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Research Article

Genotyping, Antibiotic Resistance and Biofilm Formation Ability of *Clostridium perfringens* Isolated from Raw Milk, Dairy Products and Human Consumers

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Abstract | *Clostridium perfringens* is one of the most common causes of food poisoning in the world. In this study, *C. perfringens* isolated from raw milk, milk products and human consumers were investigated for their antibiotic resistance and biofilm formation ability. A total of 460 samples (buffalo milk, cow milk, camel milk, yoghurt, Kareish cheese and soft (processed) cheese, 60 of each and 100 diarrheic stool sample of human consumers) were collected from Zagazig city, Sharkia Governorate, Egypt, and examined for the presence of *C. perfringens* strains. Twenty-four *C. perfringens* strains were isolated and all were of type A and 3 (12.5%) of camel milk origin and 4 (16.7%) from human diarrheic stool were positive for the enterotoxin associated (*cpe*) gene. Antibiotic sensitivity revealed that 87.5% of the isolates were resistant to oxytetracycline followed by amoxicillin (83.4%), ampicillin and erythromycin (75%, each). *C. perfringens* isolates from different sources were able to form biofilm at various temperatures (4˚C, 25˚C and 35˚C). In conclusions, the collected samples from the study area are considered potential sources for human infection with *C. perfringens*. Therefore, milk and dairy products should be inspected periodically to control contamination with foodborne pathogens.

Keywords | *Clostridium perfringens*, Raw milk, Dairy products, MDR

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INTRODUCTION

Clostridium perfringens is a Gram-positive, anaerobic spore-forming bacterium, it has the ability to produce a variety of toxins and enzymes that are responsible for a wide range of human and veterinary diseases including necrotic enteritis, gas gangrene, and food poisoning (Jiang et al., 2014). The bacterium can be found in diverse environment such as food, sewage, soil and gastrointestinal tract microbiota of humans and animals (Kiu and Hall, 2018; García and Heredia, 2011).

According to the production of four major toxins (alpha, beta, epsilon, and iota toxins); *C. perfringens* is classified into five types (A, B, C, D, and E). Recently, two other types were reported (F and G) based on the detection of CPE and NetB toxins (Rood et al., 2018). A small percentage (1% to 5%) of *C. perfringens* isolates, primarily of type A, can produce an enterotoxin (CPE) that causes food poisoning, antibiotic-associated diarrhea, and sporadic diarrhea in humans and animals (Brynestad and Granum, 2002; Heikinheimo, 2008). In most developed countries, *C. perfringens* type A food poisoning ranks as the second most common foodborne illness (Doyle et al., 2020).

Antibiotics are used for the control of bacterial infections

in humans and animals. Antimicrobial resistance has increased among anaerobes, thus decreased susceptibility of the clinical isolates has been resulted (Sherwood et al., 2011). The reason of increased resistance is the uncontrolled use of antibiotics in animal production as growth promoters, this lead to the emergence of multiple-drug resistant (MDR) isolates and transfer of resistance determinants from animals to humans posing risk to consumers (Jang et al., 2020).

Biofilms are communities of bacteria that are encased in an extracellular polymeric substance (EPS) that is primarily composed of polysaccharides, nucleic acids, and proteins (Mayer et al., 1999). Biofilms are capable of surviving host's immunity and different chemotherapeutic agents and they can resist hostile environmental conditions (de la Fuente-Núñez et al., 2013). *C. perfringens* is one of biofilm producer microorganisms which enhance survival in different environments and cause biofilm-related infections. Biofilms enhance the resistance to antibiotics compared with planktonic cells; for instance, cells in biofilms are 10-1000 times more resistant to antimicrobial agents (Mah and O'Toole, 2001). The current study investigated the *C. perfringens* isolated from raw milk, milk products and human consumers for their antibiotic resistance and biofilm formation ability.

