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Molecular Epidemiology of Antibiotic-Associated Diarrhoea Due to Clostridium Difficile and Clostridium Perfringens in Ain Shams University Hospitals

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ABSTRACT

Background: As we are living in the era of antibiotic overuse, antibiotic associated diarrhea (AAD) is considered now a distinct health problem with a need for more attention.
Aim of the Study: was to perform a highly specific detection and definition of pathogenic *Clostridium perfringens* and *Clostridium difficile* related AAD in children compared to adults and geriatircs.
Patients and Methods: One hundred and fifty patients diagnosed for AAD were included in this study (50 children, 50 adults and 50 geriatric patients).
All of them were subjected to full medical history including complete therapeutic history of antibiotics and collection of stool sample during the attack for detection of *Clostridium perfringenes* enterotoxin (CPEnt) and *Clostridium difficile* cytotoxin by (EIA) kit. PCR detection of *Clostridium perfringenes* cpe gene (Coding gene for CPEnt) was performed as well.
Results: Results showed that prevalence of *Clostridium difficile* cytotoxin was 24% while *Clostridium perfringenes* enterotoxin was 12% as detected by EIA in faecal specimens as a whole. Detection of cpe gene by PCR was positive in 16% of all cases. Children (OR: 4.2, 95% CI: 1.3-14.8, P_0.01) and geriatric patients (OR: 3.4, 95% CI: 1.2-13.5, P_0.02) were significantly more prone to *Clostridium difficile* AAD compared to adults. Also, childhood was a significant risk for *Clostridium perfringens* AAD (OR: 2.1, 95% CI: 0.54-7.4, P_0.04).
In Conclusion: children and geriatric patients are more vulnerable to develop AAD with antibiotic abuse compared to adults.
Abbreviations: *AAD=Antibiotic associated diarrhea, CI=Confidence interval, ELISA=Enzyme-linked immunosorbent assay, OR=Odd ratio, PCR=Polymerase chain reaction.*

Key Words:

Antibiotic-associated diarrhea, children, Clostridium perfringens, Clostridium difficile.

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INTRODUCTION

Antimicrobial agents are the most frequently prescribed medicines in children, because acute infectious diseases are prevalent in this age group. The demonstration that acute infections are primarily of viral origin has not reduced the use of antibiotics, nor has the fact that antibiotics afford only marginal alleviation of the clinical symptoms.[1]

Antimicrobial treatment may disturb the colonization resistance of gastrointestinal micro flora, which may induce clinical symptoms, most commonly diarrhea. The incidence of diarrhoea in children receiving antimicrobial treatment is unclear.[2]

The discrepancies in the incidence may have been attributable to differences in the definition of diarrhoea, the antimicrobial agent used, the number of daily doses, the duration of the treatment, and the time from previous antimicrobial treatments.[3]

The severity of antibiotic-associated diarrhoea may range from a brief, self-limiting disease to devastating diarrhea with electrolyte disturbances, dehydration, crampy abdominal pain, pseudomembranous colitis, toxic megacolon, or even death.[4]

The objective of this cross sectional controlled study was to evaluate the molecular incidence of Clostridium Perfringens and Clostridium Difficile related diarrhea after antimicrobial treatment in children compared to adults and geriatrics.

PATIENTS AND METHODS

Patients:

Antimicrobial agents were prescribed for the treatment of all the included subjects in the hospital.

Children age (<18 years) ranged from 2 weeks to 17.8 years of age (Mean: 4.5 years, Median: 6 years of age). Adult age (18-60 years) ranged from 19 years to 53 years old (Mean: 32 years, Median: 34 years) and geriatric age (Over 60 years) with mean; 65 years and median; 68 years.

All study subjects met the inclusion criteria: they had not received any antimicrobial medication during the previous 2 weeks before admission, they did not suffer from gastrointestinal disorders, and they all needed antimicrobial treatment, for different non gastrointestinal indications. All of them were hospitalizedat Ain Shams University Hospitals and had diarrhoea≥3 days post admission.

Fifty two hospitalized patients (Children, adults and geriatrics) were included as controls. All of them had received antimicrobial therapy within 2 weeks without developing diarrhea.

Study Design:

All patients received the same information and the follow-up was conducted in a similar manner. A daily symptom diary recorded stool frequency and consistency (Solid, loose, watery).

