

Occurrence of *Clostridium perfringens* Types A, E, and C in Fresh Fish and Its Public Health Significance

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ABSTRACT

Fish remains among the most traded of food commodities, and Egypt is one of the emerging countries being recognized as an important world fish exporter. *Clostridium perfringens* is an important foodborne pathogen to consider in fish trade, as it has been implicated as the causative organism of two fish outbreaks. The aim of the present study was to investigate the occurrence and toxin diversity of *C. perfringens* associated with fresh and canned fish and to examine the public health significance of *C. perfringens* infection in fish. Isolation and identification of *C. perfringens* showed a significantly higher prevalence of the bacterium in fresh fish collected from aquaculture (54.5%) and from markets (71%) as well as in humans in contact with fish (63%) compared with water used for keeping fresh fish (27.3%) and water used in canned fish (17.8%). The isolation level was significantly higher in samples from the external surface of fresh fish (31.8% in aquaculture, 45.6% in markets) than from the intestinal contents of the same fish (9.1% in aquaculture, 6.7% in markets). Thus, markets represent a risk factor for contamination of the external surface of fish from the surrounding environment. Genotyping of the *C. perfringens*-positive isolates by using multiplex PCR revealed that type A enterotoxin-negative (CPE⁻) is the predominant strain among fish (fresh and canned), humans, and water in contact with fresh fish. Interestingly, *C. perfringens* types A enterotoxin-positive (CPE⁺) and C were found only in fresh fish, and these two strains have great health importance in humans. Strikingly, *C. perfringens* type E strain was detected for the first time in fish, humans, and water in contact with fresh fish. Our results demonstrate for the first time that fish act as a reservoir for *C. perfringens*, particularly for types A CPE⁺, C, and E. The external surface of fish represents a vehicle for contamination of fish from the surrounding environment as well as a source of infection of humans, thereby representing a public health hazard.

Key words: Bacterial zoonotic diseases; Canned fish; Fish; Type C *Clostridium perfringens*; Type E *Clostridium perfringens*

Fish is a vital source of food as about one billion people worldwide depend on fish as their primary source of animal protein (10). To fulfill this demand, there has been a rapid growth rate in fisheries and aquaculture. For example, Egypt became a million-ton producer of farmed fish production in 2012 (10). Nile tilapia (*Oreochromis niloticus*) represents the majority of the aquaculture harvest in Egypt, followed by flathead grey mullet (*Mugil cephalus*). Marine fisheries in Egypt produce a wide variety of species, the most important of which are sardine, shrimp, anchovy, brush-tooth lizard-fish, mullets, and round scade (9). The widely accepted fish species in the Egyptian market are the inland common species Nile tilapia and flathead grey mullet and marine species such as tuna, sardine, and mackerel (11).

The increase in intensive aquaculture systems and the global distribution of aquatic animals and their products could result in the emergence of many diseases. Among them, bacterial diseases constitute a serious threat to aquaculture systems and public health (25, 27). Most food outbreaks associated with fish are derived from consumption

of raw or insufficiently heat-treated fish, which might be contaminated with bacteria (26). *C. perfringens* is one of the most common foodborne pathogens that thrives in high-protein food or canned food worldwide (20). *C. perfringens* is widely distributed in nature compared to other pathogenic bacteria; because it is highly prevalent in the intestinal tracts of humans and animals as well as in the soil (7, 21). In addition, *C. perfringens* has the ability to produce highly resistant spores (20).

C. perfringens causes several human diseases ranging from necrotic enteritis to wound infection and life-threatening gas gangrene (23). The virulence of this bacterium results from the toxins produced by some strains. There are five strains of *C. perfringens* (A to E) that are classified according to the major extracellular toxins produced—alpha, beta, epsilon, and iota—as well as other minor toxins (28). Alpha toxin is produced by all five strains, beta toxin is found in types B and C, epsilon toxin is made by types B and D, and iota toxin is produced only by type E (21). Other toxin genes, such as enterotoxins, are not associated with a specific strain, but they relate to the overall virulence and differences among *C. perfringens* strains (28). In humans, the disease is usually caused by type A and type C strains. Type A is typically

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associated with uncomplicated food poisoning (20), whereas type C strains cause necrotic enteritis (17). Type A enterotoxin-positive (CPE⁺) strains that carry the enterotoxin gene (*cpe*) cause foodborne illnesses, sporadic diarrhea, and antimicrobial drug-associated diarrhea (2, 24).