MATERIAL AND METHODS

Sampling

A total of 460 samples of milk and milk products were collected from Zagazig city, Sharkia Governorate, Egypt. The samples comprised of buffalo milk, cow milk, camel milk, yoghurt, Kareish cheese and soft (processed) cheese (60, each) and 100 diarrheic stool samples from human consumers. The collected samples were immediately transported to the laboratory for bacteriological analysis. The study was approved by the Committee of Animal welfare and Research Ethics, Faculty of Veterinary Medicine, Zagazig University, Egypt.

Isolation and bacteriological identification

Ten milliliters from raw milk samples and 10 g of cheese were homogenized with 90 ml of cooked meat media (CMB; TM MEDIA, ISO 9001). Sterile swabs from diarrheic stool samples were immersed in tubes containing CMB. The CMB tubes were then incubated anaerobically at 37°C overnight in anaerobic jar with gas generation kits (AnaeroGen, OXOID, Ltd, England). A loopful of the enriched culture was streaked onto the surface of Reinforced Clostridial Agar plates (Oxoid, CM0151) and incubated anaerobically at 37°C overnight.

The suspected colonies were purified and then identified morphologically and biochemically using Gram staining and biochemical screening tests including oxidase, catalase test, nitrate reduction, blood hemolysis test, indole production, urea hydrolysis test, H_2H_2S production on triple sugar iron agar (HIMEDIA, MM021), lecithinase test and sugar fermentation test.

Molecular identification of *C. perfringens* isolates

The bacterial DNA was extracted by QIAamp DNA Mini kit (QIAGEN, GmbH, Hilden, Germany, Catalogue no.51304) following the manufactures guidelines. Molecular confirmation of *C. perfringens* isolates, was further carried out using primer sets specific for alpha toxin (Yoo et al., 1997) and enterotoxin (Kaneko et al., 2011) genes with the respective sequences F: 5'- GTT GAT AGC GCA GGA CAT GTT AAG -3' and R: 5' – CAT GTA GTC ATC TGT TCC AGC ATC -3 for alpha toxin gene and F: 5'- ACA TCT GCA GAT AGC TTA GGA AAT -3' and R: 5' – CCA GTA GCT GTA ATT GTT AAG TGT -3 for enterotoxin gene. A reaction mixture with no added DNA was run in the PCR as a negative control, and a positive control DNA from *C. perfringens* strains (ATCC 13124) was also run in the reaction.

Antibiogram analysis

C. perfringens isolates were subjected to antibiotic sensitivity testing using Kirby-Bauer disc diffusion method. Müeller-Hinton medium (HIMEDIA, M173) plates were swabbed with Müeller-Hinton broth (OXOID, CM0405) inoculated with the isolates (adjusted to match a McFarland obesity tube No. 0.5 by adding sterile saline, 1.5× 10^8 10^8 CFU|ml) and antibiotic disks were placed, and the plates were incubated at 37°C overnight. The used antibiotics were amoxicillin (AML, 25 µg), ampicillin (AM, 10 µg), enrofloxacin (EN, 10 µg), erythromycin (E, 15 µg), gentamicin (GM, 10 µg), neomycin (N, 30 µg), streptomycin (S, 10 µg), norfloxacin (NOR, 10 µg), novobiocin (NB, 30 µg) and oxytetracycline (OX, 30 µg). These antimicrobial agents were chosen on the basis of their importance for the treatment of human clostridial infections. Interpretation was done according to interpretation criteria recommended by the British Society for Antimicrobial Chemotherapy (BSAC, 2011). The multiple antibiotic resistance index (MAR) was calculated as the ratio of the number of antibiotics to which *C. perfringens* isolates displayed resistance to the number of drugs to which *C. perfringens* isolates were exposed (Krumperman, 1983). The multidrug resistance (MDR) was defined as an isolate resistance to at least one agent from three or more antibiotic classes (Magiorakos et al., 2012).

