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TRANSFERABLE ANTIBIOTIC RESISTANCE IN *ESCHERICHIA COLI* ISOLATED FROM URINARY-TRACT INFECTIONS: HOSPITAL *v.* COMMUNITY PATIENTS

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Summary

In order to study the prevalence of plasmid-mediated antibiotic resistance in *E. coli* causing urinary-tract infections (UTI) specimens of urine were obtained, from a total of 125 hospital and community patients, and examined bacteriologically and analysed. Thirty-nine (80%) of forty-nine, and thirty-three (65%) of fifty-one hospital and community patients respectively were infected by multiple resistant *E. coli*. Of these multiple-resistant strains more than three-quarters carried transferable R-plasmids. Antibiotic resistance was transferred *en bloc* or singly to known sensitive recipient mainly by conjugation by a technique that ensured cell-to-cell contact. Resistance markers in both the donor and the transcipient strains were eliminated by treatment with aminoacridine dyes and ethidium bromide. The result emphasises the need for bacteriological diagnosis of UTI and sensitivity testing prior to antibiotic therapy.

Résumé

Pour étudier la courante des résistances antibiotiques, à la médiation de plasmide dans *E. coli*, impliquer comme causatif des infections de tract urinoir chez 125 malades hôpitaux et communautés. On a fait un examination et analyse bactériologique. Quatre-vingt et 65% des malades hôpitaux et communautés respectivement ont eu la résistance multiple de *E. coli*. De ces strains de résistance multiple, plus de trois-quarts ont porté R-plasmides transférables. Les résistances antibiotiques étaient transférées en bloc ou séparément dans les récipients avec la sensitivité commu par conjugation par un technique qui assure le contact cellule-à-cellule. Les indicateurs de résistance dans les donneurs et les récipients été éliminés par trâitement avec les teintures aminoacridine et ethidium bromide. Les résultats souligne la besoin pour les diagnoses bactériologiques d'UTI et la teste du sensitivité avant la thérapie antibiotique.

Introduction

The increasing antibiotic resistance, seen these days in medicine, is one of the major problems of bacterial infections and the discovery of transferable drug resistance factor (*R*-plasmids) in practically all Gram-negative and some Gram-positive organisms has added a new dimension to our understanding of the problem. Plasmids are heterogeneous extra-chromosomal; deoxyribonucleic acid (DNA) molecules which replicate in synchrony with the chromosome so that a ratio of 1:1 is always maintained between the two. There is increasing evidence that the emergence of antibiotic resistance R-plasmid is directly related to the amount of antibiotic usage in the environment (Datta *et al.*, 1971; Levy, Fitzgerald & Macone, 1976). Transferable drug resistance was first found in multiple drug resistant strains of *Shigellae* in Japan in 1959 (Akiba *et al.*, 1960), in *Salmonella*

typhimurium in Europe (Datta, 1962) and in enteropathogenic *E. coli* in U.S.A. and Germany (Kabins & Cohen, 1966; Labek, 1963). The extent of the transferable R-plasmid is such that it has been found practically everywhere it has been sought.

Most reports on the incidence of transferable drug resistance have been in the enterobacteria isolated from the intestine of healthy people (Datta, 1971; Mann & Gedebou, 1966; Salzman & Klemm, 1966). There are, however, very few reports on the incidence of *E. coli* carrying R-plasmids, in both hospital and domiciliary practice, isolated from infections, particularly urinary-tract infections (UTI). Our experience at LUTH shows that the majority of the UTI pathogens in a hospital environment have a high level of drug resistance (Rotimi, Saliu & Odugbemi, 1981). This study was undertaken to determine the incidence of drug-resistant *E. coli* carrying R-plasmids isolated from hospital and community patients suffering from UTI.