C. perfringens was determined to be the causative organism in outbreaks of food poisoning after the consumption of boiled salmon (14) and tuna salad (15). But, little information is known about the occurrence of toxin genes in *C. perfringens* isolated from fish. Our hypothesis is that fish act as a reservoir of infection with *C. perfringens*. Therefore, the objectives of our study are to determine the occurrence and toxin diversity of *C. perfringens* in fresh and canned fish; to examine their association with humans such as aquaculture workers and fish handlers who are in contact with fish; and to investigate the public health significance of the infection of fish with this bacterium.

MATERIALS AND METHODS

Samples. The study groups included samples from apparently healthy fresh whole fish ($n = 268$), canned fish ($n = 135$), water ($n = 11$), and humans in contact with fresh fish ($n = 30$) as well as samples from human patients with gastrointestinal (GI) complaints ($n = 79$). The samples were collected from November 2013 to November 2014.

Fresh fish samples were derived from aquaculture ($n = 88$) and markets ($n = 180$) and were randomly selected from El-Fayoum and El-Giza governorates in Egypt. The species of fresh fish were as follows: Nile tilapia ($n = 158$), flathead grey mullet ($n = 70$), and sardine ($n = 40$). Two types of samples were collected from each fish: swabs from the external surface and whole intestinal contents. The whole external surface of fish was swabbed from head to tail by using cotton swabs moistened with peptone water. The intestines were cut into pieces and used for bacterial isolation.

Canned fish samples were collected from different supermarkets, grocery stores, and retail stores. Three fish species were studied: sardine ($n = 40$), mackerel ($n = 25$), and tuna ($n = 70$).

Water samples were collected from aquaculture ponds ($n = 2$) and from tanks holding live fish and from containers holding water used for washing hands of fish handlers at markets ($n = 9$) where fish samples were collected. The water samples were collected in sterile glass bottles containing sodium thiosulfate. The samples were then filtered through 0.45- μ m pore size nitrocellulose filters (Sartorius, Aubagne, France) to trap bacteria. Next, we placed the filters in tubes containing peptone broth and vortexed the samples to recover any bacteria (5). Finally, the filters were removed from the tubes, and the broth containing the bacteria was used for bacterial isolation.

Hand swabs were collected from aquaculture workers and fish vendors working at the same markets where the fish samples were collected. The samples were taken by rubbing swabs in the interdigital spaces, nails, and palms and on the back of the hands. One swab was used for all parts of the hands of each individual.

Stool specimens (2 to 10 g) were collected from persons who had GI complaints with ($n = 29$) or without ($n = 50$) diarrhea at the sampling time and who had visited public hospitals and private diagnostic medical laboratories. Ethical clearance to use human subjects was obtained from the designated health facilities after official correspondence was submitted. Written consent to use the samples was obtained from each person.

Isolation and biochemical identification of *C. perfringens*.

Each sample was inoculated into a tube of sterile, freshly prepared, cooked meat medium. The tube was incubated anaerobically in jars by using anaerobic gas-generating kits (Oxoid, Cairo, Egypt) at 37°C for 24 to 48 h. A loopful of sample from the previously incubated tube was streaked onto the surface of a plate containing 10% sheep blood agar with neomycin sulfate (200 μ g/ml). The plate was then incubated anaerobically at 37°C for 24 to 48 h. Subcultures from the suspected colonies of *C. perfringens* were examined microscopically and biochemically according to Kone-man et al. (16).

Molecular identification of *C. perfringens*. *C. perfringens* isolates obtained from all samples were subjected to genotyping by using multiplex PCR.

DNA extraction. Pure colonies of *C. perfringens* that showed double zone of hemolysis on blood agar and that were confirmed biochemically were grown overnight in 5 ml of brain heart infusion with 16% glycerol (Oxoid) at 37°C under anaerobic conditions. A rapid boiling method was used to prepare DNA template from the isolated bacterial strains according to Sheedy et al. (30).