Biofilm formation

Biofilm formation ability of *C. perfringens* isolates at different storage temperatures was determined by microtiter plate assay (MtP) as previously described (Kırmusaoğlu, 2019). The optical density (OD) of the stained adherent bacteria was determined with an ELISA reader (model: sunrise R4, serial no: 610000079) at wavelength 570 nm (OD 570 nm) after adjustment of the negative control to zero. This experiment was carried out in triplicate and the data are represented as mean and the standard deviation was calculated. The cut off value (ODc) was calculated by the formula: ODc = Average OD of negative control + (3 × standard deviation (SD) of negative control). The OD for each isolate = Average OD of the isolate – ODC.

The strains were classified as non, weak, moderate and strong biofilm producers according to equations explained by Saxena et al. (2014): Non biofilm producer (0) OD ≤ ODc;
Weak biofilm producer (+ or 1) = ODc < OD ≤ 2 x ODc;
moderate biofilm producer (++ or 2) = 2 × ODc < OD ≤ 4 x ODc
and strong biofilm production (+++ or 3) = 4 × ODc < OD.

Statistical analysis

Kruskal-Wallis H One-Way Analysis of Variance (ANOVA) and post hoc Bonferroni correction were performed to estimate the differences in biofilm formation degrees at the three different temperatures. The results were calculated by SPSS version 22 (IBM Corp. 2013, Armonk, NY). Data are presented as mean ± SD and significance was considered at $P < 0.05$.

Table 1: Proportion of *Clostridium perfringens* isolates identified in different samples by biochemical tests and confirmed by PCR.

Samples	Number of samples	Positive	%
Buffalo milk	60	4	6.67
Cow milk	60	2	3.33
Camel milk	60	3*	5
Yoghurt	60	3	5
Kareish cheese	60	4	6.67
Soft (processed) cheese	60	1	1.67
Human consumers	100	7*	7
Total	460	24	5.22

* Three isolates from camel milk and four from human diarrheic stool were positive for the enterotoxin associated gene.

Table 2: Antimicrobial susceptibility of *Clostridium perfringens* isolates to different antibiotics.

Antimicrobial (abbreviation)	*C. perfringens* isolates (n=24)		
	R	I	S
Amoxicillin (Ax)	20(83.4%)	2(8.3%)	2(8.3%)
Ampicillin (AM)	18(75%)	1(4.2%)	5(20.8%)
Enrofloxacin (ENR)	10(41.7%)	-	14(58.3%)
Erythromycin (E)	18(75%)	-	6(25%)
Streptomycin (S)	13(54.2%)	2(8.3%)	9(37.5%)
Gentamicin (CN)	13(54.2%)	-	11(45.8%)
Neomycin (N)	13(54.2%)	2(8.3%)	9(37.5%)
Norfloxacin (NOR)	13(54.2%)	2(8.3%)	9(37.5%)
Novobiocin (NV)	13(54.2%)	2(8.3%)	9(37.5%)
Oxytetracycline (T)	21(87.5%)	-	3(12.5%)

R: resistant, I: intermediate, S: sensitive

Table 3: Frequency distribution of multidrug resistant *Clostridium perfringens* isolates and MAR index

Resistance pattern	No. of *C. perfringens* isolates	Percentage of *C. perfringens* isolates	MAR index
Resistant to 4 antibiotics	1	4.2 %	0.4
Resistant to 5 antibiotics	2	8.3 %	0.5

Resistant to 6 antibiotics	5	20.8 %	0.6
Resistant to 7 antibiotics	7	29.2 %	0.7
Resistant to 8 antibiotics	3	12.5 %	0.8
Resistant to 9 antibiotics	2	8.3 %	0.9
Resistant to 10 antibiotics	1	4.2 %	1

Multiple Antibiotic Resistance (MAR) index is calculated as the ratio of number of antibiotics to which the organism is resistant to the total number of antibiotics which the organism is exposed.
MAR = a/b, where "a" represents the number of antibiotics to which the test isolate depicted resistance and "b" represents the total number of antibiotics to which the test isolate has been evaluated for susceptibility.

