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# Detection of *Clostridium difficile* by Cultural and Gas Liquid Chromatography Techniques among Diarrheic Children in Khartoum (Sudan)

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## Abstract

**Background:** *Clostridium* species are part of the intestinal indigenous microbiota of young children and they can produce several endogenous infections. *Clostridium difficile* (*C. difficile*) frequently colonizes the human large intestine when the normal colonic flora is disturbed by antibiotic therapy. The result of colonization may be asymptomatic, or it may lead to illness, ranging from mild diarrhoea to pseudo-membranous colitis. *C. difficile* is a major nosocomial pathogen, responsible for up to 20% of cases of antibiotic-associated diarrhoea in industrialized countries, and is an emerging problem in developing countries.

**Objective:** To detect *Clostridium difficile* by cultural and gas liquid chromatography techniques among diarrheic Children in Khartoum (Sudan).

**Materials and methods:** The study investigated 552 children aged from 15 days to 8 years. 351 faecal specimens were collected from diarrheic children, and 201 specimens were collected from non- diarrheic children carriers of *C. difficile* who were at risk in whom antibiotic-associated diarrhoea may develop after using chemotherapeutic agents. A total of 202 cooked meat carbohydrates selective (CMC+S) broth were inoculated with faecal samples and were tested for detection of *Clostridium difficile*. Screening for *Clostridium difficile* in stool was performed by fatty acid analysis using gas liquid chromatography technique (Shimadzu GC-1020, Japan) to detect isocaproic acid from faecal specimens.

**Results:** One isolate of *Clostridium difficile* was identified by colonial morphology, spore shape and position, biochemical tests, and gas liquid chromatography (GLC) technique. and *Clostridium difficile* (1/1.61%) was detected on selective medium (CCFA). Fatty acid analysis by GLC technique was used for detection of isocaproic ($iC_6$) peak in height of $\geq$ 2mm, and also valeric and isovaleric acids.

**Conclusion:** Detection of *Clostridium difficile* by cultural methods is insignificant. Detection by gas liquid chromatography technique is more reliable.

**Key words:** *Clostridium difficile,* Diarrheic children, Culture, GLC, Fatty acid analysis.

## Introduction

*Clostridium* species are widely distributed in the environment, inhabiting both human and animal gastrointestinal tract. They are members of the family of *Clostridiaceae*. They are eterogeneous, Gram-positive rods, anaerobic, fermentative, and spore-bearer, inhabiting the human intestinal indigenous microbiota. *Clostridium* species include: C. *perfringens,* C. *septicum,* C. *novyi* (type A), C. *bifermentans,* C. *histolyticum,* C. *sordellii,* C. *tetani,* C. *botulinum,* C. *difficile,* C. *ramosum,* and C. *bifermentans. Clostridium difficile* is a strict obligate anaerobe, spore-former, motile bacillus, rod shaped with sub terminal or terminal, non-bulging oval spores. Cells stain uniformly Gram-positive in young cultures, but may become Gram-negative after 24-48 hours. Electron microscopy shows peritricous flagella The spores do not germinate unless suitable reducing conditions are available and oxygen is absent. The spores are unusually resistant[1].

The organism gained its name *difficile* (means difficult) because of the difficulties encountered in its isolation. It grows very slowly. It is also particularly difficult to eradicate from surfaces and equipment because spores are very difficult to be cleaned off or killed and microscopic spores from faeces can survive on surfaces for months. It may be found in marine sediment and in sand, in camel, horse and donkey dung, faeces of dogs, cats and birds, human genital tract, intestinal tract of humans and in their faeces and generally in hospital environment. *Clostridium difficile* was discovered by Hall and O'Tools in 1935 as a member of the intestine of normal infants, and subsequently received little attention from microbiologists until the mid 1970s when it was reported that colitis occurred in 10 % of clindamycin -treated patients. It is now recognized as the primary pathogen responsible for antibiotic associated' colitis (AAC) and 15 % to 25 % of cases of antibiotic diarrhoea[2]

*Clostridium difficile* was not known to be pathogenic until it was implicated as the cause of antibiotic associated membranous colitis. The toxin found in the faeces of patients was attributed to be produced by C. *difficile.* This toxin possession had enterotoxic and cytotoxin activities which were subsequently separated from each other and designated as toxin A and toxin B, respectively[3] .

