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DETECTION OF ENTEROTOXIGENIC CLOSTRIDIUM PERFRINGENS TYPE A IN CAMEL MEAT

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SUMMARY

Clostridium perfringens enterotoxin (CPE) is an important sporulation associated virulence factor in several illnesses of humans and domestic animals.

In the present invistigation169 camel meat samples were collected from small butcher's shop where *C. perfringens* type A was identified with an incidence of 33.7% only 5.9% had detectable enterotoxin. The meat samples tested had MPN per gram values ranging from 0 to 35, determination of spore heat resistance for *cpe* positive *C. perfringens* type A isolates varied between logs 1.3 to log 5.07 at different time intervals. The current study has used PCR assays to detect cpe production in gene as a method for determining the enterotoxigenicity of *C. perfringens* isolates. Results showed that PCR is suitable for detection of *C. perfringens* enterotoxin from raw meat.

INTRODUCTION

C. perfringens spores are present in small numbers in human faecal specimens and raw food samples. Human food poisoning caused by *C. perfringens* is due to an enterotoxin produced during sporulation of the bacteria in the intestine following ingestion of vegetative cells in large numbers (Kilic et al., 2002).

The Gram-positive, spore-forming, anaerobe *Clostridium perfringens* is a notorious toxin-producing pathogen. One biomedically important toxin expressed by some *C. perfringens* isolates is *C. perfringens* enterotoxin (CPE), which has been implicated as a virulence factor in several human gastrointestinal illnesses (Collie and

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McClane,1998). These CPE-associated illnesses include *C. perfringens* type A food poisoning, as well as non food borne human gastrointestinal (GI) diseases, such as antibiotic-associated diarrhea, and may also contribute to the pathogenesis of some veterinary diarrheas (Bean et al., 1996).

The enteropathogenic effects of CPE are primarily mediated through a multistep cytotoxic action, which initiates when CPE binds to a proteinaceous receptor(s) (Cornillot et al ., 1995) *Clostridium 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 *C. 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).

A recent study suggests that the strong association between type A isolates carrying a cpe gene and *C. 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 (Varga et al., 2004).

There were two reasons for our focus on testing camels meat, this food is epidemiologically important as a most common food vehicle of *C. perfringens* type A and as a cheapest meat used mon in Egypt and our samples were raw which migh favor detection of *C. perfringens* isolates.

MATERIALS AND METHODS

Collection of meat samples. 169 camel mea samples were obtained from small butcher shop of Cairo, Giza and kalubia governorates.

Preparation of meat samples. (Food and Dru Administration, 1998).

A sample (10 g) was collected aseptically an placed into sterile plastic bag for transfer to th laboratory. Food samples were typically pro cessed immediately.

Processing of each food sample started with a ho mogenization step using sterilized surgical scis sors. Ten milliliters of sterile fluid thioglycolat (FTG) medium were then added to the 50 ml flas containing the minced meat. An aliquot (1 ml) c each FTG meat suspension was added to each c two tubes containing 10 ml of sterile FTG. To en rich for any *C. perfringens* spores present in th meat sample, one of those two tubes was hea shocked at 72°C for 20 min before incubation 37°C for 18 to 24 h. The other tube was direct incubated at 37°C for 18 to 24 h to enrich primar ly for *C. perfringens* vegetative cells present that meat sample.

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Each FTG enrichment culture showing growth was streaked onto one plate of tryptose-sulfite-cycloserine agar containing 10% egg yolk (TSC with egg yolk) and a second plate of brain heart infusion agar containing 10% sheep blood and 40 µg/ml neomycin . Both plates were then incubated for 18 h at 37°C in an anaerobic jar. When a meat sample did grow presumptive *C. 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 *C. 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 *C. perfringens* per gram in meat.

A three-tube most probable number (MPN) method was used to investigate *C. perfringens* levels in meat samples (Lin and Labbe, 2 003). Briefly, a 10 g aliquot of a meat suspension (prepared as described above) was diluted by 10 fold increments (from 10^{-1} 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 of differential reinforced clostridial broth medium (DRCM). After incubation at 37°C for 24 h, cultures testing positive for *C. perfringens* produced a unique black precipitation in this DRCM. Statistical analyses were performed (Koburger , 1975).

