DETECTION OF *CPE*GENE IN STOOL SAMPLES OF FOOD POISONING PATIENTS IN MOSUL/IRAQ

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ABSTRACT

In this study, 27 stool samples collected from food poisoning patients in Mosul city were analyzed by both bacteriological standard methods and molecular methods. The bacteriological methods used for enumeration of *C. perfringens* while the nested PCR technique was used for detection *cpe* gene directly in stool samples in a method consists of a combination of nested PCR reaction with enrichment of the sample. Two pairs of oligonucleotide primers were used: the first primer pair amplifies a 425-bp fragment and the second pair amplifiesa199-bp within the 425-bp. Results showed that the number of *C. perfringens* were less than 10^3 cell/g gave a 199-bp amplified fragment which indicated that those samples contain *cpe* gene. When we compared nested PCR result with the number of *C. perfringens*,7 out of 9 samples(77. 8%) which have greater than $10^3$of *C. perfringens* per g were positive for *cpe* gene and 8 out of 18 samples(44. 4%) which have less than 10^3 of *C. perfringens* per g also gave *cpe* gene positive result.

Keywords. Clostridium perfringens. Food poisoning. Nested PCR. Cpe gene.

INTRODUCTION

Clostridium perfringens, an anaerobic Gram-positive bacterium is ubiquitous in the intestinal flora of human and animals, and it is also commonly isolated from environmental materials such as soil and water(Matches *et al*.,1974;McClane,2005) . Individual isolates of this bacterium are classified into five type (A to E), based upon their production of four toxins(alpha, beta, epsilon, and iota) (Rood,1998;Petit *et al*.,1999;McClane*et al*.,2006) . Each *C. perfringens* type is associated with certain human or veterinary disease. In addition ~5% of *C. perfringens* isolates produce another toxin, named *C. perfringens* enterotoxin (CPE). CPE is biomedically important, although it not part of the toxin typing classification scheme(McClane, 2007). The causative factor of *C. perfringens* food poisoning is an enterotoxin. It is unusual in that it is a spore- specific protein. The enterotoxin is synthesized by sporulating cells in association with the late stages of sporulation(Jay,2000).*C.perfringens* food poisoning is caused by the ingestion of food that contain large number of vegetative cells of *cpe* gene- positive *C. perfringens* strains usually belonging to type A. These *cpe* – positive cells sporulate in the intestinal tract, producingCPEwhichisresponsiveforthediarrhealsymptomsofthedisease(McClane,2001). The detection method for *C. perfringens* from stool or food samples is the use of selective culture followed by biochemical tests and final confirmation of entertoxigenic strains by serological analysis, enzyme-linked immunosorbent assay (ELISA) , Latex agglutination or vero cell assay for the differentiating the entertoxigenic strains from the nonentertoxigenic ones(McClane and Strouse,1984;Berry *et al*., 1988). However, conventional isolation of *cpe +C. perfringens* from the feces of affected individuals is occasionally complicated by the presence of *cpe -C. perfringens* as part of the normal microbial population found in stool(Eisgrüber and Hauner, 2001;Lukinmaa*et al*.,2002;Nakamura *et al*., 2003) . The isolation of the causative agent from stool samples by direct plating is challenging, particularity if several days have passed since the onset of the illness (Nakamura *et al*., 2003). Miwa *et al*. (1996) have reported a method of nested polymerase chain reaction (nested PCR) for detecting enterotoxigenic *C. perfringens* in animal feces containing both enterotoxigenic and nonenterotoxigenic *C. perfringens*. This research was aimed to use rapid detection and identification of enterotoxigenic *C. perfringens* strains directly from human stool samples which represents the first report in Mosul city- Iraq about this subject.

MATERIALS AND METHODS:

Stool samples:

The stool samples were collected in sterile containers and separated into two portions. One portion was used immediately for bacteriological analysis while the second portion of each sample was stored at -70°C for nested PCR analysis.

Bacteriological analysis:

For bacteriological analysis,0. 5-1gof stool sample was diluted with (4.5-9 ml) of peptone water medium to obtain 1: 10 dilution. The diluted samples were divided into three portions. The first portion was heated at 80°C for 10 min, the second one was heated at 100°C for 30 min while the third portion was not subjected to any treatment.

For each portion, serial dilution were prepared in peptone water medium. One ml of each dilution for the three portions was placed in a sterile petri dish and then 10-15 ml of selective media was added (TSC agar, SPS agar and TSN agar) and allowed to solidify under anaerobic condition. Then, solidified agar was overlaid with a further 10 ml of selective media. The plates were incubated anaerobically by using anaerogen (oxoid) in 37 and 46°C for 24-48 hours. After incubation, the sulfite- reducing clostridium cells were counted. Colonies surrounded by the characteristic black precipitate were identified biochemically (By using lactose fermentation, nitrate reduction, gelatinase production and motility test) .

