

Isolation and Identification of *Clostridium perfringens* and its Enterotoxin in Food poisoning Patients

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Abstract:

Background: *Clostridium perfringens* enterotoxin (CPE), which is one of the most common cause's foodborne illnesses and contribute to diarrhea that is associated with broadspectrum antibiotic treatment.

Objectives: This study focuses on diagnosis of *Clostridium perfringens* enterotoxin (CPE) from patients suffering from food poisoning and diarrhea associated with antibiotic treatment cases in stool samples and to determine the resistance of isolated against antibiotics.

Methods: Samples were taken during the period of first of June 2015 until the end of April 2016 from Baghdad hospitals. Enzyme Linked Immunosorbent Assay (ELISA) was used to detect *Clostridium perfringens* enterotoxin in stool samples. Api 20A kit and culture to confirm isolation and identification was used, disk diffusion was performed for antibiotic resistance.

Results: The infection cases increased among old adult age group, were (8.7%) and their age range was (64 \geq) years old, and children (5.3%) their age range was (15 \leq) years old. Overall positivity was (23%) in present studied groups and infection increased with causes of food poisoning (61.5%).

Conclusion: This study revealed that the majority percent from age \geq 64year (8.7%) and this percent decreased under this age. The future advances research should explain the epidemiology of enterotoxigenic *C. perfringens* and also participate to the prevention of *C. perfringens* food poisoning outbreaks and other CPE-associated human diseases.

Keywords: ELISA, *Clostridium perfringens*, foodborne illnesses, diarrhea, antibiotic.

Introduction:

Clostridium perfringens enterotoxin (CPE) is responsible for causing of the several food and nonfood borne human gastrointestinal diseases that are a global clinical concern. *Clostridium perfringens* (*C. perfringens*), spore-forming an anaerobic, gram-positive bacillus, this bacterium is well known as a causative agent of several forms of enteric disease, including the major cause of food poisoning, and it is responsible for approximately (5–15%) of all cases of diarrhea after antibiotic treatment, and fatal enterotoxemia [1,2,3]. There are five types of *C. Perfringens*: A, B, C, D and E, classified according to the production of four major exotoxins (alpha, beta, epsilon and iota). The most CPE-positive strains classification as type A, although types C and D strains producing this enterotoxin are also properly common. The most happen on type a (alpha-toxin-producing type) strain causes gas gangrene (myonecrosis) and food-borne illness with cramps and diarrhea in addition to enterotoxemia in humans [4, 5]. Type B (alpha-, beta-, and epsilon-toxin positive) and type D (alpha- and epsilon positive) strains are the causative factors of fatal enterotoxemia in animals and humans [6]. Type C (alpha- and beta-toxin positive) also causes fatal enterotoxemia in humans [7].

Type E (alpha- and iota-toxin positive) has rarely been isolated in humans and thus, its pathogenicity remains unclear. The enterotoxin gene (*cpe*) is situated on either the chromosome (for most *C. perfringens* type A food poisoning strains) or large conjugative plasmids (for the remaining type A food poisoning and generality, if not all, other CPE-producing strains). In all CPE-positive strains, the *cpe* gene is highly linked with insertion sequences that may help to assist its mobilization and spread. During disease, CPE was generated when *C. Perfringens* sporulates in the intestines, the action of CPE onset with its binding to claudin receptors to form a small compound, and then oligomerize to create a hexameric prepore on the membrane surface. Beta hairpin loops from the CPE molecules in the prepore assemble into a beta barrel that inserts into the membrane to form an active pore that promotes calcium flow, causing cell death. This cell death results in intestinal damage that causes liquid and electrolyte loss. Gastrointestinal disease caused by *C. perfringens* enterotoxin was described by sudden onset of abdominal pain followed by diarrhea, and less usually by vomiting and fever. A short incubation period is usual (range 6-24 hours). Adequate heat inactivates *C. perfringens* vegetative cells, spores germinate in contaminated food under circumstances of poor temperature control.[8,9]. Increases in the happening of foodborne diseases, often linked with outbreaks, threaten international public health safety and elevate international importance. [10, 11].