Materials and methods

Urine samples

Mid-stream urine (MSU) samples were collected from sixty consecutive patients who attended four private clinics, situated at various locations in the Surulere, Ilupeju and Ikeja areas of Lagos; they all had clinical signs and symptoms of UTI and had not used any antibiotics for their ailment prior to visiting the clinics. MSU or catheter stream urine (CSU) samples were also obtained, as appropriate, from another sixty-five patients who were already on admission but who developed signs and symptoms of UTI while still in the hospital. All urine samples were collected in sterile plastic universal containers (Sterilin Products, Teddington, Middlesex, U.K.) and processed immediately on arrival in the laboratory. Where urine samples could not be transported immediately to the laboratory, they were kept at 4°C and, in any case, were processed within 2 h of collection.

Media

The following media were used: MacConkey agar (oxoid), blood agar (blood agar base (oxoid) + 7% sterile human blood obtained from the blood bank), DST agar (oxoid) + 5% lysed horse blood (oxoid); brain heart infusion (BHI) broth (oxoid); all media were freshly prepared daily. The selective media were prepared by adding various concentrations of antibiotics to MacConkey agar as appropriate – e.g. Mac + streptomycin (Strept. 30 mg/l) and tetracycline (Tet. 10 mg/l), Mac + Strept. (30 mg/l) and ampicillin (Amp. 10 mg/l), Mac + rifampicin (Rif. 25 mg/l) + Amp. (10 mg/l) or Tet. (10 mg/l).

Laboratory investigation of urine

All urine samples were processed according to the standard method (Stokes & Ridgway, 1980). A semi-quantitative method was used to perform bacterial count of urine and bacteriological diagnosis of UTI was recorded when the bacterial count was $>10^5$ CFU/ml of uncentrifuged urine (Kass, 1956). Urine samples were also inoculated onto enriched non-selective media (Mac) using a standard method of plating (Gilles & Dodds, 1976) and incubated in ordinary incubator at 37°C for 24 h. The lactosefermenting (LF) colonies on the non-selective media, morphologically resembling *E. coli*, were picked and identified by standard methods (Cowan, 1974). The isolates were then sensitivity tested.

Sensitivity test

Antimicrobial susceptibility of the bacterial isolates was determined initially by the disk-diffusion method with inocula of 10^4 CFU/ml. About five different LF colonies on a purity plate were emulsified in 2 ml of sterile peptone water in sterile 3 × 1½ inch tubes to give the opacity of No. 1 Brown's tube. A sterile cotton-wool swab was dipped into the suspension and used to inoculate the surface of well-dried DST agar using Stoke's method. Multidisk filter paper (oxoid), containing sulphamethoxazole (Sul. 300 μg), nitrofurantoin (NF, 200 μg), tetracycline (Tet. 30 μg), ampicillin (Amp. 25 μg), cotrimoxazole (TS, 25 μg), nalidixic acid (NA, 30 μg), was carefully placed onto the surface of the seeded plate. A reference standard *E. coli* strain (NCTC 10481) was included in each test as a control.

Plates were then incubated at 37°C in air for 24 h. After incubation, zones of inhibition around individual disks, for test and control strains, were compared and recorded as sensitive (S) or resistant (R). Inhibitory concentrations (MICS) were determined by inoculating 1:100 dilution of overnight cultures of the test strains onto DST-agar plates containing doubling dilutions of filter-sterilized antibiotic solutions (0.025–128 mg/l), the MIC being the lowest concentration that did not permit the visible growth of bacteria after incubation for 24 h.

Transfer experiments

Multiple-resistant *E. coli* strain – i.e. resistance to two or more antibiotics – were used as donors; the strains were designated H (hospital) and C (community) strains. The recipient strains used were known competent sensitive strains:

(i) *E. coli* K12 strain J53, plasmid-free, LF, Rif.^{r}, and requiring proline (pro-) and methionine (met-) for growth,

(ii) *E. coli* K12 strain CSH1, plasmid-free, NLF, streptomycin-resistant (Str.^{r}) and requiring tryptophan (try-) and thiamine (thi-) for growth.

Mating experiments

The centrifugation technique previously described (Rotimi, Duerden and Hafiz, 1981) and the modified filter-membrane technique (Brefort *et al.*, 1977) were used.

