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A STUDY OF RELATIONSHIP BETWEEN ANTIBIOTIC RESISTANCE AND MOLECULAR CHARACTERISTICS OF *ESCHERICHIA COLI* ISOLATES OBTAINED FROM DIFFERENT HUMAN CLINICAL SPECIMENS AGAINST MULTIPLE ANTIBIOTIC RESISTANCE (MAR) INDEX IN BAREILLY (INDIA) REGION

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ABSTRACT

To analyse the situation of antibiotic resistance, a total of 77 *E. coli* isolates from urine, pus, sputum and endo-tracheal aspirate were screened for their antibiograms for antibiotic resistance, multiple antibiotic resistance (MAR) index for evaluating the spread of resistance and plasmid profiles for the presence and characterization of plasmids. Very high resistance level (>90%) was detected against ampicillin, amoxicillin, ceftazidime, norfloxacin, tetracycline while imipenem and amikacin recorded the least resistance levels of 2.3% and 13.9% respectively among the isolates. An increased resistance to amoxicillin, tetracycline, cotrimoxazole and norfloxacin were observed in this geographical area which however displayed a lower resistance in other countries. The MAR index varied considerably, the lowest was 0.18 and the highest was 0.89. Plasmids of 10 size ranges were detected in the isolates. Some isolates possessed single sized plasmid while other possessed multiple plasmids. Isolates with high multi- antibiotic resistance profiles were found to possess multiple plasmids. This study shows that regular antimicrobial sensitivity surveillance is necessary and acquisition of plasmid could greatly contribute in the antibiotic resistance and poses a significant risk of the spread of microbial resistance in this community.

INTRODUCTION: *Escherichia coli* is a multi-talented, very adaptive, enteric gram-negative bacillus, which belongs to the family Enterobacteriaeceae. Most strains of *E. coli* live as commensal, many perhaps all are opportunistic pathogens of humans. *E. coli* is one of the main causes of both nosocomial and community acquired infections in humans. The organism is therefore of clinical importance and can be isolated from various clinical specimens. It is one of the organisms most frequently isolated from blood [1, 2, 3]. *E. coli* has now been established as etiological agents of human gastroenteritis, enteric fever, septicemia, localized infections and diarrhea disease of humans [4].

The antibiotic susceptibility of bacterial isolates is not constant but dynamic and varies with time and environment [5]. Indeed, high resistance rate to several antimicrobial agents have been observed in commensal bacteria in developing countries [6].

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E. coli is highly resistant to ampicillin, amoxicillin, tetracycline and trimethoprim – sulfamethoxazole [7]. *E. coli* became resistant as a result of genetic mutations or acquisition of pre-existing genes that confer resistance [8] which occur either in the deoxyribonucleic acid (DNA) of the bacteria chromosomes or plasmids [9]. Thus antibiotic resistance can be disseminated to other bacteria by the plasmid during conjugation [10].

The rapid spread of antibiotics resistance genes in bacterial population is due to selective pressure resulting from the intensive and the indiscriminate use of antibiotics in human therapy [11]. This rapid dissemination of drug-resistant bacteria is an increasing global concern, as it seriously complicates the treatment of infections [12, 13]. The association of these pathogenic organisms with diseases in humans has increased the importance of epidemiological studies. This demands the need for periodic screening of common bacterial pathogens for their antibiotic susceptibility profiles in different communities.

Therefore, *E. coli* could serve as an indicator bacterium for so called 'alert organism surveillance'[14]. Historically, serotyping has been extensively used for identification of Salmonella, Pseudomonas and *E. coli* for epidemiological purposes but several other methods, including phage typing, biotyping, antibiotic resistance determination and plasmid profile analysis are now available. A number of plasmid screening procedures which vary in subtle ways have been used for the detection of plasmid [15].

