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RESEARCH ARTICLE

Transferable drug-resistant coliforms in fish exposed to sewage

Sutapa Sanyal, Samir Banerjee

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Abstract. In this study, the thermotolerent fecal coliform (Th FC) bacterial population (n = 81) in a waste-fed aquaculture system was examined for multiple antibiotic resistance and the possession of transferable drug resistance factors (R factors). Multiple antibiotic resistant (MAR) coliforms were found to be common in the sewage-fed pond environment, with 83% of the screened MAR isolates harboring plasmids of > 10 kilo base pair (kb). The transfer of resistance was confirmed by mating experiments in 92% of plasmid-positive MAR coliforms with a nalidixic acid (NA) resistant strain, *Escherichia coli* ATCC (American Tissue Type Cell Culture) 14948 recipient in the presence of DNase (Deoxyribonuclease). Antibiotic resistance profiles of some mated progenies (70.83%) indicated that transfer was unidirectional. DNase-treated cell-free supernatants did not transform, which excluded transduction. A DNase-resistant conjugation-like mechanism probably plays a major role in the transfer of resistance factors. Physical evidence of transmissible resistance factors in fish suggests a potential health risk to humans and animals, including farmed fish.

Keywords: fish, health risk, multiple-antibiotic-resistant isolates, transferable drug resistance factors

S. Sanyal [✉]
Department of Zoology, Bethune College, 181 Bidhan Sarani
Kolkata - 700006, West Bengal, India
Tel. + 91 9874075312; e-mail: sutapa2007.sanyal@gmail.com,

S. Banerjee
Department of Zoology, University of Calcutta, 35-Ballygunge
Circular Road, Kolkata 700019, West Bengal, India

Introduction

Wastewater-fed aquaculture provides an opportunity to treat wastewater with integrated material-flow recycling. Several goals are achieved simultaneously: on the one hand valuable goods such as foodstuffs, animal feeds, raw materials, ornamental plants and animals, mainly fish, are produced, and on the other utilizable gray water, or purified and hygienized wastewater, is produced. Nowadays, recycling and reusing wastes through aquaculture present several concerns that are mainly related to health hazards generated by the use of water recovered from sewerage systems. This reclaimed water contains parasites, bacteria, viruses, various types of pharmaceuticals such as antibiotics, estrogens, and active ingredients of drugs at relatively low concentrations. Although individual antibiotic concentrations are low, there are so many different ones that when combined they can pose serious health and environmental problems (Koplin 2002). These lower concentrations of antibiotics are sufficient to affect susceptible bacteria (Al-Ahmad et al. 1999). Therefore, the occurrence of such antibiotic concentrations in sewage has the potential to select for antibiotic resistance.

In the field of aquaculture, both environmental and therapeutic problems are addressed since antimicrobial agents are released into surrounding waters of waste-fed fish ponds through domestic sewage (Aoki 1992). Some of the major concerns presented by antibiotics in the aquatic environment

are that entire trophic levels of bacteria can be eliminated in some ecosystems, or that multiple drug-resistant bacteria flourishes and make their way into the food chain. Commercial fishes residing in wastewaters can act as carriers of antibiotic resistant bacteria prompting health risks to consumers (Schwartz et al. 2003, Pathak and Gopal 2005, Newaj et al. 2008). The occurrence of antibiotic resistance might signal the occurrence of plasmid transfer in the microbial milieu of sewage systems (Schluter et al. 2003). The high concentrations of bacteria, nutrients, and suspended solids in sewage-fed ponds are all factors that enhance horizontal gene transfer (Lorenz and Wackernagel 1994). Wastewaters and the fishes inhabiting them are, therefore, a potent source of antibiotic-resistant bacteria, which, in turn, can transfer their resistance genes to nonresistant bacteria. Today, transferable drug resistance represents a major threat to the treatment of infectious diseases in both humans and animals, including farmed fish (Schwartz et al. 2003).