Table 4: Biofilm formation in *Clostridium perfringens* at 4°C, 25°C and 35°C.

Temperature	Non-producer	Degree of biofilm production (%, Average OD±SD)			Overall biofilm producers
		Weak	Moderate	Strong	
4°C	12 (50%, 0.0452±0.0487)	5 (20.8%, 0.147±0.04)	7 (29.2%, 0.295±0.0429)	-	12 (50%)
25°C	3 (12.5%, 0.078751±0.036638)	4 (16.6%, 0.188417 ±0.047107)	7 (29.2%, 0.3836±0.04725)	10 (41.7%, 0.781084±0.0574)	21 (87.5%)
35°C	-	4 (16.7%, 0.275±0.0338)	5 (20.8%, 0.368±0.026817)	15 (62.5%, 0.7135±0.0619)	24 (100%)

OD: Optical Density
SD: Standard Deviation.

RESULTS

Prevalence and toxin types of *C. perfringens* in the examined samples

In the current study, examination of 360 raw milk and cheese samples revealed the identification of *C. perfringens* in buffalo, cow and camel milk with the respective percentage of 6.67, 3.3 and 5% (Table 1). While 5% of yoghurt samples were contaminated with the organism. Kariesh and soft cheese were also contaminated with *C. perfringens* with the percentage of 6.67 and 1.67 %, respectively. Seven human diarrheic stool samples were positive for *C. perfringens* with the percentage of 7%. All the isolates were identified as Type A by the presence of alpha toxin genes determined by PCR. Out of the identified 24 isolates, 3 (12.5%) of camel milk origin and 4 (16.7%) from human diarrheic stool were positive for the enterotoxin associated gene.

Antibiotic sensitivity of *C. perfringens* isolates

C. perfringens isolates were tested for their antibiotic susceptibility to 10 antibiotics using disc diffusion method (Table 2). According to the zone diameter break points, the most common resistance was observed against oxytetracycline (87.5%) followed by amoxicillin (83.3%). However, high level of sensitivity was observed to enrofloxacin (58.3%) followed by gentamicin (45.8%). The resistance patterns and distribution of the *C. perfringens* isolates indicated that 21 isolates demonstrated multiple resistance (Table 3). The majority of the isolates (29.2 %) were resistant to seven antibiotics, while 5 (20.8%) and 3 (12.5%) were resistant to six and eight antibiotics, respectively. The MAR index of the isolates ranged from 0.4 -1 with an average of 0.7.

The ability of *C. perfringens* isolates to form biofilm

The ability of 24 *C. perfringens* isolates to form biofilm was determined by microtitre plate method (Table 4). The results revealed that the majority the tested isolates could form biofilm at various degrees. Out of 24 *C. perfringens* isolates, 15 (62.5%) were strong biofilm producers, 5 (20.8%) were moderate biofilm producers and 4 (16.6%) were weak, at 35°C. At 25°C, 21 (87.5%) were biofilm producers, of which, 4 (16.6%), 7 (29.2%) and 10 (41.7%) were weak, moderate and strong producers, respectively. While, at 4°C, only 12 isolates (50%) produced biofilm, where 7 (29.2%) and 5 (20.8%) were weak and moderate biofilm producers, respectively. There was a statistically significant difference between the different degrees produced by the isolates ($p < 0.05$).

DISCUSSION

C. perfringens is implicated in contamination of milk and milk products resulting in economic losses, equipment damage and/or reputational damage for food companies. The presence of *Clostridium* species in milk products is hazardous posing a serious health risk to milk consumers, especially in the absence of pasteurization or sufficient boiling (Aliwa and Mulwa, 2019). The aim of our study was to investigate the prevalence of *C. perfringens* in milk and dairy products and to determine the antibiotic sensitivity and biofilm formation ability of *C. perfringens* isolated during the study.

