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Transferable antibiotic resistance among thermotolerant coliforms from rural drinking water in India

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SUMMARY

A total of 231 thermotolerant coliforms was isolated from rural drinking water from four states of India. Of these, 220 isolates were resistant to ampicillin, chloramphemicol, streptomycin and tetracycline. Multiple (MAR), double and single antibiotic resistances were observed in 31·4, 48·6 and 13·7% of the isolates, respectively. Out of 177 antibiotic-resistant isolates examined for transmissibility, only 15·3% were able to transfer their resistances to *Escherichia coli* K-12 recipient. The resistances were transferred by 32·5% of MAR, 21·9% of double resistant and 7·6% of single resistant isolates. Ampicillin resistance was transferable in 14·69% strains while resistances for the rest of the antibiotics were transferable in $< 4\%$ strains. MAR strains of *E. coli* and *Klebsiella* sp. showed highest levels of R-plasmid transfer.

INTRODUCTION

Several workers have drawn attention to the incidence of antibiotic resistance among coliforms in treated and untreated drinking water [1–3]. Coliform bacteria carrying plasmid-borne antibiotic resistance are common in the intestine of man and as a result of sewage pollution they may become widely disseminated into the environment, where they are subjected to a variety of selective forces. Eventually they contaminate drinking water to varying degrees which in many parts of the world is consumed without treatment. The public health hazards involved are related to the frequency with which the ingested coliforms are able to transfer antibiotic resistance to other sensitive coliforms or enteric pathogens which they encounter in the intestine of man [4, 5].

In tropical areas where shigella and salmonella infections are endemic and becoming increasingly frequent, epidemics of multiply resistant strains are occurring. It is important to evaluate the risk of water-borne dissemination of R-plasmid bearing organisms [6]. Enhancement of the prevalence of multiple antibiotic-resistant bacteria will compromise the success of future antibiotic therapy in man and all possible precautionary measures are required to reduce the spread of multiple antibiotic resistance.

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In this study, the occurrence of antibiotic resistance among thermotolerant coliform isolates from rural drinking water in India has been investigated and compared with resistance among coliform isolates. At the same time, the R-plasmid transfer frequencies of the antibiotic resistance among thermotolerant coliforms has also been evaluated.

MATERIALS AND METHODS

Sampling and isolation procedure

Water samples from rural gravity piped supply schemes which are sometimes chlorinated were collected from South East Sikkim, Sikkim, North Tripura, Tripura and Leh, Jammu and Kashmir. Ground water from India Mark II hand pumps and dug wells was collected from Nagpur (Maharashtra) and Tripura. The water samples were collected in sterile glass bottles and transported on ice to the laboratory and processed within 6 h of collection. Purified coliform isolates were obtained by plating on MacConkey agar plates. Gas production in brilliant green bile broth and indole production at 44 °C were used to identify the thermotolerant coliforms [7]. The term 'thermotolerant' is defined as that portion of the coliform group capable of producing gas from lactose within 24 h at a temperature of around 44·5 °C. Several countries use the term 'faecal' coliforms to convey the same information.

Determination of antibiotic resistance

Coliform isolates were inoculated into 5 ml of sterile broth incubated at 37 °C for 18 h. A loopful was then diluted in 5 ml sterile phosphate buffered saline (PBS) and seeded onto Mueller Hinton Agar (Hi-Media Ltd, Bombay) using cotton swabs. Antibiotic sensitivities were determined using disks (Pasteur Biological Laboratories, Gujrat, India) which were impregnated with antibiotics (μg/ml), namely, ampicillin (20), streptomycin (20), chloramphenicol (20), tetracycline (20), kanamycin (20), gentamicin (10), co-trimoxazole (20), colistin (10), polymixin B (300U), carbenicillin (50), cephaloridine (30) and sulphatriad (300). Control sensitive strain (*Escherichia coli* K1262) and control resistant strain (a multiple antibiotic-resistant isolate of *E. coli* resistant to ampicillin, streptomycin and chloramphenicol were included in all the tests. Resistant strains were scored by their growth up to the disk. The antibiotic resistance index (ARI) for the coliform isolates was calculated as described by Hinton and colleagues [8]. This provides a useful summary of the overall proportion of the antibiotic resistance in relation to the number of antibiotics tested (ARI = Y/nx) where Y is the total number of resistant scores, n the number of isolates and x the number of antibiotics tested).

Organisms resistant to ampicillin, chloramphenicol, streptomycin and tetracycline were chosen for detailed study because of their widespread use and importance in treatment. Antibiotic-resistant thermotolerant coliforms were identified according to the methods of Cowan and Steel [9].

