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Original Article

Widespread Occurrence of Possible Antibiotic Efflux Mechanism in Multidrug Resistant Intestinal *E. coli* Strains Isolated from Healthy Human Subjects in Bangladesh

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A total of 50 isolates of *Escherichia coli* obtained from healthy adult human subjects were studied and tested for possible presence of efflux mechanism in resistance determination and possible correlation of plasmids with resistance.Minimal inhibitory concentration (MIC) of the antibiotics amoxicillin, azithromycin, ciprofloxacin, chloramphenicol and tetracycline were determined by agar dilution method with or without the H^+/K^+ proton pump inhibitor omeprazole. Plasmids were extracted by rapid alkaline plasmid extraction method and analyzed by agarose gel electrophoresis. Many strains showed 5 – 10 fold reduction of MIC values in the presence of omeprazole; a few strains showed up to 100-fold MIC reduction. Plasmid analysis of these 50 isolates revealed the presence of both plasmidless and plasmid containing strains, the latter with plasmid number varying from one to seven. However, the plasmids apparently had no relationship with high level antibiotic tolerance as indicated by the observation that some plasmidless strains had very high MIC values, while other strains containing several plasmids had very low MIC. Decrease in MIC in the presence of omeprazole apparently indicates existence of an efflux mechanism. Evidence of the efflux of ethidium bromide was noted in some strains that had been grown in ethidium bromide containing agar plate with and without omeprazole. These results suggest that reduction of MIC caused by omeprazole may be related to possible inhibition of efflux pump activity by omeprazole in the isolates studied.

Key words: Efflux mechanism, multidrug resistance, *E. coli*

Introduction

Soon after the widespread use of antibiotics began in clinical practice during the 1940s bacteria resistant to antibiotics began to emerge in increasing frequency, and in subsequent years the problem became truly worrisome. Studies led to identification of several mechanisms of resistance that included enzymatic inactivation of antibiotic, barrier to entry of antibiotic into the cell and modification of the molecular target of antibiotic in host bacteria. Later in the early 1990s, a pioneering study led to the discovery of a novel mechanism involving efflux of antibiotic by bacteria; tetracycline resistant bacteria were found to possess a molecular pump which when activated could efficiently pump out tetracycline[1]. Since then efflux mechanism continued to be reported in different species of bacteria. Some bacteria were resistant to several different antibiotics. The multiple drug resistance (MDR) character resulted from accumulation of several resistance genes usually in mobile genetic elements such as plasmids and transposons[2]. Later, efflux pumps structurally complex and functionally versatile were found that could expel simultaneously several different antibiotics resulting in MDR phenotype. Genes encoding the proteins of these pumps and their regulatory elements were located in plasmids, conjugative or transformable transposons and integrons. Many excellent reviews are available on molecular basis of MDR phenomenon in bacteria[2-3].

Many clinical isolates of *Escherichia coli* are multidrug resistant and they also carry many plasmids that play no obvious role in antibiotic resistance. Such cryptic plasmids are also common in environmental *E. coli* isolates and also in isolates obtained from intestine of healthy human subjects where they occur as part of the normal enteric bacterial flora. We examined 50 *E. coli* isolates obtained from stool specimen of adult healthy human subjects. Antibiotic sensitivity data and plasmid profile analysis of the isolates failed to show any correlation between plasmids and high MIC values. We then examined possible basis of antibiotic resistance in these bacteria by using a proton pump inhibitor as

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to its effect on reducing MIC *in vitro*. Our preliminary study demonstrated the existence and widespread occurrence of antibiotic efflux mechanism in intestinal *E. coli* in healthy human subjects related to high level antibiotic tolerance *in vitro.* To the best of our knowledge no study has so far been reported from Bangladesh on the existence antibiotic efflux mechanism in intestinal *E. coli*.

One well studied efflux pump is the H^+/K^+ proton pump which expels out H^+ from mammalian parietal cells in stomach lining. This acidifies the stomach fluid that helps in digestion, but over-expression of the pump can also lead to adverse clinical conditions. The H^+/K^+ proton pump is a membrane bound ATPase activity of which the drug omeprazole is a potent inhibitor. Omeprazole is also known to inhibit other molecular efflux pumps in bacteria such as the NorA pump involved in efflux of the quinolone antibiotic norfloxacin[4-5]. In this communication we report preliminary results on potential efflux mechanism that expels unrelated antibiotics in intestinal *E. coli* isolates.

