Bacteriological and molecular studies on toxigenic *Clostridium perfringens* in milk and some milk products

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A B S T R A C T

Two hundred random samples of milk, kareish cheese, yoghurt and ice-cream (50 for each) were examined microbiologically for the presence of *Clostridium perfringens*, their enterotoxigencity and their antibiotic sensitivity. *Clostridium perfringens* was isolated from 3 (6%) milk samples, 4 (8%) kareish cheese samples and it could not be isolated from any examined samples of yoghurt and ice-cream. The majority of *C. perfringens* isolates recovered from milk and milk products were susceptible to ofloxacin, ampicillin + sulbactam and norfloxacin (100%), vancomycin, tetracycline, metronidazole and amoxicillin + clavulinic acid (83.3%) and clindamycin (66.7%). The majority were resistant to cephalothin (100%), sulphamethoxazole + trimethoprim (83.3%), oxacillin and chloramphenicol (66.7%). Molecular studies using multiplex PCR technique for detection of alpha toxin gene and *C.perfringens* types "A" enterotoxin gene revealed that the 7 isolates of *C. perfringens* (100%) were positive for alpha toxin gene and only 2 out of 7 isolates (28.57%) were positive for enterotoxin gene .

Key words: milk, *C. perfringens*, enterotoxigencity, antibiotic sensitivity, PCR.

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1. INTRODUCTION

Clostridium perfringens is a common contaminant of food and a frequent cause of food-borne illness due to the production of enterotoxin (Tseng and Labbe, 2000) and it is considered the second most common causative agent of FBD in US, after Salmonella (Brynestad and Granum, 2002; Scallan et al., 2011). Alpha toxin is the principle lethal toxin of *C. perfringens* that produced mainly by all types of the *C. perfringens* species (Alex et al., 2004) with a 370-amino acid necrotizing zinc metallo-enzyme with phospholipase C (lecithinase, PLC) activity (Hoi (Hoi et al., 2002). Certain strains of *C. perfringens* type A produce an exotoxic component known as enterotoxin which recognized as the only diarrheagenic toxin responsible for *C. perfringens* food-borne outbreaks (Monma et al., 2015). Dermonecrotic test in albino guinea pig is a helpful method for typing of *C. perfringens* isolates (McDonel, 1986; Sterne and Batty, 1975). The development of antimicrobial resistance in both human and animal bacterial pathogens has been associated with the extensive therapeutic use of antimicrobials or with their administration as growth promoters (Aestrup and Wegener, 1999). Molecular PCR has been applied for detection of the genes encoding major toxins of *C. perfringens* (alpha (α), beta (β), epsilon (ε), iota (ι) and enterotoxin). This method is more accurate and faster than sero-neutralization with mice or guinea pigs (Buogo et al., 1995). Therefore, this study was carried out for the evaluation of bacteriological patterns of *Clostridium perfringens* as one of food poisoning micro-organisms in milk and milk products.

2. MATERIAL AND METHODS

2.1. Samples collection:

A total of 200 random milk and milk products samples including kareish cheese, yoghurt and ice-cream (50 of each) were collected from different large and small dairy plants, street vendors and dairy house in El-Sharkia and Giza Governorates.

2.2. Isolation and identification of C. perfringens:

Isolation on cooked meat medium (Robertson, 1916) and neomycin sulphate sheep blood agar medium (Carter and Cole, 1990), morphological identification by Gram stain (Cruickshank et al.,

1975), biochemical tests (Macfaddin, 2000) and typing by dermonecrotic reaction for alpha toxin (Quinn et al., 2002).

2.3. In-Vitro anti-microbial sensitivity method:

Using agar diffusion method.

2.4. Molecular biology technique (PCR):

Multiplex PCR for detection of alpha exotoxin gene *(cpa)* and enterotoxin gene *(cpe)* of *C. perfringens* using specific oligonucleotide primers sequences for these genes with the length of amplified products at 1167 bp for alpha toxin and 233 bp for enterotoxin.