In this study, we investigated the resitance profile of *E. coli* isolates obtained from urine, pus, sputum and endo-tracheal aspirate to common antibiotics and the existence of plasmid DNA which confer antibiotic resistance in *E. coli*.

MATERIALS AND METHODS:

Sample collection: A total of 77 clinical specimens comprising urine, pus, sputum and endo-traceal aspirate of patients attending medicine OPD at Shri Ram Murti Samarak Institute of Medical Sciences (SRMS-IMS), Bareilly, U.P., India were screened for *E. coli*. The specimens were processed at SRMS-IMS Hospital, using standard microbiological methods[16]. Plasmid profile was carried out at the Central Research Laboratory, SRMS-IMS, Bareilly.

Antibiotic Susceptibility Testing: Susceptibility of isolates to antibiotics were tested using the disk diffusion method on Mueller Hinton agar by Bauer-Kirby Method (Hi-media, Mumbai), against the following antibiotics, namely Ampicillin (10 µg), Ceftriaxone/ Cefotaxime (30/ 30 µg), Ceftazidime (30 µg), Tobramycin (10 µg), Nitrofurantoin (300 µg), Co-trimoxazole (1.25/23.75 µg), Norfloxacin (30 µg), Tetracycline (30 µg), Gentamycin (10 µg), Amikacin (30 µg), Ciprofloxacin (5 µg), Piperacillin/ Tazobactum (100/10 µg), Cefoparazone + Sulbactam (75/10 µg), Imipenem (10 µg), Amoxicillin + Clavulanic acid (20/10 µg),. The sensitivity tests were standardized using *E. coli* ATCC no. 25922). Discs were consistently tested for efficacy against standard strains recommended by Clinical Laboratory Standards Institute [17]. Inhibition zones sizes were interpreted in accordance to Performance Standards for Antimicrobial Disk Susceptibility Tests, CLSI.

Results were interpreted as percent sensitive (% S) and percent resistant (% R) isolated derived using CLSI and WHO breakpoints. Intermediate isolates were counted as resistant to all the agents tested. A multiple drug resistance (MDR) phenotype was defined as resistance to ≥ 2 antimicrobial agents. Only *E. coli* isolates which showed MDR (58 isolates) were included for further investigation for MAR index study and plasmid profile analysis. Multiple antibiotic resistance (MAR) index is a tool to analyze health risk and is helpful to check the spread of bacterial resistance in a given population [18], where there is resistance to more that three antibiotics. It is calculated as the number of antibiotics to which test isolate displayed resistance divided by total number of antibiotics to which the test organism has been evaluated for sensitivity. The value of MAR index 0.2 differentiates the low and high risk. MAR index greater that 0.2 implies that the strain of such bacteria originate from an environment where several antibiotics are used.

Isolation and separation of plasmid DNA: Plasmids DNA isolation was done using small-scale alkaline lysis method as described by Sambrook *et al* [19]. Agarose gel electrophoresis was performed on 0.8 % (w/v) Agarose and stained with ethidum bromide (0.5µg/ml). Plasmid profiles were documented under UV light in Gel Documentation System (UVP., USA).

Determination of molecular weight of plasmid: Molecular weight of plasmids from *E.coli* isolates was determined by comparing with molecular weight DNA maker (Lambda DNA/Hind *III* digest and 1 kb DNA ladder). Images of gels were captured on DigiDoc-It™ Imaging System and molecular weight of test plasmids were determined by comparing them with the DNA marker using the Doc-It® LS image analysis software. For reproducibility testing, comparison of plasmid with DNA marker was done thrice and an average of two readings obtained for each isolate was affirmed as the final molecular weight of the test plasmid.

RESULTS: A total of 58 *E. coli* isolates selected were analyzed for resistance ability against different antibiotics. *E. coli* isolates displayed 100% resistance towards ampicillin, 93.3% isolates were resistant to ceftazidime, 91.3% were resistant to amoxicillin/ clavulanic acid, 89.4% isolates were resistant to ciprofloxacin, 78.5% isolates were resistant to cotrimaxazole, 24% isolates were resistant to nitrofurantoin, 14% isolates were resistant to amikacin and 2.32% isolates were resistant to imipenem. The results of antibiotic resistance pattern are summarized in **table 1**.