Diarrhoea was defined as at least three watery or loose stools per day for a minimum of 2 consecutive days within

2 weeks from antimicrobial intake. In the case of diarrhea, the parents were requested to bring a fecal sample for analysis. The primary outcome measure was "Diarrhoea during the first 2 weeks after the beginning of the antimicrobial treatment", because this period most likely reflects the effects of antimicrobial use.

The parents/patients were informed verbally and in writing about the nature and requirements of the study. Written informed consent was obtained from the parents/patients, and the study was approved by the ethics committees of Ain Shams University Hospital.

Collection of patients and controls data included; demographics, admission details, lengths of hospital stay before diarrhea, and histories of non antibiotic (Antacids, cytotoxics, and gut stimulants) and detailed antibiotic drug administration and procedures undertaken (Endoscopy, enema, feeding tube insertion, and surgery) within the 14 days before the onset of symptoms.

Samples:
Stool specimens were obtained from patients and controls. For patients, only diarrhoeal faecal specimens (i.e. those that adopted the contours of the vessel) were included in the study. Stool specimens were cooled immediately at 6°C to 8°C and within 24 hours *the stool specimen was divided into three compartments:*

Part 1: inoculated on thioglycolate broth, incubated aerobically at 37ºC for 24 hours, then subcultured on Colombia blood agar (Oxoid) with a 5μg metronidazole disc (Oxoid), and Egg yolk agar (Oxoid), both were incubated anaerobically 48 hours at 37ºC.

Part 2: inoculated into distilled water and then centrifuged, supernatant was taken in aliquot tubes, stored at -20ºC for further detection of *Cl. difficile* cytotoxin A/B and *Cl. perfringenes* enterotroxin with EIA supplied by Biopharm, Germany.

Part 3: inoculated into Phosphate Buffered Saline with glycerol 40%, then centrifuged and the supernatant was taken to aliquot tubes and stored at -20ºC for further DNA extraction and nested PCR for detection of cpe gene.

Microbiological analysis of samples:

(i) Toxin detection in feces:
All samples were tested for *C. difficile* cytotoxin and *C. perfringens* enterotoxin by RIDASCREEN kits supplied by Biopharm, Germany. They are enzyme immunoassays for the detection of cytotoxin A and B of Clostridium difficile and *Cl. perfringenes* enterotoxin in stool samples. The cutoff was calculated, any test sample more than 10% above it was considered+ve and any sample below it was considered-ve. Enzyme-linked immunosorbent assay (ELISA) kit was used according to the manufacturer's instructions.

(*ii) Culture of Clostridium Perfringens and Clostridium Difficile:*
Colonies were identified by:
- Colony morphology.
- Gram stain.
- Lactose fermentation.
- Lecithinase activity. According to **Collee**.[5]

(iii) Molecular finger printing cpe gene analysis:
DNA was extracted directly from each faecal specimen with QIAamp DNA stool Mini Kit «QIAGEN, Crawley,

UK» according to manufacturer's instructions. DNA extracts were stored at -20ºC before PCR. The first round primers were 5̀-TGTTAATACTTAAG GATATGTATCC-3 and 5-TCCA-TCACCTAAGGACTG-3̀ as described by Asha and Wilcox.[6]

The reaction combrised 5 min. at 94ºC followed by 34 cycles of 1 min. at 94ºC, 1 min. at 50ºC and 1 min. at 72ºC. there was a final extension step of 10 min. at 72ºC. The PCR product was used as a template for the second round PCR. Second round primers were: 5̀ATGTAATAGATAAAGGAGA TGGTT-3̀ and ATAAATTCAG AAGTAAATCCAACT-3̀. The reaction comprised 5 min. at 95ºC followed by 45 cycles of 30s at 94ºC, 30s 50ºC and 30s at 70ºC. The PCR product was 163 bp. The enterotoxigenic *C. perfringenes* strain NCTC was used as the positive control for PCR assays.

Statistical Methods:
The results are presented as means with range. The x2 test, Wilcoxon signedrank test, and analysis of variance for repeated measurements were used in statistical comparisons. Statistical analyses were conducted using SPSS (SPSS Inc., Chicago, version II.) software and Epi Info (Window version; 3.3.2- 2005) software. Age and gender data were analyzed by comparison of cases with the overall study population by use of multinomial logistic regression. Other risk factors were assessed using conditional backwards multivariate logistic regression with matched data.

