Diagnosis of Clostridium Difficile Antibiotic associated Diarrhoea Culture versus Toxin Assay

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Abstract

Objective: To compare the results of Clostridium Difficile (CD) on culture with detection of C. difficile toxin by Enzyme Immunoassay (ETA) in the stool specimens of hospitalized patients with antibiotic associated diarrhea (AAD).
Patients and Methods: The study included 80 adult patients with AAD and 20 adult patients with non-AAD. Stool specimens of all these subjects were inoculated on cycloserine cefoxitin fructose agar and incubated anaerobically to isolate C. difficile. At the same time, all the stool specimens were tested for C. difficile toxin by EIA technique using cytoclone A and B kit manufactured by Cambridge Biotech Corporation, Worecester, Massachusette.
Results: Out of 80 adult patients with AAD, thirty were females and fifty males. C. difficile was isolated on culture from stool specimen of 16 patients, while twenty-three stool specimens were positive for C. difficile toxin. From 20 control subjects, C. difficile was isolated from stool specimen of only one subject. No stool specimen from the controls was positive for toxin.
Conclusion: Diagnosis of CDAAD by culture is difficult and time consuming because of strict anaerobic nature of organism. Moreover, mere isolation of C. difficile on culture is not sufficient to establish the pathogenic role of these isolates. C. difficile toxin detection by ETA technique is a highly sensitive and specific method for diagnosis of CDAAD. Using this method, results are available in three hours time. Therefore, ETA is recommended for rapid diagnosis of CDAAD (JPMA 50:246, 2000).

Introduction

Clostridium difficile is widely distributed in nature and is particularly prevalent in the hospitals[1-4]. It has been implicated as an etiologic agent in 90-100% cases of pseudomembranous colitis and 15-25% cases of antibiotic associated diarrhoea[5,6]. Antibiotic associated diarrhoea due to C. difficile is a toxin mediated disease[7-11].
Isolation of C. difficile from stool specimen on culture and/or detection of C. difficile toxin has been used for laboratory diagnosis of C. difficile antibiotic associated diarrhoeal disease[6,12-14]
The isolation of C. difficile from stool provides only presumptive diagnosis of C. difficile associated enteric disease, because some strains of C. difficile are non-toxigenic[12,14,15] while the presence of toxin indicates that diarrhoea is really due to a pathogenic strain[8,10,16,17].
Tissue culture assay, counter current immunoelectrophoresis, enzyme linked immunosorbent assay and polymerase chain reaction have been used for detection of C. difficile toxin[7,16,18,19]. Out of these, enzyme linked immunosorbent assay has been found to be rapid and cost effective method[15,6,20-22].
Antibiotic associated diarrhoea is common in Pakistan due to indiscriminate use of antibiotics. Present study was done to compare the results of C. difficile by culture with detection of C.

difficile toxin by enzyme immunoassay in the stool specimens of hospitalized patients with antibiotic associated diarrhoea.

Patients and Methods

The present study was carried out on one hundred adult patients irrespective of sex, admitted in surgical and allied wards of Sir Ganga Ram Hospital/Services Hospital, Lahore. Out of these, 80 patients were suffering from antibiotic associated diarrhoea. They were taking antibiotics for a least 4-5 days prior to the onset of diarrhoea. From the same wards, 20 patients having non-antibiotic associated diarrhoea were studied as control subjects. The patients with diarrhoea were passing three or more stools with decreased consistency per 24 hours. The patients were included in the study irrespective of passage of mucus/blood in stool and other gastrointestinal complaints. Moreover, the patients were not subjected to sigmoidoscopic examination.

The diarrheal stool specimens of the patients were processed for isolation of C. difficile on culture and detection of C. difficile toxin with enzyme immunoassay method. For C. difficile culture a small amount of stool was added to Robertson cooked meat medium. The stool specimens were also taken to laboratory as such. In the laboratory gross examination was done and then half portion of these specimens was kept at -20°C till further use for toxin detection[23].

