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ELECTROPHORETIC CHARACTERIZATION FOR DETECTION OF *CLOSTRIDIUM PERFRINGENS* ENTEROTOXIN TYPE (A) IN MEAT

(With 2 Tables and One Figure)

By
A.M.O. EL-MAHROUK

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التوصيف الكهربائي لتحديد سموم ميكروب الكلوستريديم برفرنجنس نوع (أ) في اللحوم

عاطف محمد عمر المحروق

أجري هذا البحث على عدد مائة عينة من لحوم الجاموس تم جمعها من الأسواق المختلفة بمحافظة الجيزة وتم فحصها ظاهريا وبكتريولوجيا. ووجد أن ٣٩% من عينات اللحوم المختبرة ملوثة بميكروب الكلوستريديم برفرنجنس نوع (أ) المفرز للسموم. وتم عمل العدد الأحتمالي للميكروب في الجرام ووجد أنه يتراوح ما بين صفر حتى ٣٥. وتم عمل عد لجرثمة الميكروب على فترات مختلفة عند ١٠٠ درجة مئوية. وتم أجراء التحليل الكهربي والطبع المناعي لتحديد الأنتيجين المناعي لسموم الميكروب ووجد ان وزنه الجزيئي ٣٥ كيلو دالتون.

SUMMARY

A total of 100 buffalo meat samples were collected from different markets in Giza governorate. They were subjected to physical and bacteriological examination. Thirty nine percentage of the samples tested were found to be contaminated with *Clostridium perfringens* type A. The most probable number (MPN/gram) values ranging from 0 to 35. Also, the spore count of *Clostridium perfringens* type A at 100°C at different time interval was investigated. Electrophoretic analysis and immunobloting for detection of *Clostridium perfringens* enterotoxin type (A) was carried out for its characterization. The immunogenic band was determined with molecular weight 35 KDa.

Key words: *Electrophoresis Cl. perfringens, enterotoxin, meat*

INTRODUCTION

Most species of the genus Clostridium are saprophytes that normally grow in soil, water and decomposing plant and animal matter, playing an important part in the process of putrefaction.

Clostridium perfringens type A isolates can carry the enterotoxin gene (*cpe*) on either their chromosome or a plasmid, but food poisoning isolates usually have a chromosomal *cpe* gene. This linkage between chromosomal *cpe* isolates and food poisoning has previously been attributed; Jihong and Bruce, 2006; Lin McClane, 2006.

In vitro toxin production is an important tool not only for diagnostic purposes but also for the study of pathogenesis of *Clostridium perfringens* infections; Fernandez *et al.,* 2007.

The prevalence of the enterotoxin gene in a well-characterized collection of 71 *Clostridium perfringens* strains from 36 separate food-poisoning cases or outbreaks was analyzed with the polymerase chain reaction (PCR); Ridell *et al.,* 1998.

Meat and fish are sensitive to contamination and support growth of microorganisms. Anaerobic bacteria constitute an important group of microorganisms responsible for many health hazards to consumer from consumption of processed meat and fish products where oxygen availability is limited .The most important species of anaerobic bacteria are *Cl. botulinum and Cl. perfringens*, but some other species are also known including *Cl. butyricum, Cl. sordellii, Cl. bifermentans, Cl. sporogenes* and *Cl. Barati;* Mead, 1992.

Clostridium perfringens type A is one of the four most important bacterial agents causing food poisoning. Differential biochemical characterization appears to be important because of certain confounding species. Both the heat sensitive and resistant spore forming strains cause food poisoning; Narayan, 1982.

Cl. perfringens carried in the human and animal intestine, soil, dust and flies, ruminant meat are often contaminated. They have resistant spores and are thus able to survive well in such type of environment; Barnes, 1985.

The isolation of *Cl. botulinum* from foods is generally considered to be of less significance than the detection of the toxin; Hobbs *et al.,* 1982. *Cl. botulinum* causes a food borne intoxication known as botulism.

Seven types of *Cl. botulinum* (A, B, C, D, E, F and G) are recognized on the basis of antigenic specificity of their toxins; Pierson *et al.,*1988.

The objective of this study was to investigate the role of several methodological variables that might be encountered during the study and to fulfil the following items:

1-Isolation and identification of *Clostridium perferingens* type A from meat.

2-Detection of the ability of of *Clostridium perferingens* type A to produce toxins in culture medium.

3-Detection of the of *Clostridium perferingens* type A in foods of Reversed passive latex agglutination (PET- RPLA) test using a commercial kit.

