Prevalence of different Clostridium species in fresh fishes and water

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Abstract:

Clostridium species is considered a foodborne anaerobic pathogen popular in fish. The current study aimed to detect *Clostridium* species prevalence in fish and fresh water collected from three different Egyptian fish farms. One hundred and eighty samples were collected from gills and intestine of tilapia, catfish and dennis and 30 water samples were collected from the corresponding farms of sampled fish. Bacteriological examination was conducted for identification of different *Clostridium* species in fish and water. The overall prevalence of *Clostridium* species in fish was 28.3% (51/180). The prevalence of *Clostridium* species in tilapia was 30% (18/60), in catfish was 31.6% (19/60) and in dennis was 23.3% (14/60). The overall prevalence of *Clostridium* species in water was 56.6% (17/30). Biochemically, the *Clostridium* species were identified as *Clostridium sporogenes, Clostridium perfringens* and *Clostridium bifermentans. C. sporogenes* was detected by 16.6%, 20% from intestine and gills of tilapia, 16.6%, 13.3% from intestine and gills of catfish, 6.6%, 16.6% from intestine and gills of dennis, 16.6% from water. *C. bifermentans* was detected by 10%, 13.3% from intestine and gills of tilapia, 13.3%, 20% from intestine and gills of catfish, 10%, 13.3% from intestine and gills of dennis, 13.3% from water. *C. perfringens* was detected by 26.6% from water.In conclusion, tilapia, catfish, dennis can be potentially contaminated with *C. bifermentans* and *C. sporogenes* additionally *C. perfringens* was detected in water of fresh fish farms.

Key words: Bacteriological examination, *Clostridium* species, fish, fresh water

Introduction:

In the past few decades, the output of farmed fish and shellfish has risen globally in effort to reduce the scarcity of animal protein. Anaerobic bacteria that can infiltrate and accumulate in fish tissue and pose a risk to public health can be found in untreated organic fertilizers. **(*Mirhaj et al.*, 2014)**

The group of Clostridial species is highly diverse, ranging from diseases that produce severe toxins to harmless saprophytes, as well as from strict anaerobes to aerotolerant species. **(*MacLennan* 1962).**

Currently 23 recognized genera of heterotrophic, obligately anaerobic bacteria. Within this group are gram-negative rods and cocci and gram-positive rods and cocci. Many of the genera have representative species which are clinically important pathogens of man and animals. According to ***(Gajdács et al.* (2017),** the most well-known genus, *Clostridium*, is home to the species *C. botulinum*, *C. tetani*, and *C. perfringens*, which cause gas gangrene, tetanus, and botulism poisoning, respectively. Research conducted in Egypt has demonstrated that the primary sources of *C. perfringens* in earthen fish farms are animal waste and chicken droppings. **(*Saad et al.*, 2013).**

Numerous locations are home to *C. sporogenes*, such as soil, freshwater and marine sediments, preserved meat and dairy products, fecal matter, snake venom, and illnesses in humans and domestic animals. Although *C. sporogenes* lacks the ability to create the neurotoxin botulinum that causes disease in humans, it shares phenotypic similarities with other species of its genus, including *C. difficile* and *C. botulinum*. Therefore, the American Type Culture Collection has designated *C. sporogenes* as a harmless biosafety level I organism ***(Kubiak et al.*, 2015).** Food products contaminated with *C. sporogenes* may spoil more quickly than those contaminated with *C. botulinum* due to its proteolytic nature and capacity to create spores that are more resistant to high temperatures. This could be dangerous for the general public's health. **(*Brunt et al.*, 2014 and *Brunt et al.*, 2020).**

