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Prevalence of *Clostridium perfringens* Alpha Toxin in Processed and Unprocessed Fish

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Abstract: The current research was carried out on one hundred and thirteen random fish samples (56 processed and 57 unprocessed) collected from different localities in El-Sharkia Governorate, Egypt to obtain a complete picture of *C. perfringens*. Bacteriological and biochemical examination was done on the isolates. The total incidence of positive samples from 56 processed fish samples were 32 isolates (57.1%) and from 57 unprocessed fish samples were 34 isolates (59.6%). The Nagler's test was applied on the recovered *C. perfringens* isolates. Typing of *C. perfringens* isolates revealed that the incidence of toxigenic and non-toxigenic isolates were 84.8 and 15.2% respectively. Typing of toxigenic strains of *C. perfringens* revealed that *C. perfringens* type A was the most predominant one comparing with type D. Immune diffusion test showed that 17 and 11 toxins of types A and D gave identified reaction with incidences of 47.2 and 55% respectively. ELISA test revealed that 22 out of 25 isolates of *C. perfringens* produced toxin.

Key words: *C. perfringens* · Fish · Nagler's Test · Immune diffusion Test

INTRODUCTION

Fish are considered one of the most widely accepted and valuable food in most countries. Egypt is currently one of the fastest growing countries in the field of aquaculture to solve protein shortage problem in Egypt.This increase in aquaculture has led to the further spread of diseases [1, 2]. The presence of microbial pathogens, especially those of bacterial origin is one of the most significant factors affecting fish culture [3]. Anaerobic bacteria are important groups of microorganisms which are responsible for reduction of growth rate, increased mortality and high costs of treatment with antibiotics in addition to many public health hazards [4, 5].

C. perfringens is widely distributed in soil and intestinal contents of man and animals. It has a great effect on the human health causing food poisoning. It also causes a number of human diseases ranging from necrotic enteritis to wound infection and gas gangrene. This pathogenicity is associated with lethal extra cellular toxins which have been defined as enzyme activity as collagenase, hyaluronidase and deoxyribonuclease [6]. The colonies of *C. perfringens* are smooth, round, glistening and surrounded by double Revise zone of hemolysis [7]. *C. perfringens* are large Gram-positive rods (0.6-2.4 x 1.3-9.0 μm), encapsulated, non-motile, spore forming, fermentative and catalase negative [8]. The spores of some *Clostridia* species are high heat resistant and may survive heat treatment of canned foods. If the surviving spores germinate and the vegetative cells grow, spoilage will occur [9]. *C. perfringens* is divided into 5 types (A, B, C, D and E) on the basis of the production of 4 major toxins (α, β, ε and ι), each type had been linked to specific diseases [10]. So, the main goals of the present study were:

- Identification and typing of *C. perfringens* isolates from processed and unprocessed fish samples.
- Serological identification of *C. perfringens* toxins by toxin-antitoxin neutralization test, minimal lethal dose in mice and immune diffusion test.
- Detection of *C. perfringens* type A (alpha toxins) isolated from collected samples by ELISA test.

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MATERIALS AND METHODS

Samples: A total of 113 fish samples were collected from large supermarket and retail fish shops in El-Sharkia Governorate. The samples were 56 processed fish samples (canned salmon, canned mackerel, salted sardine, renga and feisekh) and 57 unprocessed fish samples (Nile cat fish, Tilapia nilotica, danis, fresh water sardine, rossi, macaroni and bagha).

Bacteriological Examination

Isolation and Identification of *C. perfringens:* The surface of each sample was sterilized and bean size pieces were obtained from the deeper parts of each sample and inoculated separately into tubes of freshly prepared cooked meat broth medium. All tubes were incubated anaerobically in the anaerobic jar by using anaerobic gas generating kits (Oxoid) at 37°C for 48 hours. For isolation of *C. perfringens*, a loopful from the previously incubated tube was streaked onto the surface of 10% sheep blood agar with neomycin sulphate (200 µg/ml) and incubated for 24 hours at 37°C in the anaerobic jar. The suspected colonies of *C. perfringens* were picked up and examined for their morphological and culture characters [11], microscopical examination of stained films from the suspected colonies with Gram's stain and biochemical tests were done [12].

Nagler's Test by Half Antitoxin Plate: This test was done as was previously described [13]. The attack of alpha toxin that is produced by all types of *C. perfringens*, on lecithin is inhibited by alpha antitoxin [14].