Multiplex PCR. Multiplex PCR was carried out according to Meer and Songer (22) for the primer sequences (Metabion, Planegg, Germany) (3) for the five toxin genes: alpha (*cpa*), beta (*cpb*), epsilon (*cpe*), iota (*iA*), and enterotoxin (*cpe*) of *C. perfringens*. PCR mixtures of a 25- μ l total reaction volume consisted of 2.5 μ l of template DNA from each isolate, 12.5 μ l of 2 \times PCR master mix (Vivantis, Cairo, Egypt), 1 μ l of 10 pmol of each primer, and 9 μ l of DNase- and RNase-free water. PCR amplification was carried out in an iCycler Peltier thermal Cycler (PTC-100, MJ Research) as follows: denaturation at 94°C for 4 min; annealing at 94°C, 55°C; extension at 72°C for 1 min; and final extension at 72°C for 10 min. A negative control PCR mixture with no template DNA was included. PCR products were then run on 1.5% agarose gel containing 0.5 \times Tris-borate-EDTA at 70 V for 60 min and then visualized using UV light. A DNA ladder marker was run simultaneously.

The following positive-control *C. perfringens* national strains were grown on blood agar under the above-mentioned conditions and subsequently used in PCR: types A, C, D, E, and A CPE⁺ (Veterinary Serum and Vaccine Research Institute, Abassia, Cairo, Egypt).

Statistical analysis. NCSS 9 software (NCSS, Kaysville, UT) was used for statistical analyses. A chi-square test was used to determine whether there was a difference in the occurrence of *C. perfringens* and type of sample (Table 1). A two-proportion Z test was used to compare the occurrence of *C. perfringens* between fish collected from markets and from aquaculture (Table 1) and between fish species (Fig. 1). To compare the occurrence of *C. perfringens* between external with intestinal samples, a McNemar test was used (Table 1). On each occasion, *P* values ≤ 0.05 are regarded as significant.

RESULTS AND DISCUSSION

C. perfringens is among the most common cause of foodborne illness in humans (8); however, little is known about the role of fish in the epidemiology of *C. perfringens* (1, 6). Here, we investigated the occurrence and toxin diversity of *C. perfringens* associated with the commonly

TABLE 1. Occurrence of *C. perfringens* in different types of samples

Sample	Total	<i>C. perfringens</i> -positive samples		
		No.	% ^a	
Fresh fish: aquaculture	88	Total	48	54.5
		Intestinal	8	9.1
		External	28	31.8
		Intestinal and external	12	13.6
Fresh fish: markets	180	Total	127	71*
		Intestinal	12	6.7
		External	82	45.6
		Intestinal and external	33	18.3
Humans (hand swabs)	30	19	63.3	
Water	11	3	27.3	
Canned fish	135	24	17.8	

^a Asterisk indicates significant difference from aquaculture, with *P* value of 0.0017.

consumed fish species in Egypt and also the public health significance of *C. perfringens*.

Egypt relies mainly on inland aquaculture from which fish get exported or distributed to local markets. Therefore, we examined fresh fish collected directly from aquaculture and markets. Bacteriological culture and biochemical identification (Table 1) revealed that the occurrence of *C. perfringens* was significantly lower in fresh fish from aquaculture (54.5%) than in fish collected from markets (71%), suggesting an increased risk of infection with *C. perfringens* in market fish. There was no significant difference in the occurrence of *C. perfringens* between the

different markets where fish samples were collected. The isolation rate recorded in our study is higher than that in other studies carried out on freshwater fish collected from fisheries in, for example, China (17.9%) (6) and India (18.4%) (1). This distinction might be due to differences in the source of samples, as an increase in the prevalence of diseases in aquaculture compared to fisheries was discussed previously (33). In contrast to the other two studies (1, 6) that examined only the intestinal contents of fish, we included samples from both the external surface and the intestinal contents of each fish. This approach increased the prevalence in our study, as the isolates of *C. perfringens*