## Materials and methods

**In** the present study, 552 faecal specimens were investigated for the presence of C. *difficile* from hospitalized and outpatient children in Khartoum Teaching Hospital and Omdurman Teaching Hospital. All specimens were collected from children aged 15 days to 8 years. The study population were classified into two groups: 351 faecal specimens were collected from the diarrheic children group and 201 faecal specimens were collected from the non-diarrheic children group. The stool container of each specimen was labelled with date, age, number of the specimen and the specimen group. Specimens were transferred in an ice-box as soon as possible to the laboratory for investigation.

Stool samples were inoculated on *Clostridium difficile* base medium supplemented with egg-yolk and for horse blood agar. The isolate was subcultured on Smith and Holdeman medium, blood

agar medium, lactose egg yolk agar medium, and brain heart agar medium for purification. Pure cultures were inoculated onto cooked meat medium for storage. Identification was performed by Gram stain, catalase test, lactose fermentation, lipase hydrolysis, lecithinase activity, reactions on cooked meat medium, gelatin hydrolysis, milk digestion test, indole test, urease test, nitrate reduction test, and fermentation tests. Screening for *Clostridium difficile* in stool was performed by fatty acid analysis using gas liquid chromatography technique (Shimadzu GC-1020, Japan) to detect isocaproic acid from faecal specimens (Fig. 1).

## Results

In this study, 552 faecal specimens were investigated for *Clostridium difficile.* From these, 331 specimens (60%) were collected from Khartoum Teaching Hospital, and 221 specimens (40%) were collected from Omdurman Teaching Hospital.
351 specimens (63.6%) were collected from diarrheic children and 201specimens (36.4%) were collected from non- diarrheic children. 389 (70.5%) of the children investigated were outpatients and 163 (29.5 %) were hospitalized patients.
On cycloserine-norfloxacin fructose agar medium, only 3 *Clostridium difficile* strains were isolated, and only one strain of these isolates (isolated from a non- diarrheic child) was fully identified; and 2 strains were not confirmed.
202 stool specimens were screened for C. *difficile* using *C. difficile* latex antigen detector kit. 14 specimens of these showed a positive reaction. These 14 positive samples were inoculated into cooked meat carbohydrates selective (CMCS) broth and then subjected to fatty acids analysis by GLC. 13 samples (93%) were found positive for C. *difficile.*

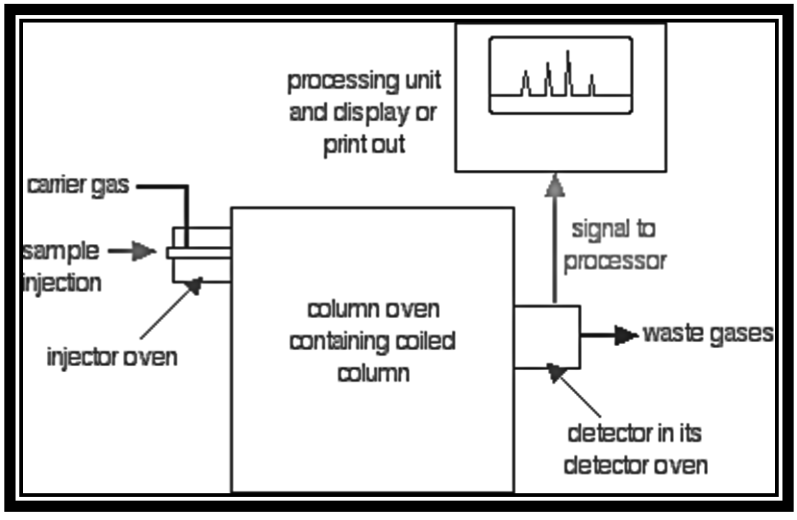


Fig. (1): A flow scheme for gas-liquid chromatography

## Discussion

Few data are available on *Clostridium* inhabiting the human gastrointestinal tract, and causing acute diarrhoea in children and colon cancer. Investigation of the intestinal clostridia is crucial for obtaining an understanding of the role of the gut microflora in health and disease. Scanty works were conducted in developing countries as regard the problem of *C. difficile.* The present study seems to be the first of its type in such countries. This could be due to difficulty in investigating anaerobes specially clostridia species. *Clostridium difficile* is an opportunistic pathogen that causes pseudomembranous colitis.