(i) The centrifugation technique. 3.5 ml of the recipients and donor were mixed and centrifuged at 3000 × *g* for 10 min. The supernatant was decanted and 1 ml of freshly prepared brain–heart infusion (BHI) broth was carefully layered onto the cell pellets. The bottle was then incubated undisturbed for 18 h at 37°C. After incubation, 0.1 ml of the mixture was seeded onto the selective media that eliminated the growth of both the donor and recipient but allowed only the growth of the transcipients – e.g. Mac + Strept. + Amp. or Mac. + Rif. + Amp. Incubation was again in air at 37°C for 24 h. The transcipients on the selective media were then identified by standard methods (Cowan, 1974), sensitivity tested and checked for the non-selective marker profiles – i.e. lactose or non-lactose fermenting, pro-, met-, and thi-.

(ii) The membrane-filter technique. Overnight broth cultures of the donor and recipients grown to a density of *c.* 4 × 10^8 CFU/ml were mixed in equal proportions of 5 ml. The mixture was filtered through a 0.45 μm Millipore filter (Millipore Corp., Bedford, Mass. U.S.A.). The filter, containing the mixed cells of donor and recipient, was then placed face down on MaC agar and incubated at 37°C for 24 h. The growth on MaC was harvested into 1 ml fresh BHI broth and then used to seed the appropriate selective media. After 24 h incubation the resultant transcipients were treated in the same way as (i) above.

Control experiments

In order to eliminate the possibility of transfer of resistance by transformation or transduction, a series of control experiments was undertaken: (i) 100 mg/l of type I DNase (Sigma Chemicals Co., Poole, Dorset), which has both endonuclease and exonuclease activity, was added to each mating mixture and to MaC agar on which the mating mixture were plated; (ii) resistant strains were cultured overnight in BHI broth and the broth culture filtered through 0.45 μm Millipore filter. The filtrate was incubated in air at 37°C for 24 h with the recipients before plating on appropriate selective media; (iii) resistant donor strains were lysed by repeated freezing and thawing and mixed with the recipients. The mixture was incubated at 37°C for 24 h; 0.1 ml of this mixture was then plated on selective media and incubated at 37°C for up to 48 h.

Curing experiments

This experiment was performed by the method of Rotimi and Duerden (1981). Subinhibitory concentrations (16 mg/l) of acriflavine, acridine orange and ethidium bromide were added to a set of three bottles of BHI broth. The donors and transcipients were seeded into each bottle and into a fourth plain BHI broth, as growth control.

The bottles were incubated at 37°C for 24 h and then subcultured onto appropriate selective and non-selective media which were then incubated at 37°C for 24 h. No growth on the appropriate selective media indicated loss of selective markers (resistance markers).

Results

A total of 125 patients with clinical signs and symptoms of UTI confirmed by bacteriological investigations were studied. Specimens from sixty community patients and sixty-five in-patients at LUTH were investigated. *E. coli* was the cause of UTI in forty-nine of the sixty-five in-patients and in fifty-one of the sixty community patients.

Bacterial sensitivity to antibiotics

Majority of the *E. coli* isolates showed varied sensitivity to the antibiotics tested. Table 1 shows the resistance pattern of the *E. coli* to antibiotics. All the positive (i.e. forty-nine) in-patients (H patients) and forty-five of the fifty-one community (C patients) had *E. Coli* resistant to sul (300 μg) as the cause of their UTI. Out of the forty-nine H patients, forty had *E. coli* resistant to ampicillin, thirty-eight had strains resistant to tetracycline, eighteen to cotrimoxazole (Septrin) and sixteen to nitrofurantoin; only three H patients had *E. coli* resistant to gentamicin. Of the fifty-one C patients cotrimoxazole-resistant *E. coli* were isolated from thirty-five, tetracycline-resistant strains from thirty-two, ampicillin resistant strains from ten. All C and H strains were sensitive to gentamicin and cefuroxime, and cefuroxime respectively.