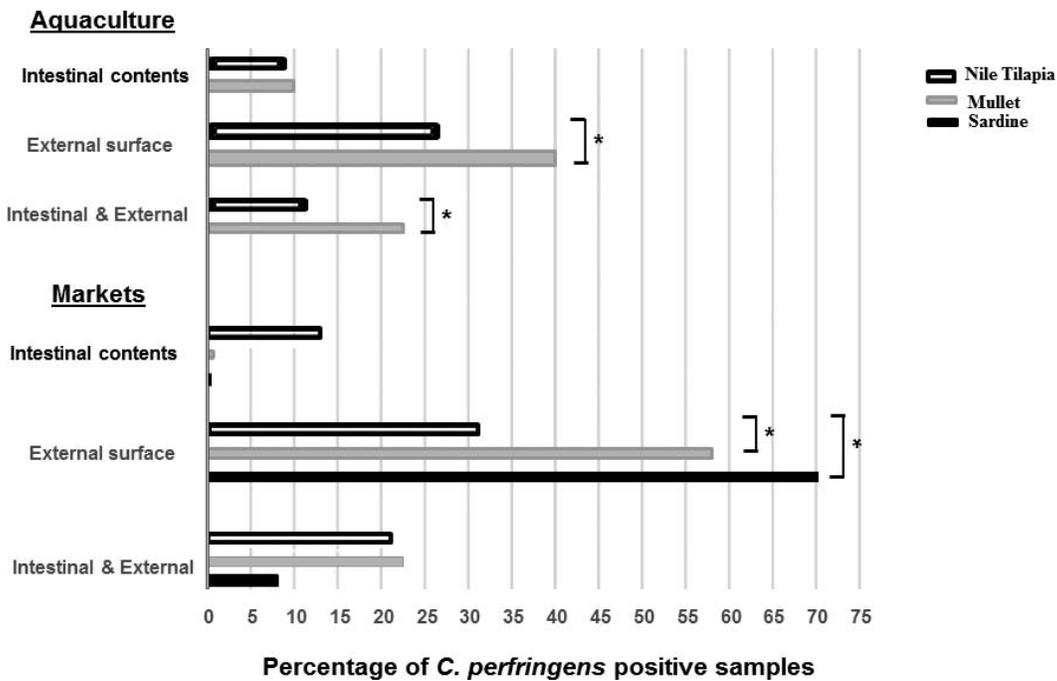


FIGURE 1. Species of fish and the proportion of *C. perfringens*. Two fish species from aquaculture were included, Nile tilapia (n = 58) and flathead grey mullet (mullet) (n = 30); three fish species from markets were included, Nile tilapia (n = 100), flathead grey mullet (n = 40), and sardine (n = 40). Intestinal contents and swabs from the external surface of each fish were collected. The results are shown as percentage of *C. perfringens*-positive samples, Intestinal contents refers to fish for which only its intestinal contents were positive; external surface refers to fish for which only the external surface was positive; and intestinal & external refers to fish for which both its intestinal and external surface were positive. The horizontal line with an asterisk (*) indicates a significant difference by using a two-proportion Z test.

were more frequently detected on the external surface (external) of fish rather than in the intestinal contents (intestinal), regardless of whether fresh fish was collected from aquaculture or from markets (Table 1). We have also reported the copresence of the bacteria in the intestinal contents and on external surface of the same fish (intestinal and external). Our results highlight the importance of the external surface of fish as a potential source of contamination and infection that needs attention when examining fish for the presence of *C. perfringens*.

Interestingly, a significantly higher percentage of fish collected from markets (45.6%) than from aquaculture (31.8%) had *C. perfringens* on the external surface (Table 1). Moreover, no significant difference was found in the intestinal contents between fish from aquaculture (9.1%) and markets (6.7%). These findings provide evidence for increased contamination of fish through the external surface either during transportation to markets or in markets.

We then determined the possible sources of contamination of fresh fish and its public health significance (Table 1). Examination of hand swabs from handlers in contact with fish collected from aquaculture or markets showed a 63% prevalence of *C. perfringens*. In contrast, the isolation rate of *C. perfringens* from aquaculture water or water tanks from markets was significantly lower (27.3%) than that via the hand swabs. Similarly to that found for red meat (29), our results suggest that contamination of the external surface of fish can occur through contaminated hands of fish handlers. But, our results do not exclude the possibility that the *C. perfringens* isolated from hands of fish handlers might originate from fish.

Furthermore, we examined whether *C. perfringens* has a favorable fish species. Figure 1 demonstrates that in aquaculture, there is no difference in the occurrence of *C. perfringens* in the intestinal contents between Nile tilapia (8.6%) and flathead grey mullet (10%). A significant increase was found in *C. perfringens* presence on the external surface in flathead grey mullet (40%) compared to Nile tilapia (25.9%). Interestingly, the copresence of *C. perfringens* on the external surface and intestinal contents in the same fish was significantly higher in flathead grey mullet (23%) compared to Nile tilapia (10%). Common fish collected from the markets included Nile tilapia, flathead grey mullet, and sardine. The occurrence of *C. perfringens* in the intestinal contents was higher in Nile tilapia (13%) than the other two species. Conversely, sardine (70%) and flathead grey mullet (58%) showed a significantly higher occurrence of *C. perfringens* than Nile tilapia (31%) in samples from the external surface. The copresence in the intestinal and external surfaces was prominent in flathead grey mullet (22.5%) and Nile tilapia (23%) and shows that there is no clear association between the occurrence of *C. perfringens* and certain fish species. The occurrence of *C. perfringens* is influenced by the source of fish (aquaculture or markets) as well as the type of samples (intestinal contents or external surface).

