British Microbiology Research Journal
14(6): 1-8, 2016, Article no.BMRJ.25725
ISSN: 2231-0886, NLM ID: 101608140

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Diversity of Plasmid Profile in Multi-drug Resistant Non- *E. coli* Intestinal Flora Lacking Association with Resistance Phenomenon

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Authors' contributions

This work was carried out in collaboration between all authors. Authors MHR and NJ designed the study, performed the statistical analysis, wrote the protocol, wrote the first draft of the manuscript and managed literature searches. Authors NJ, JM and FA managed the data acquisition of the study and literature searches. Author SI did critical revision. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/BMRJ/2016/25725
Editor(s):
(1) Grzegorz Cieslar, Department and Clinic of Internal Diseases, Angiology and Physical Medicine, Medical University of Silesia, Poland.
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Complete Peer review History: http://sciencedomain.org/review-history/14674

Original Research Article

Received 17th March 2016
Accepted 9th May 2016
Published 17th May 2016

ABSTRACT

Aims: To study the plasmid diversity of intestinal non- *Escherichia coli* community, describe their potential as a reservoir of antibiotic resistance phenomenon and to find any association of resistance phenomenon with the plasmid content if any.
Place and Duration of the Study: Department of Microbiology, Jahangirnagar University, Dhaka between June 2014 to July 2015.
Methods: Stool samples were cultured onto MacConkey agar plates for selective isolation of Gram negative bacteria. Identification of intestinal non- *E. coli* was done through conventional

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biochemical tests according to Bergey's Manual of Systematic Bacteriology. Plasmid extraction was done according to the modified hot alkaline method. Antibiogram was carried out by disc diffusion method and MIC determination by agar dilution assay.

Results: One hundred and forty four representative colonies were selected from stool culture of 60 healthy human subjects. Of 144 colonies, 90 belonged to 21 non- *E. coli* species shared by 11 genus. Majority of the isolates (81%, 73 of 90) showed resistance to one or more antibiotics while the rest 18.9% (17 of 90) were sensitive to all the antibiotics used (amoxicillin, cefixime, ciprofloxacin, gentamicin, sulfamethoxazole-trimethoprim). Highest resistance frequency was observed against amoxicillin (67%) followed by cefixime (28%), gentamicin (24%), sulphamethoxazole–trimethoprime (16%), tetracycline (14%), and ciprofloxacin (8%). A notable proportion of the bacteria (20%) were found to be multi-drug resistant. Majority of the isolates (57%) were found to contain 1 to 6 plasmids and exhibited 34 different plasmid profiles. Thirty two isolates (of 90) carried plasmids with high molecular weight (> 20 Mdal) including 4 isolates that carried large plasmids of approximately 140 Mdal size.

Conclusion: Findings of this study indicate that intestinal non- *E. coli* population may function as a reservoir of heterogeneous plasmid. Although no direct association was observed between resistance phenomenon and presence of plasmids in general, to study the role(s) of individual plasmid for encoding any particular drug resistance phenomenon and /or pathogenic gene(s) would be an interesting line of inquiry.

Keywords: Plasmid diversity; multi-drug resistance; intestinal flora.

1. INTRODUCTION

Clinical use of antibiotics perhaps exerts selection pressure that causes clustering of the resistance genes [1]. Although plasmids have been directly implicated in the acquisition of resistance to many antibiotics [2-6], several other mechanisms, such as blocking of antibiotic entry, efflux mechanism, enzymatic inactivation of antibiotics, target site alteration, and bypass mechanism etc. have been discovered [7]. Plasmids can cross many species and genus barriers, and the rate of transfer has even been shown to increase in more heterogeneous communities [8]. Plasmids thus allow resistance to spread and persist in niches that are not necessarily subject to antibiotics [9]. Although many enteric bacteria carry plasmid mediated drug resistance, the presence is not mandatory for resistance against any particular antibiotic.

As normal flora are considered non-pathogenic and their role as a reservoir of pathogenic and / or resistance genes is generally overlooked, this population remains unexplored. For this reason, limited information is available on normal intestinal flora associated antibiotic resistance in Bangladesh. Most of the studies are focused on *E. coli* associated antibiotic resistance of clinical or environmental isolates [10,11,12]. Recently, prevalence of resistance against therapeutically important antibiotics has been reported in normal enteric organisms and *E. coli* has been extensively studied for the presence of plasmids and correlation between plasmid content and drug resistance [13]. Coliform bacteria other than *E. coli* are frequently found in gut and often exhibit multidrug resistance [14-16]. This heterogeneous population is believed to participate in genetic exchange during their passage through the intestine [17-20]. Our objective was to study the plasmid diversity of non- *E. coli* community, describe their potential as a reservoir of antibiotic resistance gene. We also wanted to see the association of the resistance phenomenon of the isolates with their plasmid content.