A serious epidemic in grass carp raised in a German polyculture fish farm pond is linked to *C. bifermentans* ***(Hoffman et al.*, 1995).** However, even at 1000 times greater doses, *C. bifermentans* serovar Malaysia did not cause toxicity in goldfish. Tarantellas of Eubacterium an asporogenous, Gram-positive, anaerobic bacteria known as *E. tarantellas* was isolated in pure culture from the

brains of many dead and moribund striped mullets from Biscayne Bay that had neurological manifestations. For channel catfish, all isolates were pathogenic, but not for mice or guinea pigs. ***(Thiery et al.,* 1992)**

Because Clostridial infections cause large financial losses, widespread morbidity, and high mortality rates, they pose a persistent danger to the worldwide livestock business. Because spores from C*lostridium difficile* can survive in soil for decades and via extensive animal handling, eradication of the disease is challenging. It is difficult to find curative treatments for this virus because of its quick and deadly effects, and there is no immunological cross-protection between the different toxinotypes. ***(Uzal et al.,* 2014).**

In conclusion, to bridge the information gap in epidemiology and virulent forms of diseases connected to fish anaerobes, as well as to gain a better understanding of the pathogenic mechanisms of anaerobic bacterial infections, molecular and pathophysiological research is absolutely necessary.

Material and methods

Sample collection and processing:

One hundred and eighty fish samples (n = 180) were collected from gills and intestine of tilapia (n=30), catfish (n=30) and dennis (n=30), also 30 water samples were collected from 3 different fresh seafood from Egypt's Dakahlia Governorate. Fish specimens were gathered.

In sterile bags while water samples were collected in sterile tubes, tubes were closed by sterile pieces of cotton, samples were transported to bacteriology laboratory as soon as possible. Each type of fish was handled under complete aseptic conditions The fish under examination were arranged on the right side. Fish skin is disinfected using 70% ethyl alcohol. Using blunt, sterile scissors, the initial incision was made into the abdominal wall in front of the anus. The third incision was made from the anus to the head parallel to the center line where the first cut was done, and the second cut was made perpendicular to the first right behind the bronchial cavity.

. the internal organs become visible. Then we used small pieces of gills and small pieces of intestine for each sample and chopped into very small pieces using sterile scissors.

Isolation and Identification:

Isolation of genus *Clostridium* was made according to ***Collee et al.,* (1989)** as follows: for each sample, three tubes of freshly boiled and cooled meat medium were inoculated with each sample. The first inoculated tube was heated at 60℃˚for 15 min. The second tube was heated at 80℃ for 30 min. and the third was left unheated. All tubes were incubated anaerobically at 37℃ for 48 hours.

A loopful from each heated tubes was striped on fresh sheep blood agar and a loopful from the other unheated tube was striped on sheep blood agar plates was incubated anaerobically at 37℃ for 24 hours. The suspected colony was moved to a tube that had been recently boiled, cooled, and anaerobically incubated at 37 ºC for 24 hours in order to get a pure culture of isolates for additional identification.

which were carried out according to Bergey's manual ***(Breeds et al* 1957 and *Buchanon and Gibbons,* 1974)**

For identification of the isolates, isolates were identified according to colonial morphology and appearance, growth characteristics, haemolytic patterns, microscopically by Gram's stain and biochemically according to ***Collee et al*. (1996), *Quinn et al*. (2002) and *Boerlin et al*. (2003).**

A- Microscopic examination:

Films were made from the pure culture of isolates and stained with Gram's stain and examined microscopically for studying the stain reaction and the morphological characteristics.

B- Motility:

Semisolid agar tubes (0.5% w/v) were stabbed by isolates and incubated at 37℃ for 6 days. The diffuse zone of growth displayed by motile organisms extended beyond the line of inoculation, whereas the non-motile microorganisms growth remained confined to the line of inoculation.

C- Culture characters on 10% sheep blood agar:

Haemolysis and colonial characters.

D- Biochemical reaction:

The anaerobic spore forming isolates recovered were identified

1-Catalase test (*Baron* (2004), *Finegold* and *Martin (*1982).

2- Oxidase test (*El-Gohary,* 1989).