The potential for antibiotic exposure and resistance development in human and animal gastrointestinal tracts, coupled with relatively great abundance in waters contaminated with human and animal wastes, make the thermotolerent fecal coliform bacteria a logical focal group for studies of antibiotic resistance and transfer in waste-fed aquatic environments. Sewage-fed fisheries play a great role in improving water quality in tropical Asian countries, and it can be compared to an efficient stabilization pond as far as fish production is concerned. Moreover, potential public health hazards have never been considered although good fish production has been achieved (Strauss 1997). In view of the public health concern about the safety of fish raised in wastewater ponds, the aim of this study was to analyze the antibiotic resistance patterns in thermotolerent fecal bacteria collected from fish and the waste-fed aqueous environments they are reared in. The investigation was also undertaken to study the transfer of R factors and the frequencies of transfer under standardized laboratory conditions. Much of the work dealing with the transfer of R factors has focused on which portion of an antibiotic-resistant bacterial population (more than one antibiotic) exhibits R factors, and which portion of them is capable of transferring R factors and at what rates to sensitive organisms.

Materials and Methods

Study area

A wetland located in Bandipur, Rahara, North 24 Parganas, (22°44' N and 88°24' E), where untreated domestic wastewater is utilized for fish farming, was the study site. The raw sewage is entirely of domestic origin, and comes from the adjacent town of Titagarh, a municipal area in North 24 Parganas, West Bengal, India.

Sampling and dissection

The fish samples were caught with a net and were immediately transferred to the laboratory in containers with pond water. The major Indian carps *Labeo rohita* (Hamilton), *Cirrhinus mrigala* (Hamilton), and *Oreochromis niloticus* (Peters) were subjected to bacteriological assays. Ten live fish of each of the species were selected randomly from the catch at each sampling time (bimonthly from March 2010 to November 2010). The fish were dissected according to Buras et al. (1987). Muscles and digestive tract contents were isolated and placed in sterile glass vessels. The tissues were weighed under sterile conditions, ground in a mortar, and suspended in a sodium chloride (NaCl) physiological solution (9 ml of the solution for each 1 g of muscle or digestive tract content). The suspensions were homogenized using a Universal Laboratory Aid Type MPW-309 homogenizer, at 1000 rpm for 10 min. The homogenates were then serially diluted (10^{-1} to 10^{-8} for muscles and 10^{-1} to 10^{-10} for digestive tract contents) and inoculated into culture media. The time lag from fish collection to analyses did not exceed 6 h. Water from the sewage-supplied pond was sampled from the sub-surface, i.e., 15 to 20 cm below the water

surface, to avoid surface contamination and analyzed simultaneously with the fish sampling.

Microbiological analyses

The pond water and fish samples were examined for thermotolerent fecal coliforms using the three-tube fermentation technique (APHA 1998). Representatives of typical thermotolerent coliform isolates were selected randomly by colony morphology from the selective culture medium (as described by APHA 1998 and Sanyal et al. 2011). They were streaked aseptically several times on freshly prepared Nutrient agar plates to obtain pure isolates for antibiotic resistance tests. Pure fecal isolates were subjected to several biochemical tests which included indole production, methyl-red, and Voges Proskauer reactions, citrate utilization, and reaction in motility and urea medium. Eighty-one purified fecal bacterial isolates were tested for anti-microbial sensitivity using the disc diffusion method (Bauer et al. 1966). Antibiotic impregnated discs of 8 mm diameter were used for the test, and the disks contained the following antibacterial agents: ampicillin (AMP 10 μg); amikacin (AK 10 μg); chloramphenicol (C 30 μg); cotrimoxazole (CO 25 μg); gentamicin (GEN 30 μg); kanamycin (KAN 30 μg); streptomycin (STR 10 μg); tetracycline (TET 25 μg). Resistance was estimated by measuring the inhibition zone as per standards (NCCLS 2002). Reference strains *E. coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853, recommended by NCCLS (1997), were used as control organisms for verification of the disks on Tryptone soya agar plates. All the culture media and antibiotic discs were obtained from Hi Media Laboratories Ltd., Mumbai, India. The rationale for choosing these compounds as target antibiotics was based on previous studies, (Andersen 1993, Goñi-Urriza et al. 2000, Kim and Diana 2007, Jury et al. 2010) that reported the occurrence of AMP, AK, C, CO, GEN, KAN, STR, and TET in municipal wastewater treatment plants. This made it likely that the microbial biomass in the Bandipur waste-fed ponds also included bacteria resistant to these antibiotics.