TABLE 1: ANTIBIOTIC SENSITIVITY PROFILES OF *E. COLI* ISOLATED FROM 58 CLINICAL SAMPLES

Antibiotics	*E. coli* isolates resistance (%)
β-Lactam	
Ampicillin	58/58 (100%)
Amoxicillin	53/58 (91.3%)
Cephalosporins	
Ceftazidime	42/45 (93.3%)
Ceftriaxone/Cefotaxime	38/44 (86.3%)
Aminoglycosides	
Amikacin	6/43 (13.95%)
Gentamycin	33/58 (56.89%)
Tobramycin	29/44 (65.9%)
Quinolones	
Ciprofloxacin	51/57 (89.47%)
Norfloxacin	26/27 (96.29%)
Cycline	
Tetracycline	21/23 (91.3%)
Carbipenem	
Imipenem	1/43 (2.32%)
Others	
Nitrofurantoin	6/25 (24%)
Cefoperazone + Sulbactam	19/43 (44.18%)
Co-trimoxazole	44/56 (78.57%)
Piperacillin/ tazobactam	15/44 (34%)

Among the antibiotics, Imipenem was most effective with 100% sensitivity in the *E. coli* isolates from urine sample followed by amikacin with 14.8% resistance. In *E. coli* isolates from sputum samples, amikacin and imipenem both were equally effective with 4.5% resistance. In *E.coli* isolates from pus samples, the results were similar as of urine samples viz, Imipenem was most effective with 100% sensitivity followed by amikacin with 14.28% resistance. The results of antibiotic resistance pattern in *E.coli* isolates from urine, pus and sputum samples are summarized in **table 2**.

TABLE 2: ANTIBIOTIC RESISTANCE PATTERN IN *E.COLI* ISOLATES FROM URINE, PUS AND SPUTUM SAMPLES

Samples	Antibiotics	Resistance (%)
Urine	Imipenem	0%
	Amikacin	14.8%
	Nitrofurantoin	17.8%
Sputum	Imipenem	4.5%
	Amikacin	4.5%
Pus	Imipenem	0%
	Amikacin	14.28%

The multiple antibiotic resistance (MAR) index varied considerably the lowest MAR index was 0.18 and the highest MAR index was 0.89 (**table 3**). Overall the maximum population of *E. coli* isolates (17 in number) belongs to an MAR index of 0.6. The *E. coli* isolates from urine sample had the maximum population (20 in number) with MAR index above 0.6 when compared to isolates from other samples. It is interesting to note that majority of *E. coli* isolates belong to the groups above MAR index 0.6.

Plasmid profile revealed that a total of 10 different sized plasmids were possessed by the isolates. The smallest plasmid was of size ≤ 2.0 kb and the largest was above 23.13 kb (see **figure 1**). It was an interesting finding that the maximum number of resistant *E. coli* isolates belongs to MAR index 0.6 and 0.7 and these groups also possessed maximum variation of plasmid size (starting from 2 Kb to 23 Kb). The number of plasmid bands in low and medium resitance profile (groups with MAR index 0.5 and below) varied between 1-3, while the number of plasmid bands in the high resitance profile (group above 0.5 MAR index) varied between 4-10. Plasmid sized 21 kb was the most common plasmid which was present in 6 groups of MAR index (0.1, 0.2, 0.5, 0.6, 0.7 and 0.9) (see **table 3**).