2. MATERIALS AND METHODS

2.1 Isolation and Identification of Intestinal Non- *E. coli*

Human individual aged 22-25 years adults from student dormitories of Jahangirnagar University were randomly included in the study who volunteered to participate and agreed to self-collect the stool sample. Intestinal complications and ongoing antibiotic treatments were considered exclusion criteria for the participants. Small aliquots of (100 µL) of the diluted stool samples were plated onto MacConkey agar plates to obtain isolated and discrete colonies. After pure culture of the colonies onto fresh Nutrient agar plates, the isolates were identified by conventional biochemical tests according to Bergey's Manual of Systematic Bacteriology [21] and Cappuccino & Sherman [22]. The bacterial cultures were preserved at -80℃ with glycerol broth for further analyses.

2.2 Antimicrobial Assay

Antimicrobial susceptibility test of non- *E. coli* isolates were performed by agar disc diffusion method [23] followed by determination of MIC by agar dilution assay and interpreted according to CLSI standards and guidelines [24]. To observe their resistance pattern the isolates were subjected to susceptibility test against six different groups of antibiotics. The groups of antibiotics included penicillin (amoxicillin, AML^{10}); cephalosporin (cefixime, CFM^{5}); tetracycline (tetracycline, TE^{30}), aminoglycosides (gentamicin, CN^{10}); fluoroquinolone (ciprofloxacin, CIP^{5}); sulphonamide (sulphamethoxazole-trimethoprim, SXT^{25}).

2.3 Plasmid Extraction

Plasmids from the diverse Gram-negative population were extracted according to the modified hot alkaline method by Kado & Liu [25], electrophoresed in a 0.7% agarose gel containing ethidium bromide (0.5 μg/mL) and observed under UV illumination. *E. coli* V517 and PDK9 were used as control strains for the extraction method and plasmid size marker [26,27].

3. RESULTS

3.1 Diverse Gram Negative Bacteria Belongs to Intestinal Microbial Community

Stool samples from 60 healthy individual were cultured onto MacConkey plate to isolate gram negative normal bacterial flora from intestine. A total of 144 representative colonies were selected for pure culture and preserved at both 4℃ and -80℃ in parallel. After biochemical characterization of the isolates, 54 were identified as *E. coli* while the rest 90 isolates belonged to 21 non- *E. coli* species shared by 11 genus (data not shown).

3.2 Resistant Bacteria against Therapeutically Important Antibiotics Constitute the Major Proportion of Intestinal non- *E. coli* Community

All 90 non- *E. coli* isolates were screened for their resistance against commonly used antibiotics belonging to six different groups. The antibiotics included amoxicillin, cefixime, tetracycline, gentamicin, ciprofloxacin, and sulphamethoxazole-trimethoprim. Out of 90 non-*E. coli* intestinal isolates above 81% (n =73) showed resistant to at least one of the tested antibiotics. Table 1 shows the resistance percentage against individual antibiotics and range of MIC displayed by the resistant isolates in general. Highest percentage of resistance phenotype was observed against amoxicillin (67 %) followed by cefixime (28%), gentamicin (24%), sulphamethoxazole–trimethoprime (16%), tetracycline (14%), and ciprofloxacin (8%).

3.3 Prevalence of Multiple Antibiotic Resistance (MAR) in Non- *E. coli* Population of Intestinal Bacteria

A large proportion of intestinal Gram-negative bacteria (34%; 31 of 90) showed resistance against three or more groups of antibiotics. The predominant MAR phenotypes for the isolates were AML-CFM-CN, AML-SXT-TE and AML-CFM-CIP-CN observed in 11%, 8% and 4% of the isolates, respectively. MAR phenotype AML-CN-TE was detected in 3% of the isolates. Another two MAR phenotypes with resistance against three antibiotics were AML-CFM-TE and AML-CIP-SXT, observed in single isolates. MAR phenotype AML-CFM-SXT-TE and AML-CIP-SXT-TE were also detected in single isolates. Moreover, MAR phenotypes with resistance against different combinations of five antibiotics AML-CFM-CIP-CN-TE, AML-CFM-CIP-SXT-TE and AML-CIP-CN-SXT-TE were also carried by individual isolates (for each phenotype 1 in 90). Table 2 shows multiple antibiotic resistance phenotypes observed in intestinal flora.