Molecular typing of the bacterial isolates was performed by multiplex PCR by using five sets of primers specific for the genes encoding the *C. perfringens* toxins alpha (*cpa*), beta (*cpb*), epsilon (*cpe*), iota (*ia*), and enterotoxins (*cpe*)

(Fig. 2 and Table 2). Of the 220 total *C. perfringens* isolates from fresh fish, 211 (96%) were type A positive (208 [94.5%] CPE⁻ [enterotoxin negative] and 3 [1.4%] CPE⁺), 2 (0.9%) were type C positive, and 7 (3.2%) were type E positive. Interestingly, 66.6% of the isolates from water samples collected from aquaculture and markets were type A CPE⁻, but only one isolate from aquaculture was type E. Like the isolates from fresh fish and water, 18 (94.7%) of 19 of the isolates from human hand swabs were type A CPE⁻. Type E was also found in one isolate from aquaculture worker. Type A CPE⁺ was not found in water or human isolates. None of type B or D was found in any of the examined samples.

Our study revealed that *C. perfringens* type A CPE⁻ was the common strain in fresh fish, fish handlers, and water collected from aquaculture and markets (Fig. 2A). This finding is similar to that of a study from India (1) wherein all isolates from samples of freshwater fish were type A. Moreover, type A is known to be the predominant strain in the global *C. perfringens* population and that the majority of environmental *C. perfringens* strains are enterotoxin negative (21).

Furthermore, type A CPE⁺ was detected in only 1.4% of fish collected from aquaculture (Fig. 2B), a value similar to the estimated 2 to 5% occurrence of *C. perfringens* isolates that carry the enterotoxin gene worldwide (21, 23). None of the human or water samples collected from aquaculture carried the enterotoxin gene, suggesting that the fish is the source of this strain in the present study. This source poses a health hazard because the majority of human illnesses caused by *C. perfringens* have been correlated with the presence of enterotoxin (23). Moreover, the *C. perfringens* strains that carry the enterotoxin gene are shown to produce spores with high heat resistance (18). Therefore, *C. perfringens* foodborne outbreaks are most often associated with food subjected to poor temperature, such as may occur in commercial kitchens (15, 19). Thus, eating raw or improperly cooked fish that carry these strains can result in food poisoning. Furthermore, enterotoxin-associated nonfoodborne GI diseases were reported to spread person to person or via ingestion of environmental contaminants (24).

Strikingly, our study is the first to isolate *C. perfringens* type E (3.2%) from fresh fish (Fig. 2D). This isolation occurred only in fish from aquaculture, particularly from the intestinal contents of four fish and the external surface of three fish. There was an association between *C. perfringens* type E and aquaculture, as this type was also isolated from hand swabs of handlers and water samples collected only from aquaculture. Type E is not a human strain; it was detected postmortem in ovine and bovine intestines (32). There are only two reports about type E enterotoxemia (13) and enteritis in calves and lambs (31). This suggests that the fish might be the source of type E in our study.

Type C was detected in 0.9% of the fish isolates (Fig. 2C). Type C was also found in a study of freshwater fish in China, but with a prevalence of 77% (6). *C. perfringens* type C represents a great human risk as it cause necrotic enteritis (17). Collectively, our findings indicate a common source of infection with the strains of *C. perfringens* that is