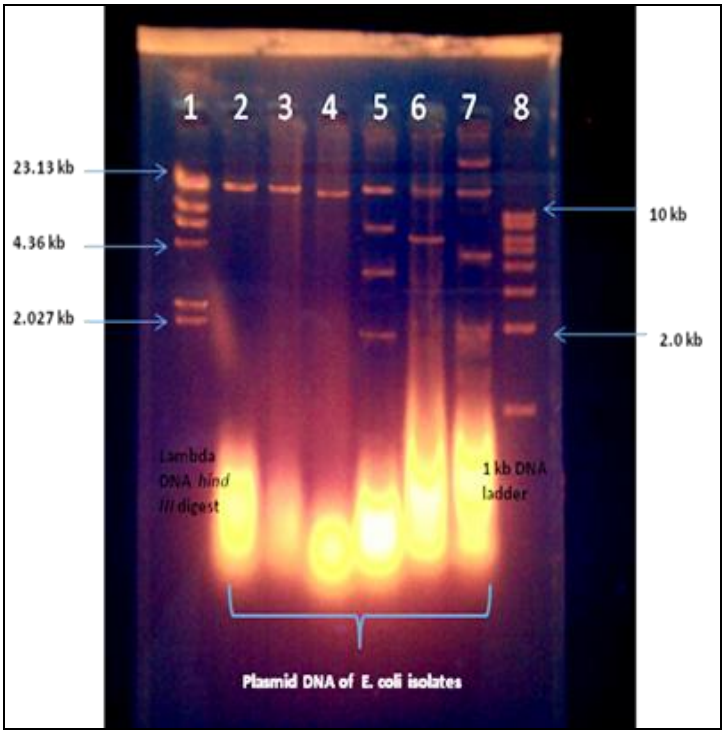

FIGURE 1: PLASMID PROFILE OF TEST ISOLATES OF *E. COLI* (LANE 1&8: DNA MARKER- LAMBDA DNA/*HIND III* DIGEST & 1 KB DNA LADDER)

DISCUSSION AND CONCLUSION: Epidemiological surveillance of antimicrobial resistance is indispensable for empirical treatment of infections, implementing control measures, and preventing the spread of antimicrobial resistant microorganisms [20].

Pathogenic isolates of *E. coli* have relatively high potential for developing resistance [1]. High resistance of *E. coli* to antimicrobial agents tested was observed in this study. This is similar to what was observed previously [7] who reported 100% resistance of their *E. coli* isolates to Ampicillin. In our study, *E. coli* isolates showed a high resistance (91.3%) to Amoxicillin than what was observed in South Africa, Israel (62%-84%) and Hong Kong, Philippines (64%-82%) [21].

Thus, the result from the present study, showed higher resistance of *E. coli* isolates to the antibiotic amoxicillin which alarms us of the possibility that the *E. coli* could have become resistant to many more antibiotics to which it displayed lower resistance.

TABLE 3: MAR INDEX, SIZES AND FREQUENCY OF PLASMIDS DETECTED IN *E. COLI* ISOLATED FROM DIFFERENT HUMAN CLINICAL ISOLATES AND CORRELATION WITH RESISTANCE PROFILES

MAR Index	Number of Isolates	Number of Plasmid bands	Level of resistance profile	Most common sized plasmid (no of MAR group in which plasmid present/ total number of MAR group)
0.1	01	1	Low	21 kb (6/9)
0.2	02	2		20 kb (5/9)
0.3	01	1	Medium	22 kb (4/9)
0.4	03	3		23 kb (4/9)
0.5	05	3		10 kb (3/9)
0.6	17	8	High	2 kb, 3kb, 4kb & 7kb (2/9)
0.7	14	10		8 kb (1/9)
0.8	08	4		9 kb (1/9)
0.9	07	5		

Densenclos *et al*., [22] reported 53% of their *E. coli* isolates were resistant to co-trimaxazole and 67% to tetracycline. Subequently, Umolu et al[23] reported an increase in resistance, showing 69% and 88% to co-trimaxazole and tetracycline respectively which now increased to 78.5% and 91.3% respectively in this study.