Culture for Clostridium Difficile

For isolation of C. difficile, the stool specimens were inoculated onto cycloserine cefoxitin fructose agar (CCFA) containing defibrinated horse blood and egg yolk. In order to eliminate overgrowth of commensal organisms present in the stool specimens, one ml of liquid or one gram of solid stool specimens was homogenized in one ml of methylated spirit and then plated out on CCFA plates within 30 minutes[24]. The plates were incubated anaerobically at 37°C for 48 hours. Robertson cooked meat enrichment broth was sub-cultured onto CCFA plates. These plates were also incubated at 37°C under anaerobic environment for 48 hours. Anaerobic conditions were produced by Oxoid anaerobic system using BR 58 (Oxiod) gas-generating kits. Preliminary identification of Clostridium difficile was done by colony morphology, gram staining, lecithinase/lipase activity, aerotolerance test and flourescence under long wave length (365 mm) ultraviolet light. Definitive identification of the isolates was done by various biochemical tests[23,25] such as urease, indole, esculin hydrolysis, gelatin digestion, motility and sugar fermentation tests.

Detection of Clostridium difficile Toxin in Stool Specimens

All the stool specimens were tested for the presence of toxin A and toxin B against C. difficile by enzyme linked immunosorbent assay (ELISA) using Cytoclone A and B EIA kit (Cambridge Biotech Corporation, Worcester, Massachusetts) following the instructions of the Manufacturer. This kit contained rnicrotiter plate, with wells being precoated with toxin A and B specific monoclonal antibodies. If either toxin was present in the stool samples, toxin monoclonal antibody and biotinylated goat polyclonal conjugate (specific for both toxins) complexes were formed which remained in the micro wells after washing. A strepta-vidin-horse radish peroxidase conjugate was then added into the wells. This got attached to biotinylated conjugate toxin complex if present. After final working step a sub-strate (urea peroxide) and chromogen (tetramethylbenzidine) mixture was added to the wells. Any bound conjugate converted the substrate chromogen to blue color. Addition of acid (stop solution) converted blue to yellow colour. The intensity of the yellowish color was proportional to the amount of toxin(s) bound in the wells. For statistical analysis of results, chi square (X^2) test with yates correction was applied.

Results

Out of 80 adult patients suffering from antibiotic associated diarrhoea, 30 (37.5%) were females and 50 (62.5%) males with male to female ratio of 1.66:1. Of the 20 subjects with non-antibiotic associated diarrhoea, 11 were females and 9 males. The age range of the patients was 18-65 years (mean of 35 years) and controls 23-60 years (mean age 43 years).
Out of 80 patients with antibiotic associated diarrhoea, the stool specimens of 4 were culture positive, toxin negative; twelve were culture positive, toxin positive while 11 were culture negative, toxin positive. Against that in control subjects only one was culture positive, while toxin was not detected in any stool specimen (Table). Based on positivity on culture and/or presence of toxin evidence of C. difficile infection was observed in twenty-seven (33.75%) patients and one control subjects (5%). Statistical analysis showed that infection was significantly higher in AAD patients ($p<0.05$) as compared to non-AAD controls.