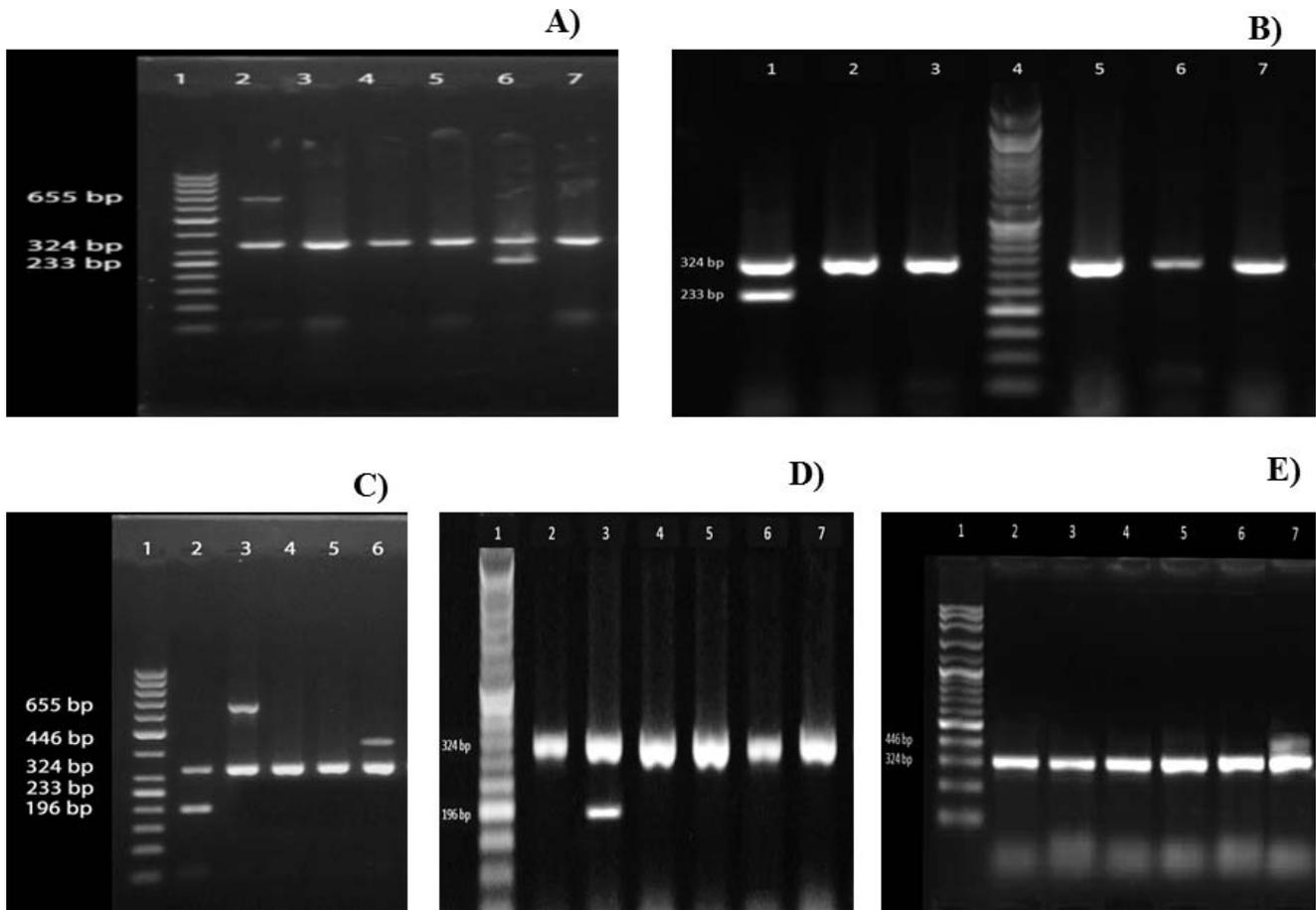


FIGURE 2. Agarose gel electrophoresis of multiplex PCR products of *C. perfringens* isolates recovered from different examined samples and positive control strains. (A) Lane 1, 50-bp DNA ladder; lane 2, *C. perfringens* type D (control strain, *cpa* gene at 324 bp and *cpε* gene at 655 bp); lane 3, *C. perfringens* type A (control strain, *cpa* gene); lane 6, *C. perfringens* type A CPE⁺ (control strain, *cpa* gene and *cpe* gene at 233 bp); and lanes 4, 5, and 7, *C. perfringens* type A (samples). (B) Lane 4, 50-bp DNA ladder; lane 1, *C. perfringens* type A CPE⁺ (samples); and lanes 2, 3, and 5 through 7, *C. perfringens* type A (samples). (C) Lane 1, 50-bp DNA ladder; lane 2, *C. perfringens* type C (control strain, *cpa* gene and *cpb* gene at 196 bp); lane 3, *C. perfringens* type D (control strain); lane 6, *C. perfringens* type E (control strain, *cpa* gene and *iA* at 446 bp); lanes 4 and 5, *C. perfringens* type A (samples). (D) Lane 1, 50-bp DNA ladder; lane 3, *C. perfringens* type C (samples); lanes 2 and 4 through 7, *C. perfringens* type A (samples). (E) Lane 1, 100-bp DNA ladder; lane 7, *C. perfringens* type E (samples); and lanes 2 through 6, *C. perfringens* type A (samples).

