PREVALENCE OF MULTIPLE ANTIBIOTIC RESISTANCE AND R-PLASMID IN *ESCHERICHIA COLI* ISOLATES OF HOSPITAL SEWAGE OF ALIGARH CITY IN INDIA

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ABSTRACT

R-plasmids that transfer antibiotic resistance are common in the non-pathogenic *Escherichia coli* of the gastro-intestinal tract of human beings and domestic animals, which intum may enter into sewage. Therefore we have isolated 30 *Escherichia coli* isolates from hospital sewage of Aligarh city. These isolates were tested for their resistance and sensitivity against 10 antibiotics. 90% isolates showed resistance against ampicillin and sulphamethizole. Of the total 30 *E. coli* isolates 86.6% were resistant to erythromycin and rifampicin but none of them was resistant to kanamycin and streptomycin. Plasmids (mol. wt. 16.5 mega daltons) were isolated from five different *E. coli* strains which harboured only a single plasmid and were characterized on the basis of antibiogram. Moreover, the transformation experiments were also performed to confirm the resistant character on the plasmid. We conclude that multiple drug resistance among most of the *E. coli* isolates is plasmid borne.

KEY WORDS: R-plasmid, *E. coli*, Sewage water, Multi drug resistance, Antibiotic resistance

INTRODUCTION

Worldwide extensive use and overuse of antibiotics has resulted in an increase of resistance to them in bacteria. Consequently this leads to increased morbidity and mortality (1,2). It has been shown that most multiple antibiotic resistance in clinical isolates of Gram negative bacteria is plasmid borne. Moreover, animal and human faeces have been recognized as reservoirs of the resistance plasmid (3,4). Therefore, we initiated our work to characterize R-plasmids in different *E.coli* strains, isolated from hospital sewage of Aligarh city in India.

MATERIALS AND METHODS

Bacteriological media were purchased from Hi-media, India. Sodium dodecyl sulphate (SDS) and Tris were purchased from SRL, India. Phenol was

distilled and stored frozen until use. The frozen phenol was liquefied at 50°C and mixed with chloroform (50:50, vol/vol). The lysing solution containing 3% SDS and 50 mM Tris (pH 12.6) was adjusted to pH 12.6 by adding 1.6 ml of 2N NaOH made up to 100 ml with distilled water, and filtered through a membrane filter (0.2µm pore size).

Isolation of *E. coli* was performed by inoculating 100µl water sample in the Mac Conkey broth of single and double strength followed by the incubation for overnight at 37°C. The freshly grown overnight culture was streaked on to the eosine methylene blue (EMB) - agar plates. Colonies were grown under the same incubation condition as described above. The green fluorescent colonies were picked from EMB-agar plates and were then identified as *Escherichia coli* by performing IMViC series of tests (5). All the *E. coli* isolates were tested for their sensitivity to antibiotics by means of disc diffusion method employing multi discs of antibiotics (Hi-media, India). The sensitivity was monitored by visualizing

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zone of inhibition around the disc (6).

A preparation of plasmids was made by the method of Kado and Liu (7) and was detected on 0.7% agarose. To avoid overheating and to resolve high molecular weight plasmids, a low salt buffer system composed of 40 mM Tris-acetate and 2 mM sodium EDTA was used. The Tris was adjusted to pH 7.9 with glacial acetic acid. Electrophoresis was carried out at 12 V/cm. The gel (5 mm thick) was stained with 0.5 µg of ethidium bromide per ml for 1 hour at 28°C (7).

Molecular weight of the plasmids were obtained by comparing their relative mobility with known DNA fragments of EcoR1 digested λ DNA on agarose gel electrophoretic profile (7).

The transfer of R-plasmid to recipient *E. coli* C600 cells was achieved by using the method of Lederberg and Cohen (8). Overnight grown C600 cells were made competent by suspending in 0.1 M MgCl₂ for 30 minutes on ice and then in 0.1 M CaCl₂ under similar conditions. To 1.0 ml competent cells, 2.0 µg of plasmid DNA was added separately and left on ice for 30 minutes. Heat shock was given to these cells at 42°C for 3 minutes. These cells were then allowed to grow in nutrient broth for 2 hours at 37°C. The cells were spread on to the different plates each of them is having single antibiotic of the same concentration as was used in the disc.