Plasmid profiles of drug-resistant thermotolerent coliforms

Drug resistant isolates were tested for the availability of plasmids. A rapid alkaline extraction procedure proposed by Sambrook et al. (1989) was followed for screening plasmid DNA. Electrophoresis was performed using a 0.8% and 1% agarose gel system (Bangalore Genei, India) in tris acetate buffer with molecular weight markers (Hi Media, Mumbai, India). The gels were stained with ethidium bromide (0.5 μg ml^{-1} Sigma). The resolved bands were visualized on a UV-transilluminator at a wavelength of 360 nm, and photographed using a UV gel documentation system (Alpha Imager, Innotech Corporation, USA). The concentrations of agarose noted above were used in electrophoresis to separate high-molecular-weight plasmid DNA (up to 25 kb).

Mating experiments on the transfer of resistance factors in bacteria

A nalidixic acid (NA) resistant strain, *E. coli* ATCC 14948, was used as the recipient in all the mating experiments. All of the environmental isolates that were not resistant to nalidixic acid were tested as donor strains in individual mating assays. Isolates demonstrating multiple-drug resistance were processed and tested separately for the ability to transfer each type of drug resistance factor. Twenty-four plasmid-positive multiple-antibiotic-resistant thermotolerent fecal coliforms isolated from the fish and the water of the waste-fed ponds were tested for transferable resistance.

Transfer of resistance factors in liquid medium

Prior to mating, individual bacterial cultures were grown overnight at 30°C in Tryptone Soya Broth (TSB) containing 30 μg ml^{-1} of the appropriate antibiotic (NA for *E. coli* ATCC 14948 and either AMP, C, STR, or TET for the environmental isolates). The bacterial cells were pelleted by centrifugation for 5

min at 8,500 g, and resuspended in fresh TSB lacking antibiotic. Mating experiments were conducted as described by Bell et al. (1983). Briefly, equal volumes (0.1 ml) of donor cells and recipient cells were mixed in a 1.5 ml tube with 0.8 ml of antibiotic-free TSB. Mated cells in broth were supplemented with DNase I and $MgCl_2$ at final concentrations of 200 μg ml^{-1} and 2 mM, respectively (Omar et al. 2007). The mixtures were incubated at 30°C for 24 h. After incubation the mixtures of donor and recipient cells were diluted 10-fold in 0.9% saline to a 10^{-7} dilution. Samples (0.1 ml) from each dilution were plated onto TSA containing dual antibiotics (NA and either AMP, C, STR or TET, all at 30 μg ml^{-1}) and incubated for 24 h at 30°C to select for recombinants. When mating progeny was achieved, the antibiotic-resistance patterns of representative recombinants were determined with multi tipped disks. Negative mating control experiments lacking either the donor or recipient bacteria were included in all mating experiments to check for the mutation of either strain to antibiotic resistance. No mutation to specific antibiotic resistance was noted with any donor strains or the universal *E. coli* ATCC 14948 recipient strain (West et al. 2011).

