

Prevalence and Antimicrobial Resistance of *Clostridium perfringens* in Milk and Dairy Products

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Abstract: In this study one hundred and fifty samples of [market milk, Damietta cheese, Milk powder, Ras cheese, kariesh cheese (25 of each) 10 condensed milk and 15 sterilized milk packs] were collected randomly from different localities of El-Dakahlia province, Egypt, for isolation and identification of *Clostridium perfringens*. *Clostridium perfringens* was isolated by the conventional method and identified by biochemical and molecular techniques. The prevalence of *Clostridium perfringens* was 20, 60, 20, 60 and 36% in raw milk, Damietta cheese, milk powder, Ras cheese and Kariesh cheese respectively. *Clostridium perfringens* was not found in sterilized and condensed milk. The isolates of *Clostridium perfringens* were screened for presence of toxins genes alpha (CPA), beta (CPB), epsilon (CPE) and iota (CPI) to detect the type of these isolates. *Clostridium perfringens* strains isolated in this study were confirmed to be type A. All (100%) *Clostridium perfringens* isolates were resistant to Colistin (CT) and Ampicillin (AM) followed by Lincomycin (MY) 91.8%, Erythromycin (E) 75.5%, Ampicillin-Sulbactam (SAM) 73.4%, Neomycin (N) 71.7%, Amoxicillin (AML) 69.38%, Streptomycin (S) 67.34%, Spiramycin (SP) 63.26%, Clindamycin (DA) and Tetracyclin (TE) 53.06%, Cephadrine (CE) 42.8%, Pefloxacin (PEF) 40.8%, Gentamycin (CN) 36.73%, Norfloxacin (NOR) 30.6% and Vancomycin (VA) 18.36%.

Key words: *Clostridium perfringens* • Milk • Dairy products • Antimicrobial susceptibility

INTRODUCTION

Milk is the most perfect food for human from birth to senility as it contains all the nutrients needed by the body for rapid growth and healthy development. There is a substantial epidemiological evidence that consumption of raw milk during childhood may protect against asthma, allergies and other immune-mediated diseases [1].

Milk is subjected to contamination from different sources; such contaminants find their way to milk and its products through faulty methods of production and handling. On the other hand soil, air, water, bedding, feeds and excreta of human and animal act as important sources of contamination [2].

Many problems are caused by the different species of anaerobic spore formers (*Clostridium* and related genera) that can be found in milk. These bacteria are found throughout the dairy farm environment and can be

toxigenic, neurotoxigenic or spoilage bacteria. This makes the presence of *Clostridium* and related spores in bulk tank milk important from both a public health and economic aspect [3].

Cells from the genus *Clostridium* are defined as Gram-positive, endospore-forming rods and most species are obligate anaerobes with varying tolerance to oxygen [4].

The presence of clostridia in milk has been a matter of public health concern since the early days of dairy industry because of its ability to produce a wide diversity of biologically active proteins, many of which have roles in provoking human and animal diseases and cause food deterioration [3]. The most important one is *Clostridium perfringens* which produces an enterotoxin that causes food poisoning outbreaks [3]. *Clostridium perfringens* is one of the most important causes of foodborne illness. It is estimated that it causes nearly a million cases of food borne illness each year. Patients infected with

Clostridium perfringens develop diarrhea and abdominal cramps within 6 to 24 hours (typically 8-12). The illness usually begins suddenly and lasts for less than 24 hours without developing fever or vomiting [3].

As milk and its products represent a potential hazardous source of *Clostridium perfringens* not only threatened the public health but also have a great economic effect on the dairy industry so the aim of this work was to investigate the prevalence of *Clostridium perfringens* in milk and dairy products by using the conventional method and molecular technique and determine the antibiotic sensitivity for *Clostridium perfringens* isolated from this study.

MATERIALS AND METHODS

Collection of Samples: One hundred and fifty samples of different dairy samples were used in this study. These samples included [market milk, Damietta cheese, Milk powder, Ras cheese, kariesh cheese (25 of each), 10 condensed milk and 15 sterilized milk packs]. All samples were collected randomly from different localities of El-Dakahlia province, Egypt in clean, dry and sterile containers then transferred to the laboratory as soon as possible to be examined.

Isolation and identification of *Clostridium perfringens*: Each sample was inoculated into a tube containing freshly prepared cooked meat medium. the tube was incubated anaerobically at 37°C for 24 hours using anaerobic jar, then was streaked onto the surface of 10% sheep blood agar with neomycin sulphate at a concentration of 200µg/ml. All plates were incubated anaerobically at 37° C for 24 hours. Suspected colonies of *clostridium perfringens* initially characterized by double zone hemolysis were picked up and maintained in cooked meat broth and identified microscopically after Gram stain which showed Gram positive bacilli with rarely central or oval non bulging endospores. Afterwards, colonies were

subsequently characterized using biochemical tests including catalase test, sugar fermentation test and indole test as previously published [5].

