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Original Research Paper

Horizontal Transfer of Antibiotic Resistance in the Marine Environment

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ABSTRACT

The discovery of horizontal dissemination of antibiotic resistance in the environment has focused the attention on bacteria carrying infectious drug resistance plasmids (R factors). In the present study, 10 water samples from different coastal marine environments of Mumbai were analysed. Twenty seven organisms including *E. coli, K. pneumoniae, P. aeruginosa and Salmonella paratyphi* B were isolated. Evaluation of the isolates for their antibiotic resistance by the disc diffusion method revealed varying patterns of resistance to the antibiotics. Plasmid encoded resistance was seen in 74% of the isolates as shown by the loss of plasmids by acridine orange curing. Organisms were further assessed for their ability to transfer the antibiotic resistance by employing sediment associated transformation and transformation in beaker microcosms using the selected representative isolates. Intergeneric transfer of resistance in the natural environment was observed among the isolates by coincubation.

INTRODUCTION

Many scientists, who were working in the beginning of the antibiotic era, were almost cavalier about the microbial resistance to the antibiotics. But, today no one can deny that resistance is a major public health threat worldwide. In 1970, antimicrobial agents were described as potential environmental contaminants. Indeed, various antibiotics have been found in municipal sewage, including fluoroquinolones, sulfonamides and erythromycin metabolites. Residues of the antibiotics administered to humans and animals reach the sewage systems in urine or faeces, in the form of either the parent compound or the degraded metabolites depending on the pharmacology of the specific antibiotic (Guardabassi & Dalsgaard 2002).

The antibiotic concentrations found in the sewage vary between 1 and 100 μg/L. Such concentrations are 100 to 1000-fold lower compared with those necessary to inhibit resistant bacteria, but are sufficient to affect susceptible bacteria. Long-term environmental exposure to the low concentrations of antibiotics at the ng/L to μg/L range causes selection and evolution of the antibiotic resistant bacteria rapidly thereby shifting the population towards resistance (Nagulapally 2007).

Sewage contains high densities of living organisms, including pathogenic, commensal and environmental bacteria. Multiple resistant bacteria occurring in the municipal sewage effluents can survive for relatively long periods and maintain their resistance properties following introduction into the natural aquatic habitats. The pathogenic organisms serve as a source from which non-pathogens can acquire genes conferring resistance, and in turn, they can become resistant by acquiring the genes from the pathogens discharged into the environment (Shahid & Malik 2004). Thus, dissemination of the resistant bacteria is not only a problem of the resistant pathogens themselves, but also availability

of the resistant genes to the pathogens via horizontal gene transfer in practically every environment (McPherson & Gealt 1986).

The proximity of slums to the beaches, the release of the untreated sewage and industrial effluents in the sea, and nonadherence to the sanitary conditions have all led to the tremendous pollution of the marine environment in Mumbai. Hence, the aim of the present investigation was to identify the antibiotic resistant microorganisms in the coastal marine environment in Mumbai city along with the assessment of the transfer of the resistant genes among species. The study was focused on gram negative organisms like *E. coli, Klebsiella* sp., *Salmonell-Shigella* group and *Pseudomonas* sp.

MATERIALS AND METHODS

All the media chemicals (AR grade), reagents and antibiotic discs were procured from Hi Media Laboratories, Mumbai. The present study was carried out from July 2007 to January 2008 and was detailed on Gram negative organisms isolated from the coastal marine environment of Mumbai.

Water samples were collected from ten different sites in 100 mL sterilized glass bottles. All the samples were processed in the laboratory within 2 h of their collection by plating on the selective/ differential media like MacConkey's agar, Citrimide agar and *Salmonella-Shigella* agar. The plates were incubated at 36±1°C for 24 h. Single well-isolated colonies were picked, purified and identified using biochemical analysis as per the standard evaluation required (Collins et al. 1989).

Antibiotic susceptibility testing of the isolates: Antibiograms for the presence of drug resistance markers were performed by using Kirby Bauer disc diffusion method on Mueller Hinton agar by using commercially available antibiotic discs of chloramphenicol (30mcg), amikacin (30mcg), tetracycline (30mcg), ampicillin (10mcg), nalidixic acid (30mcg), piperacillin (100mcg), amoxicillin (20mcg), tobramycin (10mcg), cephotaxime (30mcg), cephalothin (30mcg), carbenicillin (100mcg) and ceftazidime (30mcg). The plates were incubated at 36±1°C for 24 h (Bauer et al. 1966). Zones of inhibition were measured and resistance pattern was evaluated as per the NCCLS guidelines.