Transfer of resistance factors using cell culture supernatants

Experiments using cell-free culture supernatants as a DNA source were done as described below. After the harvested cells of donors and recipients were centrifuged for 5 minutes at 8,500 *g*, the supernatants were passed through a filter with a 0.2 μm pore size (Life Sciences Products, Inc.) and then centrifuged for an additional 3 min at 11,500 *g*. Aliquots of recipient cells (0.1 ml) were mixed in 0.8 ml of antibiotic-free TSB broth tubes together with 0.1 ml of the treated supernatants of the donor cells. These experiments were done in the presence of DNase I, as described above. The mixtures were incubated at 30°C for 24 h. After incubation, the mixtures were diluted 10-fold in 0.9% saline to a 10^{-7} dilution. Samples (0.1 ml) from each dilution were plated onto TSA containing dual antibiotics (NA and either AMP, C, STR or TET, all at 30 μg ml^{-1}) and incubated for 24 h at 30°C. When mating progeny was achieved, the antibiotic resistance patterns of representative recombinants were determined with multi tipped disks. Additional mating experiments were also performed in the presence of DNase I following the method described above with cell-free supernatants of donors that were not filtered. Further experiments were performed in the presence of DNase I with treated supernatants from co-cultures of donors and recipient cells.

Measurement of transfer frequency

Transfer frequency was estimated by dividing the number of recombinants per milliliter by the number of donors per milliliter in the mating mixture (Yutaka et al. 2004). A number of transferable resistant coliforms were characterized biochemically to detect possible effects on strain distribution caused by the discharge of domestic waste effluent in sewage-fed ponds. Biochemical characterizations of the isolates were performed to identify genus by a standard procedure used for *Enterobacteriaceae* (Kelly et al. 1995). In the present study, the presence of coliforms was confirmed using multiplex PCR for the specific amplification of the lacZ gene that encodes β-D galactosidase and a portion of the uid A gene for *E. coli* detection. For multiplex PCR, a pair of primers (5'-ATGAAAGCTGGCTACAGGAAGGCC-3' and 5'-CACCATGCCGTGGGTTTCAATATT-3', Bej et al. 1990) located within the coding region of the lacZ gene of *E. coli* and a pair of primers (5'-TGGTAATTACCGACGAAAACGGC-3' and 5'-ACGCGTGGTTACAGTCTTGCG-3', Bej et al. 1991) located within the uid A structural gene of *E. coli* were used. The primers were obtained from Messers Bangalore Genei, Bangalore, India. During the development of the PCR amplification procedure for coliform detection, total genomic DNA was extracted from the cultures with the procedure by Ausubel et al. (1996). In each PCR amplification, a buffer control, to which no DNA template was added, was included as an internal control. DNA

Table 1
Antibiotic resistance patterns in thermotolerent fecal coliforms from different sources at a waste-fed farm. Numbers of isolates in brackets. NA= not analyzed, -ve = negative for plasmid, AMP = ampicillin, AK = amikacin, C = chloramphenicol, CO = cotrimoxazole, GEN = gentamicin, KAN = kanamycin, STR = streptomycin and TET = tetracycline, MAR= Multiple antibiotic resistance

Source	No. of isolates	Single antibiotic resistant isolates	MAR isolates	Patterns and number of MAR isolates	No. of plasmid positive MAR isolates
Water	12	AMP (2)	8	AMP- TET (3)	AMP-TET (2)
				AMP-CO-TET (3)	AMP-CO-TET (3)
				AMP-CO-STR-TET (1)	AMP-CO-STR-TET (1)
				AMP-C-CO-TET (1)	AMP-C-CO-TET (1)
Oreochromis sp.	24	AMP (7)	11	AMP-C (1)	AMP-C (1)
		TET (1)		AMP-CO (1)	AMP-CO (1)
		KANA (2)		AMP-TET (1)	AMP-TET (1)
				AMP-CO-STR (1)	AMP-CO-STR (-ve)
				AMP-C-TET (4)	AMP-C-TET (4)
				AMP-C-TET (3)	AMP-C-TET (3)
Labeo sp.	24	AMP (5)	10	AMP-C (3)	AMP-C (3)
				AMP-TET (4)	AMP-TET (3, 1-ve)
				AMP-C (1)	AMP-C (NA)
				AMP-C-STR (1)	AMP-C-STR (1)
				AMP-C-CO (1)	AMP-C (-ve)
Cirrhinus sp.	21	AMP (4)	2	AMP-C (1)	AMP-C (-ve)
		C (1)		AMP-C (1)	AMP-CA (NA)
		CO (1)			
Total	81	23	31	31	24