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

To evaluate the heat resistance of *C. perfringens* meat isolates, the spore count was measured at 100°C for each isolate, (Sarker et al., 2000). Briefly, sporulating cultures of *C. perfringens* were prepared by inoculating a 0.2 ml aliquot of a FTG culture into 10 ml of Duncan-Strong (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}. Two1 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,

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10^{-2} to 10^{-7}) with sterile FTG 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 that time point per milliliter of each heated DS culture.

Detection of enterotoxins producing isolates by reversed passive latex agglutination (RPLA Kit) A portion of the sporulated culture (about 5 ml) was centrifuged for 15 min at 10,000 xg and cell free culture supernatantwas tested for enterotoxin by using RPLA Kit (Food and Drug Administration, 1998).

Extraction of DNA for cpe detection: (Kokai-Kun et al., 1994)

To prepare template DNA for *cpe* detection, a loopful of a pure FTG culture from each isolate confirmed as *C. perfringens* by biochemical tests was streaked onto a brain heart infusion (BHI) agar plate. After incubation in an anaerobic jar at 37°C for 18 h, five colonies from each BHI plate were suspended in 200 µl of sterilized phosphate-buffered saline (PBS). Those cell suspensions were microcentrifuged at 14,000 x g for 5 min, and the resultant pellet was similarly washed twice more with PBS before final resuspension in 100 µl of PCR-grade H_2O (Sigma). Microcentrifuge tubes containing each preparation of washed *C. perfringens* cells were then sealed with Parafilm and subjected to heating in a microwave (700 W) for a total of 20 min (administered as four separate 5-min heat treatments with 1-min cooling intervals) to induce bacterial lyses. The resultant lysates were cleared by microcentrifugation at 14,000 x g for 5 min, and 5 µl of each supernatant were then used directly as template DNA in PCR assay.

PCR protocol for cpe detection: (Kokai-Kun et al., 1994)

The primer sequences used in our standard PCR protocol are 5'-.TGTTAATACTTTAAGGATATGTATCC-'3 and 5'-TCCATCACCTAAGGACTG-'3

This primer pair was designed to decrease the probability of identifying isolates carrying only portions of cpe open reading frame (ORF).

PCR amplification involved incubation of 500 ng of template DNA, 0.4µM (each primer, and 0.4 mM deoxynucleoside triphosphates, 2.0 mM $Mgcl_2$ and 1.5 U of Taq DNA polymerase (promega), in a total reaction volume of 50 µl in a thermal cycler for 34 cycles, each consistign of the following: 1.5 min at 94°C, 1 min at 50°C, and 1 min at 72°C.

After PCR cycling, 20µl aliquots of the reaction products were loaded onto a 1.5% agarose gel in the presence of ethidium bromide for electrophoresis. The 935-bp PCR product of *cpe* was observed.

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RESULTS and DISCUSSION

Results from the present study provide an explanation for the association between *C. perfringens* type A isolates and cpe gene.

As shown in Table (1) 33.7% of meat samples tested in the present survey were found to be contaminated with *C. perfringens* isolates, similar result were reported by Abd El-Rahman et al . (1995) who mentioned that *C. 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 camel meat samples tested in this survey 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 5.9% of sampled meat grew *C. perfringens* after heat shocking clearly indicating they contained spores of this bacterium.

Meanwhile, 33.7% of meat samples grew *C. perfringens* only in the absence of heat shocking .It is theoretically possible that some of these samples also contained *C. perfringens* spores that spontaneously germinated in the absence of heat shocking. However, the large difference in *C.perfringens* observed between heat-shocked versus non heat -shocked meat samples (5.9% versus 33.7% positive for *C.perfringens*) strongly suggests that most of the non-heat-shocked food samples growing *C. 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 *C.perfringens* had contained spores that spontaneously germinated into vegetative cells, those samples also tested positive for *C.perfringens* after heat shocking.