Typing of *C. perfringens* Toxins by Dermonecrotic Test: Preparation of toxins and their treatment [15] and the application of the dermonecrotic test [16, 17] were carried out.

Serological Identification of *C. perfringens* Toxins: Toxin antitoxin neutralization test: This test was done as previously described [13].

Determination of Minimum Lethal Doses (MLD) of the Prepared C. Perfringens Toxins: The test was done using the prepared toxins (alpha toxin of type A and epilson toxin of type D) as described previously [18].

Immune Diffusion Test: Agar gel immune diffusion test was used to determine the relationship between antigen and antibodies [19].

Detection of *C. perfringens* Alpha Toxin by Indirect ELISA: The serum of the rabbit (50 µl/well) were diluted in 0.005% Tween 20 in PBS 1:16 were added to each well and the plate was incubated at 37°C for 2 hours. Rapid detection of *C. perfringens* type A (alpha toxin) was done by indirect ELISA technique. Alpha toxin isolated from fish used as antigen by [20].

RESULTS AND DISCUSSION

Clostridium perfringens is more widely spread than other pathogenic bacteria; its principle habitats are in the soil and the intestinal content of the man and animals. There is much evidence that obligate anaerobic organisms are probably the principal sources of infection in human beings, domestic animals and fish as mentioned by Hayed [6]. In recent decades, many surveys have been conducted on the incidence of *C. perfringens* in raw and processed meat and poultry. This report indicates wide spread occurrence of this organism in processed and unprocessed fish [21].

Incidence of *C. perfringens* Isolates in Processed Fish Samples: Table 1 shows that the prevalence of *C. perfringens* in 56 processed fish samples was 57.1%. The isolation of positivity was seen in feisekh, renga and salted sardine by percentages of 82.3, 80.0 and 75, respectively, but there was no *C. Perfringens* isolates from canned products such as mackerel and salmon.

Table 1: Incidence of *C. perfringens* in different types of processed fish samples

Processed fish samples	No. of + ve samples/Total number
Canned salmon	0/11 (0%)
Canned mackerel	0/5 (0%)
Salted sardine	6/8 (75%)
Renga	12/15 (80%)
Feisekh	14/17 (82.3%)
Total	32/56 (57.1%)

Table 2: Incidence of *C. perfringens* in different types of unprocessed fish samples

Un-processed fish samples	No. of +ve samples/total Number
Nile cat fish	8/10 (80.0%)
Tilapia nilotica	5/8 (62.5 %)
Danis	4/8 (50.0 %)
Sardine	3/8 (37.5%)
Rossi	5/8 (62.5%)
Macaroni	5/8 (62.5%)
Bagha	4/7 (57.1%)
Total	34/57 (59.6%)

Table 3: Typing of *C. perfringens* isolates recovered from fish samples

		Toxigenic *C. perfringens* isolates		
Type of fish samples	Non toxigenic *C. perfringens*/ total isolates	*C. perfringens* type A	*C. perfringens* type D	Total
Processed	5/32 (15.6%)	17/32 (53.1%)	10/32 (31.32%)	27/32 (84.43%)
Unprocessed	5/34 (14.7%)	19/34 (55.98%)	10/34(29.4%)	29/34 (85.32%)
Total	10/66 (15.21%)	36/66 (54.5%)	20/66(30.3%)	56/66 (84.8%)

These results coincide with those recorded by Kassem [22] who stated that *Clostridium* spp. could be isolated from the three types of examined salted fish (feisekh, molouha and salted sardines), while canned product is safe and free from anaerobic microorganisms. The present study results also go hand in hand with those recorded by Richardson [23].

Incidence of *C. perfringens* Isolates in Unprocessed Fish Samples: Table 2 shows that out of 57 unprocessed fish samples, 34 (59.6%) were positive for *C. perfringens*. The highest incidence of positivity was shown in Nile cat fish by a percentage of 80.0, while the lowest incidence of positivity appeared in sardine with an incidence of 37.5%.

These results are similar to that of Schoken *et al.* [24], Peterson *et al.* [25] and Marzouk *et al.* [26] who mentioned that *C. perfringens* need presence of high amount of organic pollutions of human and animal origin and strict anaerobic condition to grow and become pathogenic. They also added that the high incidence of *C. perfringens* is due to the heavy use of poultry dropping and animal manure that usually contain *C. perfringens*. On the other hand, Enany *et al.* [27] reported that Nile cat fish possess highest prevalence with *C. perfringens* as Nile cat fish are highly exposed to subclinical infection with different levels of *C. perfringens* toxin so that they become immunized without showing symptoms of illness.

