Received: 13 September 2023, Accepted: 29 October 2023

Original Research

Journal of Advanced Veterinary Research
(2023) Volume 13, Issue 9, 1809-1814

Prevalence, Antibiotic Sensitivity Testing and Molecular Characterization of Virulence and Antimicrobial Resistance Genes in *Clostridium perfringens* in Fish

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Abstract

Fish is one of the most widely advertised foods, and Egypt is now recognized as a significant global fish producer. Human and animal intestinal illnesses and significant histotoxic illnesses are caused by foodborne microorganisms as *Clostridium perfringens* (*C. perfringens*). This study was directed to monitor the prevalence, antibiotic susceptibility testing, detection of some virulence and antibiotic resistance genes of multidrug-resistant *C. perfringens* recovered from fish. A total of 300 samples were collected from gills and intestine of Catfish, Tilapia and Dennis. Bacteriological examination was conducted, the obtained *C. perfringens* strains were tested for antibiogram, PCR screening of virulence and antibiotic resistance genes. The investigated samples showed *C. perfringens* prevalence of 48.3%. *C. perfringens* isolates were resistant to several antibiotics as clindamycin (90%), cefprozil (80%), novobiocin (80%), aztreonam (80%) and erythromycin (80%). While, isolates were sensitive to nalidixic acid (90%), ofloxacin (90%), chloramphenicol (90%) and rifampicin (80%). Multidrug-resistant (MDR) *C. perfringens* was detected in 80% of tested strains. PCR proved that the obtained *C. perfringens* strains were carrying the virulence genes: *cpa*, *cpb* and *cpe* in a prevalence of 60%, 40% and 10%, respectively. As well, *bla* and *erm*B antibiotic resistance genes were detected in *C. perfringens* strains in a prevalence of 100% for both genes. In conclusion, *C. perfringens* isolated from fish was multidrug- resistant (MDR) bacteria and was harbored *cpa*, *cpb* and *cpe* virulence genes and *bla* and *erm*B antibiotic resistance genes. The development of MDR *C. perfringens* is conceived as a public health threat.

KEYWORDS
Clostridium perfringens, Antibiotic susceptibility testing, Virulence, Antibiotic resistance genes, PCR.

INTRODUCTION

Because of one billion people rely on fish as their primary source of animal protein, fish is a necessary food source (Myers *et al*., 2014). The prevalence of bacterial fish illnesses and infections is one of the most difficult health issues to manage, bacteria can enter the fish body through the gills, skin, or by remaining on the body's surface (Saad *et al*., 2013).

A variety of human and veterinary diseases are brought on by the gram-positive, rod-shaped, encapsulated, nonmotile anaerobe *Clostridium perfringens* (*C. perfringens*) (McClane *et al*., 2012). Human illnesses caused by *C. perfringens* include necrotic enteritis, wound infections, and the potentially fatal gas gangrene (Myers *et al*., 2014). *C. perfringens* pathogenicity is a result of the toxins that some strains generate. Based on the main extracellular toxins they generate, based on the production of the main extracellular toxins alpha, beta, epsilon, and iota, *C. perfringens* is divided into five strains (A to E) (Rood, 1998). Beta toxin is produced by types B and C, epsilon toxin by types B and D, and just iota toxin by type E. all five strains produce alpha toxin (McClane, 2007). Other toxin genes, such enterotoxins, are connected to variations in *C. perfringens* strains' virulence and phenotypic characteristics. (Rood, 1998). The disease in humans is mainly because of type A and type C strains. Type A is often linked to simple cases of food poisoning (Lindström *et al*., 2011), but type C can results in necrotic enteritis (Lawrence and Walker, 1976). Type A enterotoxin-positive (CPE+) strains that carried the enterotoxin gene (*cpe*) is resulted in diarrhea and Foodborne illnesses (Asha and Wilcox, 2002).

Particularly for types A CPE+, C, and E of *C. perfringens*, the fish act as a reservoir for the pathogen. According to (Sabry *et al*., 2016), the exterior surface of fish can be exploited as a point of entry for human illness as well as a means of delivering environmental contaminants into fish.