In the current study, 17 (4.7%) of the examined milk and milk products samples were contaminated with *C. perfringens*. This percentage coincides with 6.6% (Turchi et al., 2016) from ewe's milk, 5% (Abd Elaal, 2008) in yoghurt and kariesh cheese and 3.5 % (Abd El Tawab et al., 2016) in milk and dairy products in Egypt.

In our study, *C. perfringens* was identified in 6.7% buffalo milk, 3.3% cow milk and 5% camel milk. These findings are comparable with 6% reported from raw milk samples in Egypt (Abd El Tawab et al., 2016). Another study in Egypt reported the isolation of *C. perfringens* from 4.5% (16/357) of cow milk samples and 4% (1/25) from buffalo milk (Osman et al., 2009). In Italy, not only buffalo's milk is used for the manufacture of yoghurt, but also ewe's milk, Turchi et al. (2016) reported that 6.6% of ewe's milk were contaminated with *C. perfringens* spores.

Scarce literature reported the isolation of *C. perfringens* from camel milk, for instance; Aliwa and Mulwa (2019) found that 19.1% (59/148) of raw camel milk in Kenya were positive for *C. perfringens*. The contamination of raw milk could be through faecal contamination, unhygienic harvesting and handling or from contaminated environment.

The level of *C. perfringens* in Kariesh cheese depends on the initial count of clostridia spores in cheese and the ability of these spores to grow under conditions of processing such as pH, salt, temperature and moisture (Osama et al., 2015). *C. perfringens* was isolated from 6.67% (4/60) of the examined kariesh cheese samples in our study. Other findings were reported by Abd El Tawab et al. (2016), Osama et al. (2015) and El-Shater (2010) who identified *C. perfringens* in Kareish cheese with percentages of 8, 36 and 20, respectively.

Regarding yoghurt samples, 5% of the samples were positive for *C. perfringens* (5%) in the present study. In contrary, *C. perfringens* was not isolated from yoghourt in other studies (Abd El Tawab et al., 2016; Abd Elaal, 2008).

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 (Osama et al., 2015). *C. perfringens* was detected in one sample from 60 Soft (processed) cheese samples with the percentage of 1.67%. Abd Elaal (2008) reported the presence of *C. perfringens* in 12% of soft cheese samples in Egypt. This higher isolation rate could be due to insufficient hygienic conditions in production and storage of cheese.

According to our data, *C. perfringens* was isolated from 7 % of all tested diarrheic stool samples. This is lower than 34.2% reported in Japan (Nagpal et al., 2015) and 58% Iran (Akhi et al., 2015) from human stool samples. This difference could be attributed to the types of samples, collection and identification methods.

The *cpa* gene was found in all *C. perfringens* isolates in our study indicating the predominance of type A strains. This is consistent with other researches (Abd El Tawab et al., 2016; Akhi et al., 2015; Osama et al., 2015). Type A is usually isolated from the intestine of apparently healthy humans and animals, therefore, it's a role in pathogenicity is controversy (Fernandez-Miyakawa and Redondo, 2016). Enterotoxin (CPE) is produced by nearly 5% of *C. perfringens* isolates and it is responsible for the majority of the food-poisoning outbreaks and diarrheal cases because it 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 h from ingestion (Miyamoto et al., 2009; Osama et al., 2015). The CPE toxin causes the diarrhea and abdominal cramping symptoms that are associated with type A food poisoning. (Doyle et al., 2020).

In our study, the *cpe* gene was identified in 7 isolates (29.2%) of which three were from camel milk and four from human diarrheic stool. In Egypt, Abd El Tawab et al. (2016) reported that 28.57% of *C. perfringens* isolates in raw milk and dairy products were positive for the *cpe* gene. While, lower percentage of *cpe* gene detection were reported in Iran in stool specimens (Akhi et al., 2015) and Japan in human feces (Nagpal et al., 2015) with the respective percentage of 3.7 and 4.3.