Identification of *C. perfringens* Isolates: The suspected colonies of *C. perfringens* were Gram-positive, short plumb, rarely sporulated and non-motile bacilli when they were stained with Gram's stained. It was apparent that sheep blood agar with neomycin sulphate (200 µg/ml) is a perfect medium for isolation of *C. perfringens* rather than other *Clostridium* species and gave double zones of hemolysis. All the recovered strains in this work were fermentative to different sugars as glucose, maltose, lactose, sucrose and mannose with production of acid and gases, gelatin liquefiers, litmus milk positive, catalase, oxidase and indole negative. Similar results were recorded by several authors as Vaikosen and Muller [11] and Assis *et al.* [28].

Results of Nagler's Test for Identification of *C. perfringens* Isolates: The obtained results revealed that 10 out of 66 isolates were toxin producer on egg yolk agar medium. These results go hand in hand with those recorded by Smith and Holdman [13] who applied Nagler's test using half antitoxin plate to detect lecithinase activity of alpha toxin of different types of *C. perfringens*.

Typing of *C. perfringens* Isolates by Intradermal Injection of Guinea Pigs: The typing of *C. perfringens* by intradermal injection of guinea pig revealed that the incidence of toxigenic and non-toxigenic isolates were 84.8 and 15.2%, respectively as shown in table (3). The action of *C. perfringens* type "A" (alpha toxin) appeared as an irregular area of yellowish green necrosis tented to spread downward, while that of type "D" (epilson toxin) appeared as a circular whitish green necrosis with few small areas of purplish hemorrhagic necrosis [29].

Typing of the toxigenic *C. perfringens* isolates revealed that *C. perfringens* types A and D were the most predominant ones with percentages of 54.5 and 30.3, respectively. Such finding is similar to that of Enany *et al.* [27], Aschfalk and Muller [30] and Songer and Dale [31] who recorded presence of *C. perfringens* types A and D with percentage of 50 and 25 respectively in Nile tilapia samples.

Toxin Antitoxin Neutralization Test: All sixty six *C. perfringens* isolates were identified by toxin antitoxin neutralization tests. The results showed the protection of the injected albino guinea pigs because of neutralization of each toxin with its specific antitoxin. Also, the obtained results revealed that *C. perfringens* type A was the most predominant one among the total recovered isolates as shown in table 3. These results are in accordance with Joklik *et al*. [14] and Songer and Dale [31] who deserved that alpha toxin is the most important toxin produced by all types of *C. perfringens*.

Results of MLD Test in Mice: All sixty six *C. perfringens* isolates were tested for MLD in mice and the obtained results showed that the minimum lethal doses for

C. perfringens types A and D were 1/16 and 1/8, respectively. Stark and Duncan [32] found that the minimum lethal dose for *C. perfringens* is 0.014.

Results of Immune Diffusion Test: Immune diffusion test revealed that out of 36 *C. perfringens* type A toxin, 17, 7 and 12 toxin showed identity, non-identity and partial identity with percentages of 47.2, 19.4 and 33.3, respectively. Furthermore, out of 20 of *C. perfringens* type D toxin, 11, 3 and 6 showed identity, non-identity and partial identity with percentages of 55.0, 15 and 30.0, respectively. These results go hand in hand with those recorded by Aloisi [33] and Mona [34] who reported that *C. perfringens* type A toxin showed identity, non identity and partial identity with percentage of 46.2, 18.3 and 36.5, respectively. Furthermore *C. perfringens* type D toxin showed identity, non-identity and partial identity with percentages of 53.2, 17 and 29.8, respectively.

Results of ELISA Test: A total of 25 rabbit serum samples were examined. There were 22 positive samples with 88% specificity and 3 negative samples with 12%. These results agree with the finding of Aschfalk and Muller [30] and El-Idrissi and Ward [35] who suggested that ELISA assay could be used to detect *C. perfringens* type A toxin in fish by a good rapid and simple manner and fair accuracy.

In conclusion, in the present study the highest incidence of *C. perfringens* isolates was in feisekh and Nile catfish, while the lowest incidence was in canned salamon and mackerel. ELISA is a good and rapid technique for detection of alpha toxin of *C. perfringens*.

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