Molecular characterization of *Clostridium perfringens*:

Preparation of genomic DNA: Genomic DNA was extracted from tested strains using the QIAamp DNA mini kit Catalogue no.51304 from QIAGEN (USA) according to the manufacture instructions.

Molecular Characterization: PCR was conducted for the presence of toxin genes (alpha, beta, iota and epsilon toxins). Primers sets used for PCR amplification of the target toxins are listed in Table (1). PCR was carried out in a 50 µl reaction mixture containing 8 µl *C. perfringens* genomic DNA template, 1µl for each of forward and reverse primers Midland Certified Reagent Company_ oilgos (USA), 25iEmerald Amp GT PCR master mix (Takara) Code No.RR310A (Japan) (2x premix) and 9 µl PCR grade water.

After an initial denaturation at 94°C for 5 min, 35 cycles PCR amplification cycles consisting of denaturation at 94°C for 1 min, annealing at 55°C for 1 min and an extension at 72°C for 1 min. were performed followed by a final extension at 72°C for 10 min.

PCR products were visualized using ethidium bromide stain at 1.5% agarose gel electrophoresis. The separated PCR products were then visualized under UV light and photographed.

Antimicrobial Susceptibility: Antimicrobial susceptibility patterns for recovered *C. perfringens* isolates were determined by disc diffusion method using Mueller-Hinton agar (Oxoid CM 0337) (U.K). By using sterile swap, *Clostridium perfringens* was swapped across the plates then antibiotic discs were impregnated on the surface of the agar, using sterile forceps to dispense each antimicrobial disk one at a time. Plates were incubated at

Table 1: Primers used in PCR

Toxin	Primer	Sequence	Reference	Amplified product
(CPA) Alpha toxin	F	GTTGATAGCGCAGGACATGTTAAG	YOO <i>et al.</i> [29]	402bp
	R	CATGTAGTCATCTGTTCCAGCATC		
(CPB) Beta toxin	F	ACTATACAGACAGATCATTCAACC	YOO <i>et al.</i> [29]	236 bp
	R	TTAGGAGCAGTTAGAACTACAGAC		
(CPE) Epsilon toxin	F	ACTGCAACTACTACTCATACTGTG	YOO <i>et al.</i> [29]	541 bp
	R	CTGGTGCCCTTAATAGAAAAGACTCC		
(CPI) Iota toxin	F	GCGATGAAAAGCCTACACCACTAC	YOO <i>et al.</i> [29]	317 bp
	R	GGTATATCCTCCACGCATATAGTC		

37°C for 24hrs and clostridia plates were incubated anaerobically. Strains were evaluated as susceptible, intermediate or resistance [6].

The antimicrobial discs tested were Colistin(CT) 10 ug, Lincomysin (MY) 10 ug, Ampicillin (AM) 10 ug, Pefloxacin(PEF) 5 ug, Norfloxacin(NOR) 10 ug, Neomycin(N) 10 ug, Ampicillin-Sulbactam (SAM) 20 ug, Tetracyclin(TE) 10 ug, Amoxicillin(AML) 10 ug, Gentamycin(CN) 10 ug, Cephradine (CE) 30 ug, Spiramycin(SP) 100 ug, Vancomycin (VA) 5 ug, Erythromycin (E) 10 ug, Clindamycin (DA) 10 ug and Streptomycin (S) 10 ug. (Oxoid Limited, Basingstoke, Hampshire, UK).

RESULTS AND DISCUSSION

In this study *C. perfringens* was detected in 49 samples (32.6%) of all the examined samples (20% in raw milk, 60% in Damietta cheese, 20% in milk powder, 60% in Ras cheese, 36% in kariesh cheese, while it could not be detected in ultra-heat temperature milk (UHT) and condensed milk (Fig. 1). In a similar study, Al-Ashmawy and El-Toukhy, 2008 [7] could detect *C. perfringens* in 15, 30 and 15% of raw milk, milk powder and Ras cheese respectively. Also, several studies were conducted in different countries to screen milk and dairy products for the presence of *C. perfringens*.

In raw milk, Chaturvedi and Shukla [8] found *Clostridium perfringens* in 26% of the tested samples which is similar to our result. While Tandir [9] found higher incidence 32%, Gurmuet *al.* [10] recorded lower incidence 10%, this difference may be due to environmental cross-contamination of raw milk with spores and its presence considered an indication of faecal contamination.

In case of soft cheese, water activity, pH, salt concentration, temperature, nature of starter used, types of contaminating microorganisms and residual enzymes lead to great difference in the prevalence of *C. perfringens* in different types and places [11].