Transfer of antibiotic resistance

The proportion of patients from whom multiple-resistant *E. coli* strains (resistance to two or more antibiotics) were isolated is shown in Table 2. Out of the forty-nine H patients, thirty-nine had multiple resistant *E. coli*, while such strains were isolated in thirty-three of fifty-one C patients. These multiple-resistant strains (H and C strains) were used as donors. Successful transfer of antibiotic resistance from the donors to the recipient(s) was obtained more easily by the centrifugation technique than by the membrane-filter technique; only five identical results were obtained by the later method in all the parallel transfer experiments. Of the thirty-nine multiple-resistant H strains, thirty (77%) transferred their resistance *en bloc* or singly, while twenty-five (76%) of the thirty-three multiple-resistant C strains transferred theirs. Whenever transfer of resistance marker(s) occurred it included tetracycline and sulphametoxazole and cotrimoxazole in all cases, and ampicillin in most cases. When the transcipients were identified and tested they all had the phenotypic characters of the parent recipient strains (*E. coli* CSH1 or J53) but had acquired the genotypic characters of the donor strains; transcipients were either LFs or NLFs, pro- and met- or try- and thi- respectively.

TABLE 1. Resistant *E. coli* causing urinary-tract infections in hospital and community patients

Antibiotics tested	Number of patients with resistant *E. coli*	
	Hospital patients (n = 49)	Community patients (n = 51)
Ampicillin	40	10
Tetracycline	38	32
Sulfamethoxazole	49	45
Nitrofurandantin	16	5
Gentamicin	3	0
Cotrimoxazole	18	35
Cefuroxime	0	0
Nalidixic acid	6	2

TABLE 2. Multiple-resistant *E. coli* carrying transferable drug resistance isolated from individual patients

Bacterial isolates	No. of hospital patients (%)	No. of community patients (%)
Multiple-resistant *E. coli*	39 (80)	33 (65)
E. coli carrying transferable resistance	30 (61)	25 (49)

Minimum inhibitory concentrations

The MICs of the test antibiotics for the donors, recipients and transcipients are shown in Table 3. In each case, the MICs for the donors and transcipients, that had acquired resistance from the donors, were the same.

Curing of antibiotic resistance

Gentamicin resistance along with other antibiotic resistance were eliminated *en bloc* in all three multiple-resistant H strains after growth in sub-inhibitory concentrations of the aminoacridine dyes and ethidium bromide. Similarly Tet^r, Sul^r and Am^r were readily eliminated *en bloc* from both the H and C strains and their transcipients. In all the strains, resistance to nalidixic acid and nitrofurantoin were never eliminated even after prolonged incubation in the respective dyes. On sub-culture of the cured strains onto non-selective media, none reverted to resistant variants.

Control experiment

The transfer of resistance marker was not prevented by the addition of DNase (type I) 100 mg/l to the mating mixtures. The *E. coli* K12 strains J53 and CSH1 used as recipients did not acquire any resistance markers when they were mixed and incubated with cell-free filtrates or lysed donors.

Discussion

The increasing prevalence of resistance plasmids and the patterns of resistance that they mediate, and the associated pathogenicity

TABLE 3. Minimum inhibitory concentrations for donor, recipient and transcipient strain

Bacteria strains	Minimum inhibitory concentration (mg/l) of:					
	Amp.*	Tet.	Gent.	Cef.	Rif.	Str.
Donors						
H strains	64	128	64	0.5	–	–
C strains	64	64	–	0.5	–	–
Recipients						
E. coli CSH1	1	1	0.25	0.5	–	64
E. coli J53	2	1	0.25	0.25	>128	–
Transcipients						
C/J53	64	64	64	0.25	>128	–
H/CSH1	64	>128	64	0.25	–	64
H/J53	64	128	64	0.25	>128	–
C/CSH1	64	128	64	0.5	–	64

*Mean MICS. Amp. = ampicillin, Tet. = tetracycline, Gent. = gentamicin, Cef. = cefuroxime, Rif. = rifampicin, Str. = streptomycin. H strains = hospital strains; C strains = community strains. H/CSH = transcipients from H strains and *E. coli* CSH1 mating mixture. H/J53 = transcipients from H strains and *E. coli* J53 mixture. C/CSH1 = transcipients from C strains and *E. coli* CSH1 mixture. C/H53 = transcipients from C strains and *E. coli* J53 mixture.

factors which has serious clinical implications is of general interest because the future use and effectiveness of antibiotics, particularly in West Africa, could be judged against this information.

