, CAZ30, OX1 ,T30, VA30,L2 ,CES105	*cpa*	TypeA	*bla, tetK, ermB,*
10	E15, TE30 ,OX1 CLR15,CAZ30,T3,MTZ5,VA30, Prl100	*cpa,cpe*	TypeA, TypeF	*ermB*

DISCUSSION

C. perfringens has been constantly associated with various significant systemic and enteric diseases, in both humans and animals, including gas gangrene (Clostridial myonecrosis), food poisoning and non -foodborne diarrhea, and enterocolitis (Raymond etal.,2018). In small ruminants, enterotoxaemia, a severe and acute illness driven by *C. perfringens*, results in high mortality rates (Hussain et al 2022).

Phenotypic characterization of *C.perfringens* based on culture and biochemical tests were indicated that (15.1%) of total samples were positive for *C. perfringens*, on Tryptose Sulfite Cycloserine Agar (TSC) . Several authors found that TSC agar was one of the most effective media for the quantitative recovery of *C. perfringen*s while inhibiting the growth of other facultative anaerobes, (Greco et al. 2005: Downes and Ito 2001) because it includes sodium disulphite, which serves as a sulphite reduction indicator and is indicated by colonies that are black in color, also the addition of egg yolk to TSC tested the lecithinase activity, which was characterized by an opaque halo around colonies, and confirmed it as *C. perfringens*. a D-Cycloserine supplement reduces the amount of nearby bacterial flora, aiding in the selective isolation of *C. perfringens*. It also minimizes the disturbing and spreading blackening around colonies. ISO Committee's (2004) recommended it for isolation and counting *C. perfringens* from foods.

The PCR method played an important role in identification the members of the *Clostridium* genus and to investigate the links between this genus and other pathogenic and non-pathogenic bacteria.

In our study the using of species specific *16SrRNA* primers confirmed the presence of *C.perfringens* DNA in ten out of 25 isolates(40%). Different studies supported the role of *16SrRNA* gene in *C.perfringens* identification (Ateba et al., 2008 and Moschona et al., 2011). Kumar et al. (2014) detected *C. perfringens* DNA in 59.62% of sheep isolates by PCR while Nazkiet al. (2017) showed that 70.62% of isolates were positive for *C. perfringens* also .Hayati and Tahamtan (2021) noted that out of 167 suspected cases, 61% were positive for *C.perfringens*.

Extracellular enzymes and toxins that *C. perfringens* produces are thought to act together and contribute to its pathogenesis (Ohtani and Shimizu, 2016). Results of multiplex PCR for detection of toxigenic genes revealed that *cpa* gene which responsible for type A (positive only for alpha-toxin) was the predominant gene (90%) in apparently healthy and dead cases of sheep and goat, type A associated with food poisoning worldwide (Guran and Oksuztepe, 2013). Many studies reported the superiority of type A with percentage reached to100 % (Mignaqui et al.,2017 ;Hayati and Tahamtan,2021; Rasool et al., 2017 ; Karunakarnan et al., 2018).

In Egypt, Moustafa et al. (2022) showed that type A(*cpa* gene) was predominated (43.69%) in *C. perfringens* isolates, resembles that Omar et al .(2018) isolated type A (50 %) with no evidence of existence type B and D .As it is obvious that high prevalence of type A (alpha toxin gene), this posse need to further study for this type and incorporate this strains into the vaccine.

C. perfringens is a naturally occurring commensal of the gut that releases a little quantity of toxin that is eliminated by normal intestinal movement or become infected by circulating antibodies (Kiu and Hall ,2018;Uzal et al .,2016).These previous studies confirmed our finding which ascertained 70% of isolates of *C. perfringens* in relation to intestine.

Another toxinotype gene was detected in this study was *cpe* gene which responsible for enterotoxin producing by type F *C. perfringens*, and was investigated with percentages 10%. Freedman et al., 2016 found that only 5%

of *C. perfringens* isolates worldwide possess the enterotoxin gene (*cpe* gene).This toxin was associated with human food poisoning and non-food-born diarrhea (Park and Rafii, 2019). *cpe* is a small pore-forming toxin that is produced by all species of *C. perfringens*, with the exception of type B, and is either encoded on the chromosome or plasmid. It acts enterotoxically and causes histological damage (Li et al., 2013).