Detection of 'R' plasmids by acridine orange curing method: Presence of the 'R' plasmids was detected by acridine orange curing method for all the isolates. 1 mL of 18 h old culture was added in 1.9 mL of nutrient broth (pH 7.2) containing 0.1 mL acridine orange (50µ/mL) (Shahid & Malik 2004). After 24 h incubation at 36±1°C, cultures were isolated and tested for antibiotic sensitivity using Kirby Bauer disc diffusion method as described above. All the representative isolates of each genus showing presence of 'R' plasmid by the virtue of the loss of resistance to the antibiotics were used for further investigation.

Transformation mediated transfer of the antibiotic resistance: Sediment-associated transformation and transformation in beaker microcosm were designed to simulate the transfer of resistance in the natural environment. The recipients chosen in the study for each genus showed antibiotic resistant pattern differing from that of the donor, i.e., each recipient showed sensitivity to those antibiotics to which the donor possessed resistance markers and for which the transfer was sought.

Sediment-associated transformation: Builder's sand mixed with 5% commercial peat was washed extensively with sterile artificial seawater (ASW) and then sterilized by autoclaving at 121°C, 15 Lb/in for 1 h. After sterilization, the mixture was used as sediment and maintained in sterile containers under ASW. Sterile sediment samples (5.0 cm^3) were aseptically packed by gravity flow in 10 cm^3 columns. Recipient cultures, grown overnight in ASW at 36±1°C with shaking, were centrifuged at 2000 rpm for 20 min, washed twice with equal volumes of sterile ASW, and resuspended in

an equal volume of ASW. Two sets of columns were preloaded for 1 h with 5.0 mL of the recipient cell suspension, preventing flow through the column to allow association of cells with the sediments. After 1 h, cell suspensions were drained from the columns by gravity flow. The columns were then loaded with 5 mL of ASW (with and without 0.5% glucose), containing 10μg DNA from antibiotic-resistant strains. Flow was again restricted through the columns, and columns were incubated at 36±1°C. Columns were drained by gravity flow following overnight incubation, washed with 5.0 mL of sterile ASW and sediments were aseptically transferred to sterile tubes containing 5.0 mL of sterile ASW. Cells tightly associated with the sediments, were released by vigorous vortexing for 5 min. Appropriate dilutions were plated on Luria Bertani agar (tryptone 10g, yeast extract 5g, NaCl 5g, agar 10g in 1L distilled water, pH 7.2) containing the antibiotic to which the recipients were sensitive in order to select for the transformants which were further analysed for the acquisition of the antibiotic resistance markers (Stewart & Sinigalliano 1990).

Transformation in beaker microcosms: The microcosms consisted of 1 L beakers containing 400 mL of sterile (autoclaved 15 min at 15 lb) ASW. Mating experiments were performed by placing overnight cultures of the recipient (1 mL) and source DNA (100 μL) on the surface of sterile scrubbed slate discs, covered with a larger filter paper (Whatman No. 1), secured with a thread to facilitate the contact between the recipient and source of DNA in the beaker microcosm. Slate discs were incubated at room temperature for 24 h and then the filters were retrieved. The transformants were isolated on Luria Bertani agar containing the antibiotic to which the recipients were sensitive. The 18 h old transformants were further subjected to antibiotic susceptibility testing (AST) using Kirby Bauer disc diffusion method to determine the nature of antibiotic resistance acquired by them (Williams et al. 1996).

Coincubation: Coincubation experiments were designed to study intergeneric transfer of the antibiotic resistance. The mating pairs chosen belonged to two different genera and displayed differing antibiotic sensitivity patterns. Five mL of each overnight grown cultures (10^8/mL) were mixed (1:1) in test tubes containing sterile sewage and incubated, without shaking, for 24 h at 36±1°C. After incubation, the bacteria were plated on selective media and then each culture was tested for antibiotic resistance pattern by using AST (McPherson & Gealt 1986).