Measurement of R-plasmid transfer frequencies

The transfer of R factors from the antibiotic-resistant strains to the recipient strain (*E. coli* K12 62 lac^- pro^- his^- trp^- Nal^r) was performed by the technique of Walter and Vennes [10]. Overnight cultures, 0·1 ml each of donor and recipient

were separately inoculated into 10 ml of Brain Heart Infusion (BHI) broth (Hi-Media Ltd, Bombay, India), incubated for 6 h and then 0·1 ml each of donor and recipient cultures were mixed, diluted 100 times and incubated for 18 h. Decimal dilutions of the mixed culture of donor and recipient were plated on selected MacConkey's agar plates. For enumeration of the donor, each plate contained either of the antibiotics (20 μg/ml), and for transconjugant selection each plate contained nalidixic acid in addition to the respective antibiotic. The rates of plasmid transfer were expressed as the number of transconjugants formed per donor. Transconjugants obtained from mating antibiotic-resistant faecal coliforms with *E. coli* K12 recipient were used for secondary transfer of antibiotic resistance using an *E. coli* C600 $thr^{-}leu^{-}B$ lac^{-} F^{-} as recipient strain. The technique for secondary transfer was similar to that mentioned above. Transconjugants from secondary transfer showed the resistance from donor and the auxotrophic requirement of the recipient strain in synthetic medium.

Curing

Each tube containing 10 ml peptone water supplemented with 20 μg/ml acridine orange (Riedel-De Haen AG, Germany) was inoculated with 0·1 ml of overnight broth culture and incubated at 37 °C for 24 h. Appropriate dilutions of the culture were plated on nutrient agar to obtain single colony isolates after overnight incubation at 37 °C. Resulting colonies were tested for loss of antibiotic resistance on nutrient agar plates containing the appropriate antibiotics.

RESULTS

Identification of thermotolerant coliforms

Thermotolerant coliforms identified were 201 strains of *Escherichia coli* and 30 strains of *Klebsiella* sp.

Antibiotic resistance

Apart from increased resistance to chloramphenicol ($P < 0{\cdot}001$) and streptomycin ($P < 0{\cdot}0001$), the thermotolerant coliforms exhibited a lower level of antibiotic resistance than the coliforms (Fig. 1). The ARI for coliforms (197) and thermotolerant coliforms (231) were 0·083 and 0·071, respectively. Out of the 220 thermotolerant coliform strains studied 69 (31·4%) exhibited multiple resistance, 107 (48·6%) double resistance, 12 (13·7%) single resistance and 32 (14·5%), were sensitive to all the antibiotics studied (Fig. 2).

Transfer of antibiotic resistance

The transfer of antibiotic resistance was studied in detail in 177 thermotolerant coliform isolates exhibiting resistance to ampicillin, chloramphenicol, tetracycline and streptomycin (Table 1). It was found that 10 (32·2%) of the multiply antibiotic-resistant (MAR) strains, 9 (21·9%) of the strains with double resistance and 8 (7·6%) of the strains with single resistance transferred one or more of their resistances to *E. coli* K12 (Table 1). The most common resistance patterns transferred are shown in Table 1. All the conjugative MAR strains consistently

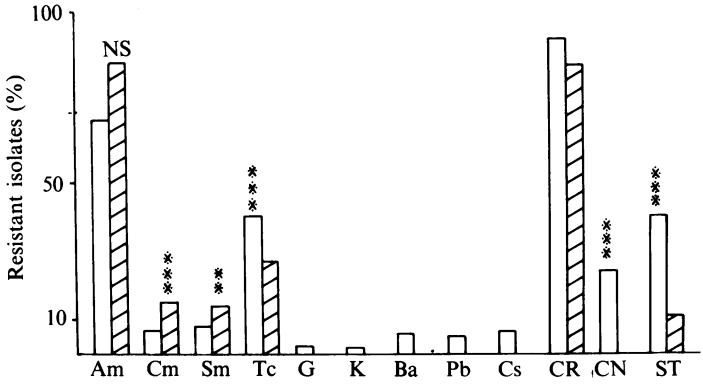

Fig. 1. Comparative incidence of antibiotic resistance among coliforms (□), and faecal (thermotolerant) coliforms (▨). **, $P < 0.01$; ***, $P < 0.001$; NS, Not significant. Am, ampicillin; Cm, chloramphenicol; Sm, streptomycin; Tc, tetracycline; G, gentamicin; K, kanamycin; Ba, cotrimoxazole; Pb, polymixin B; Cs, colistin; CR, cephaloridine; CN, carbenicillin; ST, sulphatriad.