Oligonucleotide primers:

Two pairs of oligonucleotides were used as described by Miwa *et al*. (1996) on the basis of DNA sequence of the *cpe* gene(Van Damme-Jongsten *et al*.,1989). These primers were obtained from a commercial source (Biocorp, Canada). The first PCR primers, Primer 1 sequence (5'-ACAGGTACCTTTAGCCAATC-3')and primer 2 sequence (5'-AATCTTTCTGTAGCAGCAGC-3').The second PCR primers (nested primers), primer 3 sequence (5'-GGATTTGGAATAACTATAGG-3').and primer 4 sequence (5'-CTGCAGATGTTTTACTAAGC-3').The first PCR primers used to amplify a 425-bp fragment while the nested primers were used to amplify a 199-bp fragment within the 425-bp fragment.

Primers preparation:

The stock solution for each primer was prepared in a concentration of 100 pmoles /µl by adding sterile distilled water depending on the concentration for each primer. Later, the stock solution was diluted to obtain the work solution in the final concentration 400nM.

Template preparation and enrichment procedure

DNA was extracted from stool samples depending on the enrichment procedure described by Miwa *et al*. (1996).

First PCR amplification:

The PCR reaction mixture was prepared in a final volume 50 µl by using kappa Taq Ready Mix DNA polymerase (Kapa biosystems, USA) as shown in table 1. DNA was denatured for 2 min at 95°C and amplified for 25 cycles (20 sec at 94°C, 60 sec at 55°C, 90 sec at 72°C for denaturation, annealing and extension phases , respectively) and followed by final extension for 10 min at 72°C with athermal cycler (Mastercycler personal ,Germany).

Table1: Components of PCR reaction.

Components	Volume
2x reading Mix with Mg2+	25µl
Fwdprimer	2µl
Rev primer	2µl
Template	10µlFor first PCR 5 µlFor second PCR
Water	Up to 50µl

Second (Nested) PCR amplification:

The reaction mixture and amplification condition were the same as those described in the first PCR reaction except the primer 1 and 2 were replaced by 3 and 4 and 5µl of DNA sample which is the products of the first PCR amplification was used as a template in the second amplification reaction.

Electrophoresis:

PCR Products were separated by gel electrophoresis using 1. 5% (w/v) agarose gel and ethidium bromide staining. PCR products in a volume 10 µl were subjected to electrophoresis for 60-90 min at 100 v. Amplified band were visualized and photographed under UV illumination

RESULTS:

The results of the present study revealed that the number of *C. perfringens* as determined by the standard bacteriological method were less than 10^3 bacteria per g for 18 samples out of 27 stool samples and greater than 10^3 bacteria per g for 9 samples (Figure1).

DNA samples were extracted from 27 stool samples and were amplified by nested PCR technique. Fifteen of 27 stool samples(55. 6%)

have been contained *cpe* gene and yielded amplified fragment of 199-bp fragment when compared with the DNA marker (Figure 2).
Seven of 9 samples (77. 8%) have *C. perfringens* greater than 10^3bacteria per g and the nested PCR confirmed that those samples contain enterotoxigenic*C. perfringens* while 8 of 18samples (44. 4%) which have *C. perfringens*lessthan10^3bacteriaperg have shown to be enterotoxigenic *C. perfringens* depending in the nested PCR technique (Figure 1).