Because of its capacity to produce resistant spores, ability to cause foodborne illness, widespread distribution and potential to infect humans with diseases like necrotic enteritis and fatal gas gangrene, *C. perfringens* has public health significance. The issue is made worse globally by reports of the discovery of *C. perfringens* strains that are extremely resistant to numerous widely used antibiotics (Cortés-Sánchez, 2018). Multidrug resistance (MDR) has recently become more prevalent throughout the world and is now viewed as a concern to public health (Algammal *et al*., 2022a; Algammal *et al*., 2022b).

Fish farmers and the fishing industry have suffered significant financial losses as a result of fish diseases. Antibiotics have been used to treat and prevent disease-causing bacteria, but the effectiveness of treatment depends on how susceptible the etiologic bacteria are to antibiotics. When selecting the best antibiotic, antibiotic resistance is a crucial concern that must be considered.

ISSN: 2090-6277/2090-6269/

The genetic relatedness of *C. perfringens* isolates from fish and their environment has only received limited attention internationally (Park *et al*., 2016). Hence, this study was carried out to illustrate the prevalence, antimicrobial susceptibility testing, detection of some virulence and antibiotic resistance genes of MDR *C. perfringens* recovered in Egypt's fish farms.

MATERIALS AND METHODS

Animal Ethics

The entire procedures were completed in compliance with the applicable laws. The Suez Canal University in Egypt's Animal Ethics Review Committee approved of all methods and fish handling, under an approval number of 2023050.

Collection of samples

Three Hundred samples were taken from 150 fish (50 from Catfish, 50 from Tilapia and 50 from Dennis). Gills and intestine were collected from each fish. Fish were collected from 3 different fish farms in Egypt (Gamasa fish farm, Shatta fish farm in Damietta and Manzala fish farm). Sterile bags were used for collection of samples, labeled and transported in ice tanks as quickly as possible to the laboratory.

C. perfringens isolation and biochemical identification

Each sample was placed into a tube filled with sterile, recently cooked beef medium. The tube was incubated anaerobically for 24 to 48 hours at 37°C in jars with anaerobic gas-generating kits (Oxoid). A loopful of the previously tube was streaked onto a plate of 10% sheep blood agar with neomycin sulfate (200 µg/ml) after being anaerobically incubated for 24 to 48 hours at 37°C. According to Koneman *et al*. (1992), subcultures from the suspected colonies of *C. perfringens* were investigated by microscopical examination and biochemical tests. Substantially, identification was primarily based on Gram's stain, colonial appearance, and biochemical tests: nitrate reduction, gelatin hydrolysis, catalase test, H_2S production, indole test, f*erm*entation of sugars (sucrose, mannitol, lactose, glucose, galactose, xylose, maltose, and mannose), lecithinase activity and urease.

Antibiogram of the retrieved C. perfringens isolates

Using the disc diffusion test on Muller Hinton agar (Difco, USA), the resistance patterns of the recovered *C. perfringens* isolates were examined. Oxoid, UK's eleven antibiotic discs, were used: clindamycin (CD/2µg), kanamycin (K/30µg), rifampicin (RIF/5µg), cefprozil (CPR/30µg), tetracycline (T/30µg), novobiocin (NV/30µg), nalidixic acid (NA/30µg), ofloxacin (OF/5µg), chloramphenicol (C/30µg), aztreonam (AT/30µg) and erythromycin (E/15µg). According to the guidelines provided by Patel (2015), the test results were interpreted. The examined *C. perfringens* isolates were categorized as MDR (Multidrug-resistant: resistant to at least one antimicrobial agent in at least three classes) based on their patterns of resistance.