Most of the studies on R-plasmids have been performed in the resistance selective surroundings of hospital patients. So far, in Nigeria, no work has been reported in the literature, on the occurrence of R-plasmid-mediated antibiotic resistance in pathogenic bacteria. In this study, apart from the quantitative information provided on the presence of R-plasmids outside of the antibiotic milieu of hospital, the prevalence of R-plasmid in pathogenic bacteria of UTI within the hospital itself is also reported.

Several earlier reports indicate that *E. coli* is the commonest pathogen causing UTI in both hospital and community patients (Sanford, 1964; Mulholland & Bruun, 1973; Meers *et al.*, 1981; Rotimi *et al.*, 1981). The result of this study confirms this observation and extends the earlier report on the antibiogram of UTI pathogens in LUTH (Rotimi *et al.*, 1981). As was expected, the hospital *E. coli* strains were more resistant to a variety of the test antibiotics than the community strains with the exception of resistance to cotrimoxazole. This resistance to cotrimoxazole is a reflection of the heavy usage of septrin outside the hospital. Also worthy of note is the almost total resistance of the strains to tetracycline, sulphametoxazole, ampicillin and cotrimoxazole.

As many as 80% of the H patients and 65% of the C patients got infected by multiple-resistant *E. coli* strains. Thirty (77%) of the thirty-nine and twenty-five (76%) of the thirty-three multiple-resistant H and C strains respectively carried R-plasmid-mediated antibiotics resistance that were readily transferred to sensitive recipient strains. In a similar study in London Brumfitt *et al.* (1971) isolated multiple-resistant *E. coli* from seven (19%) of their thirty-one patients; six of the seven strains of *E. coli* carried plasmid-coded resistance transfer. The source of these R-plasmid-carrying strains is usually the intestinal-tract flora of the patient (Datta, 1971), although nosocomial UTI also may occur by spread through the hands of attendants or instruments (Lowbury *et al.*, 1977; Rotimi, Esho & Emina, 1984).

There is general agreement today that the frequent use of antibiotics has created an environment where resistance appears to be selectively advantageous to the bacteria. The result of this study also reflects the unbridled usage of antibiotics in the hospital and the community judging by the level of resistance to the commonly used antibiotics and the presence of R-plasmids. The hospital environment is in reality usually contaminated by resistant organisms developed in response to widespread use of antibiotics. Although community patients denied antibiotic therapy prior to visiting the clinic, it is common knowledge that the reverse is generally the case: antibiotics can be purchased freely at chemist shops and open markets.

It is interesting to note the dominance of tetracycline, sulphametoxazole and ampicillin resistance respectively, in the pattern of transferred resistances. The three gentamicin resistant *E. coli* transferred *en bloc* seven of the eight antibiotics; they were sensitive to cefuroxime.

The result of the control experiments clearly shows that the transfer of the genetic information was by conjugation, which provided cell-to-cell contact, and not by transduction or transformation. Transfer occurred in the presence of added DNase, showing no evidence of transformation, and resistance was not acquired by the recipient cells from cell-free lysates of resistant donor strains.

Spontaneous loss of some or all of the antibiotic resistance is most likely to occur as a result of deletion of R-plasmids particularly by exposure of the resistant strains to aminoacridine dyes and ethidium bromide (Hashimoto *et al.*, 1964; Rotimi & Duerden, 1981). To show that a phenotypic trait is plasmid-mediated, it is important to correlate the presence of the plasmid with the expression of the trait. This correlation was achieved in this study by curing the various strains of their resistance genotype.

The sum total of these findings emphasizes unequivocally the need for bacteriological diagnosis and sensitivity testing before therapy, as well as controlled prescription and sales of antibiotics. In this hospital, resistance to

antibiotics is a serious clinical problem and further spread of resistance may be catastrophic particularly when multiple and plasmid-linked.

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