templates of coliform bacteria and *E. coli* extracted from reference bacterial strains (*Klebsiella pneumoniae* ATCC 27736, *E. coli* ATCC 25922) were included as positive controls.

Results

A substantial portion of bacteria isolated from the fish and pond water were resistant to ampicillin, chloramphenicol, and tetracycline (except *Cirrhinus* sp.), whereas bacteria resistant to kanamycin (except in *Oreochromis* sp.), streptomycin, and co-trimoxazole were infrequent (Table 1). No resistance was found for amikacin and gentamicin. Resistance to ampicillin alone was very common, whereas resistance to each of the other antibiotics was usually associated with multiple resistances. Twelve different MAR patterns were observed with a wide range of multi resistance distribution, to as many as five antibiotics (Table 1). Most of the isolated strains shared resistance to ampicillin and tetracycline. Of 29 MAR isolates screened, 24 (83%) harbored plasmids (Table 1). Single high molecular weight plasmid (> 10 kb) possessed by isolates was clearly observed in 0.8 and 1% agarose gel electrophoresis (Fig. 1a and 1b), while 92% of the MAR isolates that harbored plasmids were able to transfer all or part of their determinants of antibiotic resistance to *E. coli* ATCC 14948 (Table 2). However, in none of the successful mating experiments was the presence of DNase I able to prevent the development of recombinants. The addition of treated, cell-free culture supernatant of donors in the presence of DNase I did not

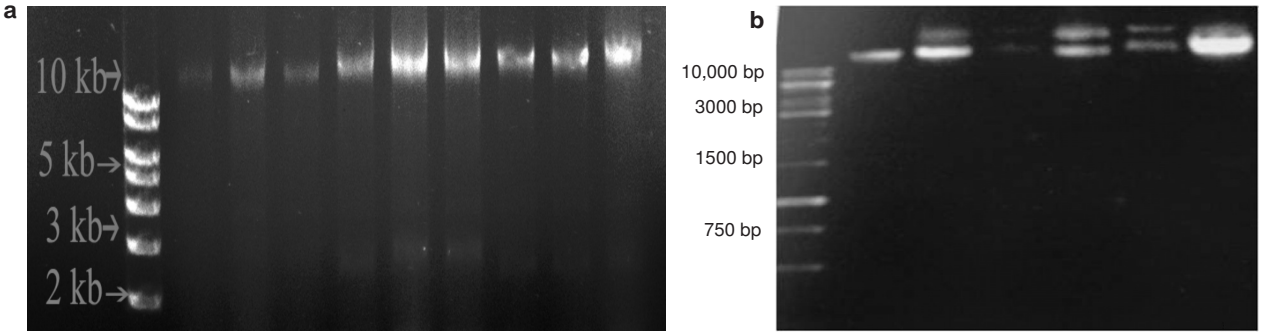

Figure 1. Plasmids of Drug Resistant Coliform isolates. 1a) 0.8% agarose gel (Plasmids of > 10 kilo base pair (kb)). 1b) 1% agarose gel (Plasmids of > 10 kilo base pair (kb)).

