Isolation and identification of antibiotic-associated diarrheagenic resistant bacteria from patients of Rajshahi medical college hospital, Rajshahi, Bangladesh


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Key words: Antibiotic-associated diarrhea, antibiotic susceptibility, resistant bacteria.

http://dx.doi.org/10.12692/ijb/5.1.155-160 Article published on July 02, 2014

Abstract

Bacteriological investigations of Antibiotic-associated diarrhoeal diseases were carried out among 45 patients using stool samples from pediatric ward of Rajshahi medical college hospital, Rajshahi, Bangladesh. Total 9 types of bacterial colonies were isolated from 45 stool samples and only 6 isolates were found resistant to antibiotics. Commercially prepared paper disc of four antibiotics viz. Azithromycin, Ciprofloxacin, Erythromycin and Tetracycline were used for antibiotic susceptibility test. The identified resistant bacteria were Escherichia coli, Yersinia enterocolitica, Klebsiella pneumoniae, Pseudomonas aeruginosa, Salmonella sp. and Staphylococcus aureus.

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Introduction
Antibiotic-associated diarrhea (AAD) is defined as diarrhea that occurs in association with the administration of antibiotics (Bartlett, 2002). Diarrhea can occur within just a few days of antibiotic use or even a few weeks later (Surawicz and Christina, 2003). The incidence of AAD has been estimated to vary between 5% and 25% in adults and between 8% and 30% in children (Conway, 2007). The disruption of the normal enteric flora caused by antibiotics may lead to overgrowth of pathogens and functional disturbances of the intestinal carbohydrate and bile acid metabolism, resulting in osmotic diarrhea (Hogenauer et al., 1998). The severity of antibiotic-associated diarrhea 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 (Bartlett, 1992). Although any antibiotic may be associated with AAD, the highest risk is found with the use of clindamycin, cephalosporins, broad-spectrum penicillins, ampicillin, and amoxicillin (Sullivan et al., 2001; Beaugerie and Petit, 2004).

As antibiotics are increasingly used and misused, the bacterial strains become resistant to antibiotics rapidly (Abeyesinghe and Wanigatunge, 2006). Resistance has emerged even to newer, more-potent antimicrobial agents (Parry, 1989). A number of epidemics have recently occurred caused by multiply resistant organisms (Frost et al., 1981; Olarte et al., 1976). The development of antibiotic resistance has become a global public health challenge which is causing ineffectiveness of antibacterial agents leading to increase in diseases and death rate (Adeshina et al., 2011).

AAD is important and increasingly frequent complications of antibiotic therapy. While these occur most often in hospitals and nursing homes, they also occur in the community (Fekeyt, 1997). Diarrhea represents a major condition responsible for pediatric mortality worldwide. The onset of diarrhea may rapidly lead to life threatening dehydration and malnutrition (Brad et al., 2011). AAD is an important health problem in Bangladesh, represents a clinical entity leading to prolonged hospital stays and diagnostic and therapeutic procedures, and results in additional costs and also antibiotic resistant bacteria are one of the major problems challenging the health care system in general. Therefore, the present research was undertaken to isolate and identify of resistant bacteria in stools of patients with AAD.

Materials and methods
Sample collection
Around 45 stool samples were collected in sterile screw-capped tube from AAD affected patient from pediatric ward of Rajshahi medical college hospital, Rajshahi, Bangladesh. These samples were obtained from diarrhea affected patients (different ages) during the first two weeks after the starting of the antibiotic treatment. Enumeration for colony started within 5-6 hr of collection using a serial dilution technique. The samples were then cultured following Holt et al., (1994) by plated onto MacConkey agar and Nutrient agar at 37ºC for about 24 hours.

Antibiotic susceptibility test
Antibacterial susceptibility test of the isolated organisms was done by disc diffusion method using the Kirby-Bauer technique (Bauer et al., 1966) and as per recommendation of National Committee for Clinical Laboratory Standards (NCCLS) (NCCLS, 1997). Bacterial inoculum was prepared by suspending the freshly grown bacteria in 4-5 ml sterile nutrient broth and the turbidity was adjusted to that of a 0.5 McFarland standard. The antibacterial susceptibility test was performed using Mueller-Hinton medium. Commercially prepare paper disc (Oxoid Ltd., Basingstoke, and Hampshire, England) of four antibiotics namely Azithromycin (15 μg), Ciprofloxacin (5 μg), Erythromycin (15 μg) and Tetracycline (30 μg) were used. Hundred μl of 18 hours old culture of inoculums of each tested bacteria was spread onto Mueller-Hinton agar plate. The surface of Mueller Hilton agar plate was inoculated by spreader. Then Antibacterial discs were placed on the surface of the agar plate using forceps. Gently pressed
down each disc to ensure complete contact with the agar surface. The plates were incubated at 37°C for 18-24 hours. The zones of inhibition were measured and compared with NCCLS guidelines (NCCLS, 1997).