4-Western Blotting or immunoblotting for identification of Enterotoxigenic isolates of *Cl. perfringense* type A

MATERIALS and METHODS

Samples: A total of 100 buffalo meat samples were collected from different markets in Giza governorate (types of sample, site of collection, date) were reported on each sample. The samples (10g) were collected aspectically in clean plastic bag and kept in ice box where transferred to the laboratory of animal Health Research Institute (AHRI), Dokki, Giza, Egypt. The samples were immediately subjected to physical and bacteriological examination without delaying..

Cultural conditions: Processing of each food sample started with a homogenization step using sterilized surgical scissors. Ten milliliters of sterile fluid thioglycolate (FTG) medium were then added to the 50 ml flask containing the minced meat. An aliquot (1 ml) of each FTG meat suspension was added to each of two tubes containing 10 ml of sterile FTG. To enrich for any *Cl. perfringens* spores present in the meat sample, one of those two tubes was heat shocked at 72°C for 20 min before incubation at 37°C for 18 to 24 h. The other tube was directly incubated at 37°C for 18 to 24 h to enrich primarily for *Cl. perfringens* vegetative cells present in that meat sample.

Each FTG enrichment culture showing growth was streaked onto one plate of tryptose-sulfitecycloserine agar containing 10% egg yolk (TSC with egg yolk) and a second plate of brain heart infusion agar containing 10% .sheep -blood and 40 Ug /ml neomycin . Both plates were then incubated for 18 h at 37°C in an anaerobic jar. When a meat sample did grow presumptive *Cl. perfringens*, those colonies were inoculated into 10 ml of FTG medium, which was then incubated for an overnight at 37°C. To confirm the identity of those presumptive FTG cultures as *Cl. perfringens*, standard methods were used; Food and Drug

Administration, 1998. A loopful of each culture was stabbed into a tube of motility nitrate and lactose-gelatin media. Those tubes were then incubated at 37°C for 18 to 24 h. Toxin type of the isolates were determined by neutralization test in mice; Stern and Batty, 1975.

Determination of MPN of *Cl. perfringens* per gram in meat:

A three-lube most probable number (MPN) method was used to investigate *Cl. perfringens* levels in meat samples; Lin and Labbe, 2003. Briefly, a 10 g aliquot of a meat suspension (prepared as described above) was diluted by 10 fold increments (from 10-' to 10-5) in FTG, and then 1 ml aliquots of each dilution from a single sample were inoculated into three tubes containing 10 ml , pf differential reinforced clostridial broth medium (DRCM). After incubation at 37°C for 24 h, cultures testing positive for *Cl. perfringens* produced a unique black precipitation in this DRCM.

Statistical analyses were performed; Koburger, 1975.

Determination of spore heat resistance for CPE-positive *Cl. perfringens* type A meat isolates.

To evaluate the heat resistance of *Cl. perfringens* meat isolates, the spore count was measured at 100°C for each isolate; Sarker *et al.*, 2000.

Briefly, sporulating cultures of *Cl. perfringens* were prepared by inoculating a 0, 2 ml aliquot of a FTG culture into 10 ml of Duncan-Slrong (DS) sporulation medium. After an overnight incubation at 37°C, the presence of sporulating cells in each DS culture was confirmed by phase contrast microscope. Those DS cultures were then heat shocked at 72°C for 20 min to kill any remaining vegetative cells and to facilitate spore germination. A 0.1 ml aliquot of each heat-shocked DS culture was then serially diluted with sterile FTG medium to obtain dilutions ranging from 10^{-2} to 10^{-7}. Two ml aliquots of each dilution were duplicate plated onto BHI agar plates in order to establish the number of viable spores present per milliliter of DS cultures at the start of heating (i.e., at the zero time point of the experiment).

The remainder of each heat-shocked DS culture was then heated at 100°C for time periods ranging from 1 min to 2 h- At each time point, the boiled DS culture was mixed, and a 0.1-ml aliquot was withdrawn and diluted (dilution range, from 10^{-2} to 10^{-7}) with sierilo rTG medium. Each dilution was then duplicate plated onto BHI agar plates, which were incubated anaerobically at 37°C for 18 h. Colonies developing from germinated spores that survived heating, were then counted to determine The number of viable spores present at th ime point per milliliter of each healed DS culture.

Detection of enterotoxins producing isolates by reversed passive latex agglutination (PET-RPLA Kit)

A porlion of the sporulated culture (about 5 ml) was centrifuged for 15 min at 10,000 xg and cell free culture supernatant was tested for enterotoxin by using RPLA Kit; Food and Drug Administration, 1998.