Table 2
Resistance pattern observed after gene transfer for non-nalidixic acid isolates.* Marks indicate mated progeny with unidirectional transfer. AMP = ampicillin, AK = amikacin, C = chloramphenicol, CO = cotrimoxazole, GEN = gentamicin, KAN = kanamycin, STR = streptomycin and TET = tetracycline

Antibiotic resistance pattern	Donor	No. of isolates with plasmid	No. of isolates transferring resistance	Resistance pattern genetically transferred	Rate
AMP-C-TET	*Citrobacter* spp.	1	1	AMP*	1×10^{-3}
AMP-C	*Enterobacter* spp.	2	2	AMP*	3×10^{-3}
					9×10^{-4}
AMP-C-STR		1	1	AMP-C-STR	6×10^{-3}
AMP-CO-TET		1	1	AMP-CO-TET	1×10^{-2}
AMP-TET	*E. coli*	5	3	TET*	6×10^{-1}
					3×10^{-2}
					5×10^{-1}
			1	AMP-TET	2×10^{-2}
AMP-C		2	2	AMP*	1×10^{-3}
					1×10^{-3}
AMP-C-TET		3	3	AMP-C*	9×10^{-3}
				C-TET*	6×10^{-2}
				AMP-C-TET	2×10^{-2}
AMP-CO-TET		1	1	AMP-TET*	3×10^{-2}
AMP-C-TET		3	2	AMP*	4×10^{-2}
					6×10^{-2}
AMP-CO-STR-TET				AMP-CO-STR	6×10^{-3}
AMP-CO	*Klebsiella* spp.	1	1	AMP*	3×10^{-4}
AMP-TET		1	1	AMP*	6×10^{-3}
AMP-CO-TET		1	1	AMP-TET*	2×10^{-3}
AMP-C-CO-TET		1	1	AMP-CO-TET*	9×10^{-3}
Treated cell-free supernatant of donors				No recombinants	
Untreated cell-free supernatant of donors				No recombinants	
Supernatant from co-culture of donors and recipient cells				No recombinants	

transform the recipient cells (Table 2). Additional experiments were performed with supernatants that were not filtered. The results were the same as those observed with the filtered supernatants. Mating experiments were performed with supernatants from co-cultures of donors and recipients, and the results were the same again (Table 2), with 45% of the isolates (10 out of 22) capable of achieving antibiotic resistance transfer rates greater than 10^{-3} per donor organism (Table 2). The following numbers of combinations of fecal coliforms with transferable R+ determinants were found: 13 in the *E. coli* populations, 4 in the *Enterobacter* populations, 4 in the *Klebsiella* populations, and 1 in the *Citrobacter* populations (Table 2).

The PCR amplification of oligonucleotide primers yielded a detectable DNA fragment of an expected molecular weight (876 bp for coliform bacteria; 876 bp and 147 bp for *E. coli*) only in the presence of their respective DNA templates. However, the amplified products of Klebsiella spp. with lacZ primer were larger than those of *E. coli*, *Citrobacter* spp., and *Enterobacter* spp., indicating a difference between the target lacZ among these organisms (Fig. 2).