PCR for virulence and antibiotic resistance gene detection

PCR was used to monitor three virulence toxin genes (alpha (*cpa*), beta (*cpb*) and enterotoxin (*cpe*)) and two antibiotic resistance genes (erythromycin resistance gene (*erm*B) and beta-lactamase gene (*bla*) of *C. perfringens*. The PureLink DNA Extraction Kit (Life Technologies, Renfrew, UK / Cat. No. K182001) was used to extract DNA. Positive and negative controls (DNA-free reactions) were used in each experiment. The cycling conditions and the utilized primers (Biobasic, Canada) are shown in Table 1. Additionally, agar gel electrophoresis was used to separate the PCR products. After then, the gel was photographed.

Statistical analysis

All data were collected, calculated, tabulated, and statistically evaluated using the following statistical tests. A normality test (Shapiro-Wilk) was performed to make sure the samples' distribution was normal.

Frequencies (n) and percentages (%) were used to present qualitative data. The significance of the correlation between categorical variables was examined using the Chi-square test. The statistical package for social science, SPSS software for Windows version 26.0 (IBM Corp., Armonk, NY), was used for all analysis, and a significant level was set at 0.05 (P- Value ≤0.05).

RESULTS

C. perfringens prevalence in fish samples

After bacteriological examination, 145 *C. perfringens* isolates were isolated from 300 samples obtained from 50 Tilapia, 50 Catfish and 50 Dennis. *C. perfringens* prevalence was 48.3% (145/300) in the examined fish samples. Thirty-three (33%) and 19 (19%) isolates were obtained from gills and intestine of Tilapia, respectively, while 25 (25%) and 31 (31%) isolates were obtained from gills and intestine of Catfish respectively, moreover 18 (18%) and 19 (19%) isolates were obtained from gills and intestine of Dennis, respectively. Statistically, the distribution of *C. perfringens* in the examined organs derived from the fish samples is not significantly different., as shown in Table 2 and Figure 1 (p value > 0.05%).

Table 1. Primers sequences and PCR cycling conditions.

Toxin	Gene	Nucleotide sequence (5′-3′)	Amplicon Size (bp)	Primary denaturation	Secondary denaturation	Annealing	Extension	No. of cycles	Final extension	References
alpha	*cpa*	F:GCTAATGTTACTGCCGTTGA R:CCTCTGATACATCGTGTAAG	324	95 ˚C 10 min	94 ˚C 45 S	55 ˚C 30 S	72 ˚C 1.5 min	35	72 ˚C 10 min	Ahsani *et al*. (2010)
beta	*cpb*	F:GCGAATATGCTGAATCATCTA R:GCAGGAACATTAGTATATCTTC	196	95 ˚C 10 min	94 ˚C 45 S	55 ˚C 30 S	72 ˚C 1.5 min	35	72 ˚C 10 min	
Enterotoxin	*cpe*	F:GGAGATGGTTGGATATTAGG R:GGACCAGCAGTTGTAGATA	233	95 ˚C 5 min	94 ˚C 1 min	55 ˚C 2 min	72 ˚C 3 min	30	72 ˚C 5 min	Lin *et al*. (2003)
Antibiotic resistance genes	*erm*B	F:GAA AAG GTA CTC AAC CAA ATA R:AGT AAC GGT ACT TAA ATT GTT TAC	638 bp	94˚C 5 min.	94˚C 30 sec.	57˚C 40 sec.	72˚C 45 sec.	35	72˚C 10 min.	Soge *et al*. (2009)
	bla	F:ATGAAAGAAGTTCAAAAATATTTAGAG R:TTAGTGCCAATTGTTCATGATGG	780 bp	94˚C 5 min.	94˚C 30 sec.	50˚C 40 sec.	72˚C 45 sec.	35	72˚C 10 min.	Catalán *et al*. (2010)