RESULTS AND DISCUSSION

The release of the non-disinfected wastewaters into marine environments, a common worldwide practice, exerts enormous pressure on marine environment. Unprecedented increase of human activities, in and around Mumbai, has imposed considerable stress on the surrounding marine environment. In recent times Mumbai's burgeoning population and industries have grown to a larger extent, resulting in the generation of over 2700 MLD of wastewater including sewage, a large part of which is released without any treatment in the marine environment of Mumbai (Sawant et al. 2004). The seas are, thus, constantly infused with wastewater bacteria, among them predominant are the highly pathogenic ones. Their numbers vary from a few per litre to about 10^8 per mL. Bacteria are abundant in near shore regions, where organic pollution as well as microbial pollution from the surrounding environment occurs (Nagulapally 2007).

In the present investigation, the samples were collected from the shorelines in the vicinity of the hospitals, residential complexes, slums, or near sewage outfalls. Amongst the isolates obtained (n=27), the distribution of organisms was as follows: 7.4% *E. coli* (n=2), 25.9% *K. pneumoniae* (n=7), 29.6% *Pseudomonas aeruginosa* (n=8) and 37 % *Salmonella paratyphi* B (n=10). Such pathogenic

microorganisms can infect humans through the primary contact such as swimming, secondary contact such as boating or fishing, and ingestion of the contaminated shellfish. Shellfish concentrate bacteria and viruses from the sewage. Consuming raw or partially raw shellfish can lead to the transmission of diseases. Since, shellfish are at the bottom of the food chain they affect other fish species as well (U.S.EPA 2001). The diseases caused by these pathogens around the world are cause of great concern, and antibiotic resistance is an increasing problem in the treatment of these diseases.

Evaluation of all the isolates for their antibiotic resistance showed resistance to two or three of the first line of antibiotics. Resistance to mainly tetracycline, ampicillin and chloramphenicol was the most prominent in all the isolates tested. Amongst the second line of antibiotics, resistance to nalidixic acid was the most common. *K. pneumoniae* 4, *P. aeruginosa* 4, 5, 6 and *S. paratyphi* B 5, 6 showed resistance to all the four second generation antibiotics. All the isolates were observed to be resistant, on an average, to 3-4 of the third generation antibiotics. Resistance to cephalothin, carbenicillin and ceftazidime was predominant amongst all the isolates. The most shocking result was shown by *K. pneumoniae* 4 with resistance to all the twelve antibiotics tested.

Simultaneous resistance in one bacterium to three or more classes of the antibiotics by various resistance mechanisms generally encoded by different genes is defined as the multi drug resistance (MDR). Isolates were characterized as multiple antibiotics resistant if they showed resistance to three or more antibiotics. Antibiotic resistance to at least one third generation antibiotic was a prerequisite for an isolate to be considered as MDR. 8.33% isolates were resistant to four antibiotics, 25% to six antibiotics, 75% to seven antibiotics, 50% to eight antibiotics, 33.33% to nine antibiotics, 16.66% to ten antibiotics, and 8.33% to all the twelve antibiotics tested.

Coastal samples collected from various countries have shown the presence of antibiotic resistance markers amongst the isolated pathogens. Resistance to ampicillin, erythromycin and ciprofloxacin was detected in the isolates of enterococci in the samples collected off the coast of Greece. In samples collected from the coasts of Spain, Egypt, Puerto Rico, Maryland, North Carolina, and in the Gulf of Mexico, sites receiving runoff from the sewage treatment plants, had a higher percentage of resistant organisms than sites with less human impact. In the Apalachicola Bay of Florida, 82% of *E. coli* isolates were resistant to at least one antibiotic (Gangle 2005).

One of the major causes of antibiotic resistance amongst organisms is the presence of R-plasmid. A single plasmid may carry separate genes for the resistance to a number of antibiotics. Resistance factors (R factors) have now been found in a host of different environments and in a variety of different organisms but mainly in the Enterobacteriaceae (Shahid & Malik 2004). Among the multitude of substances occurring in the sewage, heavy metals and biocides have the potential to select for the antibiotic resistance, even though they are not antibiotics. Genes encoding resistance due to the heavy metals and antibiotics often co-exist on plasmids and integrons. Also, the co-selective effect of the biocides for antibiotic resistance could be particularly marked when these substances are dispersed in the environment, because of dilution and formation of the concentration gradients (Guardabassi & Dalsgaard 2002). In the present investigation, such a condition was presented by the selected sites, all of which were contaminated by sewage and domestic wastes.