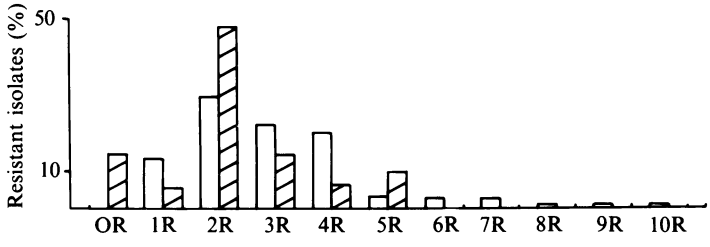

Fig. 2. Incidence of resistant coliforms (□), and faecal (thermotolerant) coliforms (▨), to different combinations of antibiotics. OR, sensitive strain; 1R, resistant to one antibiotic; 2R, resistant to two antibiotics; 10R, resistant to ten antibiotics.

Table 1. *Resistance transfer in thermotolerant coliforms*

Resistance pattern of the donor strain (Total no. strains, 177)	Number of strains studied for transfer (%)	Transfer pattern	Number of strains showing transfer of resistances (%)
MAR			
Am, Cm, Sm, Tc	18 (10·2)	Am, Cm, Sm, Tc	3 (16·6)
		Am, Cm	2 (11·1)
		Am	1 (5·6)
Am, Cm, Sm	5 (2·8)	Am, Cm, Sm	2 (40·0)
		Am	1 (20·0)
Am, Cm, Tc	8 (4·5)	Tc	1 (12·5)
2R			
Am, Sm	5 (2·8)	Am	2 (40·0)
Am, Tc	35 (19·8)	Am	7 (20·0)
Sm, Tc	1 (0·6)	—	—
1R			
Am	101 (57·1)	Am	8 (7·9)
Tc	4 (0·2)	—	—

MAR, Multiple antibiotic resistance; 2R, resistant to two antibiotics; 1R, resistant to one antibiotic.

Concentration of antibiotics used: 20 μg/ml.

Am, ampicillin; Cm, chloramphenicol; Sm, streptomycin; Tc, tetracycline.

Table 2. *Comparative transmissibility of resistances in* Klebsiella *sp. and* E. coli

	Total number of strains (%)	Number of strains showing resistance transfer (%)	Frequency of transfer
Klebsiella sp.			
MAR	1 (3·3)	1	$7{\cdot}0 \times 10^{-2}$
2R	24 (80·0)	8 (33·3)	$3{\cdot}6 \times 10^{-5}$ to $2{\cdot}6 \times 10^{-6}$
1R	5 (16·7)	2 (40)	$5{\cdot}0$ to $5{\cdot}7 \times 10^{-4}$
Total	30	11 (36·6%)	
E. coli			
MAR	30 (20·4)	9 (30)	$1{\cdot}5 \times 10^{-6}$ to $1{\cdot}0 \times 10^{-1}$
2R	17 (11·6)	nil	—
1R	100 (68·0)	2 (2)	$1{\cdot}0 \times 10^{-4}$
Total	147	11 (7·48%)	

MAR, Multiple antibiotic resistance; 2R, resistant to two antibiotics; 1R, resistant to one antibiotic.

Plasmid transfer frequencies were obtained after 18 h mixed incubation of donor and recipient.

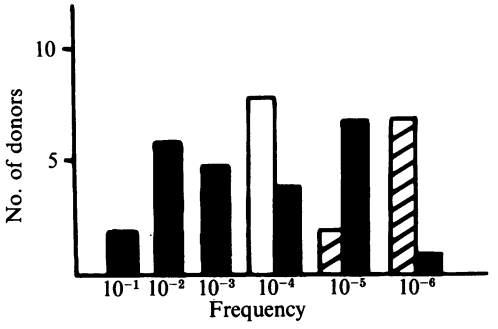

Fig. 3. Number of donors showing transfer frequencies of their R factor. □, 1R; ▨, 2R; ■, MAR.

transferred the same markers to the *E. coli* K12 recipient, irrespective of the selective antibiotic used. In strains resistant to two antibiotics, only ampicillin resistance was transferred.

Ampicillin resistance was transferred in 14·69% of the conjugation experiments whereas chloramphenicol, streptomycin and tetracycline were transferred in 2·82, 3·95 and 2·25% of the crosses, respectively.

With the thermotolerant *E. coli*, transfer of antibiotic resistance was observed in a significant percentage (30%) of MAR strains only, whereas thermotolerant *Klebsiella* spp. showed a significant level of transfer in double and single resistance isolates also (only one MAR strain was identified as *Klebsiella* spp.) (Table 2).