3- Sugar fermentation test.

4- Indole test (*Colier et al.,* 1998):

5- Nitrate reduction test (*Brewer G.H.,* 1955).

6- H2S production test.

7- Gelatine liquefaction test.

8-Nagler's test.

Table (1): Differential characteristics of Genus *Clostridium*:

species	Indole	H2S	Catalase	Gelatine	Mannito	Lactose	Nagler	Oxidase	Nitrate	Glucose
C.perfringens	-	V	-	+	-	+	+	-	V	+
C.sporogenes	-	-	-	+	-	-	-	-	V	+/-
C.bifermentans	+	+/-	-	+	-	-	-	-	V	+

+ = positive reaction - = Ngative reaction V = Variable

-/+ = Most strain ngative, occasional strain positive

Results

The overall prevalence of *Clostridium* species was 28.3% (51/180). The prevalence of *Clostridium* species in tilapia was 30% (18/60), in catfish was 31.6% (19/60) and in dennis was 23.3% (14/60). Eight isolates (26.6%), and 10 isolates (33.3%) were recovered from tilapia's intestine and gills respectively, whereas 9 isolates (30%), and 10 isolates (33.3%), were acquired from catfish's intestine and gills, respectively. Furthermore, 5 isolates (16.6%), and 9 isolates (30%), were recovered from dennis's intestine and gills, respectively as shown in Tables (2 and 3). Concerning water samples, out of 30 water samples, *Clostridium* species were detected in 17 samples with a prevalence of 56.6%.

Identification of Clostridium spp:

Based on colonial appearance, microscopical examination, biochemical identification, isolates were identified as *C. perfringens, C. sporogenes* and *C. bifermentans* as shown in Table (4). Where *C. sporogenes* was detected by 16.6% (5 isolates), 20% (6 isolates) from intestine and gills of tilapia while it was detected 16.6% (5 isolates), 13.3% (4 isolates) from intestine and gills of catfish but it was detected by 6.6% (2 isolates), 16.6% (5 isolates) from intestine and gills of dennis. Additionally, it was detected by 16.6% (5 isolates) from water. Concerning *C. bifermentans,* it was detected by 10% (3 isolates), 13.3% (4 isolates) from intestine and gills of tilapia but it was detected by 13.3% (4 isolates), 20% (6 isolates) from intestine and gills of catfish. Moreover, it was detected by 10% (3 isolates), 13.3% (4 isolates) from intestine and gills of dennis. Additionally, it was detected by 13.3% (4 isolates) from water. Concerning *C. perfringens*, it was detected by 26.6% (8 isolates) from water.

Table (2): *Clostridium species prevalence in fish samples under examination based on fish type.*

	Intestine		Gills		
	No. of collected samples	**No. (%) of positive samples**	**No. of collected samples**	**No. (%) of positive samples**	
Tilapia	30	8 (26.6%)	30	10 (33.3%)	18 (30%)
Catfish	30	9 (30%)	30	10 (33.3%)	19 (31.6%)
Dennis	30	5 (16.6%)	30	9 (30%)	14 (23.3%)
Total	90	22 (24.4%)	90	29 (32.2%)	51 (28.3%)

Table (3): *Clostridium* species prevalence in the samples under examination of fish farms based on the source of samples from different farms.

			Intestine	**Gills**
Metsallol Farm				
Tilapia	22	8	3	5
Catfish	24	8	4	4
Dennis	19	6	3	3
Water	12	7	-	-
Manzalla Farm				
Tilapia	18	5	3	2
Catfish	19	6	2	4
Dennis	23	3	1	2
Water	8	4	-	-
Shatta Farm				
Tilapia	20	5	2	3
Catfish	17	5	3	2
Dennis	18	5	1	4
Water	10	6	-	-
Total	210	68	22	29

Table (4*): Prevalence of C. perfringens in water, C. sporogenes, and C. bifermentans in fish and water samples.*