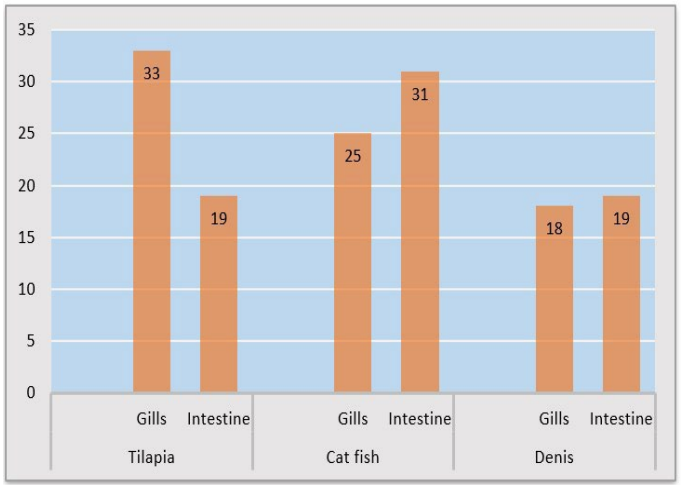

Figure 1. Prevalence of *C. perfringens* isolated from examined fish samples.

In-vitro antibiotic susceptibility of C. perfringens

The tested strains of *C. perfringens* (as shown in Table 3 and Figure 2) were resistant to clindamycin (90%), cefprozil (80%), novobiocin (80%), aztreonam (80%) and erythromycin (80%). While, isolates were sensitive to nalidixic acid (90%), ofloxacin (90%), chloramphenicol (90%) and rifampicin (80%). MDR *C. perfringens* was detected in 80% of tested strains (as shown in Table 5). Statistically, the recovered *C. perfringens* strains differed significantly (p 0.05) in their sensitivity profiles to the different tested antimicrobial classes (p<0.05).

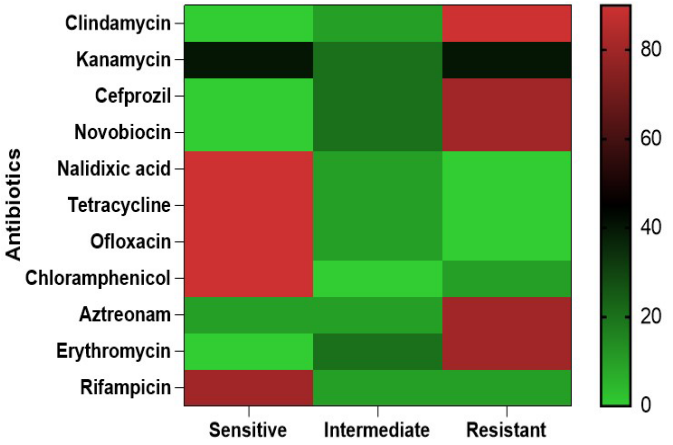

Figure 2. Heat map illustrates *C. perfringens* antibiotic susceptibility of the recovered strains from fish samples.

Virulence and antibiotic resistance genes detection by PCR

Three *C. perfringens* pathogenicity toxin genes, alpha (*cpa*), beta (*cpb*), and enterotoxin (*cpe*), were monitored using PCR. In con-

Table 2. The prevalence of *C. perfringens* isolated from examined fish samples.

Sample	Total no. of collected samples		Positive samples of *C. perfringens*	
			No.	%
Tilapia	100	Total	52	52
		Gills	33	33
		Intestine	19	19
Catfish	100	Total	56	56
		Gills	25	25
		Intestine	31	31
Dennis	100	Total	37	37
		Gills	18	18
		Intestine	19	19
Chi square		8.97		
p value		0.1102 ^{NS}		

Table 3. The in-vitro antibiotic susceptibility of *C. perfringens* (n=10).

