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

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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 (Abeysinghe 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 37°C 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) H_2S production.

Results

Counting and Isolation

The highest number of colony was recorded 54±0.33 cfuplate^{-1}. In the present investigations, total 9 types of bacterial colonies were isolated from 45 samples (Table 1).

Table 1. Selected bacterial isolates.

Bacterial isolates	Colony morphology on MacConkey agar	Colony morphology on Nutrient agar
BMLRU 1	Pink	Whitish
BMLRU 2	Pale pink	Colorless
BMLRU 3	Whitish	Pale yellow
BMLRU 4	Pink	Colorless
BMLRU 5	Pink	Yellow
BMLRU 6	Colorless	Greenish blue
BMLRU 7	Colorless	Colorless
BMLRU 8	Pale pink	Whitish
BMLRU 9	no growth	Whitish

BMLRU = Biotechnology and Microbiology Laboratory Rajshahi University

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.

Bacterial isolates	AZM	CIP	E	TE
BMLRU 1	S (19)	R (14)	R (12.5)	R (13.5)
BMLRU 2	R (13)	R (12.5)	R (12)	R (12)
BMLRU 4	S (20.5)	I (17.5)	I (18.5)	R (13)
BMLRU 6	R (12)	R (13)	I (20)	R (12)
BMLRU 7	S (13)	R (14)	R (12.5)	R (13)
BMLRU 9	R (18.5)	R (14.5)	R (12)	R (12.5)

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 dificile* 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.

Bacterial Isolates	Cell Shape	BIOCHEMICAL TEST											Identified bacteria
		Gram stain	CA	OX	NIT	MO	UR	MR	VP	IN	CIT	H_2S	
BMLRU 1	Rod	-	+	-	+	+	-	+	-	+	-	-	*E. coli*
BMLRU 2	Rod	-	+	-	+	-	+	+	-	+	-	-	*Y. enterocolitica*
BMLRU 4	Rod	-	+	-	+	-	+	-	+	-	+	-	*K. pneumoniae*
BMLRU 6	Rod	-	+	+	+	+	+	-	+	+	+	-	*P. aeruginosa*
BMLRU 7	Rod	-	+	-	+	+	-	+	-	-	+	+	*Salmonella* sp.
BMLRU 9	Coccus	+	+	-	ND	ND	+	-	-	-	+	-	*S. aureus*

CA = Catalase, OX = Oxidase, NIT = Nitrate, MO = Motility, UR = Urease, MR = Methyl red, VP =Voges-Proskauer, IN = Indole, CIT = Citrate, H_2S = Hydrogen sulfide production, + = Positive, - = Negative, ND = Not determined.

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