SDS-PAGE and Western Blotting; Vernon *et al.*, 1996.

Solubilized cells (0.4 gm/ml) were mixed with an equal volume of 4X sample buffer (3 % Tris, 20% b-mercaptoethanol, 10% SDS, 0.02% bromophenol blue and 40% glycerol; PH6.8) heated at 95^{0}C for 3 min, and then centrifuged to remove any remaining insoluble material. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed. Protein marker contains 5 proteins with molecular weight ranged from 94-20 KDa used as molecular weight standards (Sigma-Aldrich).

After electrophoresis, the separated proteins were transferred to microcellulose membranes at 360 mA for 4 hours at 4^{0}C. Membranes were then blocked overnight with a blocking buffer including 1% (W/V) gelatin in Tris-buffered saline (TBS: 20 mM Tris-HCL PH 7.4, 0.5 M Nacl). The membranes were rinsed three times with TBS containing 0.05% Tween 20 and then incubated for 3 hours with either preimmune serum or polyclonal antiserum obtained after infection. Each serum sample was diluted (1:500) in blocking buffer. Memmbranes were rinsed three times and incubated with goat anti-rabbit IgG peroxidase conjugate (diluted 1:2,500 in blocking buffer) for 3 hours (Sigma, St. Louis, Mo.). Immunoreactivity was detected by incubating blots with TBS containing H_2O_2 and 4-chloro-1-napthol.

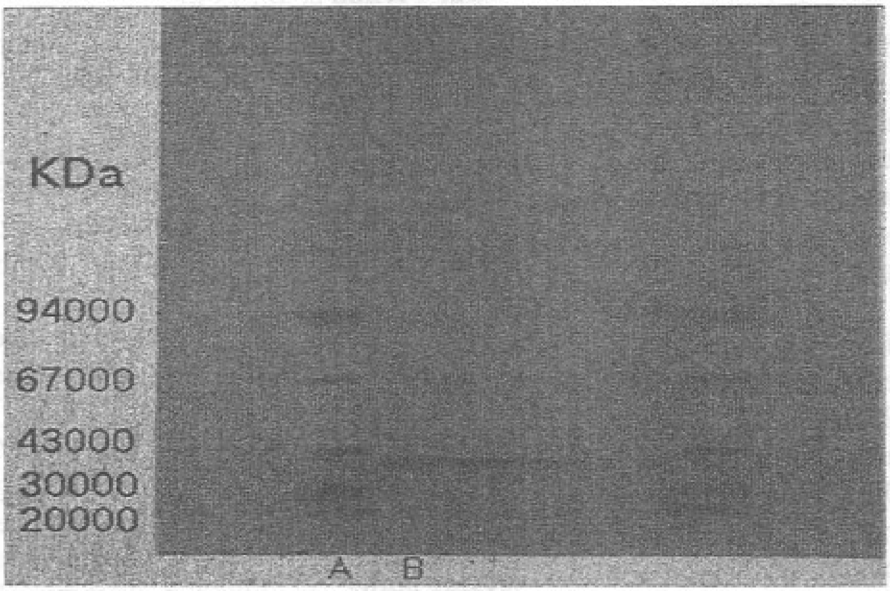
RESULTS

Table 1: Prevalence of *Cl. perfringens* in buffalo meat

No. of total samples examined	No. of positive vegetative cell of *C. perfringens*	No. (%) of spore cell of *C. perfringens*	MPN/g range	No. of samples tested with RPLA kit	
				Type A toxin	Positive CPE
100	39 (39.0)	4 (4.0)	0-35	39	4

Table 2: Spore count of *Cl. perfringens* type A after heating at 100°C at different time interval.

Isolates	0	30 min.	60 min.	90 min.	120 min.
1	7	3.4	0	0	0
2	7	3.31	2.2	1.3	0
3	7	4.0	1.1	0	0
4	7	3.19	0	0	0



Western Blot analysis of CPE, Lane A: Molecular weight marker; Lane B: Immunogenic band (35 KDa)

DISCUSSION

Clostridium perfringens isolates are commonly classified into five types (types A to E) based on the production of four typing toxins (alpha, beta, epsilon, and iota toxins); Songer, 1996. Type A isolates, the most abundant toxinotype, produce alpha toxin, but not beta, epsilon, or iota toxin; Immerseel *et al.,* 2004. Some type A isolates also produce another toxin, *Cl. perfringens* enterotoxin (CPE). These enterotoxigenic type A strains cause several human enteric diseases, including *Cl. perfringens* type A food poisoning, which is among the three most common food-borne illnesses in the United States, and some cases of non-food-borne human gastrointestinal disease, including antibiotic-associated diarrhea and sporadic diarrhea; Carman, 1997.