Antimicrobial class	Antibiotics	Sensitive		Intermediate		Resistant	
		No.	%	No.	%	No.	%
Lincosamide	Clindamycin	0	0	10%	10%	9	90%
Aminoglycoside	Kanamycin	4	40%	2	20%	4	40%
Cephalosporin	Cefprozil	0	0	2	20%	8	80%
Coumarin glycosides	Novobiocin	0	0	2	20%	8	80%
Quinolone	Nalidixic acid	9	90%	1	10%	0	0
Tetracycline	Tetracycline	9	90%	1	10%	0	0
Fluoroquinolone	Ofloxacin	9	90%	1	10%	0	0
Chloramphenicol	Chloramphenicol	9	90%	0	0	1	10%
Monobactams	Aztreonam	1	10%	1	10%	8	80%
Macrolide	Erythromycin	0	0	2	20%	8	80%
	Rifampicin	8	80%	1	10%	1	10%
Chi-square		42.34		35.22		36.45	
p value		<0.0001**		<0.0001***		<0.0001***	

trast to *cpb*, which was found in 40% of investigated strains, *cpa* was found in 60% of them, while *cpe* was found in 10%. Additionally, PCR was utilized to find genes (*erm*B, *bla*) associated with antibiotic resistance, and all tested strains (100%) tested positive for these genes (Table 4 and Figure 3). The statistical analysis revealed no appreciable variation in the distribution of genes associated with virulence and antibiotic resistance in the isolated *C. perfringens* strains.

Table 4. Virulence and antibiotic resistance genes prevalence of *C. perfringens* (no=10).

Genes type		No	%	Chi-square *p* value
Virulence-determinant genes	*cpa*	6	60	3.45 (0.177 NS)
	cpb	4	40	
	cpe	1	10	
Antibiotic resistance genes	*bla*	10	100	0 (1.00 NS)
	*erm*B	10	100	

C. perfringens multidrug resistance pattern

The current results (Table 5) investigated that 8/10 (80%) of tested strains phenotypically were multidrug resistant (MDR) where showed resistance to one antimicrobial agent in at least three classes. While all strains 10/10 (100%) were carried *bla* (100%) and *erm*B (100%) resistance genes genotypically. Hence, *bla* gene is responsible for resistance of isolates to cefprozil and aztreonam (80% for each resist, 20% int*erm*ediate sensitive) phenotypically. Cefprozil belongs to cephalosporin and aztreonam belongs to monobactams, cephalosporin and monobactams are beta lactams antibiotics. Concerning erythromycin phenotypically, 8/10 (80%) of strains were resist and 2/10(20%) were int*erm*ediate sensitive. This result proved the responsibility of *erm*B gene to erythromycin resistance in *C. perfringens* phenotypically.

DISCUSSION

For humans to attain survival, growth, and development as well as to carry out their everyday activities, food is a priority need. However, microbial contaminations can occur during food production and at any point in the food chain, and these can act as carriers for biological risks to human health that result in a variety of diseases. Foodborne illness in humans is frequently occurred by *C. perfringens*, however, few information is precepted on the function of fish in the epidemiology of *C. perfringens* (Sabry *et al*., 2016). Egypt's primary source of fish for export or distribution to local markets comes from inland aquaculture. Hence, the following data investigated the prevalence, antimicrobial susceptibility testing, virulence and antibiotic resistance genes detection in MDR *C. perfringens* recovered from fish farms in Egypt.

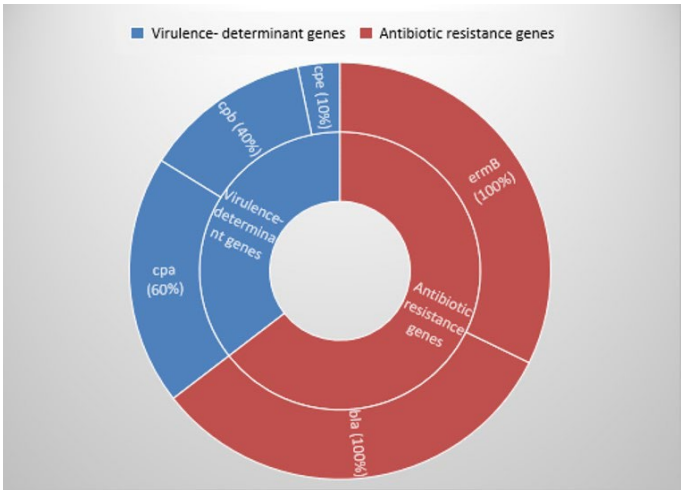

Figure 3. Virulence and antibiotic resistance genes prevalence of *C. perfringens*.