Discussion

Antibiotic associated diarrhoea/colitis is caused by Clostridium difficile and is a toxin mediated disease[7-9,26,27]. Use of broad spectrum antibiotics alter the colonic flora allowing the C. difficile to flourish in the human colon[6,10,15,28,29]. When the organism is established in the colon, pathogenic strains of C. difficile produce antibiotic associated diarrhoea through the action of two toxins, toxin A and toxin B[9,26,30,31].
The isolation of Clostridium difficile from the stool is used for presumptive diagnosis of C. difficile associated enteric disease[12,14,15,17]. However, it is a less efficient method of establishing laboratory diagnosis because some strains of C. difficile are non-toxigenic[5,15,19]. The only way to establish the diagnosis is to detect toxin production by the isolates (toxigenic culture)[13,16,33]. However, this is a time consuming process[14,15,33]. The other method used for diagnosis of this disease is direct detection of C. difficile toxin(s) in the stool specimen of these patients[13,16,18,20].
Successful recovery of Clostridium difficile by culture is challenging[13]. As it requires the proper selection of media for primary isolation, strict anaerobic environment for incubation and specific confirmatory methods[24,25] are needed. As far as toxin detection is concerned, one ff the earlier methods available to detect the toxin B and toxin A was tissue culture cytotoxicity assay[7,18,34]. Limitations of the cytotoxicity assay are that the tissue culture facilities are expensive and expert personnel with specialized laboratory equipment are required[5,21,33]. Keeping in mind these difficulties, efforts were made to device some faster methods for the detection of Clostridium difficile toxin A, toxin B or both. Some of the methods which were tried, are latex agglutination test, gas liquid chromatography, countercurrent imm unoelectrophores is and enzyme linked immunosorbent assay[35-40].
Enzyme linked immunosorbent assay (ELISA) has proved to be a good prospect[32,38,40]. Monoclonal antibodies of high specificity and sensitivity to Clostridium difficile toxins were developed and coated on microtitre plates[41]. ELISA takes about four hours which is a considerable saving of time when compared with the time required for cytotoxicity assay and/or toxigenic cultures[15,16,20,22,23].
Proper laboratory diagnosis of Clostridium difficile antibiotic associated diarrhoea includes both culture and toxin assay for C. difficile[42-44]. In the present study, out of 80 stool specimens of antibiotic associated diarrhoea patients.[27] cases showed evidence of C. difficile infection. Out of these, four cases were culture positive, toxin negative. Eleven cases were culture negative, toxin

positive while 12 cases were culture positive as well as toxin positive. The finding that stools of antibiotic associated diarrhoea patients can be culture negative, toxin positive, have also been observed by many other workers[18,27,45-47].

In the present study, out of 27 cases showing evidence of Clostridium difficile infection, four antibiotic associated diarrhoea patients were culture positive, toxin negative. This observation is in agreement with that of other workers[42,48-50]. Stools of 6-21% antibiotic associated diarrhea patients in these studies were found to be positive for C. difficile culture but negative for its toxin.

In the present study, C. difficile isolates were not tested for toxin production in vitro. Therefore, it is difficult to say whether these isolates represent colonization by nontoxigenic strains or by toxigenic strains. According to Butterworth et al (1998), C. difficile isolates from 73% of culture positive, toxin negative cases, showed toxin production in vitro. Keeping this finding in view, culture positive toxin negative patients in the present study may have infection due to toxigenic C. difficile and not colonization by non-toxigen ic strains.

Out of 20 control subjects in the present study, only one case was positive for Clostridium difficile on culture, while the toxin was not detected in stool specimen from any subject. This finding is in agreement with findings of other workers[17,50,52]. Their studies show that 5-11.5% hospitalized patients having non-antibiotic associated diarrhoea were positive on culture and negative for C. difficile toxin.

Cytotoxin tissue culture assay is widely accepted “gold standard’ test for detection of Clostridium difficile toxin[20,47,53-55]. However, it requires at least 24 hours to obtain results by this method[39,43]. Therefore, rapid methods are required to diagnose this disease. Out of these rapid methods, enzyme immunoassay and latex agglutination tests are good alternatives[13,21,38,39,53,55]

Latex agglutination test can be performed in three minutes. However, it has low sensitivity as well as spcificity[13,21,34,56]. Moreover, this method based on detection of glutamate dehydrogenase antigen which is not exclusive to toxigenic strains of C. difficile, may give false positive results[15,21,38].

ELISA can be performed in three hours. Thus, it is a rapid method of detection of Clostridium difficile toxin in stool specimens. Moreover, it has been proved to be a sensitive as well as specific test[15,20,34,47,57]. Therefore, enzyme immunoassay test is recommended for rapid diagnosis of C. difficile antibiotic associated diarrhoea disease. This will help in timely treatment of patients with this disease and consequently reducing its morbidity and mortality.

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