RESULTS

AAD Pathogens:
At least one AAD pathogen was detected in 51 of 150 (34%) AAD faecal samples. *C. difficile* cytotoxin and/or *C. perfringens* enterotoxin were detected in 24% (no-36/150) and 12% (no-18/150) of samples, respectively. Three samples (5.8% of positive specimens) had evidence of both *C. difficile* and *C. perfringens*. Of the (36/150) *C. difficile* cytotoxin positive specimens, 30 (83%) samples yielded positive culture, and of (18/150) *C. perfringens* enterotoxin positive samples, only 12 (67%) yielded positive culture for *C. perfringens*.

(a) EIA results are shown in (Table 1):

Table 1: *Cl. difficile and Cl. perfringenes* EIA-results:

	Cl. difficile Cytotoxin		**Cl. perfringenes Enterotoxin**	
	+ve No.(%)	-ve No.(%)	+ve No.(%)	-ve No.(%)
Children	16(32%)	34(68%)	8(16%)	42(84%)
Adults	5(10%)	45(90%)	5(10%)	45(90%)
Geriatrics	15(30%)	35(70%)	5(10%)	45(90%)
Controls	0	52(100%)	0	52(100%)

(b) Molecular finger printing:

Table (2) showed comparison between results obtained by EIA result was 18/150+ve cases (12%) while by PCR the result was 24/150+ve cases (16%), which was not significantly different (OR: 0.72, 95% CI: 0.35-1.45; *P*-0.41) table (2). Of the 24 positive PCR cpe gene fecal specimens, 11 were detected in children, 6 in adults and 7 in geriatrics.

- ***Analysis of risk factors:***

Pediatric patients and those more than 60 years of age had a significantly increased risk of infective AAD (Odds ratios [OR], 3.61 and 2.98, respectively; P-0.001) compared to adults. This age related risk was consistent for *C. difficile* (OR: 4.2, 95% CI: 1.3-14.8, P-0.01 in children) and (OR: 3.4, 95% CI: 1.2-13.5, P-0.02 in geroatrics). The agerelated risk for *C. perfringens* was documented only in pediatric patients (OR, 2.1, 95% CI: 0.54-7.4, P-0.04), but no significant agerelated risk was detected in geriatrics. Gender was not an independent risk factor for infective AAD (P-0.74), also there was no significant agesex interaction (P-0.46).

Table 2: Comparison between EIA and PCR as different diagnostic methods for detection of toxigenic *Cl. perfringenes* (Using Mann Whitney U test).

	EIA No. %	PCR No. %	Z	P	OR (95%CI)
+ve cases	18 12%	24 16%	0.81	0.41 (NS)	0.72 (0.35-1.45)
- ve cases	132 88%	126 84%			

Significant results from the conditional multiple logistic regression analyses for infective ADD development were assessed. The major risk factors for infective AAD were administration of cytotoxic drugs and broad spectrum cephalosporins [OR (95%CI): 8.933 (7.28–10.59) and 2.511 (1.87–3.15) respectively].

We found that 32% of AAD cases in this study were associated with using broad spectrum penicillins as ampicillin, amoxicillin-clavulanate [OR 2.277 (1.22–4.1)], 30% of cases were associated with cephalosporins and 16% with both types [OR 2.594 (2.84–5.61)].

The mean length of hospital stay prior to the onset of diarrheal symptoms was significantly longer for patients than for controls (19.2±4.1 versus 10.5±3.7 days; paired t test, t-3.91; df-154; P-0.01) – OR (95% CI) 1.017 (1.01–1.03).

DISCUSSION

The most common complication of antimicrobial therapy is antibiotic-associated diarrhea.[6] It is likely that a substantial proportion of AAD cases are not due to infective causes and instead

represent either direct gut toxicity of antibiotics or other medical etiologies. Nevertheless, studies of the infective aetiology of AAD have concentrated almost solely on *C. difficile*, and thus, in the great majority of cases, a pathogen has not been identified.[7]

We chose to analyze only the diarrhea episodes that occurred during the first 2 weeks after the beginning of the antimicrobial treatment, because the later that diarrhea occurs, the more unlikely it is caused by an antimicrobial agent.[8]

C. difficile was the predominant infective cause of AAD and was approximately from 4 to 60 times more common than *C. perfringens* in many studies.[7-9] In this study, for every 10 cases of *C. difficile* AAD, we identified 5 cases of *C. perfringens* AAD, most of them were children. However, we did not consider other putative infective causes of AAD, such as *Candida* and *Klebsiella* species, because of the given relatively weak evidence base for these organisms as pathogens.[10,11]

In the present study prcentage of toxigenic *Cl. difficile* detection by EIA in stool samples of hospitalized diarrheal patients was 24% (36/150) [Divided as 16 patients from children group, 5 patients from adult group and 15 patients from geriatric group] versus 0% (No isolation of *Cl. difficile* from controls).