Results demonstrated that *C. perfringens* prevalence was 48.3% (145/300) in the examined fish samples. Thirty-three isolates (33%), 19 isolates (19%) were obtained from gills and intestine of Tilapia, respectively, while 25 isolates (25%), 31 isolates (31%) were obtained from gills and intestine of Catfish, respectively. Moreover, 18 isolates (18%), 19 isolates (19%) were obtained from gills and intestine of Dennis, respectively. The prevalence in the current data is lower than prevalence in previous research on freshwater fish obtained from fisheries as Sabry *et al.* (2016) who detected a 54.5% prevalence of *C. perfringens* in fresh fish collected from aquaculture and higher than Yadav *et al.* (2017) who detected *C. perfringens* by 23.1% from fish. This disparity in *C. perfringens* prevalence might be due to variations in the samples' sources.

The use of antibiotics is one of the most effective therapeutic techniques in medicine. However, an increase in antibiotic-resistant organisms is caused by the excess use and improper administration of antibiotics, particularly in developing nations (Tiwari *et al*., 2013). Antibiotic resistance, which is associated with greater rates of morbidity and mortality as well as higher treatment costs, is one of the major global public health challenges (Lin *et al*., 2015). The isolation and profiles of resistance to various antimicrobials by pathogenic bacteria that originate from various natural habitats, such as soil, food, animals, and people, have been reported by several research conducted across the world. These researches provide information about a more rational use

Table 5. The resistant type of recovered isolates (n=10)

Strain	CD	K	CPR	NV	NA	TE	OF	C	AT	E	RIF	Resistant type	Antibiotic resistance genes
1. *C. perfringens*	R	R	I	R	S	S	S	S	R	R	S	Multidrug resistant (MDR)	*bla, erm*B
2. *C. perfringens*	R	I	R	R	S	S	S	S	R	R	S	Multidrug resistant (MDR)	*bla, erm*B
3. *C. perfringens*	R	R	R	R	S	S	S	S	R	R	S	Multidrug resistant (MDR)	*bla, erm*B
4. *C. perfringens*	R	S	R	S	S	S	S	R	I	R	S	Multidrug resistant (MDR)	*bla, erm*B
5. *C. perfringens*	R	S	R	I	S	S	S	S	S	I	I	Drug Resistant	*bla, erm*B
6. *C. perfringens*	I	R	I	R	S	S	S	S	R	R	S	Multidrug resistant (MDR)	*bla, erm*B
7. *C. perfringens*	R	R	R	R	I	S	S	S	R	R	S	Multidrug resistant (MDR)	*bla, erm*B
8. *C. perfringens*	S	I	R	S	S	S	I	S	S	I	S	Drug Resistant	*bla, erm*B
9. *C. perfringens*	R	S	R	R	S	I	S	S	R	R	R	Multidrug resistant (MDR)	*bla, erm*B
10. *C. perfringens*	R	S	R	I	S	S	S	S	R	R	S	Multidrug resistant (MDR)	*bla, erm*B

R: Resistant, I: Int*erm*ediate, S: Sensitive

of antibiotics in the clinical, agricultural, livestock, fisheries, and aquaculture sectors, as well as the identification of strains with antimicrobial resistance, which aids in warning us about potential genetic transfer mechanisms in nature and creates control strategies (Elhadi, 2014).