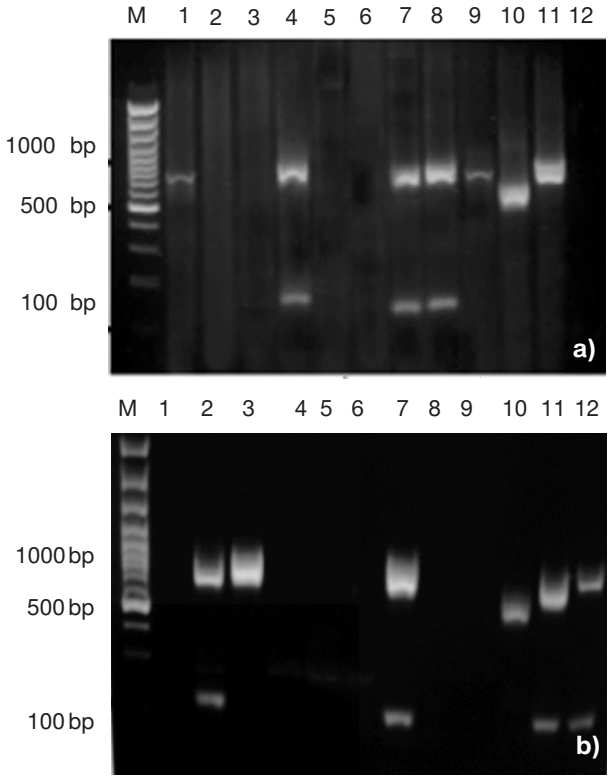

Figure 2. Agarose gel (2%) electrophoresis of PCR amplified products from various pure bacterial DNAs, using optimized multiplex PCR. 2a) Lane M: molecular size marker (100 base pairs {bp} DNA ladder); lane 1 *Klebsiella* spp., lanes 4, 7 and 8 *E. coli,* lane 9, *Klebsiella* spp., lanes 10 and 11 *Citrobacter* spp. respectively. 2b) Lane M: molecular size marker (100 bp DNA ladder); lane 2 positive control, lane 3 *Enterobacter* spp., lane 7 *E. coli,* Lane 10 *Citrobacter* spp., lanes 11 and 12 *E. coli.*

Discussion

A high resistance to ampicillin reflects the influence of humans on the environment (Andersen 1993). Interestingly, in the present study the fish exhibited high antibiotic resistance to chloramphenicol and tetracycline, which are used commonly in aquaculture. There are no reports available on the use of these drugs applied either as feed supplements or as drugs used in aquaculture practice in the Bandipur waste-fed farm. It can be presumed that antimicrobials, antimicrobial residues, and antimicrobial-resistant bacteria enter the fish ponds with the sewage effluents, thus, these anthropogenic factors might have influenced the fecal coliforms in their acquisition of resistance. Fish and pond water are not expected to have high resistance to chloramphenicol because of its genotoxicity, embryo- and fetotoxicity, carcinogenic potential, and the lack of a dose response relationship for aplastic anemia in humans. In addition, the Aquaculture Authority of India banned 19 antibiotics, including chloramphenicol, from aquaculture (Aquaculture News 2003). On the other hand, even when there is high cotrimoxazole resistance in the water, low resistance was reported in fish (Table 1). This indicates that although the drug is effective, it exerts low selection pressure (Ogbondeminu and Olayemi 1993). The sensitivity exhibited for gentamicin is a signal of the effectiveness of broad-spectrum antibiotics of the present generation (Pathak and Gopal 2005).

The high recovery rates of thermotolerent fecal coliforms from *Oreochromis* sp. and *Labeo* sp. noted

in the present study, with the simultaneous resistance to three to four antibiotics, suggest changes in nutritionally beneficial microflora with unexpected consequences for fish health (Miranda and Zemelman 2001). The high recovery rate of antibiotic-resistant bacteria from all the three fish species has immense ecological and public health implications; specially, if the resistance is plasmid mediated, then there could be a problem associated with the transfer of resistance determinants to human pathogenic bacteria that could enter the human population through fish consumption (Miranda and Zemelman 2001). The high proportion of multiple antibiotic-resistance in *Oreochromis* sp. in comparison with the detritivorous *Cirrhinus* sp. suggests that the fecal bacterial populations in *Oreochromis* sp. were subjected to conditions that fostered the acquisition of multiple-resistance determinants.