Materials and Methods

Isolation and identification of intestinal E. coli

Bacterial strains were isolated from 50 healthy individuals from Dhaka aged 23 – 27, who volunteered to participate in the study and agreed to self-collect stool samples. Cases with intestinal ailment and ongoing antibiotic treatment were excluded. Stool sample was diluted up to 10^{-5} and 100 ml plated onto MacConkey agar plates to obtain well separated colonies. Suspected *E. coli* colonies were picked and identified by conventional biochemical tests according to Bergey's Manual of Systematic Bacteriology[6]. The isolates were stored in 15% glycerol broth at –20ºC; working cultures were maintained in nutrient agar slant in the refrigerator at 4ºC.

MIC and efflux assay of the antibiotics with proton pump inhibitor

MIC assay was done by agar dilution method[7]. Presence of possible efflux mechanism was determined by decrease in MIC in resistant strains in the presence of omeprazole, an inhibitor of H^+/K^+ proton pump. For this purpose strains were grown for 18 h at 37ºC and 100 ml volumes were spotted onto antibiotic-containing nutrient agar plates with or without 100 mg/ml omeprazole. Bacterial growth was observed after overnight incubation of the plates at 37ºC.

Ethidium bromide efflux assay

Efflux of ethidium bromide was tested in a few strains by ethidium bromide (EB) cartwheel procedure[8]. Freshly grown colonies were swabbed (using sterilized cotton swab) on nutrient agar plates containing 1.5 mg/ml ethidium bromide (control) and in plates with ethidium bromide and omeprazole (100 mg/ml). The plates were incubated overnight at 37ºC and observed under UV light for orange fluorescence.

Plasmid extraction

Plasmids form all the 50 strains were isolated by the rapid alkaline denaturation method[9]. Crude plasmid extracts were electrophoresed in a 0.7% agarose gel containing ethidium bromide (0.5 mg/ml) and observed under UV illumination.

Results

MIC decrease in the presence of omeprazole

MIC with or without omeprazole was determined by agar dilution method using all the 50 isolates. For this experiment, five different antibiotics,namely amoxicillin (β-lactam), azithromycin (macrolide), ciprofloxacin (quinolone), chloramphenicol (phenicols) and tetracycline (aromatic acetogenin). The results of this experiment are presented in Table 1. Data obtained with ciprofloxacin are not included in the table, because out of the 50 strains tested 38 strains had low MIC (1 µg/ml or less). The remaining 12 strains had MIC ranging from 20-100 µg/ml, but none of these strains showed significant decrease in MIC (more than 10-fold lower compared to control) after treatment with omeprazole. With the four other antibiotics significant decrease in MIC was found in many strains which varied from 5 to 100-fold decrease compared to controls depending on strains and antibiotics. We considered MIC decrease of 10-fold or more as indicating strong efflux activity. These strains are indicated in table 1 in boldface. With the antibiotic azithromycin 100-fold decrease in MIC was found in 4 strains and 20-fold in 13 strains. With amoxicillin and chloramphenicol, 1 strain each showed 100-fold decrease in MIC. However, with tetracycline no strain was found exhibiting MIC drop to this extent; only 2 strains showed significant MIC reduction (one strain 50-fold and the other 10-fold reduction). Data in table 1 indicates that among the 50 strains 11 strains showed simultaneous high level (200 µg/ml) antibiotic tolerance to 3-4 antibiotics. In the case of ciprofloxacin similar level of resistance was not found in any of the 50 strains tested (data not shown).

Plasmid profile and antibiotic sensitivity

Analysis of plasmid in 50 strains showed sixteen different plasmid profiles (Figure 1). From the results it appears that there is no correlation between the level of antibiotic tolerance and plasmid content. There were strains containing no detectable plasmids such as strain 14b but these strains gave high MIC values for several antibiotics, conversely in many strains carrying multiple plasmids the MIC values were low. The results would support the view that genetic determinants for efflux of antibiotics are probably located in the chromosome, not in extra-chromosomal elements.

Table 1. *Effect of omeprazole on MIC reduction in intestinal E. coli isolates.Isolates in which omeprazole (100 mg/ml) caused at least ten-fold reduction in MIC compared to control (without omeprazole) of the antibiotics amoxicillin, azithromycin, chloramphenicol and tetracycline are highlighted using bold text.*