Bacterial species	**Tilapia**		**Catfish**		**Dennis**		**Water**
	Intestine No. (%)	Gills No. (%)	Intestine No. (%)	Gills No. (%)	Intestine No. (%)	Gills No. (%)	No. (%)
C. sporogenes	5(16.6%)	6(20%)	5(16.6%)	4(13.3%)	2(6.6%)	5(16.6%)	5(16.6%)
C. bifermentans	3(10%)	4(13.3%)	4(13.3%)	6(20%)	3(10%)	4(13.3%)	4(13.3%)
C. perfringens	-	-	-	-	-	-	8 (26.6)

Discussion

Food is a basic human necessity for survival, growth, and development as well as for carrying out everyday tasks. However, microbial contamination can occur throughout food production and throughout the food chain. These can act as biological dangers to health, resulting in the development of various diseases ***(Li et al.,* 2022).**

Egypt mostly depends on inland aquaculture, from which fish are supplied to regional markets or exported. As a result, we looked at fresh fish that were taken straight from aquaculture.

***(Arunava and Adarsh* 2012).** Hence, the current data was aimed to detect C*lostridium* species prevalence in fresh water and fish gathered from three separate Egyptian fish farms.

This current data detected overall prevalence of *Clostridium* species in fish by 28.3% (51/180). This result was lower than ***Saad et al.* (2021)** who detected *Clostridium* species in fish by 40% and higher than ***Jarosz et al.,* (2022)** who detected *Clostridium* species by 24%. The current data detected the overall prevalence of *Clostridium* species in water was 56.6% (17/30). This result was higher than ***Sabry et al.* (2016)** who detected *Clostridium* species in water by 27.3%. The differences in isolation rate of *Clostridium* species from fish and water samples may be attributed to differences in sources of samples collection and level of water contamination by untreated organic fertilizers.

Biochemically, in the following study the *Clostridium* species were identified as *Clostridium sporogenes, Clostridium perfringens* and *Clostridium bifermentans. C. sporogenes* was detected by 16.6% (5 isolates), 20% (6 isolates) from intestine and gills of tilapia while it was detected 16.6% (5 isolates), 13.3% (4 isolates) from intestine and gills of catfish but it was detected by 6.6% (2 isolates), 16.6% (5 isolates) from intestine and gills of dennis. Additionally, it was detected by 16.6% (5 isolates) from water. ***Saad et al.* (2021)** detected *C. sporogenes* presence in fish by 42.6% also ***Jarosz et al.* (2022)** detected *C. sporogenes* from canned fish. Numerous settings are home to *C. sporogenes*, including soil, sediment in freshwater and marine environments, preserved meat and dairy products, fecal matter, snake venom, and illnesses in humans and domestic animals ***(Kubiak et al.,* 2015),** *Clostridium sporogenes* is a toxin non-producer but used as an indicator of food contamination ***(Teramura* and *Ogura* (2020)).**

Concerning *C. bifermentans* in the current study, it was detected by 10% (3 isolates), 13.3% (4 isolates) from intestine and gills of tilapia but it was detected by 13.3% (4 isolates), 20% (6 isolates) from intestine and gills of catfish. Moreover, it was detected by 10%

(3 isolates), 13.3% (4 isolates) from intestine and gills of dennis. Additionally, it was detected by 13.3% (4 isolates) from water. *C. bifermentans* was also recovered from South Africa's Gauteng area is home to unofficial marketplaces where imported dried fish is sold. ***(Nkosi,* 2022)** also ***Saad et al.,* (2021)** detected *C. bifermentans* presence in fish by 14.3%.