**Biochemical tests**

Isolated bacteria were subjected to biochemical tests for tentative identification (Holt et al., 1994; Raghuraman et al., 2013; Konuku et al., 2012). Following biochemical tests were carried out: (a) Gram staining, (b) Catalase, (c) Oxidase, (d) Nitrate Reduction, (e) Motility, (f) Urease, (g) Methyl red & Voges-Proskauer, (h) Indole, (i) Citrate, (j) H₂S production.

**Results**

**Counting and Isolation**

The highest number of colony was recorded 54±0.33 cfu/plate. In the present investigations, total 9 types of bacterial colonies were isolated from 45 samples (Table 1).

**Table 1.** Selected bacterial isolates.

<table>
<thead>
<tr>
<th>Bacterial isolates</th>
<th>Colony morphology on MacConkey agar</th>
<th>Colony morphology on Nutrient agar</th>
</tr>
</thead>
<tbody>
<tr>
<td>BMLRU 1</td>
<td>Pink</td>
<td>Whitish</td>
</tr>
<tr>
<td>BMLRU 2</td>
<td>Pale pink</td>
<td>Colorless</td>
</tr>
<tr>
<td>BMLRU 3</td>
<td>Whitish</td>
<td>Pale yellow</td>
</tr>
<tr>
<td>BMLRU 4</td>
<td>Pink</td>
<td>Colorless</td>
</tr>
<tr>
<td>BMLRU 5</td>
<td>Pink</td>
<td>Yellow</td>
</tr>
<tr>
<td>BMLRU 6</td>
<td>Colorless</td>
<td>Greenish blue</td>
</tr>
<tr>
<td>BMLRU 7</td>
<td>Colorless</td>
<td>Whitish</td>
</tr>
<tr>
<td>BMLRU 8</td>
<td>Pale pink</td>
<td>Colorless</td>
</tr>
<tr>
<td>BMLRU 9</td>
<td>no growth</td>
<td>Whitish</td>
</tr>
<tr>
<td>BMLRU</td>
<td>Biotechnology and Microbiology</td>
<td>Laboratory Rajshahi University</td>
</tr>
</tbody>
</table>

**Antibiotic susceptibility test**

Only 6 out of 9 isolates were found resistant to antibiotics (Table 2). The resistant rate to azithromycin 44.44 % (4/9), ciprofloxacin 77.78 % (7/9), erythromycin 66.67 % (6/9) and tetracycline 100 % (9/9) was observed among 9 isolates. The resistance rate was highest to tetracycline (100%) and lowest to azithromycin 44.44 %.

**Identification of resistant bacteria**

By microscopically characteristics and biochemical studies, total six isolates were identified viz. *Escherichia coli*, *Yersinia enterocolitica*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Salmonella* sp. and *Staphylococcus aureus*. The results are summarized in Table 3.

**Table 2.** Antibiotic resistant profile of 6 isolates.

<table>
<thead>
<tr>
<th>Bacterial isolates</th>
<th>AZM</th>
<th>CIP</th>
<th>E</th>
<th>TE</th>
</tr>
</thead>
<tbody>
<tr>
<td>BMLRU 1</td>
<td>S (19)</td>
<td>R (14)</td>
<td>R (12.5)</td>
<td>R (13.5)</td>
</tr>
<tr>
<td>BMLRU 2</td>
<td>R (13)</td>
<td>R (12.5)</td>
<td>R (12)</td>
<td>R (12)</td>
</tr>
<tr>
<td>BMLRU 4</td>
<td>S (20.5)</td>
<td>I (17.5)</td>
<td>I (18.5)</td>
<td>R (13)</td>
</tr>
<tr>
<td>BMLRU 6</td>
<td>R (12)</td>
<td>R (13)</td>
<td>I (20)</td>
<td>R (12)</td>
</tr>
<tr>
<td>BMLRU 7</td>
<td>S (13)</td>
<td>R (14)</td>
<td>R (12.5)</td>
<td>R (13)</td>
</tr>
<tr>
<td>BMLRU 9</td>
<td>R (18.5)</td>
<td>R (14.5)</td>
<td>R (12)</td>
<td>R (12.5)</td>
</tr>
</tbody>
</table>

