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

**Ashraf A. Abd El Tawab1, Ahmed M. Ammar2, Fatma I. El-Hofy1, Hoda A. Aideia3, Eman A. Hammad4**

*1 Bacteriology, Immunology and Mycology Dept., Fac. Vet. Med. Benha Univ. 2 Microbiology Dept., Fac. Vet. Med. Zagazig Univ. 3 Animal Health Research Institute Dokki, Giza. 4General Authority for Vet. Services.*

**ABSTRACT**

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* wasisolated from 3 (6%) milk samples, 4 (8%) kareish cheese samples and itcould 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 7isolates of *C. perfringens* (100%) were positive for alpha toxin geneand only 2 out of 7 isolates (28.57%) were positive for enterotoxin gene .

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

**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 and 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 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 (Sterne and Batty, 1975 and McDonel, 1986). 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 seroneutralization 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 samples of milk and milk products 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 7isolates 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).

**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.

**5. REFERENCE**

Abdel-Rahman, A.A., Moustafa, F.A. and Hamd, N.A. 2006. Detection of the prevalence and pathogenicity of *C. perfringens* and *C. spiroform* associated diarrhea in rabbits. Assiut Vet. Med. J., 52(108): 321-335.

Abdel-Rahman, N.G. 2015. Phenotyping and genotyping of *Clostridium perfringens* associated with necrotic enteritis in broiler. Ph. D. Thesis (Microbiology), Fac. Vet. Med., Suez Canal University.

Aestrup, F.M. and Wegener, H.C. 1999. The effects of antibiotic usage in food animals on the development of antimicrobial resistance for humans in *Campylobacter* and *Escherichia coli*. Microbes. Infect., 1: 639-644.

Alex, S.J., Juan, B., Inma, G.A., Piedad, N., Maryse, G., Michel, R.P. and Mireia, M.S. 2004. Effect of epsilon toxin–GFP on MDCK cells and renal Tubules in vivo. J. Histochem.Cytochem., 52: 931–942.

Amer, A.A. and El-Mossalami, H. 2006. Quality assessment of sweetened condensed and evaporated milks in Alexandria Governorate. Assiut Vet. Med. J., 52(108):97-108.

Amer, I.H., Shelaih, M.A. and El-Sayed, M.S. 1986. Clostridial organisms in milk and some dairy products. Assiut, Vet. Med. J., 16(31): 213-217.

Augustynowicz, E., Gzyl, A. and Slusarczyk, J. 2002. Detection of enterotoxigenic *C. perfringens* with a duplex PCR. Journal of Medical Microbiology, 51 (2): 169.

Brynestad, S. and Granum, P.E. 2002. *Clostridium perfringens* and food borne infections. Int. J. Food Microbiol. 74: 195-202.

Buogo, C., Capaul, S., Hani, H., Frey, J. and Nicolet, J. 1995. Diagnosis of *C. perfringens* type C enteritis in pigs using a DNA amplification technique (PCR). Journal of Vet. Med. Series, 42 (1): 51.

Carter, G.R. and Cole, J.R. 1990.Diagnostic procedures in veterinary bacteriology and mycology. 5th Ed., Academic press., Harcourt. Boace Jov., Publishers, NewYork, Boston, Tokyo, Toronto.

CLSI (Clinical and Laboratory Standards Institute) 2011. Performance standards for antimicrobial susceptibility testing, Twenty First Informational Supplement. Wayne, 34 (1):111.

Cruickshank, R., Duguid, J.P., Marmoin, B.P. and Swain, R.H. 1975.Medical microbiology. The practice of medical microbiology page 434. 12th Editions, Vol.II. Churchill, Edinburgh.

Das, B.C., Gupta, G.N. and Phukan, A. 1997. Experimental production and treatment of necrotic entiritis in fowl. Indian J. of Poult. Sci., 32(1):59-66.

El-Bassiony, T.A. 1980. Occurrence of *Cl. perfringens* in milk and dairy products. J. Food Prot., 43 (7):: 536-537.

El-Shater, N.S.L. 2010. Evaluation of immunological and bacteriological patterns of some food poisoning micro-organisms (*Staphylococcus aureus and Clostridium perfringens*). Ph.D. Thesis (Bacteriology), Fact. of Vet. Med. Zagazig University.

Gatti, M., Bottari, B., Lazzi, C., Neviani, E. and Mucchetti, G. 2013. Invited review: Microbial evaluation in raw milk, long ripened cheese produced using undefined natural whey starters. J. Dairy Sci., 97(2): 573-591.

Hoi, H., Nelson, J.P., Oommen, S., Kanlic, E. 2002. Gas gangrene,eMedicine. <http://www.emedicine.com/med/topic843.htm(2002)>.

Kra, A.K., Adjéhi, T.D., Kouadio Florent, N.G., Koffi, M.D. and Yao, G.L. 2014. *Clostridium perfringens* and *Clostridium difficile* in ooked beef sold in Côte d'Ivoire and their antimicrobial susceptibility. Anaerobe, 28:90-94.

Macfaddin, J.F. 2000. Biochemical test for identification of medical bacteria. 3rd Ed. Lippin Cott Willians and Willions, Washingtion, Philadelphia, USA.