In this study only one strain of *Clostridium difficile* was isolated and confirmed among 552 children. This may be due to various factors. Firstly, the routine metronidazole treatment prescribed to diarrheic children. Secondly, it is generally difficult to obtain samples from the targeted children since it was cumbersome to obtain stool specimens from symptomatic patients and even more difficult to sample asymptomatic children. Thirdly, the spreading use of yoghurt by parents for treatment of diarrhoea in children; since such a procedure may give protection against *C. difficile.*

In this study, 5 children (38%) were asymptomatic carriers of C. *difficile*. One of them was 3 months old. Interestingly, neonates had an isolation rate up to 70%, yet symptomatic infection in children was very low despite the high carrier rate of C. *difficile.* This may be attributed to the lack of expression of colonic membrane receptors, or because of the immunoglobulin A immunity derived from the mother's milk[4] .

4 carrier children aged 9 months - 4 years were at risk of acquiring C. *difficile.* This is because patients without diarrhoea are considered at high risk for acquiring C. *difficile,* resulting from increasing child age, serious illness, prescription of antibiotics given to treat other conditions (pneumonia, otitis, cystitis, sinusitis, etc). All these factors may upset the child immune balance leading to more multiplication of *C. difficile*[5].

There are several theories to explain why such children do not exhibit symptoms, despite the high colonization rates of *C. difficile*. Alonso and his colleagues[6] (1999) suggested that the absence of disease might be related to the immaturity of the toxin receptor sites, which are unable to bind the toxins.

Neonates may encounter *C. difficile* infection either from their mothers' vaginal flora or from direct contact with patients suffering from *C. difficile.* In this study, the carrier prevalence rate of *C. difficile* in children with diarrhoea was 8 cases (3.9%). This finding was similar to that reported by Soyletir and his co-workers (1996)[7] who found 4.9 % carrier rate among Turkish children with diarrhoea.

Another study in Brazil conducted by Al-Saif and his co-authors (2003)[8] revealed a frequency rate of only 5.5 % for *C. difficile* in diarrheic children.

In the present context, among 8 children suffering from diarrhoea, 3 of them were outpatient cases receiving antibiotics and only one of them was receiving amoxicillin. The remaining 5 children were hospitalized cases receiving antibiotics intravenously. This finding supported the work of Belanger and his colleagues (2009)[9] who reported a potentially life-threatening, non-antibiotic associated *C. difficile* diarrhea case in a healthy 7-weeks old child. This may be

attributed to nutritional conditions, immunological alterations resulting from hospitalization, age, genetic factors, pathologies, or antimicrobial therapy, which interfere with *C. difficile* isolation[10]. Also, in this study only 3 strains of *C. difficile* had grown on CCFA medium, and only one of them was found positive. This may be due the fastidious nature of *C. difficile* and to reliance on cultures of stool specimens[11].

Identification by conventional biochemical techniques, is often confronted by the fastidious nature of the organism. Detection by gas-liquid chromatography is more reliable. The positive samples in this study had produced a strong agglutination with *C. difficile* latex antigen (14/7 %); and yielded high peaks of $iC_6$ acid 2: 2 mm, indicating the presence of *C. difficile* in these samples. This was attributed to the ability of *C. difficile* to produce isocaproic acid in vitro. This finding was similar to that reported by Biller (1995)[12] who found that all CMC+S broth media inoculated with faecal samples had produced high peak of $iC_6 \geq 2$ mm together with positive cytotoxicity.

**Conclusion:** Detection of *Clostridium difficile* by cultural methods is insignificant. Detection by gas liquid chromatography technique is more reliable.

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