Materials and Methods:

Three hundred forty one (341) stool sample were collected from patients(children at ages from

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newborn to 15 years old and adults at age 16 to 64 years and more) from Baghdad hospitals (Child protection teaching hospital ,Baghdad teaching hospital ,Private Nursing Home Hospital ,Children teaching hospital) suffering diarrhea from food poisoning and diarrhea associated with antibiotic therapy cases in Baghdad hospitals, Sample were taken during the period of first of June 2015 till the end of April 2016. For in vitro diagnostic use, the enzyme immunoassay RIDASCREEN® *Clostridium perfringens* Enterotoxin (biopharm,Germany) provides the qualitative identification of *Clostridium perfringens* enterotoxins in human stool samples. For ELISA test stool samples were collected into a clean container with no preservative. All stool specimens were stored at (2-8 °C) and tested as soon as possible. Ideally, stool specimens were tested within 24 hrs. But stored at (2-8°C) for up to 72 hrs. Prior to testing. If specimens were not tested within 72 hrs. They were frozen immediately upon receipt at (-20 °C) or colder. *Clostridium perfringens* Enterotoxin Test employs specific antibodies in a sandwich-type method. Monoclonal antibodies to epitopes of the *C. perfringens* enterotoxine attached to the well surface of the microwell plate. A pipette was used to place a suspension of the stool sample(After diluting a stool sample in sample dilution buffer 1:11 , (protein-buffered NaCl solution) to be examined as well as control specimens into the well of the microwell plate together with biotinylated anti-enterotoxin antibodies (Conjugate 1) for incubation in at room temperature (20–25 °C) for 60 minutes. After a washed step, streptavidin poly-peroxidase conjugate (Conjugate 2) was added and it was incubated again at room temperature (20–25 °C) for 30 minutes. Another washed step removed the unattached streptavidin poly-peroxidase conjugate. After added the substrate(Hydrogen peroxide/TMB) then incubated the plate for 15 minutes in darkness at room temperature (20–25 °C), the attached enzyme changes the colour of the previously colourless solution in the wells of the microwell plate to blue if the test was positive. Addition of a stop reagent (1 N sulphuric acid) changes the color from blue to positive yellow. Developed colour was measured at 450 nm in an ELISA reader (Orgnon,Germany). The cut-off value was calculated by extinction for the negative control + 0.15. specimen was positive if the extinction rate was more than 10 % higher than the calculated cut-off value and with extinctions more than 10 % below the calculated cut-off must be considered negative. The measure extinction at (450)nm , adjust the zero point in the air, that is without the microwell plate. The extinction is proportional to the concentration of CPE found in the specimen. For quality control purposes, positive and negative controls must be used each time the test was carried out, The test has been carried out correctly if the extinction rate (OD) for the negative control was less than (0.2) at (450) nm and the measured value for the positive control was greater than (0.8) at (450)

nm . To confirm identification tested isolation of *C. perfringens* was performed by using culture. About one gram individual stool samples were diluted in normal saline(1:10), the bath temperature was maintained at 80°C for 10 min. in order to eliminate the non-spore-forming bacteria, subsequently they were cultivated on T.S.C (Tryptose sulphit cycloserin) medium incubated anaerobically by Gas pack at 37°C for 24 to 48hrs. in jar. and on TSN agar (Tryptone Sulfit Neomycin) (SIGMA-ALDRICH,USA) ,inoculate medium with sample and incubate at $46 \pm 1^\circ\text{C}$ for 18-24 hrs. by anaerobic jar (Oxoid Anaerobic Jar with Anaerogen (AN0025,OXOID,UK)gas back Kit .[10,11,12]. The obtained typical colonies transferred into sheep blood agar and incubated anaerobically at 37°C for 24 - 72 hours. The obtained typical colonies with double zone of hemolysis were identified after conducting the anaerobic tolerance test by different biochemical tests such as (lecithinase) test on egg yolk salt agar, motility test, and fermentation of sugars like glucose, lactose and mannitol. The pure bacterial suspensions were used for API 20A biochemical to confirm identification tests [13,14]. The API 20 A system (API 20A KIT,bio Merieux ,Inc.USA) the system for the identification of anaerobes, enables 21 tests to be carried out quickly and easily for the biochemical identification of anaerobes (Table:3). Other tests such as colonial and microscopic morphology, Gram stain, Malachite Green, should be performed and the results used to confirm or complete the identification.[15,16]. Antibiotic susceptibility testing: Antibiotic disk diffusion methods were performed with Oxoid disks (Oxoid, UK). Antimicrobial susceptibility testing to seven antimicrobials (Colistin, , Clindamycin , Gentamycin, Erythromycin, Ampicillin, Chloramphenicol, and Metronidazol) were carried out for (40) isolated for *C. perfringens* by the disk diffusion method on Muellere Hinton agar with 5% sheep blood(HiMedia, India). The plates were incubated at 37°C for 24 h.anaerobically. The inhibition zone was measured for each antibiotic were determined according to BSAC methods for antimicrobial susceptibility testing.[17,18,19].