In recent years, use of fluorquinolones has increased in many countries and emergence of resistance of bacterial isolates to fluoroquinolones has been observed. Consistent step up in *E. coli* resistance to ciprofloxacin was observed from 1995 (0.7%) to 2001 (2.5%) [24].

Ciprofloxacin resistance in Portugal was 25.8% and Italy 24.3% while in Germany and Netherlands it was 15.2% and 6.8% respectively [25]. The percentage of ciprofloxacin resistance observed in this study was 89.4%. Similar high resistance of *E. coli* to Norfloxacin (96.2%) was also observed.

The reason for the high resistance to antibiotics may be due to increase in an irrational consumption rate, transmission of resistant isolates between people, self-medication and non-compliance with medication and sales of substandard drug.

Isolates in this study were highly sensitive to nitrofurantoin (76%). Extreme sensitivity of *E. coli* isolates to nitrofurantoin has earlier been reported [26]. However, in this study we found that the *E. coli* isolates were extremely sensitive to Imipenem (97.6%) and Amikacin (86%). Our results are in contrast to Uma *et al* [27], who reported high resistance to imipenem. It is interesting to note that the antibiotics Imipenem and Amikacin are only available for intravenous administration and provided on prescription only.

Hence, the route of administration of these antibiotics may have reduced its misuse which had led to the reduction in the emergence of resistant bacterial strains. Our results are in contrast to previously reported in the present study, *E. coli* isolates from UTI patients were highly sensitive to Nitrofurantoin (82.2% sensitive) which correlates to those reported earlier [24]. However, we found that Imipenem was extremely effective in *E. coli* isolates from UTI patients (100% sensitive), pus samples (100% sensitive) and sputum samples (95.5% sensitive). This was followed by Amikacin with sensitivity 85.2% in UTI patients, 85.8% sensitive in pus samples and 95.5% sensitive in sputum samples.

The Multiple Antibiotic Resistance Index data revealed interesting finding that majority of *E. coli* isolates belong to the group with MAR index above 0.5 (see table 3). This indicates that a very large proportion of the bacterial isolates have been exposed to several antibiotics. It was an interesting finding that the maximum number of resistance *E. coli* isolates had MAR index between 0.6 and 0.7 and these isolates possessed maximum variation of plasmid size (starting from 2 Kb to 23 Kb). Plasmids of 10 size ranges were detected in the isolates.

Some isolates possessed single sized plasmid while other possessed multiple plasmids. Isolates with high MAR index (above 0.5) were found to possess multiple plasmids (see table 3). Thus, this study shows that the antibiotic resistance in *E. coli* is controlled by the plasmid number and plasmid size.

Since antimicrobial resistance patterns are constantly evolving and present global public health problem, there is the necessity for constant antimicrobial sensitivity surveillance. This will help clinicians in providing safe and effective empirical therapies. Moreover, this study shows a good prospect for further research to investigate the exact cause of antibiotic resistance and to understand the mechanism of rapid development of resistance to the newly synthesized antibiotics to which the bacteria were never exposed previously.

CONCLUSION: This study showed that antibiotic like Imipenem which display high resistance in other regions across India is still very effective in our environment while conversly an increased resistance to amoxicillin, tetracycline, cotrimoxazole and norfloxacin were observed. In our study, MAR index proved to be helpful in analyzing health risk and the spread of antibiotic resistance. The MAR index data revealed that isolates with lowest and highest MAR index are present in our environment which is a major health risk.

As, antimicroboal resistance pattern are constantly evolving in our region. There is a necessity for constant antimicrobial sensitivity surveillance and susceptibility testing to be conducted prior to antibiotics prescription in India. It was also observed that route of antibiotic could contribute in checking antibiotic resistance. Plamid profile revealed that the antibiotic resistance in this geographical area is plasmid borne.

Hence, our data will help clinician in this region provide safe and effective emperic therapies and could contribute to decrease in emergence of resistance.

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