3.4 Plasmid Profile of Intestinal Non- *E. coli* Isolates

Plasmid extraction revealed that 57% (51 of 90) were found to contain plasmid with 34 different patterns (Fig. 1). Each DNA band in the gel was considered to represent one plasmid. Number of bands varied from one to six. A notable proportion of the isolates (36%; 32 of 90) carried plasmids with high molecular weight (> 20 Mdal). Four isolates (of 90; 4.4%) carried a large plasmid of approximately 140 Mdal size. The Table 3 shows plasmid patterns, plasmid contents of each pattern with their approximate molecular weight as shown in Fig. 1.

Table 1. Resistance frequency and MIC range of non- *E. coli* intestinal flora against therapeutically important antibiotics (n=90)

Antibiotic	AMX	CFM	CN	SXT	TE	CIP
Percent resistant	**67**	**28**	**24**	**16**	**14**	**8**
MIC range (µg/ml) of the resistant isolates	≥20 to ≥ 200	≥ 10 to ≤ 40	≥10 to ≥ 20	≥ 41.5/8.5 to >166/34	≥20 to ≥ 100	≥10 to ≥ 20

AMX, Amoxicillin; CFM, Cefixime; CIP, Ciprofloxacin; CN, Gentamicin; SXT, sulphamethoxazole–trimethoprime; TE, Tetracycline

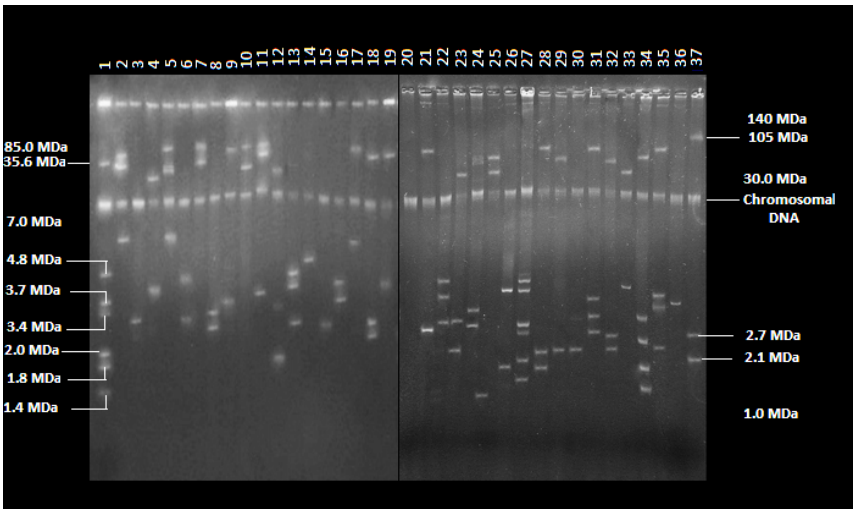

Fig. 1. Plasmid profiles of intestinal isolates of non- *E. coli* obtained from healthy human subjects

Lane 1 and 37 represent plasmid DNA markers obtained from E. coli K-12 strains V-517 and PDK-9. Lane 2-19 and Lane 21-36 represents 34 different plasmid profile exhibited by 90 non- E. coli isolates obtained from healthy human subject. Lane 20 represents 39 plasmidless non- E. coli shared by both all-sensitive and multidrug resistant isolates. Plasmid profiles of lane 3,11,12,13,21,27,28 and 31 are shared by all-sensitive isolates while plasmid profiles of remaining lanes represent non- E. coli isolates of diverse antibiogram pattern.

Table 2. Multiple antibiotic resistant phenotypes detected in gram-negative intestinal bacteria (n=90) from healthy human volunteers

Phenotype	No of isolate (n)	Percentage (approx.)
AML-CFM-CN	10	11
AML-CFM-TE	01	1
AML-CIP-SXT	01	1.
AML-CN-TE	03	3
AML-SXT-TE	07	8
AML-CFM-CIP-CN	04	4
AML-CFM-SXT-TE	01	1
AML-CIP-SXT-TE	01	1
AML-CFM-CIP-CN-TE	01	1
AML-CFM-CIP-SXT-TE	01	1
AML-CIP-CN-SXT-TE	01	1

4. DISCUSSION

The widespread and inappropriate use of antibiotics results in the development of a progressively antibiotic resistant microbial ecosystem in Bangladesh [28,29]. We studied 90 non- *E. coli* intestinal isolates and describe their resistance frequency against therapeutically important antibiotics to get an indication of the future risk and challenge for combating infectious diseases in Bangladesh. In this study, highest occurrence of resistance phenotype was observed against amoxicillin, followed by cefixime, getamicin, sulphamethoxazole-trimethoprime, tetracycline and ciprofloxacin. Antibiotics are most frequently prescribed for acute respiratory tract infections, acute watery diarrhoea, acute trauma and gastrointestinal symptoms in Bangladesh [30]. Ceftriaxone, cefixime and amoxicillin are topping the list of

frequently prescribed antibiotics in our country [31]. This probably explains why cefixime and amoxicillin resistance gene accumulated in intestinal flora. In a previous study with intestinal *E. coli* from healthy adults, highest resistance was found against amoxicillin followed by tetracycline, sulfamethoxazole-trimethoprim, gentamicin, ciprofloxacin and cefixime [13]. This disagreement in resistance frequency suggests that antimicrobial resistance of non- *E. coli* population may differ with *E. coli* population.