TABLE 2. Genotyping of *C. perfringens* isolates by using multiplex PCR

Type of <i>C. perfringens</i> ^a	Positive toxin gene	No. of <i>C. perfringens</i> -positive isolates							
		Fresh fish (n = 220)				Humans			
		Aquaculture		Markets		Water (n = 3)		Hand swabs (n = 19)	
		External (n = 40)	Intestinal (n = 20)	External (n = 115)	Intestinal (n = 45)	Aquaculture (n = 2)	Markets (n = 1)	Aquaculture (n = 2)	Markets (n = 17)
A CPE ⁻	<i>cpa</i>	35	13	115	45	1	1	1	17
				Total = 198 (94.5%)				Total 18 (94.7%)	
A CPE ⁺	<i>cpa, cpe</i>	1	2	—	—	—	—	—	—
				Total = 3 (1.4%)					
B	<i>cpa, cpb, cpε</i>	—	—	—	—	—	—	—	—
C CPE ⁻	<i>cpa, cpb</i>	—	2	—	—	—	—	—	—
				Total = 2 (0.9%)					
D	<i>cpa, cpε</i>	—	—	—	—	—	—	—	—
E	<i>cpa, iA</i>	4	3	—	—	1	—	1	—
				Total = 7 (3.2%)					
Total		40	20	115	45	2	1	2	17

^a CPE⁻, enterotoxin negative; CPE⁺, enterotoxin positive; —, negative results.

most probably the fresh fish, because some strains (types A CPE⁺ and C) were found mainly in fresh fish, but not in humans or water. This possibility poses a public health hazard, as fish and its environment may act as potential sources of infection to humans via handling and consumption. The prevalence of *C. perfringens* in human hand swabs and water collected from markets was not higher than that from aquaculture. Therefore, the increased contamination found in fish collected from markets might be due to exposure to environmental contaminants such as soil or from the containers used to keep fish during transport to markets.

C. perfringens is found in undercooked and canned food (20). Canned fish is popular worldwide, as it represents a quick and easily prepared food. Therefore, it was of interest to determine the prevalence of *C. perfringens* in canned fish. Tuna, sardine, and mackerel are among the favored canned fish species. The presence of *C. perfringens* in canned fish was significantly lower than that in fresh fish (17.8%; Table 1), but similar to that of canned beef (4). There was no difference between the three examined fish species (tuna, sardine, and mackerel). Moreover, all the isolates collected from canned fish were *C. perfringens* type A CPE⁻. Our results contradict those of Hamza and Ghoneim (12) who detected *C. perfringens* type A CPE⁺ in canned fish. This difference might be attributed to the source of the canned fish used in our study.

The public health significance of *C. perfringens* in persons with GI problems was examined. Stool specimens were collected from patients suffering from GI problems with diarrhea (diarrheic) or without diarrhea (nondiarrheic) at the time of sampling. Of 79 stool specimens, 52 (66%) were positive for *C. perfringens* (data not shown). Interestingly, comparing GI patients with and without diarrhea showed no difference in the occurrence of *C. perfringens* between the two groups: diarrheic, 59% and nondiarrheic, 70%. All the isolates were type A CPE⁻ (data not shown). Our results revealed widespread *C. perfringens* type A among patients suffering from GI problems. Furthermore, none of the isolates carry the enterotoxin gene, the most important virulence factor when *C. perfringens* type A causes GI problems (21, 23). This might explain the occurrence of *C. perfringens* in nondiarrheic patients.

In conclusion, we found high prevalence of toxigenic *C. perfringens* in fresh fish; the most prevalent strain was type A CPE⁻. Strikingly, we showed for the time that fresh fish act as a reservoir for types A CPE⁺, C, and E strains. Furthermore, we found that the occurrence of *C. perfringens* is not influenced by the species of fish, but rather by the source of fish—aquaculture or markets—thus posing a public health hazard for aquaculture workers, fish handlers, and consumers.

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