Plasmids are eliminated from the host bacteria after exposure to the sublethal concentrations of the intercalating dyes such as acridine orange, ethidium bromide, etc. In the present investigation, acridine orange was used at sublethal concentration of 50µg/mL. Curing with acridine orange inhibits the replication of the plasmid without interfering with the bacterial cell replication leading to the

production of the plasmid-free cells. Loss of the plasmid is concomitant with the loss of resistance to the antibiotic encoded by the plasmid (Shahid & Malik 2004).

After curing, 74% of the isolates showed sensitivity to some of the antibiotics to which they were resistant before the curing treatment, indicating the R-plasmid mediated antibiotic resistance. In most cases the resistance to chloramphenicol, nalidixic acid, carbenicillin and ceftazidime was plasmid encoded. *K. pneumoniae* 4 and *S. paratyphi* B 9 showed antibiotic resistance to be plasmid mediated for most of the antibiotic resistance markers (9 each) carried by them (Table 1). It is reported that a single plasmid may code for the resistance up to ten different antibiotics simultaneously (Committee for Veterinary Medicinal Products, U.K. 1999).

There is ample evidence to suggest that horizontal transfer has spread genes farther than was previously thought possible. Indeed, water-borne outbreaks of enteric pathogens carrying multiple antibiotic resistance plasmids or 'R' factors consequently promote transfer of antibiotic resistance from resistant to the completely antibiotic sensitive bacteria and intergeneric gene transfer. This has already led to large numbers of deaths due to the diagnostic problems and the failure of an adequate response to the antibiotic treatment (Alcaide & Garay 1984).

Transformation experiments were designed in order to elucidate the risk to public health presented by the marine pathogens via horizontal gene transfer. An increasing body of evidence points at natural genetic transformation as an important mechanism of the horizontal exchange of genes (Lorenz & Sikorski 2000). In the present study, four isolates corresponding to each genus were chosen as donors (*P. aeruginosa* 2, *K. pnemoniae* 4, *P. aeruginosa* 4 and *Salmonella paratyphi* B5). The corresponding recipients (*E. coli* 1, *K. pnemoniae* 1, *P. aeruginosa* 6 and *Salmonella paratyphi* B2) showed absence of antibiotic resistance markers possessed by the donor. Except in case of *E. coli* 1, for which DNA of *P. aeruginosa* 2 was used to bring about transformation, all the donor recipient pairs belonged to the same genera. The DNA from the donors was added in the respective sand columns (Table 2).

Transformation of the recipients was observed in both the sets of the sand columns with and without glucose. All the recipients acquired resistance pattern of the donors. This could be attributed to the presence of DNA in the artificial sea water, leaked from the cells during the incubation. Dissolved DNA may be an important component of the DOM (dissolved organic matter) for microbial growth, because of its enrichment in nitrogen and phosphorus, and also as a source of nucleic acid precursors, which are energetically expensive to synthesize *de novo*. DeFlaun et al. (1987) have reported dissolved DNA concentrations ranging from 0.2 to 44 pg/L for a variety of marine, estuarine, and freshwater environments. Dissolved DNA could be genetically important, encoding for gene sequences with the potential to transform microbial populations. The studies on the transformation of *Bacillus subtilis* loaded onto the sand columns by the exogenous DNA have been reported by Lorenz & Wackernagel (1987).

Several authors have studied natural transformation in the laboratory based microcosm experiments. Paul et al. (1991) demonstrated the uptake of plasmid DNA by a marine *Vibrio* strain in the marine water and sediment microcosms. *P. fluorescens* transferred plasmids to marine isolates, in microcosms and on plates, even when in the viable but non-culturable (VBNC) state. In the present investigation microcosms were set up by placing overnight cultures of the recipient (each of the four representative genera of *E. coli* 1, *K. pnemoniae* 1, *P. aeruginosa* 6 and *Salmonella paratyphi* B 2) and source DNA from the donors (*P. aeruginosa* 2, *K. pnemoniae* 4, *P. aeruginosa* 4 and *Salmonella paratyphi* B5) on the surface of sterile scrubbed slate discs, covered with Whatman No.1 filter paper.