Plasmid extraction revealed that 57% (51 of 90) of the non- *E. coli* isolates were found to contain plasmid with 34 different profiles. The occurrence of plasmids in non- *E. coli* community is a bit lower in comparison with the *E. coli* population of intestinal normal flora according to our previous report that showed that 74% (34 of 50) of the later population carried plasmid [13]. A notable proportion of the non- *E. coli* population (36%; 32 of 90) carried large plasmid (> 20Mdal). However, occurrence of large plasmid was reported to be much higher (60%; 30 of 50) in *E. coli* population of healthy human volunteer (data not shown) [13]. Four isolates (of 90; 4%) carried a large plasmid of approximately 140 Mdal size indicating possible presence of large virulence plasmid. Large plasmids present in the family enterobacteriaceae are found to encode

Table 3. Plasmid pattern and number of isolates hosting the plasmid

Pattern	Lane in Fig. 1	Plasmid content (MDa)	Number of isolates hosting the pattern.	Total isolates
1	2	85, 30, 6	7	
2	3	3.2	1	
3	4	15, 4.3	1	
4	5	110, 35, 30, 6	1	
5	6	4.8, 3.2	1	
6	7	130, 35	1	
7	8	3.4, 2.7	1	
8	9	105, 3.7	1	
9	10	110, 30	1	
10	11	110, 100, 4	1	
11	12	18, 3.5, 2.1	1	
12	13	4.8, 4.3, 3	1	
13	14	5	1	
14	15	3	1	
15	16	4.3, 4	1	90
16	17	105, 5.8	1	
17	18	85, 2.7, 3	3	
18	19	85, 4.3	2	
--	20	No plasmid	39	
19	21	105, 3	1	
20	22	5, 4.5, 3.4	1	
21	23	15, 3.4, 2.3	1	
22	24	35.6, 30, 4, 3.2	1	
23	25	35.6, 15, 1.3	6	
24	26	4.6, 1.9	1	
25	27	5, 4.6, 3.4, 2.7, 2.1, 1.6	2	
26	28	100, 2.4, 1.8	1	
27	29	35.6, 2.4	1	
28	30	2.4	1	
29	31	100, 4.3, 3.4, 2.9	1	
30	32	35.6, 2.7, 2.4	1	
31	33	15, 4.8	1	
32	34	85, 3.4, 2.7, 1.6, 1.4	1	
33	35	100, 4.3, 4, 2.3	1	
34	36	4.3	1	

diverse virulence factor determinants and antibiotic resistance. Several large plasmids are transferable and shown to mobilize resistance gene among the other enteric member [8,32,33,34]. Wide spread occurrence of large plasmids including plasmids of approximately 140MDal in normal enteric flora indicates that they may contain equivalence plasmids with pathogenic genes and thus may functions as a reservoir for virulence genes in normal flora. Screening of the large plasmids for the presence of pathogenic gene is required to prove this assumption. In this study, we reported nine isolates that were sensitive to all the six antibiotics tested. Those isolates carried plasmids in number ranging from 1 to 3. Conversely, seven isolates, which were resistant to three or more antibiotics carried no plasmid. This observation obviously suggests that multi-drug resistance phenomenon in normal enteric flora lacking association in general with wide spread occurrence of diverse plasmid population.

5. CONCLUSION

The study-findings indicate that normal intestinal non- *E. coli* population is a reservoir of heterogeneous plasmid. In general, the non- *E. coli* population is found quite comparable to *E. coli* population regarding hosting multidrug resistance phenomenon against therapeutically important antibiotics. Although no direct correlation was observed between drug resistance phenomenon and presence of heterogeneous population of plasmid, to study the role of these plasmids would be an interesting line of inquiry to understand the pathogenic evolution of diverse enteropathogens that passes throughout our gut.

ETHICAL APPROVAL

All authors hereby declare that all experiments have been examined and approved by the Biosafety and Biosecurity and Ethical Committee, Faculty of Biological Science, Jahangirnagar University and have therefore been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki.

ACKNOWLEDGEMENT

This research was funded in part by a grant from University Grants Commission, Bangladesh. Nazneen Jahan was a recipient of MS fellowship from Ministry of Science and Technology, Government of the People's Republic of Bangladesh.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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Peer-review history:
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