AZM- azithromycin, CIP- ciprofloxacin, E- erythromycin, TE- tetracycline, R- Resistant, I- Intermediate susceptible; S- Susceptible

**Discussion**

Enteric diseases are a major cause of morbidity & mortality in poor & developing countries (Kumari and Ambasta, 2013). AAD is a major health problem (Ackermann et al., 2005). The resistance of enteric pathogens to currently used antimicrobial agents has increased the world over as a result of the widespread use of antimicrobials (Sang et al., 2012). From this research identified resistant bacteria were *E. coli*, *Y. enterocolitica*, *Salmonella* sp., *K. pneumoniae*, *P. aeruginosa* and *S. aureus*. Several studies have been conducted with a view to establish the importance of different enteric bacteria in the etiology of acute diarrhea. It has been reported that *K. pneumoniae*, and *S. aureus* were isolated from the cultures of AAD stool (Song et al., 2008). In the patients with AAD *E. coli*, *Salmonella* sp. and *Klebsiella* sp. were observed by Hovius and Rietra (1982), Hogenauer et al. (1998) and Bartlett (2002). Brad et al. (2011) reported that *P. aeruginosa*, *Y. enterocolitica*, *Salmonella* sp., *E. coli* are the responsible for AAD. Vaishnavi et al. (2008); Boyce et al. (2005), Ackermann et al. (2005) and Gravet et al. (1999) also isolated *S. aureus*
bacteria from AAD stool samples. Ayyagari et al. (2003) demonstrated that S. aureus, Salmonella spp. are responsible for AAD. In one report, multidrug resistant Salmonella newport (resistance to ampicillin, carbenicillin and tetracycline) has been linked with AAD (Holmberg et al., 1984). Only 10%-20% of all cases of AAD are caused by infection with Clostridium difficile (Hogenauer et al., 1998; Brad et al., 2011; Fekeyt, 1997; Kelly et al., 1994; Katz et al., 1996). Culture-based methods provide an incomplete picture of the various microbial populations of the gut because many bacteria are difficult to culture. Multiple laboratories have reported that only 10-20% of stool specimens are positive with Clostridium difficile toxin testing (Kelly et al., 1994; Gorenek et al., 1999; Hull and Beck, 2004; Wilcox, 2003).

From this finding it can be concluded that our data will help to proper treatment of AAD and reduces prolonged hospital stays and additional costs.

Table 3. Morphological feature and Biochemical test results.

<table>
<thead>
<tr>
<th>Bacterial Isolates</th>
<th>Cell Shape</th>
<th>Gram stain</th>
<th>CA</th>
<th>OX</th>
<th>NIT</th>
<th>MO</th>
<th>UR</th>
<th>MR</th>
<th>VP</th>
<th>IN</th>
<th>CIT</th>
<th>H2S</th>
<th>Identified bacteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>BMLRU 1</td>
<td>Rod</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>E. coli</td>
</tr>
<tr>
<td>BMLRU 2</td>
<td>Rod</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>Y. enterocolitica</td>
</tr>
<tr>
<td>BMLRU 4</td>
<td>Rod</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>K. pneumoniae</td>
</tr>
<tr>
<td>BMLRU 6</td>
<td>Rod</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>ND</td>
<td>ND</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>P. aeruginosa</td>
</tr>
<tr>
<td>BMLRU 7</td>
<td>Rod</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>Salmonella sp.</td>
</tr>
<tr>
<td>BMLRU 9</td>
<td>Coccus</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>ND</td>
<td>ND</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>S. aureus</td>
</tr>
</tbody>
</table>

CA = Catalase, OX = Oxidase, NIT = Nitrate, MO = Motility, UR = Urease, MR = Methyl red, VP = Voges-Proskauer, IN = Indole, CIT = Citrate, H2S = Hydrogen sulfide production, + = Positive, - = Negative, ND = Not determined.

Acknowledgement
The authors would like to thank the Department of Pathology, Rajshahi medical college hospital, Rajshahi, Bangladesh, for collecting samples.

References