The presence of multidrug resistant C. *perfringens* in a range of animals and animal products raised serious concerns for the general public's health (Feng et al., 2020). In this study the antibiotic susceptibility profile of C. *perfringens* type A and F were showed resistance for different families of antibiotics. Phenotypically, our findings were observed a high multidrug resistance (resistance ≥ three classes of antibiotic), these results in consistency to previous study (Wen et al., 2018; Khan et al.,2019; Hussain et al,.2017; Rahaman et al.,2013; Jang et al ,2020; Elgoas et al.,2020; Ahmed et al., 2022).

The predominance of MDR among *C. perfringens* strains back to two main ways of developing resistance to antibiotics: either by changing its inhered genes or by acquiring resistance genes.

Our study declared the problem of *C. perfringens* resistance to many types of antibiotics, especially high resistance to tetracycline and penicillin and erythromycin these may be due to the misuse of these antibiotics as medication and growth stimulant in animal feed. In addition, the presence of multiple genes linked to these drugs resistance among various *C. perfringens* isolates (Slavi et al., 2011).

The demonstration of tetracycline resistance phenotypically in our finding parallels with Hosseinzadeh et al. (2018); Mohiuddin, et al .(2020) .The great prevalence may be explained by the ongoing use of tetracycline as a growth stimulant in animal feed that cause increasing prevalence of MDR *C. perfringens*.

Here, *tetk* genes was detected in 30% of *C. perfringens* isolates that coincided with Silva et al. (2014), who mentioned that 27.8% were considered resistance high than Hosseinzadeh et al. (2018) who reported 52% of the *C. perfringens* type A isolates were tetracycline-resistant in sheep carcasses.

The PCR results revealed that *erm* (B) gene were harbored in 40% of *C.perfringens* isolates by PCR while all isolates (100%) showed resistance to erythromycin by disk diffusion method. These may be due to the presence of extra genes that contribute to resistance to these antimicrobials (Lepuschitz, etal.,2019).

β-lactam antibiotics are one of the largest and most important antibiotic including penicillin, cephalosporines, carbapenems and monobactams (Worthington and Melander,2013) When necrotic enteritis outbreaks occur, these antibiotics are utilized as metaphylactic and prophylactic treatment (Wollschlaege et al., 2009).

In our results, *C.perfringens* isolates showed a high resistant to β-lactam antibiotics phenotypically (100%) while genotypically, *bla* gene was found in 70% of isolates ,it was worth noting that *bla* gene the mist common resistance genes among isolates aligned with phenotypic detection, Resistance to β-lactams can develop through mutations in genes

encoding naturally occurring chromosomally encoded-lactamases or by acquisition of foreign DNA encoding new -lactamases (King et al., 2017; Bush and Bradford , 2020).

Our finding showed that our isolates harbored many resistance genes such as; *bla*, *erm*b and *tet*k, these were in agreement with those reported by Anju et al. (2020). While *erm*B results greater than that reached by Abd El-Tawab and Hofy (2021). Our findings showed that some isolates had resistant genes but had a sensitivity phenotype; this could be because the resistance genes were largely not expressed or because these genes had point mutations. (Gholamiandehkordi et al.,2009).

C. perfringens could cause antibiotic-associated diarrhoea by creating a biofilm in the small intestine and supporting bacterial persistence during antibiotic therapy (Varga et al., 2008). In our finding *C. perfringens* isolates showed moderate and weak biofilm formation, while Ahmed et al. (2022) demonstrated that *C. perfringens* produce a strong (28%) and moderate (60%) biofilm, also Gharieb et al. (2021) reported that 7% of isolates were weak, 7% moderate and 10% strong biofilm producers. The difference in percentages between isolates in ability for biofilm producer back to the molecular processes that control biofilm formation even between strains of the same species (Monds and O'Tool .,2009).

The statistical analysis showed significant relationship ($P \leq 0.01$) between antibiotic resistance and biofilm formation (supplementary table 2) , this result supported by Kaplan, (2011) who mentioned that low doses of antibiotics especially penicillin G increased survival of *C. perfringens* cells and increase the ability of biofilm formation and that play a critical role in the development and maintenance of biofilm communities (Daniel et al.,2010)

Conclusion

C. perfringens detection and antibiotic resistance pose a threat since it can infect people through contaminated materials. Effective molecular methods, like PCR, provide for quicker identification than other conventional methods, which makes it easier to take early preventative action. These methods are therefore essential for the early detection of these microorganisms. *C. perfringens* isolates shown several virulence and resistance genes to antibiotics that indicated to be harmful for animal and human.

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