Frequency of transfer of antibiotic resistance

The frequencies of transmission of resistance from the donor strains to the sensitive *E. coli* K12 recipient is presented in Fig. 3 and is the mean of two or more matings. It was interesting to note that the frequency of transfer of resistances from *E. coli* K12 transconjugants (which represented donors in secondary transfer)

to C600 was similar to the values obtained in the primary transfer using *E. coli* K12 recipient. A significantly higher level and range of transfer frequencies was observed among MAR strains compared with the other strains.

Although 27 strains had conjugative R plasmids and transferred the resistances at a frequency greater than the mutation frequency of the recipient to the selecting antibiotic used, only MAR strains transferred drug resistance at a frequency greater than 10^{-4} (Fig. 3).

A wide range of the transfer frequency was observed in MAR strains, i.e. $1{\cdot}5 \times 10^{-6}$ to $4{\cdot}0 \times 10^{-1}$. Strains resistant to two antibiotics transferred at a lower rate varying between $2{\cdot}6 \times 10^{-6}$ and $3{\cdot}6 \times 10^{-5}$.

Curing of antibiotic resistance

Curing studies of strains with both transferable and non-transferable resistances revealed elimination of tetracycline resistance among 60% of the strains, while ampicillin, chloramphenicol and streptomycin resistances were cured in 52·5, 47·5 and 37·5% of strains, respectively. Three strains which showed cotransfer of Am, Cm, Sm and Tc resistances could not be cured of their resistances. In cases where curing occurred it was surprising to note that out of 100 cells screened for loss of resistance, 10–100% cells of each strain showed curing. Transferable resistance for ampicillin, chloramphenicol and streptomycin were cured among 24–30% strains, while none of the strains with transferable tetracycline could be cured.

DISCUSSION

Antibiotic resistance among coliform bacteria in drinking water, as reported by various workers, has ranged from 70 to 96% [2, 11, 12].

Bell and colleagues [13] observed that between 20 and 33% of faecal coliforms isolated from river water were resistant to three or more antibiotics, comparable to the incidence reported here among isolates from drinking water in rural India. In an earlier study, it was found that 6·1% of coliform strains from drinking water exhibited multiple antibiotic resistance of which only 1·5% were able to transfer the R-factors [11].

Multiple antibiotic resistance observed in our studies in drinking water appears to be more prevalent among thermotolerant coliforms than among coliforms. Other workers have reported similar findings for coliforms from various other sources [10, 13, 14].

Transfer of resistance has been reported in 34–75% of resistant strains whereas our studies show only between 15·3% strains capable of transferring their resistances [10, 15, 16].

In this study transfer frequencies of the order 10^{-3} were observed in 50% of our strains which is higher than the frequencies of 10^{-4} per donor cell and transfer rates above 10^{-3} in 25% of strains as described earlier [10, 17, 18].

The present study shows that acridine orange was able to eliminate all the four resistances but the resistance pattern of the individual strains did not influence the percentage of cells cured. Singh and Yadava [19] suggested that it is the characteristic of the plasmid which determines the curing frequencies. Although loss of antibiotic resistance on exposure to acridine was demonstrated in 60·0% of

the antibiotic-resistant strains studied, no relationship was observed between the strains exhibiting transfer of antibiotic resistance and those strains showing curing.

Recently between 11 and 50 thermotolerant coliforms/100 ml were found in 33·2% of rural drinking water samples in our studies. Consequently, it may be anticipated that $1–2 \times 10^3$ thermotolerant coliforms may be ingested daily in drinking water of which about 150 will have the potential of transmitting their R-plasmids to other gut bacteria [21]. A minimum of 10 bacteria is required for colonization and even 10 bacteria may not be able to colonize the gut [20, 21], which indicates that there is a negligible risk associated with the MAR strains contaminating rural drinking water. The hazard of MAR strains in drinking water is mainly associated with thermotolerant coliforms and more stringent criteria for water quality are not required while the guideline for safe water is < 1 thermotolerant coliform/100 ml which also allows for a very considerable safety margin.

Surveillance of the prevalence of MAR thermotolerant coliforms and frequency of resistance transfer, however, must not be overlooked in drinking water sources in the tropics where sanitation is limited and there are serious constraints in the provision of adequate water treatment. The ambient water temperatures in the tropics lie in the range reported for enhanced transfer efficiencies [22, 23]. It has been suggested by Cooke [24] that natural water environments produce advantages for the selection of bacteria with R-factors.

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