To date, there are at least two explanations for the strong association between type A isolates carrying a chromosomal *cpe* gene and food poisoning. First, a recent study; Wen and McClane, 2004 that evaluated the presence of *cpe*-positive isolates in American retail foods showed that all 13 *cpe*-positive type A isolates recovered from the foods surveyed had a chromosomal *cpe* gene. The findings indicated that, at least in part, chromosomal *cpe* isolates are the predominant cause of food poisoning because they are the *cpe*-positive type A isolates that are most often present in food.

As shown in Table (1) 39.0% of meat samples tested in the present study were found to be contaminated with *Cl. perfringens* isolates, similar result were reported by Abd El-Rahman *et al.,* 1995 who mentioned that *Cl. perfringens* is present in high incidence in meat and meat product. Table (1) showed that low contamination frequency is consistent with MPN results indicating that the meat samples tested in this study had MPN / gram values ranging from 0 to 35, this observation were in agreement with that mentioned by Lin and Labbe 2003 and Wen and McClane 2004 who tested MPN/gram in American retail foods and found that its value ranged from 0 to 32. About 4.0% of *Cl. perfringens* from meat samples grew after heat shocking obviously indicative of they restrained spores.

In the provisional, 39.0% of meat samples grew *Cl. perfringens* only in the absence of heat shocking .It is hypothetically achievable that some of these samples also contained *Cl. perfringens* spores that spontaneously germinated in the absence of heat shocking. However, the large difference in *Cl. perfringens* observed between heat-shocked versus non heat -shocked meat samples strongly suggests that most of the non-heat-shocked food samples growing *Cl. perfringens* had been contaminated with vegetative cells which were killed by heat shocking, rather than spores. In most of the non -heat -shocked samples yielding *Cl. perfringens* had contained spores that spontaneously germinated into vegetative cells, those samples also tested positive for *C.perfringens* after heat shocking and this agree with that mentioned by Varga *et al.,* 2004 who suggests that the strong association between type A isolates carrying a *cpe* gene and *Cl. perfringens* type A food poisoning is attributable (at least in part) to the exceptional heat resistance of those isolates, which should favor their survival in incompletely cooked or improperly held foods

Little is known about the mechanisms responsible for the specific heat resistance of chromosomal *cpe* isolates, although a recent study;

Raju and Sarker, 2005 showed that (i) the heat resistance of chromosomal *cpe* isolates is not dependent on the presence of a functional *cpe* gene and (ii) the heat sensitivity of plasmid *cpe* isolates is not dependent on the presence of a *cpe* plasmid.

With the ultimate goal of better controlling *Cl. perfringens* type A food poisoning, workers have begun investigating why this food-borne illness is so strongly associated with type A isolates carrying a chromosomal *cpe* gene; Jihong *et al.,* 2006. CPE is clearly responsible for the symptoms of *Cl. perfringens* type A food poisoning; Sarker *et al.,* 1999, but the relationship between food poisoning and chromosomal *cpe* isolates does not appear to involve isolates that produce either a more potent CPE or larger amounts of CPE than plasmid *cpe* isolates produce; Collie *et al.,* 1998, and McClane, 2001. Since temperature abuse is the leading factor responsible for *Cl. perfringens* type A food poisoning outbreaks; McClane, 2001.

The enteropathogenic effects of CPE are primarily mediated through a multi step cytotoxic action, which initiates when CPE hinds to a pertinacious receptor (s); Cornillot *et al.,* 1995 *Clostridiuin perfringens* uses its potent arsenal of 14 toxins to cause enteric and histotoxic infections in humans and domestic animals.

Deaths from *C perfringens* type A food poisoning are not common but do occur in the elderly and debilitated. CPE toxin is both necessary and sufficient for the enteric virulence of *Cl. perfringens* type A food poisoning isolates; Sarker *et al.*, 1999. Ingestion of purified CPE by human volunteers was determined to be sufficient for reproducing the cramping and diarrheic symptoms of the natural food poisoning; Skjelkvale and Uemura, 1977.

Results from the present study provide an explanation for the association between *Cl. perfringens* type A isolates and cpe gene and for this purpose all samples proved to toxigenic by RPLA were undergo investigation using immunoblott and it is worthy to mention that all the isolates proved to be toxigenic by RPLA was also toxigenic by immunoblott with an additionally 2 strains which give a clear idea about the sensitivity and accuracy of the test and this observation in agreement with that mentioned by Guennadi *et al.,* 2004.

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