Strain ID	MIC (µg/ml) Amoxicillin		MIC (µg/ml) Azithromycin		MIC (µg/ml) Chloramphenicol		MIC (µg/ml) Tetracycline	
	Control	Omeprazole	Control	Omeprazole	Control	Omeprazole	Control	Omeprazole
1b	200	200	10	10	20	2	10	2
2c	2	2	10	10	2	2	2	2
11a	200	200	>200	200	100	2	>200	200
11b	200	200	200	200	100	2	>200	200
12a	200	200	50	10	20	2	>200	100
12b	200	200	10	10	50	2	>200	100
13a	200	200	>200	200	200	10	>200	100
14a	200	200	20	10	10	2	2	2
14b	200	200	100	10	20	20	200	200
15a	200	200	50	10	20	2	200	100
15b	200	200	50	10	20	2	100	100
16b	200	200	>200	200	20	2	200	100
17a	200	200	>200	10	20	2	200	100
17b	200	200	>200	10	200	2	200	100
18a	200	10	200	10	10	2	2	2
18b	200	200	200	10	10	2	200	100
18c	200	200	200	10	20	2	200	100
19b	50	2	>200	>200	10	2	20	20
20a	200	2	50	2	10	2	10	2
22b	200	200	>200	>200	20	2	10	2
22c	200	200	>200	>200	20	2	10	10
26a	200	100	>200	10	10	2	200	100
26b	200	100	>200	10	20	2	50	50
27a	200	10	>200	10	10	2	10	2
28a	200	20	>200	2	20	2	10	2
30c	200	20	>200	>200	20	2	10	10
32a	>200	>200	>200	2	10	2	200	100
32b	>200	>200	>200	>200	10	2	10	2
33a	10	2	>200	2	10	2	10	2
33c	20	2	>200	2	10	2	200	100
35a	20	2	>200	10	10	2	2	2
35c	200	100	50	10	20	2	10	2
36a	200	200	>200	20	20	2	10	2
37a	10	2	>200	10	20	2	100	100
38c	20	10	>200	10	10	2	100	2
39a	20	2	50	10	10	2	20	2
40a	20	20	50	10	10	2	200	100
41a	20	2	50	10	10	2	2	2
42a	20	2	50	10	20	2	2	2
42b	20	10	100	10	10	2	2	2
43a	20	10	50	10	10	2	10	2
44c	20	20	20	10	10	2	2	2
45b	>200	>200	>200	10	10	2	10	2
46a	>200	>200	>200	10	10	2	200	100
46b	50	2	50	20	20	2	2	2
47b	20	2	20	10	10	2	2	2
48a	2	2	20	20	20	2	2	2
50a	20	10	20	10	10	2	2	2
50b	20	10	20	10	10	2	2	2
50c	2	2	20	10	10	2	2	2

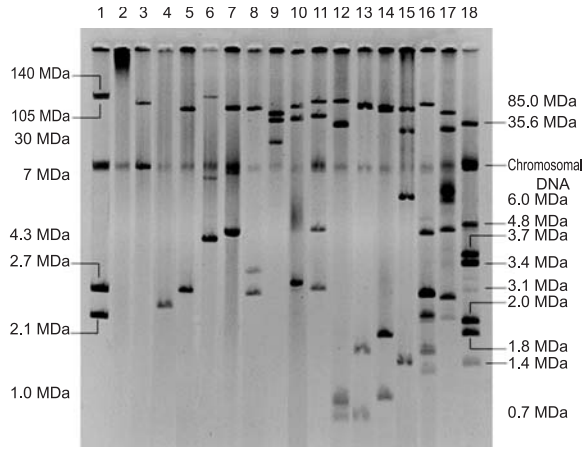

Figure 1. *Plasmid profiles of intestinal isolates of E. coli obtained from healthy adult human subjects. Lane 1 and 18 represent plasmid DNA markers obtained from E. coli K-12 strains PDK-9 and V-157, respectively. Lane 2 represents 13 plasmidless E. coli of both all-sensitive and multidrug resistant isolates. Lane 3 and 4 show single plasmid of ~85 MDa shared by seven isolates and ~ 2.5 MDa shared by a single isolate respectively. Lane 5 was shared by five isolates containing two plasmids with molecular weight of ~ 2.7 and 60 MDa. Lane 6 - 10 represent 1, 3, 3, 2 and 2 isolates, each of those harbored three plasmids with various molecular weight ranges from ~ 2.7 to 120 MDa. Lane 11 represents a single isolate carrying four plasmids, sizes ranges from ~ 2.8 to 80 MDa. Lane 12 – 15 represent 1, 5, 1 and 1 isolates respectively, each carrying four plasmids ranges from ~ 0.7 to 85 MDa. Lane 16 represents a single isolate carried seven plasmids, band size ranges from ~ 1.3 to 85 MDa. Lane 17 shared by 3 isolates, carried seven plasmids with size ranges from ~ 2 to 60 MDa. Plasmid profiles of lane 4, 7, 10, 11 and 16 are shared by all-sensitive isolates while plasmid profiles of lane 3, 5, 6, 8, 9, 12, 13, 14, 15 and 17 represent E. coli isolates of diverse antibiogram pattern.*