Antibiotic resistance is not only important among aerobic, but also among anaerobic bacteria. The availability of antibiotics contributes to increased inappropriate usage of antimicrobial drugs including the extensive therapeutic use of antimicrobials or with their administration as growth

promoter leading to the development of antibiotic resistant bacteria. Antimicrobial drug resistance (AMR) results in the emergence of antibiotic resistance strains which pose a serious public health risk and global problem (Aarestrup, 1999; Ahsanullah et al., 2019).

Ten antimicrobials were chosen during the current study to assess the susceptibility of the isolated *C. perfringens* strains (n=24) against them. In current study, 87.5% of the isolates were resistant to oxytetracycline followed by amoxicillin (83.4%), ampicillin and erythromycin (75%, each). These results are consistent with a similar study conducted by Osama et al. (2015) where all *C. perfringens* isolates were resistant to ampicillin followed by lincomycin 91.8%, erythromycin 75.5%, neomycin 71.7%, amoxicillin 69.38%, streptomycin 67.34%, tetracycline 53.06%, gentamycin 36.73%, and norfloxacin 30.6% Aliwa and Mulwa (2019) reported resistance of *C. perfringens* isolated from raw camel milk in Kenya against tested antibiotics, in decreasing order, ampicillin 61.02%, streptomycin 44.07% and gentamycin 35.59%.

C. perfringens is a source of resistance genes transferring to other species of bacteria; therefore, it is advised to monitor the patterns of antibiotic resistance periodically (Martel et al., 2004). Out of our isolates, 87.5% were multidrug resistance (MDR) with MAR index ranged from 0.4 to 1.

Biofilms are exopolymer matrix of bacteria in different surfaces which allow the organisms to survive and persist in the environment and in the infected host (Grantcharova et al., 2010). The centers for diseases control and prevention (CDC) reported that bacterial biofilms are causative agents for an estimated 65% of all reported infections (Lewis, 2007). Different studies reported a correlation between biofilm production on polystyrene plates and different surface materials used in food facilities (Vestby et al., 2009). It is recorded that biofilms of *C. perfringens* may play an important role in resistance to environmental stresses and many antimicrobial agents (Kırmusaoğlu, 2019; Varga et al., 2008). The number of strong biofilm producers was significantly higher at 35˚C than at 25˚C and 4˚C ($p \leq 0.05$). Only few studies have been published on *C. perfringens* biofilm formation, for this reason, the obtained results were compared to other studies on different micro-organisms. In Egypt, Mohamed et al. (2019) showed that 96% of *Aeromonas hydrophila* isolates were biofilm producers, at 35ºC, 16 (64%) and 8 (32%) showed strong and moderate biofilm production ability, respectively, at 25ºC, 21 (84%) were biofilm producers, of which, 8 (32%), 7 (28%) and 6 (24%) were strong, moderate and weak, respectively, while at 4ºC, decreased biofilm production ability was noticed 13 (52%), where 8 (32%) and 5 (20%) were moderate and weak biofilm producers, respectively. In Spain, out of 61 *Salmonella* isolates from poultry, 57.4% were classified as weak, 36.1% moderate and 19.8% strong biofilm producers after overnight incubation at 37˚C (Díez-García et al., 2012). In addition, Nair et al. (2015) reported that 85% *Salmonella* isolates were biofilm producers on polystyrene microtiter plates at 37˚C, of which, 67.5% and 17.5% were weak and moderate producers.

CONCLUSION

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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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

AUTHORS CONTRIBUTION

EYTE, HAA: Participated in designing the study. HAA, AAHK: Participated in the practical part and analysis of the data. HAA, AAHK, REM: Drafting the manuscript. EYTE, HAA: Revising the final version. All the authors read and approved the final manuscript.

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