Heikal *et al.* [12] and Sharaf *et al.* [13] could not detect *C. perfringens* in Damietta cheese, while Chaturvedi and Shukla [8] could detect it in 4% of the tested samples. All of these studies showed lower incidence of *C. perfringens* than our study. This may be due to bad hygienic conditions in production and storage of cheese.

Clostridia are considered as naturally occurring microflora in dry milks, clostridia spores can survive for a very long time in dried milk products because it resists drying and pasteurization process [14, 15]. Milk powder can be contaminated with clostridia for processing from low quality raw milk with high clostridial number or post processing from an air born source due to improper sealing of the package.

In the present work, 20% of the examined milk powder samples were positive for *C. perfringens*, Ibrahim [16] and El-Sakaan [17] isolated *C. perfringens* from 16% of the tested samples which agree with our result. While lower incidence was obtained by Gurmuet *al.* [10] and Saad [18] whom found *Clostridium perfringens* in (0 & 14%) of the tested samples respectively.

The level of *C. perfringens* in Kariesh cheese depends on the initial count of clostridia spores in cheese milk and the ability of these spores to grow under the conditions of processing such as pH, salt, temperature and moisture [14]. Thirty-six%% of the examined Kariesh cheese samples were positive for *C. perfringens*, this result agrees with Abd El-Hakim [19] 30% of the tested samples were positive for *C. perfringens*. Hassan and

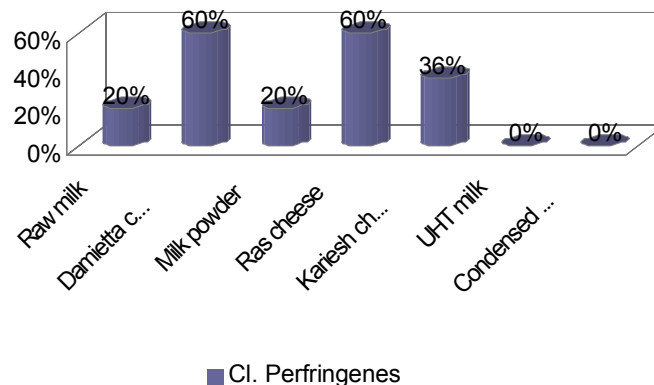


Fig. 1: Prevalence of *C. perfringens* in milk and dairy products

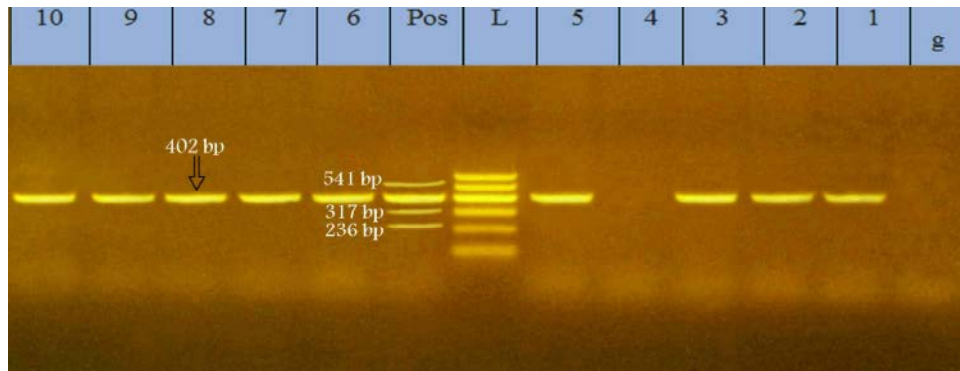


Fig. 2: Agarose gel electrophoresis of (alpha, beta, iota and epsilon toxins) amplicons specific for *Clostridium perfringens*.

L: ladder, Pos: control positive and Neg: control negative.

Lanes from 1 to 10 are representative run for some examined samples showing positive result except number 4.

Affi [20] and Ibrahim [16] obtained lower incidence of *C. perfringens* (6 and 16.4%) respectively while disagree with the result of the present study.

The extensive proteolysis which occurs during aging of cheeses causes the release of amino acids and increase in pH. These factors stimulate the growth of clostridia spp. Especially *Clostridium tyrobutyricum* and the production of gas and butyric acid in Ras cheese [21]. El-Sakaan [17] and Ibrahim [16] found the incidence of *C. perfringens* in the tested samples was (20 and 12.7%, respectively), All of these studies show lower incidence of *C. perfringens* than our study.

Amer and El-Mossalami [22] and Asaduzzaman *et al.* [23] could not detect *C. perfringens* in the examined condensed milk samples which agree with the result obtained in our study; this gave an indication about the good quality of company production because the

presence of anaerobes in the condensed milk is an index of faecal or soil contamination [24]. While, Korashy and Sabreen [25] found *Clostridium perfringens* in 6% of the examined samples.

UHT milk samples were free from *C. perfringens* and these results agree with Sharaf *et al.* [13] indicating the good quality of production and effective heat treatment.