The DNA of all the donors was able to transform the corresponding recipients conferring them with the antibiotic resistance to which they were sensitive earlier. This indicated ability of the recipients in biofilm on the available surface to develop competence required for the transformation process and get transformed. The DNA also seemed to exist in a biologically active form for a sufficient period of time to be taken up and expressed by the introduced species. According to Murray (1997), the transfer as well as the emergence of new combinations of resistance genes will happen most frequently in compartments with high bacterial density, i.e., biofilms presenting no taxonomic barrier to this horizontal genetic transfer. High bacterial concentrations increase the chance of the donor and the recipient cells come in contact with each other (Guardabassi & Dalsgaard 2002).

In the present investigation, the intergeneric transfer of the antibiotic resistance was also studied by using mating pairs belonging to different genera showing different antibiotic resistance pattern. For example, the organism showing sensitivity to certain antibiotics was mated with the isolate of a different genera displaying resistance to these antibiotics (Table 3). Assessment of the horizontal antibiotic resistance transfer after coincubation for 24 h showed that the resistance transfer occurred between all the mating pairs reiterating the fact that difference in the genera did not affect the transfer of the resistance in any way. In fact, in pairs of *E. coli-S. paratyphi* B5, *K. pneumoniae* 1-*P. aeruginosa* 2, *K. pneumoniae*1-*S. paratyphi* B 5, resistance transfer occurred in both the partners of the mating pair acquiring resistance to the antibiotics to which they were sensitive earlier. For example, chloramphenicol, amikacin and tobramycin resistance was observed in *S. paratyphi* B5 and *P. aeruginosa* 2, whereas resistance to tetracycline was observed in *E. coli* 1 and *K. pneumoniae* 1.

The antibiotic resistance transfer observed could be attributed to both the transformation as well as conjugation. It appears that some strains have a higher potential for gene acquisition than others developing low or no competence. It can not be excluded that strains, including those with low competence under standardized high-nutrient conditions, may have an increased capability of DNA uptake in the environment (Lorenz & Sikorski 2000).

The ability of the plasmid DNA to transfer or be mobilized between different strains of the same species or between bacterial species has been repeatedly demonstrated by many workers. Using green fluorescent protein (GFP), conjugative transfer was demonstrated between *P. putida* and indigenous bacteria in both the artificial seawater and seawater samples (Dahlberg et al. 1998), even under nutrient-limiting conditions. Mating experiments showed that resistance plasmids could be transferred from *E. coli* to *V. parahaemolyticus in vitro* (Dutta & Pan 2002). Fontaine & Hoadley (1976) investigated incidence of the antibiotic-resistant faecal coliforms in raw and treated municipal wastes. Antibiotic resistance was transferable to *Escherichia coli* and *Salmonella cholerae*-suis recipient strains from 90.9% of resistant isolates from municipal wastes, and 45.9% of all isolates from municipal wastes. Kralikova et al. (1986) demonstrated gentamicin resistance transfer by Enterobacteriaceae and *Pseudomonas* in a sewage sludge strain of *Klebsiella pneumoniae* resistant to seven antibiotics and in two multiresistant isolates from the River Danube.

These findings show that the release of municipal wastewater in marine environments may function as the reservoir of strains bearing the determinants of the transferable resistance. In the countries of South Asia, this problem has been particularly important in enteric pathogens, with both India and Bangladesh reporting outbreaks of *Shigella* with multiple antimicrobial resistance (Adhikari et al. 2000). The present investigation emphasizes the need to assess the relative importance and contribution of the resistant bacteria in the aquatic environment and the consequent risk posed by them to the community.

Table 1: Effect of plasmid curing on the loss of antibiotic markers in various isolates.