Many studies reported that carriage of *Cl. difficile* is common among antibiotic treated hospitalized patients, as it was detected in 32% of faecal specimens from AAD in Johnston et al.[12]

Our results agreed with[10,13-15] who reported that *Cl. difficile* prevalence ranged from 15-25% of hospitalized patients receiving antibiotics.

Our data add to the evidence base that *C. perfringens* is a significant cause of nosocomial AAD.[1,12] Laboratory identification of such cases can be justified in at least two ways. First, antibiotic treatment of *C. perfringens* AAD may aid symptom resolution.[16] While there are no data to determine optimal therapy, it is plausible that either oral metronidazole or vancomycin could be effective. Second, as discussed below, diagnosis of cases could be important to identify clusters and/or limit nosocomial transmission. ELISA-ased methods should therefore be employed to optimize the laboratory diagnosis of *C. perfringens* AAD.[17,18]

The ELISA-negative PCRpositive samples may represent a group of patients who are carrying enterotoxigenic *C. perfringens* but in whom CPEnt is not produced. Thus, the cpe gene may be present but not expressed. Alternatively, CPEnt may not be captured by the ELISA system, for example, if it was a modified toxin or neutralised by host factors.[19]

Interesting enough, children have a significant more risk to develop *C. perferingens* AAD compared to adults and geriatrics. This could be related to the intestinal internal environment and the innate resistance of anaerobic gut flora specific for children.[10]

Many AAD risk factors studies have been flawed due to limited size, poor choice of controls and thus potential for

confounding, and choice of laboratory methodology.[20] Also, most previous studies have focused solely on *C. difficile* as the etiological agent.[21] Our study was designed to address these deficiencies. Confounding factors were reduced by matching cases and controls for date and location. Times and locations were matched to account for possible fluctuations in *C. difficile* endemicity, particularly the prevalence of environmental contamination and thus the risk of patient and healthcare worker exposure.[22]

However, matching of patient locations did provide some basic adjustment for this potential confounding variable. Controversy about optimal control populations for AAD risk factor studies was highlighted in a recent systematic review.[23]

It is recognized that the risk of AAD can be reduced by substituting the administration of highrisk antibiotics, such as broadspectrum cephalosporins and clindamycin[13], with relatively lowrisk drugs such as penicillin, trimethoprim, gentamicin[17], ciprofloxacin[24], and ureidopenicillins.[21]

Identifying additional risk factors for AAD permits interventions to reduce disease burden. Risk factors identified for AAD are influenced greatly by risk factors specific to *C. difficile* AAD due to the predominance of this pathogen.[25]

Notably, each additional day of hospital stay equated to an 2% increased risk of infection.[7] However, some studies have pointed to a protective effect of broad spectrum penicillin and Aminoglycosides, as they were associated with a reduced risk of overall AAD, which was probably related to the innate resistance of anaerobic gut flora.[12]

Recent data suggest that broadspectrum cephalosporins may stimulate toxin production by *C. difficile*.[15] Also, increasing evidence points to the importance of the antibiotic resistance of *C. difficile*.[18] The roles of such factors in *C. perfringens* AAD remain to be elucidated.

Our data suggest that a limited number of *C. perfringens* genotypes cause the majority of AAD cases, although, unlike what is seen for *C. difficile*, there is no predominant genotype.[26]

We did not examine the environment for *C. perfringens*, but it is feasible that spore contamination similar to that seen in areas of *C. difficile* endemicity occurs secondary to fecal soiling by incontinent patients.[23]

Strains with serotypes the same as those of fecal isolates were found in the hospital environment and on the hands of infected patients during an outbreak of *C. perfringens* diarrhea.[26]

This is further evidence that *C. perfringens* may be acquired nosocomially. It is likely that measures to limit nosocomial dissemination of *C. difficile*, including case isolation and attention to environmental hygiene, will also be of benefit in the treatment of enterotoxigenic *C. perfringens*.

The most effective way to prevent antibiotic-associated diarrhea which is evident to be more risky for children and geriatrics is still critical use of antimicrobials, as recommended

recently for the evidenced based treatment of children. However, when antimicrobial treatment is indicated, *Lactobacillus GG* could be a novel promising adjunctive therapy[18,23] to prevent diarrhea. Further randomised controlled studies are clearly required here.

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