The antibiotic susceptibility results of the retrieved *C. perfringens* strains were resistant to clindamycin (90%), cefprozil (80%), novobiocin (80%), aztreonam (80%) and erythromycin (80%). These results are discovered to be consistent with other researchers that reported a high emerging resistance against several tested antibiotics (Osman and Elhariri, 2013; Sarkar *et al*., 2013; Slavić *et al*., 2011). Anju *et al*. (2021) found lower resistance of *C. perfringens* to erythromycin (40%). Moreover, the recovered isolates were sensitive to nalidixic acid (90%), ofloxacin (90%), chloramphenicol (90%) and rifampicin (80%). Similarly, *C. perfringens* sensitivity to ofloxacin by 86.67% was also reported by Udhayavel *et al*. (2017). The results was in contrast to Kouassi *et al*. (2014) who found *C. perfringens* resistance to nalidixic acid and chloramphenicol. *C. perfringens* showed unsatisfactory susceptibility patterns in the current study, because 80% of the isolates were MDR. Recent reports stating that the majority of *C. perfringens* strains were MDR strains (Ngamwongsatit *et al*., 2016, Ma *et al*., 2018). The key factors in the evolution of *C. perfringens* resistance patterns were antibiotics employed as growth promoters in animal feed, Due to the regular use of antibiotics, the bacteria have resistance (Arnold *et al*., 2004). Wherefore, Strong regulations is needed to guarantee the secure production of food products derived from animals.

In the present study, bacterial isolates were molecularly typed by PCR using three primers sets specific for the genes encoding *C. perfringens* toxins: alpha (*cpa*), beta (*cpb*), and enterotoxins (*cpe*). PCR evidenced that the tested *C. perfringens* strains frequently carried *cpa* gene in a prevalence of 60% of tested strains while *cpb* was detected in a prevalence of 40% but *cpe* was detected by 10%. Nearly identical results were revealed by Yadav *et al*. (2016) who detected *cpa* gene by 70.83%, 71.42% in *C. perfringens* strains isolated from fresh water fish and fish products, respectively. According to Cai *et al*. (2008), 17.3% of isolates were positive for α toxin gene, 77.3% of isolates were positive for α , β toxin genes (*C. perfringens* type C) but beta 2 toxin gene is present in 62.70% of isolates, all isolates were negative for *cpe*. The current data detected *cpe* gene in low incidence since, *cpe* gene in *C. perfringens* is infrequent, was only detected in a small percentage (less than 5%) of the *C. perfringens* type A isolates worldwide (Ezzeldeen *et al*., 2016; Wang *et al*., 2011). The reports on the genotypic characterization of antibiotic resistance genes in *C. perfringens* isolated from fish in Egypt are few. Hence, the current study used PCR to detect antibiotic resistance genes (*erm*B, *bla*), the results detected *bla*, *erm*B genes in all the tested strains (100%). The results found that phenotypically resistant isolates to erythromycin were displayed evidence of *erm*B antimicrobial resistance gene genotypically, the findings were in line with (Soge *et al*., 2009) who said that the most prevalent gene for macrolide resistance in *C. perfringens* is the *erm*(B) gene by showing the presence of the *erm*B antimicrobial resistance gene genotypically in phenotypically resistant isolates to erythromycin.

Concerning *bla* gene, the current study confirmed that this gene is responsible for resistance of isolates phenotypically to beta lactams antibiotics as cefprozil and aztreonam (80% for each resist, 20% int*erm*ediate sensitive) phenotypically and this was in line with Adams *et al*. (2018) and Hecht (2006) who detected *bla* gene in isolates that was phenotypically resistant to beta lactam antibiotics.

CONCLUSION

C. perfringens was isolated at high prevalence in fish samples collected from Egyptian fish farms. Antibiotic resistance is a major issue in the world today and resistance is recorded in *C. perfringens*. *C. perfringens* isolated from fish are resistant to clindamycin, cefprozil, novobiocin, aztreonam and erythromycin but are sensitive to nalidixic acid, ofloxacin, chloramphenicol and rifampicin. MDR *C. perfringens* was detected in 80% of tested strains. To limit the spread of MDR strains, control measures should be defined for the usage of antimicrobial agents. The examined strains of *C. perfringens* frequently possess the *erm*B and *bla* antibiotic resistance genes as well as the *cpa*, *cpb*, and *cpe* virulence genes. To increase the information on the genes implicated in *C. perfringens* resistance, more research is required to further record both phenotypic and genotypic antimicrobial resistance.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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