Marchand-Austin, A., Rawte, P., Toye, B., Jamieson, F.B., Farrell, D.J. and Patel, S.N. 2014. Antimicrobial susceptibility of clinical isolates of anaerobic bacteria in Ontario, 2010-2011. Anaerobe. 28:120-125.

McDonel, J.L. 1986. Toxins of *Clostridium perfringens* types A, B, C, D and E.In Phamacology of Bacterial toxins ed.p. 477. Oxford: Pergamon Press.

Monma, C., Hatakeyama, K., Obata., Yokoyama, K., Konishi, N., Itoh, T. and Kai, A. 2015. Four food-borne outbreaks caused by a new type of enterotoxin-producing *Clostridium perfringens.* Journal of clinical Microbiology., 53-59.

Osman, K.M., El-Enbaawy, M.I., Ezzeldeen, N.A. and Hussein, H.M. 2009. Mastitis in dairy buffalo and cattle in Egypt due to *Clostridium perfringens*: prevalence, incidence, risk factors and costs. Rev Sci Tech; 28(3): 975-986.

Quinn, P.J., Markey, B.K., Carter, M.E., Donnelly, W.J., Leonard, F.C. and Maguire, D. 2002.Veterinary microbiology and microbial disease. 2nd Ed., Blackwell Science, 84.

Robertson, M. 1916. Using cooed meat medium for isolation of *Clostridium* spp. J. Path. Bact. 20: 327.

Rodrigo, O.S., Francisco, C.F., Marcus, V.R., Carlos, A.O., Nelson, R.D and Francisco, C.F. 2014. Genotyping and antimicrobial susceptibility of *Clostridium perfringens* isolated from Tinamidae,Cracidae and Ramphastidae species in Brazil. Cienc. Rural vol.44 (3), Santa Maria.

Scallan, E., Hoekstra, R.M. and Angulo, F.J. (2011): Foodborne illness acquired in the United States-major pathogens. Emerg. Infect. Dis. 17:7-15.

Silva, R.O., Salvarani, F.M.; Assis, R.A., Martins, N.R., Pires, P.S. and Lobato, F.C. 2009. Antimicrobial susceptibility of *Clostridium perfringens* strains isolated from broiler chickens. Braz. J. Microbiol,, 44(33): 1065-1073.

Sterne, M. and Batty, I. 1975. Criteria for diagnosis clostridial infection.In pathogenic Clostridia ed. pp. 79 – 122. London: Butterworths.

Teng, L.J., Hsueh, P.R., Tsai, J.C., Liaw, S.J., Ho, S.W. and Luh, K.T. 2002. High incidence of cefoxitin and clindamycin resistance among anaerobes in Taiwan. Antimicroial Agents and Chemotherapy. 46:2908-2913.

Tseng, W.J. and Labbe, R.G. 2000. Characters of sporulation stimulating factor from *C*. *peifringens* type A. Lert. In Appl. Microbiol. 30: 254.

Table 1. Prevalence of *C. perfringens* in milk and some milk product:

|  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| 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 |

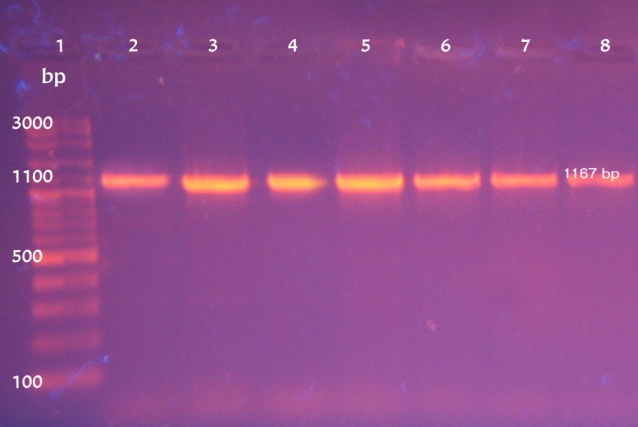
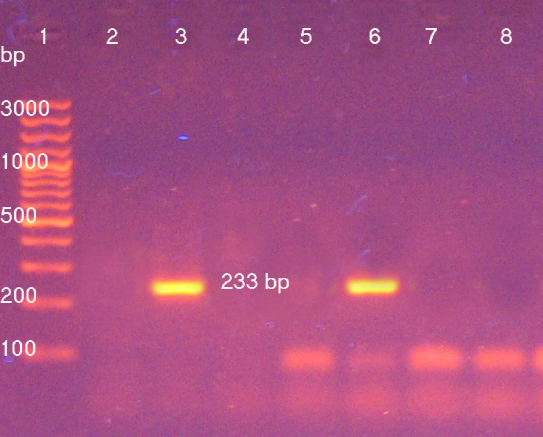
\*Percentage in relation to total number of isolated *C. perfringens*

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*

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

Lanes 1: DNA molecular size marker Lanes 1: DNA molecular size marker

(100-bp ladder) (100-bp ladder)

Lanes 2- 8: positive samples Lanes 2,4,5,7 and 8: Negative samples

Lanes 3 and 6: positive samples