The simultaneous resistance of thermotolerent bacteria to beta-lactam, chloramphenicol and aminoglycoside could be the result of the dissemination of antibiotic-resistant plasmids in the aquatic environment (Miranda and Zemelman 2001). Of the multidrug-resistant isolates, most exhibited resistance to combinations of antimicrobial drugs that included ampicillin. This is an indication that multiple-resistance genes may coexist on one plasmid (Sayah et al. 2005), a single conjugative transposon (Pembroke et al. 2002), or an integron (Mazel 2006). This condition is particularly disconcerting given that exposure to one antibiotic agent may result in resistance to others without previous exposure (Sayah et al. 2005) or cost to bacterial fitness (Aminov et al. 2002). Plasmids, conjugative transposons, and integrons make it possible for new antibiotic resistance genes to spread through bacterial populations by the process of lateral gene transfer (Scott 1993). High rates of success of resistance gene transfer, as was observed in the present assay (92% overall), suggests that regardless of the physical location of resistance genes, i.e. chromosome, plasmid or integrons within transposons, these environmental isolates have the ability to move copies of themselves from one bacterial cell to another (Jury et al. 2010). The concentration of DNase I (200 $\mu g\ ml^{-1}$) was so high in the present experiments that it completely destroyed extracellular DNA and prevented transformation. DNase-treated cell-free supernatants did not transform, ruling out transduction, at least for the isolates studied (Table 2). To confirm that the DNase-resistant recombination was not due to protein-coated DNA or DNA present in membrane blebs, experiments were performed with cell-free supernatants that were not filtered. The results were the same as those observed with filtered supernatants. To exclude the possibility that the results were because of a phage induced by the co-culturing of strains, experiments were also performed with supernatants from co-cultures of donors and recipients. The results were the same again (Table 2); the transfer was not affected by the presence of deoxyribonuclease I, which suggested the conjugative nature of it. The unidirectional process of DNA transfer was confirmed in 70.83% plasmid positive resistant progeny in the presence of DNase I by resistance to secondary antibiotic markers, which is consistent with natural conjugation (Table 2). Mating experiments were not performed to confirm that the development of resistant progeny in the presence of DNase required cell-to-cell contact. A similar process of DNase-resistant transformation not involving conjugal plasmids has been described for *Neisseria gonorrhoeae* (Catlin 1981).

The results for the frequencies of transfer for the different mating experiments were in general agreement with those of Bell et al. (1983). Most of the isolates were capable of achieving resistance transfer rates within a range of 10^{-1} to 10^{-4} (Table 2), which is consistent with the results of Mach and Grimes (1982). Transfer rates varied between biotypes of the same genus used as donors and between different genera (Walter and Vennes 1985). Intragenetic transfer might be the reason for the high transfer potentiality of *E. coli* noted in the present investigation (Table 2); however, the limited number of strains used in the mating experiments does not permit drawing broad conclusions on the frequency of such transfers occurring in nature. In the development of a resistant clone under natural conditions, the amount of R plasmid donor cells would be substantially smaller than that in the present mating

mixtures. However, the number of donor cells would increase over time under continued antimicrobial pressure. While in present investigation transfers were achieved with only a few strains, more strains might be positive under different conditions or with other recipients.

The mating experiments were performed at 30°C, a temperature that is common in tropical aquatic systems, thus, their transfer in voided excreta, sewage, and polluted rivers is a definite possibility. However, the intent was to use a method in which laboratory manipulation has the least impact on both resistance genes and bacteria, and which provides a better indication of what can happen in the environment. Coliforms, which are generally regarded as harmless indicators, can transfer drug resistance to pathogens with detrimental consequences for both fish and humans (Grabow and Prozesky 1973). The fish used in the current investigation contained high levels of fecal coliforms with transferable drug resistance factors when eaten raw or insufficiently cooked; this could result in gut microflora becoming resistant to many drugs without any symptoms of disease. During a subsequent illness, the pathogen could become resistant by transfer from existing gut microflora (Cooke 1976). The uncontrolled use of antibiotics and the common practice of self-medication typical in India could place selection pressure on the wastewater and fishes in favor of organisms possessing genes that code for resistance (Sanyal et al. 2011). Thus, the R factors in the fecal coliforms of the present study can spread among opportunistic pathogens originating in humans, animals, and fishes that are unrelated either evolutionarily or ecologically and constitute a significant public concern.

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Author contributions. S.S. conceived of and designed the experiments, performed the experiments, and analyzed the data, S.S. and S.B. interpreted the data and wrote the paper.

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