The *C.perfringens* heat resistant isolates collected from the 169 camel meat in our study and tested by RPLA techniques were subjected to PCR to determine whether they carry the *cpe* gene or not. Our result indicates that 5.9% of the camel meat samples were CPE positive *C.perfringens* type A isolates. All of that *cpe* positive type A isolates grow from heat shocked cultures and these were present in meat as spores (photo 1)This finding agree with Wen and McClane (2004) who detected that about 4.3 % of all *C. perfringens* food isolates carry *C.perfringens* and they detected that spores of type A isolates carrying cpe gene exhibit exceptional heat resistance even when present in raw foods (i.e prior to cooking) this finding indicates that heat resistance appears to be an intrinsic property of many *C.perfringens* type A isolates carrying cpe gene

Inclusion of our heat shocking to help germinate spores in meat samples proved important as all of

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the *cpe* positive type A isolates detected in meat form heat - shocked cultures. This clear identification of spores of type A isolates carrying a CPE gene in meat has important implications for preventing *C. perfringens* type A food poisoning, this observation agrees with that reported by Varga et al. (2004) who discussed the relation between the sporulation and interotoxin gene in *C. perfringens.*

Table (2) fig.(1) summarized the result of *C. perfringens* spore count of the collected samples and it is evident that the spore count varied between logs 1.3 to log 5.07.

This variation may be attributed to variation of isolate enterotoxigenicity and also contributed to increasing anaerobic load, nearly similar finding were reported by EL-Lawendy (1996) and Hassan (1999) who reported higher spore counts ($5x10^5$).

It is notable from our survey that 5.9% of all *C.perfringens* isolates obtained from camel meat were CPE positive type A isolates This results agree with survey of Lin and Labbe (2003) who had demonstrated that raw meat and poultry are epidemiologically important as the most common food vehicles of *C.perfringens* type A food poisoning and to be the most heavily contaminated with *C.perfringens* isolates and detected that raw meat might favor detection of *C.perfringens* isolates

This study evaluated the ability of *cpe* PCR assays to identify enterotoxigenic *C.perfringens* isolates.

PCR assays were 100% specific and sensitive for distinguishing enterotoxogenic strains.

It might indicate the absolute sensitivity of *cpe* PCR, I.e. the smallest amount of DNA which produces a visible PCR product.

Determining the *C.perfringens* enterotoxin is epidemiologically significant since *C.perfringens* type A food poisoning is nearly always caused by CPE positive type A isolates.

CONCLUSION

Our result purposely focused on testing meats because this food are epidemiologically important as the most common food vehicles of *C. perfringens* type A food poisoning and it is the most heavily contaminated with *C.perfringens* isolates .

Heat shocking to help germination of spores in meat samples proved important as 5.9 % of the CPE positive type A isolates detected in meat during our research were detected from the heat shocking cultures. This clear identification of spores of type A isolates carrying *cpe* gene in camel meat has important implication for understanding and preventing *C.perfringens* and food poisoning.

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Table (1): prevalence of *C. perfringens* in camel 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
169	57 (33.7)	10 (5.9)	0-35	57	10

Table (2): Spore count of Clostridium perfringens type A after heating at 100°C at different time intervals.

Isolates	0	30 min	60 min	90 min	120 min
1	7	3.322	-	-	-
2	7	4.11	-	-	-
3	7	5.07	2.11	1.30	-
4	7	5.04	-	-	-
5	7	4.47	2.85	1.6	-
6	7	4.30	1.32	-	-
7	7	3.30	-	-	-
8	7	4.14	-	-	-
9	7	3.07	-	-	-
10	7	3.17	-	-	-