Results:

The total stool samples (n= 341) were collected from patients(children and adults) that suffering from cases(food poisoning and diarrhea after broad spectrum antibiotic treatment) from Baghdad hospitals. Seventy eight samples (23%) were positive for *C. Perfringens* enterotoxin, maximum positive cases were in 64 \leq years age group (8.7%), and children (5.3%) their age range was(15 \leq) years old. (Table: 1),(figure:1) .The distribution of *C. Perfringens* infection according to the causes,the result shown a highly significant increase infection at foodpoisoning (61.5%),while (38.5%) at antibiotics therapy.(Table : 2) .Culture examination was done on blood agar under anaerobic conditions colonies appeared with double zone of hemolysis were

identified, after staining by Gram and Malachite Green stain, all isolates were gave gram positive, spore forming (sub terminal spores) rod shaped cells, occur in pairs or short chains [20]. Identification by ELISA the presence of entero toxin. Figure(2). Identification by using Biochemical test to further confirmation with the bacteria, API 20A kits (bio Mérieux)was used . After 48 hrs of incubation all the isolates were confirmed, (Table:3) .

(Table1): Distribution of *C. perfringens* Infections According to Age Groups Human Samples by Elisa, Culture and Biochemical test.

Age groups	Samples No.	%	Positive Infection %	No.
15 and less	77	22.5	18	5.3
16-31	44	12.9	6	1.7
32-47	30	8.7	9	2.6
48-63	65	19.1	15	4.4
64 and more	125	36.6	30	8.7
Total	341	100	78	23

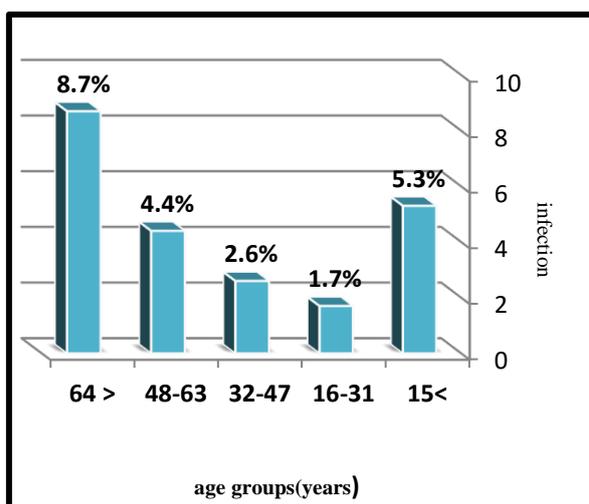


Figure (1): Distribution of *C. perfringens* Infections According to Age Groups Human Samples.

Table :(2): Distribution of *C. perfringens* infections according to causes.

causes	Positive infections	
	No.	%
Foodpoisoning	48	61.5
Antibiotic therapy	30	38.5
Total	78	100

Table (3):Api20A identification results of 78 isolates.

Test	Results
Indole (IND)	-
Urease (URE)	-
Glucose (GLU)	+
Manitol (MAN)	-
Lactose (LAC)	+
Saccharose (SAC)	+
Maltose (MAL)	+
Salicin (SAL)	-
Xylose (XYL)	-
Arabinose (ARA)	-
Hydrolysis(protease) (gelatin)GEL	+
Hydrolysis(β-glucosidase) (esculin)ESC	-
Glycerol (GLY)	±
Cellobiose (CEL)	-
Mannose (MNE)	+
Melezitose (MLZ)	-
Raffinose (RAF)	-
Sorbitol (SOR)	-
Rhamnose (RHA)	-
Trehalose (TRE)	+
Catalase (CAT)	-
Spores (SPOR)	+
GRAM reaction	+
COCC morphology	-

± variable \ - negative\ +positive

Table 4. Distribution of Sample Study According to Antibiotic Sensitive.

Antibiotic µg\disc	Sensitive No. (%)	Intermediate No. (%)	Resistance No.(%)
Metronidazole,5	29(72.5)	00	11(27.5)
Clindamycin,10	25(62.5)	00	15(37.5)
Chloramphenicol,10	33(82.5)	00	7(17.5)
Gentamycin,10	0(0)	00	40(100)
Erythromycin,15	0(0)	00	40(100)
Colistin ,10	0(0)	00	40(100)
Ampicillin,25	30(75)	00	10(25)

The resistance of 40 *C. perfringens* isolates to seven types of antibiotic used to treat infections caused by this microorganism is shown in (Table: 4). High prevalences of resistance were observed in: Gentamicin , Erythromycin and Colistin (100%), and lower resistance to Clindamycin (37.5%) , Metronidazole (27.5%), Ampicillin (25%), Chloramphenicol (17.5%). The resistance patterns and distribution of the *C. perfringens* isolates indicated that all 40 isolates demonstrated multiple resistance . (Figure : 2).