3. RESULTS

Table (1) revealed that *C. perfringens* was isolated from 7/200 (3.5%) of the examined samples represented as 3/50 (6%) from milk samples (0 from large scale dairy plants, 0 from small scale dairy plants, 2 from farmers houses and 1 from street vendors), 4/50 (8%) from kareish cheese samples (0 from large scale dairy plants, 0 from small scale dairy plants, 2 from farmers houses and 2 from street vendors) and *C. perfringens* were not isolated from any examined samples of yoghurt and ice-cream. The results of in-vitro sensitivity test for the isolated *C. perfringens* (Table, 2) showed that the majority of the isolated strains were susceptible to ofloxacin, ampicillin + sulbactam and norfloxacin (100%), vancomycin, tetracycline, metronidazole and amoxicillin + clavulinic acid (83.3%), clindamycin (66.7%). Moreover, the majority were resistant to cephalothin (100%), sulphamethoxazole + trimethoprim (83.3%), oxacillin and chloramphenicol (66.7%). Confirmation of 7 selected *C. perfringens* isolates from milk and milk products using multiplex PCR (Table, 3) revealed that the 7 isolates of *C. perfringens* (100%) were positive for alpha toxin gene (Photo 1) and only 2 out of 7 isolates (28.57%) were positive for enterotoxin gene (Photo 2).

Table 1. Prevalence of *C. perfringens* in milk and milk products (n=50):

Type of samples	No. of samples	Dairy plants				Farmers houses		Street vendors		Total	
		Large scale		Small scale							
		No./10	%*	No./10	%*	No./15	%*	No./15	%*	No./50	%**
Milk	50	-	-	-	-	2	13.33	1	6.67	3	6
Kareish cheese	50	-	-	-	-	2	13.33	2	13.33	4	8
Yoghurt	50	-	-	-	-	-	-	-	-	-	-
Ice-cream	50	-	-	-	-	-	-	-	-	-	-
Total	200	-	-	-	-	4	6.67	3	5	7	3.5

*percentage in relation to No. of each examined samples. ** percentage in relation to total No. of each 50 examined samples.

Table 2. In-Vitro antimicrobial sensitivity test for isolated *C. perfringens* (CLSI, 2011):

Antimicrobial agent	Sensitive		Resistant	
	No. of *C. perfringens* isolates	%*	No. of *C. perfringens* isolates	%*
Ofloxacin	6	100	-	-
Ampicillin+ Sulbactam	6	100	-	-
Norfloxacin	6	100	-	-
Metronidazole	5	83.3	1	16.7
Vancomycin	5	83.3	1	16.7
Amoxicillin+ Clavulinic acid	5	83.3	1	16.7
Tetracycline	5	83.3	1	16.7
Clindamycin	4	66.7	2	33.3
Oxacillin	2	33.3	4	66.7
Chloramphenicol	2	33.3	4	66.7
Sulphamethoxazole-Trimethoprim	1	16.7	5	83.3
Cephalothin	-	-	6	100

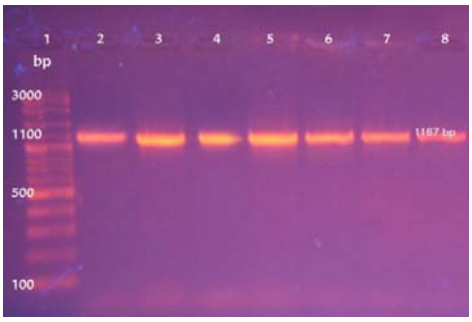
*Percentage in relation to total number of isolated *C. perfringens*

Table 3. Incidence of C. *perfringens alpha* toxin and enterotoxin genes in the seven examined samples of milk and milk products by PCR:

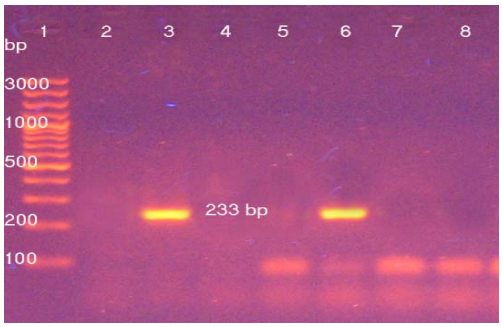
Examined *C. perfringens* for	No. of + ve samples	%*
Alpha toxin	7	100
Enterotoxin	2	28.57

*Percentage in relation to total number of isolated *C. perfringens*

Photos 1 and 2. Agarose gel electrophoresis patterns of *C. perfringens:* (1) Alpha toxin gene (2) Enterotoxin gene



Lanes 1: DNA molecular size marker (100-bp ladder)
Lanes 2- 8: positive samples



Lanes 1: DNA molecular size marker (100-bp ladder)
Lanes 2,4,5,7 and 8: Negative samples
Lanes 3 and 6: positive samples

4. DISCUSSION

Clostridium perfringens was isolated from 3/50 (6%) milk samples. Other findings were reported by Osman et al. (2009) at which *C. perfringens* isolated from 16/375 (4.48%) of milk samples from cows and 1/25 (4.0%) of samples from buffalo, but (Amer and El-Mossalami, 2006) could not detect *C. perfringens* in any of the examined milk samples. *Clostridium perfringens* could be isolated from 4/50 (8%) kareish cheese samples. Other findings were reported by El-Bassiony (1980) and El-Shater (2010) at which *C. perfringens* was detected in kareish cheese with percentages of 30% and 20%, respectively. *Clostridium perfringens* could not be isolated from any examined samples of yoghurt and ice-cream. On the other hand, El-Bassiony (1980) detected *C. perfringens* in 10% and 56% in the examined yoghurt and ice-cream samples, respectively.

In the present work, sensitivity of C. *perfringens* isolates to antimicrobial agents in-vitro was studied. As shown in Table (2). It was noticed that, they were highly sensitive to ofloxacin, ampicillin + sulbactam and norfloxacin (100%), vancomycin, tetracycline, metronidazole and amoxicillin + clavulinic acid (83.3%) and clindamycin (66.7%). These results are in general dis-agreement with Abdel-Rahman (2015) at which *C. perfringens* isolates were resistant to clindamycin and tetracycline and in general agreement with Teng et al. (2002) at which *C. perfringens* isolates were sensitive to sulbactam, clindamycin and metronidazole, Silva et al. (2009) observed that (89.1%) of *C. perfringens* isolates were sensitive to tetracycline. Metronidazole and penicillin G were the most potent agents against *C. perfringens* reported by Kra et al. (2014). Marchand-Austin *et al.,* (2014) stated that *C. perfringens* isolates were sensitive to metronidazole. Rodrigo et al. (2014) mentioned that all isolates were susceptible to vancomycin and metronidazole.

However, *C. perfringens* isolates were resistance to cephalothin (100%), sulphamethoxazole + trimethoprim (83.3%), oxacillin and chloramphenicol (66.7%) this is in general agreement with Das et al. (1997) and Abdel-Rahman et al. (2006) at which *C. perfringens* isolates were resistance sulphamethoxazole + trimethoprim. The recorded results of multiplex PCR Table (3) revealed that, 7 isolates of *C. perfringens* (100%) were positive for alpha toxin gene, while only 2 out of 7 isolates (28.57%) were positive for enterotoxin gene. These results are in line with several authors as Augustynowicz et al. (2002) and El-Shater (2010).

This study declared that, the presence of toxigenic *C. perfringens* in raw milk and milk products constitute public health hazards to consumers, which need proper milking, handling and inspection of bacterial pathogens to reduce risk to the public health.

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