Ethidium bromide efflux

We examined efflux of ethidium bromide (EB) in a few strains. At relatively high concentration of ethidium bromide (1.5 mg/ml) some strains failed to produce the characteristic intense orange fluorescence of ethidium bromide suggesting possible efflux of ethidium bromide from the cell. However, when these strains were grown in the presence of omeprazole strong fluorescence was observed indicating possible inhibition of the efflux process by omeprazole. Strain 33a in Figure 2 was an example where no fluorescence was seen in the absence of omeprazole (plate A) but strong fluorescence was seen in the corresponding omeprazole containing plate (plate B). There were strains which did not show this effect of omeprazole such as seen strains 44c and 45b in Figure 2. We did not, however, examine in this study the correlation between omeprazole induced efflux inhibition of ethidium bromide and MIC drop of antibiotics. Since we included a large number of strains and five different antibiotics and sensitivity of the ethidium bromide assay varies with strains we did not attempt to refine the assay conditions for all strains and antibiotics and find out the correlation. However, it would be of interest to obtain this information.

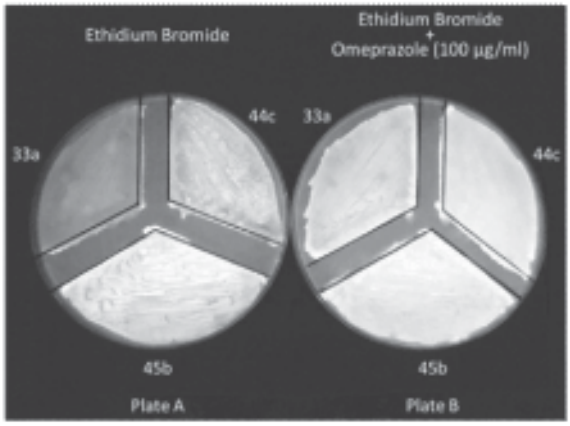

Figure 2. *Apparent efflux of ethidium bromide by omeprazole in a strain of E. coli (strain 33a). Both the plates contain 1.5 mg/ml ethidium bromide. Plate A is the control plate without omeprazole and plate B contains omeprazole (100 mg/ml). The strains 44c and 45b apparently lack strong efflux ability thus showing the same level of ethidium bromide accumulation independent of omeprazole.*

Discussion

Ability of pathogenic bacteria to expel antibiotics by using highly efficient molecular pump is now well recognized as a significant mechanism for antibiotic resistance that severely limits use of antibiotics in the treatment of infectious diseases. It is known that bacteria have the ability to accumulate different antibiotic resistance genes in efficient mobile unit called integron that confers both multidrug resistance trait to the host bacteria and ensures efficient mobility of the resistance gene cluster across bacterial species. Also many bacteria have been found to carry functionally versatile molecular pumps that simultaneously expel multiple antibiotics from the cell[2-3].

We examined 50 non-pathogenic *E. coli* isolates from healthy human subjects for possible efflux potential exploiting the well-known H^+/K^+ proton pump inhibitor omeprazole. This agent is also known to inhibit other molecular efflux pumps such as the NorA pump that expels quinolone antibiotics from bacterial cells[4-5].

Results presented in table 1 indicated that omeprazole caused significant reduction in MIC of different antibiotics in many of these isolates. It was determined that omeprazole used in concentration of 100 µg/ml had no effect on bacterial growth and there was no indication that the substance significantly inactivated the antibiotics or prevented antibiotic entry into the cell. The MIC depression was particularly pronounced in some isolates with some antibiotics. The reduction would indicate active

efflux of the antibiotic in the absence of omeprazole, and that presence of omeprazole significantly inhibited efflux with concomitant decrease in MIC.

Efflux of ethidium bromide was tested in some strains. Some strains showed no fluorescence at relatively high EB concentration (1.5 µg/ml) indicating absence of EB accumulation which could be due to a possible efflux mechanism in operation because the strain showed high level EB accumulation and consequently intense fluorescence in the presence of omeprazole (Figure 2). The lack of EB accumulation in cells grown in the absence of omeprazole and significant accumulation of EB in the presence of omeprazole, would suggest that the observed MIC depression in many of the strains (Table 1) may be related to inhibition of an efflux pump by the proton pump inhibitor omeprazole.

It is known that over-expression of resistance-nodulation-cell division (RND)-type of efflux pump is correlated to resistance to quinolone, b-lactam, tetracycline and chloramphenicol resistance[10-12]. Results of this preliminary study suggest that non-pathogenic strains of intestinal *E. coli* also carry varying abilities to expel important clinically relevant antibiotics. Further study on the extent of efflux mechanism in pathogenic microbial flora in Bangladesh and molecular basis of the potential efflux phenomena and its inhibition would be an interesting line of inquiry both from scientific and clinical perspectives.

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