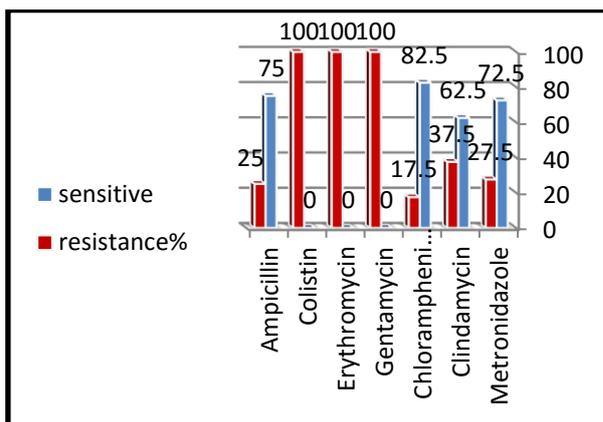


Figure (2): Distribution of Sample Study According to Antibiotic Sensitive(%) .

Discussion:

C. perfringens strains (*C. perfringens* enterotoxin, a clinically important enterotoxin) which is responsible for most of the *C. perfringens* food-poisoning outbreaks and diarrhea cases [21], under conditions, the organism can cause food-poisoning and gastrointestinal illnesses including antibiotic-associated diarrhea, sporadic diarrhea, and nosocomial diarrheal diseases in humans. The virulence of this Gram-positive, anaerobic bacterium is heavily dependent upon its prolific toxin-producing ability. [22,23,24]. These results were consistent with many international studies: Enterotoxigenic *C. perfringens* was responsible for nearly (70 %) of *C. perfringens* food-poisoning outbreaks and (20 %) of all non-food-borne gastrointestinal diseases [25,26,27]. *C. perfringens* has been found to be more abundant in children and elderly than in adults, it might be correlated to the maturation of the gastrointestinal microbial ecosystem and the associated with immune system [28,29]. It was clear from these results that the rate of infection increases with age, clearly and significantly and may be due to the weakness of immunity as a result of aging, in addition to the used of antibiotics and chemicals in the treatment of chronic diseases and malignant in many elderly and isolates were resistant to a wide range of antibiotics, these results were consistent with several global studies. The increased risk may be due to the increased number of comorbid illnesses in elderly people and perhaps more age-related colonization of *Clostridium* species in the intestine [30,31]. Cancer and immunosuppression were consistently reported as the major comorbidities in patients infected with *C. Perfringens*, humans serve as an important reservoir of *C. perfringens* cpe-positive, introducing a contamination risk into foods through handling [32]. The incidence of *C. perfringens* diarrhoea is expected to increase with the growing population of immunocompromised individuals and the increased use of antibiotic intake [33]. *Clostridium perfringens* enterotoxin (CPE) has significant medical importance due to its participation in several common human gastrointestinal diseases, causes the gastrointestinal a food poisoning, which is

the most common bacterial foodborne illness and outbreaks is challenge, a risk reduction programme should be taken, especially in vulnerable populations such as elderly. [34,35,36]. These results coincides with pervious study which shown resistance to many antibiotics. Antibiotic exposure was an important risk factor for diarrhea that associated with antibiotic but the risk was different among different antibiotic classes, avoid the excessive and misuse of antibiotics and antibiotic resistance [37,38]. *Clostridium perfringens* enterotoxin (CPE) has significant medical importance due to its participation in several common human gastrointestinal diseases. *C. perfringens* enterotoxin with high counts of toxigenic organisms in patients with diarrhea after antibiotic treatment. The incidence of *C. perfringens* diarrhea is expected to increase with the growing population of immunocompromised individuals and the increased broadspectrum antibiotic therapy[39].

Conclusion:

This study revealed that the majority percent from old age and this percent decreased under this age. The future advances research should explain the epidemiology of enterotoxigenic *C. perfringens* and also participate to the prevention of *C. perfringens* food poisoning outbreaks and other CPE –associated human diseases.

Author's contribution:

Study design and conception: luma 60%, Nisreen 40%

Acquisition of data: luma 50%, Nisreen 50%

Analysis and interpretatin of data: luma 60%, Nisreen 40%

Drafting a amnuscript: luma 60%, Nisreen 40%

Critical revision: luma 60%, Nisreen 40%

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