Concerning *C. perfringens* in the current data, it was detected by 26.6% (8 isolates) from water. *C. perfringens* was isolated from the environmental samples such as fresh water, sediment and sewage which act as reservoirs for such pathogens as reported in previous studies ***(Mueller-Spitz et al.,* 2010; *Hafeez et al.,* 2022).**

Conclusion

Fish types (tilapia, catfish, dennis) and their environment can be potentially contaminated with *C. perfringens, C. bifermentans* and *C. sporogenes.*

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مدى انتشار انواع الكلوستريديم المختلفة من الأسماك والمياه العذبة
الملخص العربى

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4 قسم الميكروبيولوجى - معهد بحوث الصحه الحيوانيه – المنصورة – مصر

تعد الأسماك من أكثر الأطعمة شعبية، وتُعرف مصر حاليًا كمنتج رئيسي للأسماك على مستوى العالم. ميكروب الكلوستريديوم بيرفرنجينز هو بكتيريا لاهوائية إيجابية الجرام، على شكل عصيات، مغلفة، غير متحركة وتسبب مجموعة متنوعة من الأمراض للانسان و الحيوان. يعد التهاب الأمعاء النخري والتهابات الجروح والغرغرينا الغازية المميتة من بين الأمراض المنقوله للانسان و التى تنتجها الكولستريديوم بيرفرينجينز. المسبب الرئيسى لتلك الامراض هو السموم التى تنتجها بعض سلالات الكولستريديوم بيرفرينجينز. تسبب الكلوستريديوم بيرفرينجنز الكثير من المشاكل فى الاسماك مثل فقدان الشهيه و فقدان التوازن و القلق الشديد و اضطراب الحركه مع احمرار الخياشيم و تهتك العضلات. كذلك وجود تورمات و موت الانسجه مع التحلل السريع لها. استهدفت هذه الدراسه تقصى مدى انتشار اصناف الكلوستريديوم فى الاسماك و المياه تم استخدام 180عينة من الأسماك تمثلت 60 من البلطي (30 أمعاء، 30 خياشيم)، 60 من أسماك القراميط (30 أمعاء، 30 خياشيم) و 60 من دينيس (30 أمعاء، 30خياشيم) و 30 عينة من المياه العذبة التى نمت فيها هذه الاسماك من 3 مزارع سمكية. و اظهرت النتائج التى تم الحصول عليها انه بعد الفحص البكتريولوجى ، بلغ معدل الانتشار العام لأنواع كلوستريديوم فى الاسماك 28.3٪ (180/51) فى الاسماك أما فى حالة المياه كانت بنسبه 56.6.(17/30) %

بعد اجراء الاختبارات البيوكيمائية المختلفه للتعرف على أنواع الكلوستريديم المختلفة تم تحديد كلوستريديم سبوروجينز و كلوستريديم برفرنجنز و كلوستريديم بايفرمنتانس . تم عزل كلوستريديم سبوروجينز بنسبة 16.6% (5 عزلات)، 20% (6 عزلات) من الأمعاء والخياشيم لسمك البلطي بينما كانت بنسبة 16.6% (5 عزلات)، 13.3% (4 عزلات) من الأمعاء والخياشيم لسمك القراميط وكانت بنسبة 6.6% (2 عزلات)، 16.6% (5 عزلات) من الأمعاء والخياشيم للدينس. كما تم عزلها بنسبة 16.6% (5 عزلات) من الماء. أما بالنسبة للكلوستريديم بايفرمنتانس فقد تم عزلها بنسبة 10% (3 عزلات)، 13.3% (4 عزلات) من الأمعاء والخياشيم لسمك البلطي ولكن تم عزلها بنسبة 13.3% (4 عزلات)، 20% (6 عزلات) من الأمعاء والخياشيم من سمك القراميط. كما تم عزلها بنسبة 10% (3 عزلات)، 13.3% (4 عزلات) من الأمعاء والخياشيم للدينس. كما تم عزلها بنسبة 13.3% (4 عزلات) من الماء. أما بالنسبة لكلوستريديم برفرنجنز فقد تم عزلها بنسبة 26.6% (8 عزلات) من الماء.