RESULTS AND DISCUSSION

A total of 30 well isolated colonies were identified as *E. coli* (5) and their antibiotic sensitivity and resistance tested (6) by methods stated above. Of the total 30 isolates 27 were found to be multiple antibiotic resistant (Table 1). 100% isolates were sensitive to kanamycin and streptomycin. In contrast, resistance to ampicillin and sulphamethizole was exhibited by 90% isolates. 86.6% isolates were resistant to erythromycin and rifampicin. These isolates showed moderately high resistance against norfloxacin and novobiocin (63.3% and 40% respectively). A small percentage of the isolates was found to exhibit resistance against chloramphenicol and tetracycline (Table 1).

The indiscriminate use of these antibiotics is one of the major factors that resulted in the emergence

of antibiotic resistant bacteria (9). Reports from various parts of the world indicate highest rate of 97.5% in Hung Shui Kiv river and the lowest, 5.3% in the Kam Tin river (10). Our earlier studies also revealed that ampicillin resistance was maximum in the *E. coli* isolates of both drinking water as well as domestic sewage of Aligarh city (11).

The next highest resistance to erythromycin, observed in our isolates, was also supported by Katayama et al (12). The high level of resistance of these isolates against ampicillin, sulphamethizole, erythromycin and rifampicin (Table 1) seems to reflect their widespread chemotherapeutic use in the city population.

These *E. coli* isolates were classified on the basis of types and numbers of antibiotic classes. The number of antibiotics against which resistance was found, ranged from 2 to 8. Thus out of 30 *E. coli* isolates, 12 different strains were identified on the basis of antibiogram. Moreover, of the 12 *E. coli* strains, 7 were further categorized, based upon the number of antibiotics against which resistance was conferred. *E. coli* HSW25, among the 12 *E. coli* strains, showed resistance to only one antibiotic while HSW21 was resistant to 8 different antibiotics (Table 2).

The progressive appearance of a number of bacterial species with multiple antibiotic resistance is the characteristic of plasmid (13). All the 12 aforementioned *E. coli* HSW strains were found to harbour single plasmid (data not shown). Figure 1a shows the agarose gel electrophoretic profile of plasmids isolated from 5 distinct *E. coli* HSW strains. A single plasmid was found to present in majority of multiple resistant isolates (14). No difference was observed in their relative mobility and hence a common plasmid pattern was shown. Molecular weight of plasmids was calculated as 16.5 M dal by plotting the relative mobility on the standard curve (Figure 1b). Our data support the previous findings in which plasmids isolated from different sources, showed molecular weight in the range of 0.5 to 48 M dal (15). In the earlier studies it was found that a single plasmid could permit and spread in the *E. coli* population of chicken (16).

Table 1. Antibiotic sensitivity and resistance profile of *E. coli* isolates of hospital sewage water. Sensitivity and resistance against antibiotics

<i>E. coli</i> isolates	Am(25)	Ch(30)	Er(15)	Ka(30)	Nv(30)	Nx(10)	Rf(30)	St(25)	Sm(300)	Tr (30)
HSW1	-	+	-	++	+	-	-	+	-	+
HSW2	-	-	-	++	-	-	-	+	-	+
HSW3	-	+	-	++	+	-	-	+	-	+
HSW4	-	-	-	++	-	-	-	++	-	+
HSW5	-	+	-	+++	-	+	-	++	-	+
HSW6	-	-	-	++	+	-	-	++	-	+
HSW7	-	+	-	+++	+	+	-	++	-	+
HSW8	-	-	-	+++	-	+	-	++	-	+
HSW9	-	+	-	+++	-	-	-	++	-	+
HSW10	-	+	-	+++	-	-	-	+	-	+
HSW11	-	+	-	+++	+	-	-	++	-	+
HSW12	-	+	-	+++	+	-	-	++	-	+
HSW13	-	-	-	+++	+	+	-	++	-	+
HSW14	-	-	-	+++	+	-	-	++	-	+
HSW15	-	+	-	++	+	-	-	++	-	-
HSW16	-	+	-	+++	+	+	-	++	-	-
HSW17	-	+	-	+++	-	+	-	++	-	+
HSW18	-	-	-	+++	+	-	-	++	-	+
HSW19	-	+	-	+++	+	-	-	++	-	-
HSW20	-	+	-	+++	-	+	-	++	-	+
HSW21	-	-	-	++	-	-	-	+	-	-
HSW22	+++	++	+++	+++	++	++	+++	++	+++	++
HSW23	-	+	-	+++	-	-	-	++	-	+
HSW24	-	+	-	++	-	-	-	+	-	+
HSW25	+++	-	+++	++	++	+++	+++	++	+++	++
HSW26	++	+++	+++	+++	++	+++	++	+++	++	++
HSW27	-	+	-	+++	-	-	-	++	-	-
HSW28	-	++	+++	++	+	++	+++	+	-	+
HSW29	-	+	-	+++	+	-	-	++	-	+
HSW30	-	+	-	+++	+	-	-	++	-	+
%resistance against individual antibiotic	90	30	86.6	00	40	63.3	86.6	00	90	16.6