All *C. perfringens* isolates were examined for presence of (alpha, beta, iota and epsilon) toxins (Table 1), 49 isolates were confirmed as *C. perfringens* type A. *C. perfringens* is 5 types from (A to E) based on the type of its extracellular toxins (alpha, beta, iota and epsilon). The cases of food poisoning were caused by *C. perfringens* type A. in recent years, *C. perfringens* has been recognized as much more common cause of gastroenteritis. *C. perfringens* is a strict anaerobe and its enterotoxin gene is always found in food poisoning

Table 2: Antimicrobial susceptibility pattern of *C. perfringens* (no=49)

Antimicrobial agent	S		I		R	
	No	%	No	%	No	%
Colistin (CT)	-	-	-	-	49	100%
Lincomysin (MY)	-	-	4	8.16%	45	91.8%
Pefloxacin (PEF)	-	-	29	59.18%	20	40.8%
Ampicillin (AM)	-	-	-	-	49	100%
Norfloxacin (NOR)	20	40.8%	14	28.5%	15	30.6%
Neomycin (N)	6	12.2%	8	16.32%	35	71.7%
Ampicillin-Sulbactam(SAM)	-	-	13	26.5%	36	73.4%
Tetracycline (TE)	5	10.2%	8	16.3%	26	53.06%
Amoxicillin (AML)	-	-	15	30.6%	34	69.38%
Gentamycin (CN)	-	-	31	63.2%	18	36.73%
Cephadrine (CE)	-	-	28	57.1%	21	42.8%
Spiramycin (SP)	14	28.5%	4	8.16%	31	63.26%
Vancomycin (VA)	4	8.16%	36	73.4%	9	18.36%
Erythromycin (E)	-	-	12	24.4%	37	75.5%
Clindamycin (DA)	-	-	23	46.9%	26	53.06%
Streptomycin (S)	-	-	16	32.6%	33	67.34%

Table 3: Antimicrobial susceptibility pattern of *C. perfringens* (no=49)

No. of isolates	Antimicrobial agent	MAR average
4	CT,MY,PEF,AM,NOR,N,S,DA,SAM,TE,AML,CN,CE,SP,VA,E	1
3	CT,MY,PEF,AM,NOR,N,SAM,AML,CN,CE,SP,E,DA,S	0.875
5	CT,MY,PEF,AM,N,SAM,TE,AML,CE,SP,E,DA,S	0.812
6	CT, MY, AM, N, TE, CN, CE, SP, E, DA, S	0.687
4	CT,MY,AM,SAM,TE,AML,CN,SP,VA,E	0.625
7	CT,MY,AM,N,SAM,TE,AMLE,S	0.562
8	CT,MY,PEF,AM,N,SP,E,DA,S	0.562
5	CT,MY,AM,N,SAM,AML	0.375
4	CT,AM,SAM,AML,S	0.312
3	CT,MY,AM	0.187

isolates the enterotoxin (CPE) is produced in the intestine after ingestion of food contaminated with at least 10^7 *C. perfringens* cells and symptoms of food poisoning appears after 6-12 hr from ingestion. Symptoms start with severe abdominal cramps, nausea and diarrhea.

All isolates recovered from raw market milk, Damietta cheese, milk powder, kariesh cheese and Ras cheese were tested for antimicrobial resistance against 16 antimicrobial agents shown in Tables [2, 3].

In this study (Table 2&3), 100% of *Clostridium perfringens* isolates were resistant to Colistin CT and Ampicillin (AM) followed by Lincomycin (MY) 91.8%, Erythromycin (E) 75.5%, Ampicillin-Sulbactam (SAM) 73.4%, Neomycin(N) 71.7%, Amoxicillin (AML) 69.38%, Streptomycin(S) 67.34%, Spiramycin (SP) 63.26%, Clindamycin (DA) and Tetracycline (TE) 53.06%, Cephadrine (CE) 42.8%, Pefloxacin (PEF) 40.8%, Gentamycin (CN) 36.73%, Norfloxacin (NOR) 30.6% and Vancomycin(VA) 18.36%. These results are consistent with a similar study conducted by Osman and Elhariri [26] where 100% of *C. perfringens* isolates were resistant to Erythromycin and Lincomycin followed by Colistin 94% and Neomycin 93%; Gholamiandehkordi *et al.* [27] showed that 66 and 61% of isolates were resistant to tetracycline and lincomycin, respectively. These are according to Varghese and Roymon [28].

CONCLUSIONS

Using high quality raw milk for the manufacture of milk products, proper cleaning and sanitization of equipment, employment healthy workers with health certificate in dairy industry and effectiveness of sanitation in dairy industry are considered as recommendations that should be undertaken to prevent contamination of milk and other dairy products by clostridia organisms.

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