Antibiotic	Isolates for which curing was observed
Chloromphenicol	*P. aeruginosa* 2, 4, *K. pneumoniae* 4, *S. paratyphi* B 5, 7, 9,10
Amikacin	-
Tetracycline	*E. coli* 1, *K. pneumoniae* 1, *S. paratyphi* B 9
Ampicillin	-
Nalidixic acid	*E. coli* 1, *K. pneumoniae* 1-5, *S. paratyphi* B 2, 5-10
Piperacillin	*E. coli* 1, *K. pneumoniae* 1, *P. aeruginosa* 9, 6, 8, *S. paratyphi* B 2, 8-10
Amoxycillin	*K.pneumoniae* 4, *S.paratyphi* B2,5,6 and 9
Tobramycin	*P. aeruginosa* 2, 3, 4, *K. pneumoniae* 4, *S. paratyphi* B 5-7
Cephotaxime	*K. pneumoniae* 3, 4, *S. paratyphi* B 5
Ceprofloxacin	*P. aeruginosa* 2, 4, 6, 8, *S. paratyphi* B 7-10
Carbenicillin	*E. coli* 1, *K. pneumoniae* 1, *P. aeruginosa* 4, 6, 8, *S. paratyphi* B 2-10
Cefoxitin	*E. coli* 1, *K. pneumoniae* 1,3-5, *P. aeruginosa* 2, 4, 6, 8, *S. paratyphi* 2-10

Table 2: Transformation of the resistant markers from donors to the recipients.

Donors	Markers	Recipient	Markers	Transferred markers
P. aeruginosa 2	C^R, Ak^S,T^R, A^R, Na^S, Pc^S, Ac^S, Tb^R, Ce^S, Ch^R, Cb^R, Ca^R	*E. coli* 1	C^S, Ak^S, T^R, A^R, Na^R, Pc^R, Ac^S, Tb^S, Ce^S, Ch^R , Cb^R, Ca^R	Chloramphenicol, Tobramycin
K. pnemoniae 4	C^R, Ak^R, T^R, A^R, Na^R, Pc^R, Ac^R, Tb^R, Ce^R, Ch^R, Cb^R, Ca^R	*K. pnemoniae* 1	C^S, Ak^S, T^R, A^R, Na^R , Pc^R, Ac^S, Tb^S, Ce^S, Ch^R, Cb^R, Ca^R	Amoxycillin, Tobramycin
P. aeruginosa 4	C^R, Ak^S, T^R, A^R ,N^R, Pc^R, Ac^R, Tb^R, Ce^S, Ch^R, Cb^R, Ca^R	*P. aeruginosa* 6	C^S, Ak^S, T^R, A^R, Na^R , Pc^S, Ac^S, Tb^S, Ce^S,Ch^R , Cb^R,Ca^R.	Chloramphenicol, Tobramycin
Salmonella paratyphi B5	C^S, Ak^S, T^S, A^R , Na^R, Pc^R, Ac^R, Tb^R, Ce^R, Ch^R, Cb^R, Ca^R.	*Salmonella paratyphi* B2	C^R, Ak^S, T^R, A^R, Na^R , Pc^R , Ac^S , Tb^S , Ce^S , Ch^R , Cb^R, Ca^R.	Amoxycillin, Tobramycin

Key: Chloramphenicol (C), Amikacin (Ak), Tetracycline (T), Ampicillin (A), Nalidixicacid (Na), Piperacillin (Pc), Amoxicillin (Ac), Tobramycin (Tb), Cephotaxime (Ce), Cephalothin (Ch), Carbenicillin (Cb), Ceftazidime (Ca)

Table 3: Antibiotic resistance transfer by coincubation.

Mating pair	Resistance Pattern								Resistance Transfer in
	Before Coincubation				After Coincubation				
	C	Ac	Tb	T	C	Ac	Tb	T	
E. coli 1+	S	S	S	S	R	R	R	R	*E. coli* 1
K. pneumoniae 1	R	R	R	R	R	R	R	R	
E. coli 1 +	S	S	S	S	R	R	R	R	*E. coli* 1
P. aeruginosa 2	R	R	R	R	R	R	R	R	
E. coli 1 +	S	S	S	R	R	R	R	R	*E. coli* 1 and *S*
S. paratyphi B 5	R	R	R	S	R	R	R	R	*paratyphi* B5 both
K. pneumoniae 1 +	S	S	S	R	R	R	R	R	*K. pneumoniae* 1 and
P. aeruginosa 2	R	R	R	S	R	R	R	R	*P. aeruginosa* 2 both
K. pneumoniae 1 +	S	S	S	R	R	R	R	R	*K. pneumoniae* 1 and
S.paratyphi B5	R	R	R	S	R	R	R	R	*S. paratyphi* B5 both

Key: Chloramphenicol (C), Tetracycline (T), Amoxicillin (Ac), Tobramycin (Tb), Resistant (R), Sensitive (S)

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