HSW designates hospital sewage water, (-) indicates resistance against antibiotics, (+) least sensitive (diameter of zone of inhibition, 9-14 mm); (++) moderately sensitive (diameter of zone of inhibition, 15-20 mm); (+++) highest sensitive (diameter of zone of inhibition, 21-32 mm); Am (ampicillin), Ch (chloramphenicol), Er (erythromycin), Ka (kanamycin), Nv (novobiocin), Nx (norfloxacin), Rf (rifampicin), St (streptomycin), Sm (sulphamethizole), Tr (tetracycline). Number in parenthesis against each antibiotic indicates its concentration in µg/disc.

Table 2. Antibiotic resistance pattern of 12 *E. coli* HSW strains isolated from hospital sewage water.

<i>E. coli</i> Strains isolated	Resistance against antibiotic*	No. of antibiotics against which resistance was observed
HSW1	Am; Er; Nx; Rf; Sm	5
HSW2	Am; Ch; Er; Nv; Nx; Rf; Sm	7
HSW5	Am; Er; Nv; Rf; Sm	5
HSW6	Am; Ch; Er; Nx; Rf; Sm	6
HSW7	Am; Er; Rf; Sm	4
HSW9	Am; Er; Nv; Nx; Rf; Sm	6
HSW13	Am; Ch; Er; Rf; Sm	5
HSW15	Am; Er; Nx; Rf; Sm; Tr	6
HSW16	Am; Er; Rf; Sm; Tr5	
HSW21	Am; Ch; Er; Nv; Nx; Rf; Sm; Tr	8
HSW25	Ch	1
HSW28	Am; Sm	2

* Total number of antibiotics used are 10

Figure 1. a: Agarose gel electrophoresis of plasmid isolated from *E. coli* HSW strains. Marker : Eco R1 digested λ DNA

b: Log of molecular weight vs mobility plot of the test and standard DNA.

Symbol: Unknown Molecular weight ($\leftarrow$), 16.5 M dal, Standard DNA (.)

To provide evidence that the resistance markers were actually on plasmid DNA, we carried out the transformation experiments employing plasmids isolated from test *E. coli* strains. The expression of resistance markers in all transformant suggested that the antibiotic resistance was plasmid mediated. The transformation frequencies of C600 cells with R-plasmids (pHSW2, pHSW6, pHSW9,

pHSW13, pHSW15) are shown in table 3. These frequencies obtained with recipient strains in *E. coli* C600 were comparable to those obtained by Bopp et al (17) in case of *E. coli* recipients, transformed with *P. putida* derived Rp4 plasmids. All the test *E. coli* isolates transferred four or more resistance marker out of 5 to 8 marker (Table 3).

Table 3. Transformation frequency of *E. coli* C600 with plasmids pHSW2, pHSW6, pHSW9, pHSW13, pHSW15 isolated from five *E. coli* strains.

S.No.	Plasmid cells/ml	No.of resistant	Total no. of antibiotic transformants per plate (.1 ml)	Transformation frequencies	Resistance marker present
1	pHSW2	5X10 ⁷	2.6X10 ²	5.2X10 ⁻⁴	Am, Er, Nv, Nx
2	pHSW6	5X10 ⁷	3X10 ³	6X10 ⁻⁴	Am, Nx, Rf, Sm
3	pHSW9	5X10 ⁷	1.5X10 ²	3X10 ⁻⁴	Am, Er, Rf, Sm
4	pHSW13	5X10 ⁷	1.8X10 ²	3.6X10 ⁻⁴	Am, Ch, Er, Rf, Sm
5	pHSW15	5X10 ⁷	4.2X10 ²	8.4X10 ⁻⁴	Am, Er, Nx, Rf, Tr

This research provides supportive evidence in favour of the prevalence of multiple drug resistance and R-plasmid in the *E. coli* isolates of hospital sewage. Present study also has clinical dimension, in view of the increasing incidence of multiple drug resistance in pathogens, which is conceivable due to the observed transmissible nature of the R-plasmid. These findings call for particular concern

towards the spread of multiple drug resistance in the environment through these microorganisms.

Acknowledgement

This research was supported by the internal funds from Interdisciplinary Biotechnology Unit, AMU, Aligarh, India.

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