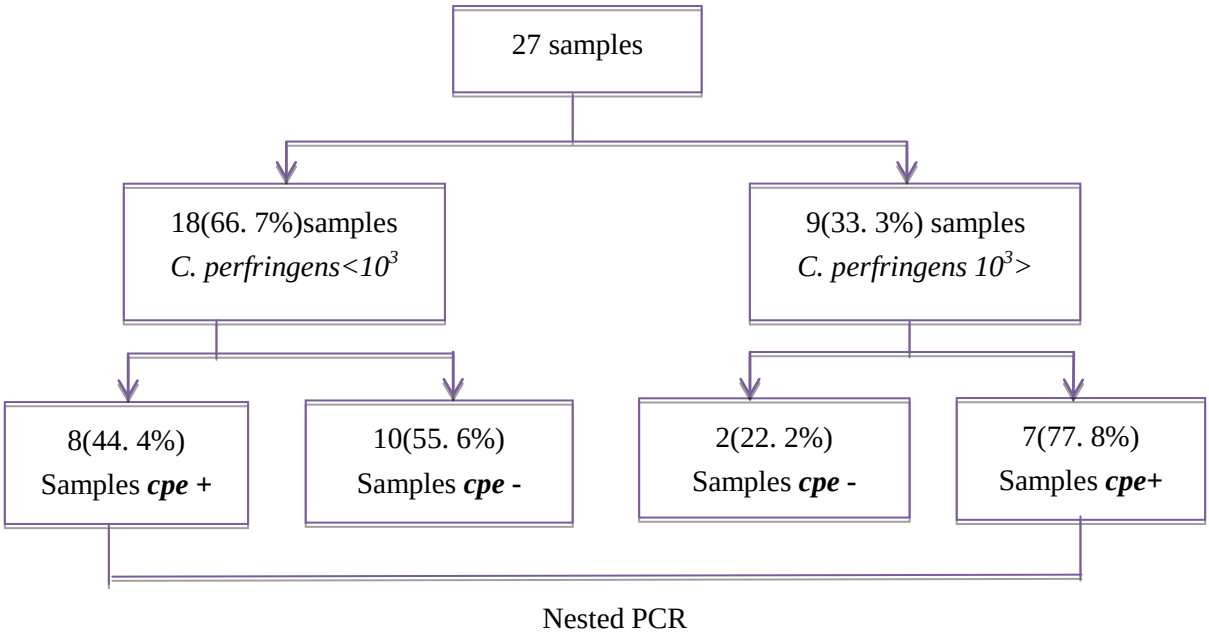

Figure 1: Summary of nested PCR results and standard bacteriological analysis

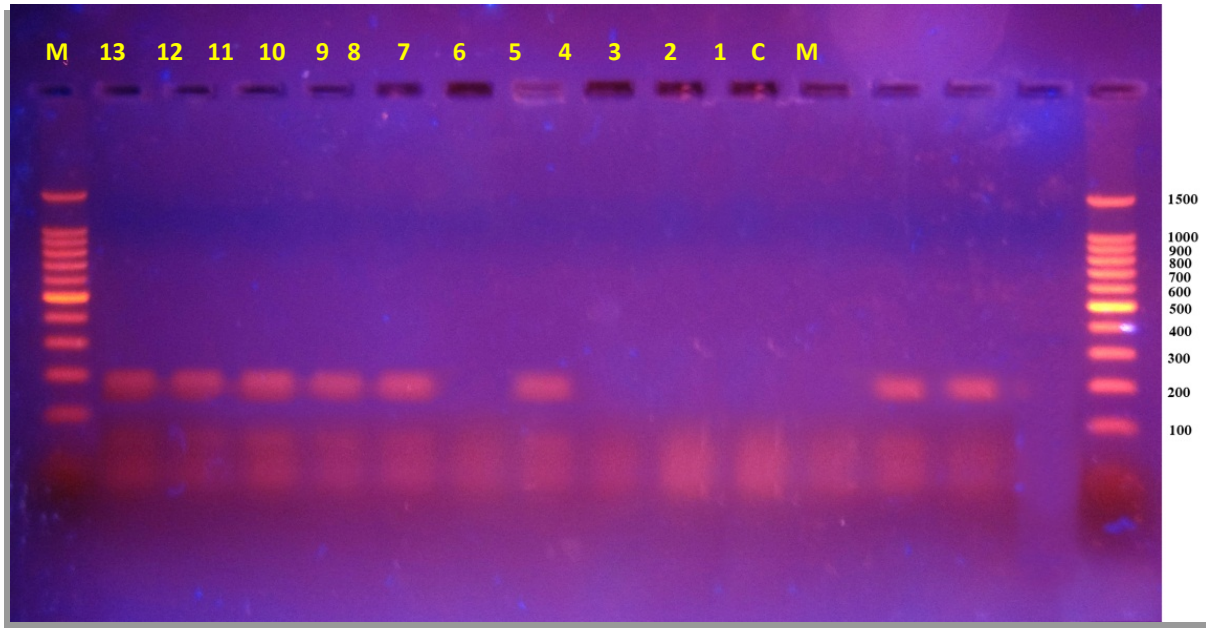

Figure 2: Agarose gel electrophoresis showing *cpe* 199-bp products of nested PCR assay
M: molecular size markers .
C:negative control
Lanes:1, 2, 7, 9, 10, 11, 12 and 13*cpe* positive results
Lanes: 3, 4, 5,6 and 8*cpe* negative results

Discussion:

The results of this study showed that the nested PCR is a useful technique for the analysis of clinical samples. Enterotoxigenic *C. perfringens* was detected directly in 15 of 27 stool samples from patients suffering from food poisoning. The probability of *cpe* positive result was increased as the number of *C. perfringens* cells from stool samples increased. In 9 samples which have *C. perfringens* 10^3>the *cpe* positive samples is 77. 8%, comparing with the 18 samples group which have *C. perfringens*$<10^3$ *and cpe*genewasdetectedin44. 4%. This result agree with those of Fach and Popoff (1997) in which 1 of 4 stool samples(25%) from food poisoning patients have *C. perfringens* $<10^4$andhave*cpe* positive result while 17 of 19 stoolsamples(89. 5%) have *C. perfringens*10^4~10^7 alsohave*cpe*positiveresult. The high percentage of *cpe* positive in this study indicates indicatesthat the enterotoxigenic *C. perfringens*has a relationship with food poisoning in these patients because the *cpe* + strains were rarely present in healthy population. Sunagawa*et al*. (1987) and Monma*et al*. (1994) reported that the *cpe* + isolates are rare in the healthy people,whilelahti*et al*. (2008) reported a higher rate (91%) of *C. perfringens* carried *cpe* which were isolated from food poisoning outbreaks. Traditionally, a spore count of>10^6/g in stool samples of patients have been regarded as the criteria for diagnosis (APHA, 1985) .However, the carriage of *C. perfringens* in the stool varied considerably between individuals (Stringer *et al*.,1985) where spore count of >10^6/g may be found in stool samples taken from institutionalized well patients or healthy people (Yamagishi*et al*.,1976).The quantitative fecal spore counts and biochemical testing of several colonies after secondary subculture also led intensive, high cost and time-consuming(Transuphasiri, 2001) . The prescence of the *cpe* gene or CPE production by isolates is normally carried out only in diagnostic procedures of food poisoning investigations (Smedley and McClane, 2004; Le Marc*et al*.,2008)

Detecting the enterotoxigenicity offecal *C. perfringens*isolatedbyserologicassaytodemonstra teitsabilitytoproduce CPE in vitro is helpful in epidemiological investigations of *C. perfringens*food poisoning outbreaks (Transuphasiri, 2001). However, this phenotypic assay may produce false negative result because CPE expression is associated with sporulation and many clinical isolates of *C. perfringens*sporulate and produce CPE *invivo* but cannot be induced to sporulate*invitro*, so these problems in the identification of enterotoxigenic*C. perfringens* can be avoided by the use of PCR methods to detect the *cpe* gene. There are several reports of direct detection of pathogenic microorganisms in samples by PCR (Gumerlock*et al*.,1993;Varela *et al*.,1994).most of these methods require purification of DNA extracts to avoid inhibition by the substances found in the samples (Widjojoatmodjo*et al*.,1992;Gumerlock*et al*.,1993;Varela *et al*.,1994) . Feces contain many compounds such as bilirubin and bile salts which can inhibit PCR analysis (Widjojoatmodjo*et al*.,1992) . In a study by Asha*et al*. (2002), some stool samples failed to yield *cpe* positive result by nested PCR, So they suggested that enrichment stool samples may be helpful in avoiding negative result. Nested PCR and enrichment culture method were used in the current study to avoid most of these problems and also to increase the DNA samples. In addition the detection of *cpe* gene directly from clinical samples by nested PCR was helped in direct detection of *cpe*+ strain which found in mixed population of microorganisms.

In conclusion, the enrichment nested PCR assay was a suitable method for the rapid and specific detection of enterotoxigenic*C. perfringens*directlyfromfoodpoisoningpatients. Alsoitconsideredavery good method for time and cost saved compared to conventional method essentially for epidemiological investigations of food poisoning or other gastrointestinal (GI) diseases caused by *cpe+ C. perfringens* strain.

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الخلاصة:

تضمنت الدراسة تحليل بكتريولوجي وجزيئي ل 27 عينة خروج من مرضى التسمم الغذائي في مدينة الموصل حيث استخدم التحليل البكتريولوجي لغرض تعداد *C.perfringens* ب ينما استخدمت تقنية Nested PCR لغرض التحري المباشر عن جين *cpe* في العينات بالاعتماد على طريقة تجمع بين تفاعل Nested PCR مع طريقة الاغناء للعينات . استخدم زوجين من البادئات : يضخم الزوج الاول من البادئات قطعة بحجم 425–bp ويضخم الزوج الثاني قطعة بحجم 199-bp ضمن القطعة 425-bp.بينت النتائج ان عدد *C.perfringens* اقل من 10^3خلية /غرام في 18 عينة فيما كان عددها اكثر من 10^3خلية/غرام في 9 عينات.كما تبين ان 15من مجموع 27 عينة خروج (55.5%) تحتوي على جين *cpe* بدلالة وجود القطعة المضخمة bp -199. وعند مقارنة نتائج تفاعل Nested PCR مع تعداد جرثومة *C.perfringens* تبين ان 7 من مجموع 9 عينات تمتلك تعداد اكثر من 10^3خلية /غرام بنسبة (77.8%) تمتلك جين *cpe* بينما 8 عينات من مجموع 18 عينة تمتلك تعداد اقل من 10^3 خلية/غرام بنسبة (44.4%) اعطت نتيجة موجبة لجين *cpe*.