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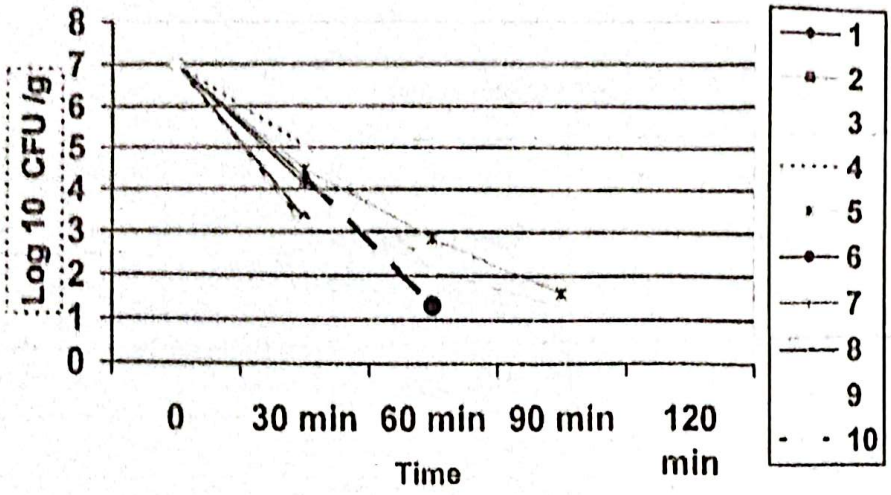

Spore count of *C. perfringens* type A after heating at 100°C at different time intervals

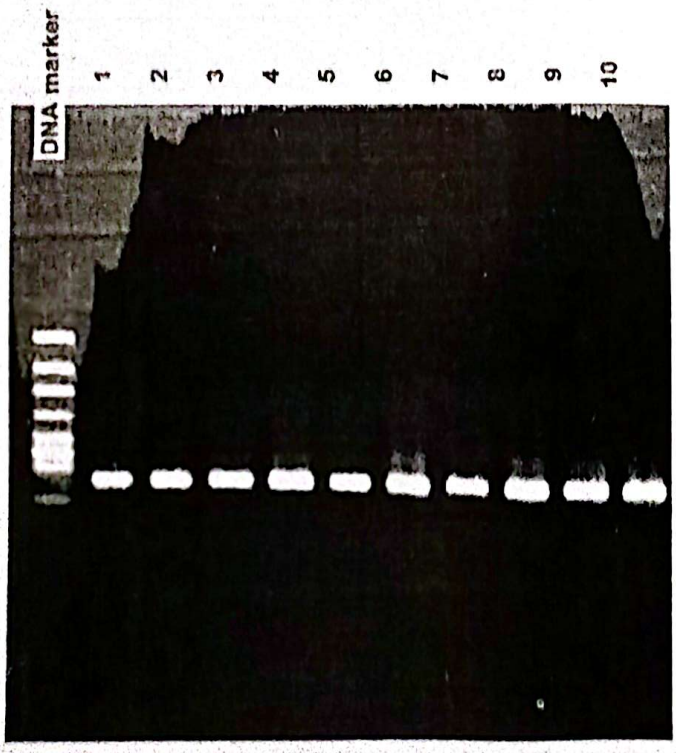

Photo (1): 1% agarose gel showing the pattern of CPE PCR genotyping analysis of CPE positive *C.perfringens* type A meat isolates.
M= 100 bp molecular weight marker ladder.
Lanes (1, 2, 3, 4 ,.. 10): PCR product of *cpe* gene

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تحديد سمية ميكروب كلوستريديم بيرفرنجينز نوع (أ) فى لحم الجمال

د/ بسمة شلبى ، د/ عاطف المحروق
معهد بحوث صحة الحيوان - الدقى - الجيزة

فى هذا البحث تم تجميع عدد ١٦٩ عينة لحم جمال وذلك من محلات الجزارة بمحافظات القاهرة والجيزة والقليوبية وتم عزل ٥٧ عترة من ميكروب كلوستريديم بيرفرنجينز نوع (أ) بنسبة (٧، ٣٣٪) وتم تعيين العدد الكلى لميكروبات الكلوستريديم بيرفرنجينز فى جرام اللحم المختبر كما تم تعيين قدرة المعزولات على مقاومة(MPN) الحرارة وتكوين الجرثومات (التحوصل) وتم تحديد قدرة المعزولات على تكوين السموم (CPE) وذلك بإختبار (RPLA).

كما تم فصل الحامض النووى للعترات وإجراء إختبار تفاعل البلمرة المتسلسل (PCR). للتعرف على جين (cpe). وتم مناقشة النتائج.

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