



Prevalence of Transferable Oxytetracycline Resistance Factors in *Aeromonas hydrophila* in Fish Hatcheries

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Abstract

The distribution of motile aeromonads and prevalence of transferable oxytetracycline resistance factors in *Aeromonas hydrophila* of Indian major carp and catfish hatcheries in West Bengal, India were studied. Counts of motile aeromonads in carp hatching pool water ($4.23 - 4.54 \log \text{cfu ml}^{-1}$), carp larval rearing water ($3.36 - 3.93 \log \text{cfu ml}^{-1}$) and catfish larval rearing water ($5.41 - 5.85 \log \text{cfu ml}^{-1}$) of different hatcheries did not vary much. The motile aeromonad counts in the range of $2.81 - 4.28 \log \text{cfu carp larva}^{-1}$ and $6.08 - 6.69 \log \text{cfu g}^{-1}$ catfish larvae were recorded. The sensitivity of 48 *Aeromonas* strains to six antibiotics was observed to be in the following descending order: gentamycin, chloramphenicol, ciprofloxacin, nitrofurantoin, oxytetracycline and co-trimoxazole. The incidence of multiple antibiotic resistance (MAR) in motile aeromonads was significantly high ($p < 0.05$) in catfish hatcheries (95%) than in carp hatcheries (41%). The prevalence of MAR in *A. hydrophila* (69%) was high, but not significant ($p > 0.05$) than in *A. caviae* (55%). The mutation frequency of *A. hydrophila* strains to oxytetracycline ($25 \mu\text{g ml}^{-1}$) was in the range of $6.71 \times 10^{-9} - 1.60 \times 10^{-6}$. The oxytetracycline resistance factors of size 20.13 and 25.41 kb from *A. hydrophila* were transferred to *Escherichia coli* K12 at a frequency of $2.70 \times 10^{-6} - 5.35 \times 10^{-3}$. It is probable that these bacteria carrying resistant factors may be the source of spreading antibiotic resistance to other environmental and pathogenic bacteria, which share the same aquatic environment.

Keywords: *Aeromonas hydrophila*, fish hatchery, antibiotic resistance, oxytetracycline, mutation frequency, R-plasmids

Introduction

West Bengal is the pioneer in production of freshwater fish seeds, which contributes to about 75% of the total fish seed production of India. The larvae of Indian major carps and catfishes are susceptible to various diseases during the hatchery rearing due to biotic and abiotic factors (Milwain et al., 2002). Variation in survival, growth and quality of hatchery-produced larvae is a common problem and mass mortalities can occur even under optimal abiotic factors. A wide variety of aquadrugs are, therefore, used to combat diseases and to reduce mortalities. The extensive use of antibiotics in aquaculture for treating bacterial diseases has been associated with the development of resistance in fish pathogens such as *Aeromonas hydrophila*, *A. salmonicida*, *Edwardsiella tarda*, *Pasteurella piscicida* and *Yersinia ruckeri* and other aquatic bacteria (DePaola et al., 1995; Sorum et al., 2003; Serrano, 2005; Penders & Stobberingh, 2008). Members of the genus *Aeromonas* are ubiquitous in most aquatic environments. Resistance in *Aeromonas* and other fish bacterial pathogens is attributed to exposure to antibiotics, which is mediated either by R - plasmid, transposons, integrons and gene cassettes or by mutation. The rapid spread of antibiotic resistance genes in bacterial population, a consequence of indiscriminate use of antibiotics, can be partly attributed to plasmid-mediated horizontal transfer (Mukherjee et al., 2005). Tetracyclines have been used exclusively in aquaculture to control diseases (Serrano, 2005). Inevitably, resistance to oxytetracycline emerged and was found to be plasmid encoded (Shotts et al., 1976; Schmidt et al., 2001; Agerso et al., 2007). The emergence of bacteria that are

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resistant to antibiotics represents a continuing ecological battle to achieve a natural host-pathogen balance. Controlled studies are needed to determine the effect of antimicrobial therapy on the ecology of antibiotic resistant bacteria in fish hatchery environments. The prevalence of antibiotic resistant bacteria in freshwater fish hatchery systems of India is not documented well. This communication reports the distribution of motile aeromonads and prevalence of transferable oxytetracycline resistance factors in *Aeromonas hydrophila* of Indian major carp and catfish hatcheries in West Bengal, India.

Materials and Methods

Samples comprising 1 - 2 days old Indian major carp larvae, 7 - 30 days old African catfish *Clarias gariepinus* larvae, hatchery source water, carp hatching pool water, carp larval rearing water and catfish larval rearing water were collected from fish hatcheries in Naihati (Lat 22° 54' 10" N; Long 88° 25' 1" E), North 24 Parganas district, West Bengal, India during 2007. The larval samples in oxygen filled polythene bags and the water samples in 300 ml sterile plastic containers were brought to the laboratory within 2 - 3 h of collection.

The water samples were diluted by 10 fold serial dilution in sterile physiological saline. The medium containing Indian major carp larvae was first filtered through sterilized plankton net (pore size 10 mm) to separate the larvae for the enumeration of motile aeromonads. Ten unwashed larvae were aseptically collected and transferred to test tube containing 10 ml sterile saline. The larvae were ground to fine paste using a sterile glass rod, mixed thoroughly in a cyclomixer and serial decimal dilutions were made with sterile saline. *Clarias gariepinus* larvae were aseptically cut in to small pieces using a sterile scissor and 10 g of cut pieces was transferred to 90 ml of sterile saline, homogenized thoroughly in Stomacher (Steward, 400C) serial decimal dilutions were made.

Motile aeromonads were enumerated by spread plating on starch ampicillin agar (Palumbo et al., 1985) consisting of phenol red agar base (Hi-Media, Mumbai), soluble starch 10% (w/v) and ampicillin 10 µg ml⁻¹ medium (SAA). Aliquots (0.10 ml each) of appropriately diluted larvae and water samples were spread on to SSA plates in duplicate and incubated at 30±2°C for 24 h. The seeded SAA plates after 24 h incubation were flooded with iodine solution. The ampicillin resistant, amylase positive

and yellow colonies were counted as presumptive motile aeromonads. The aeromonad counts are presented as colony forming unit (cfu) ml⁻¹ water or cfu carp larva⁻¹ or cfu g⁻¹ catfish larvae. Typical colonies with distinct colony morphology were picked, purified by repeated streaking on tryptic soy agar (TSA, HiMedia, Mumbai) and maintained on TSA slants. Taxonomic keys proposed by Arcos et al. (1988) were followed for *Aeromonas* identification.

A total of 48 *Aeromonas* strains comprising *A. hydrophila* (n = 26) and *A. caviae* (n = 22) from water and larvae were screened for their sensitivity to six broad-spectrum antibiotics (HiMedia, Mumbai), viz., chloramphenicol (30 µg), ciprofloxacin (5 µg), co-trimoxazole (25 µg), gentamycin (10 µg), nitrofurantoin (300 µg) and oxytetracycline (30 µg) by agar disc diffusion assay (Bauer et al., 1966) on Mueller Hinton agar. The resistance profiles and resistance pattern were determined from the antibiogram data. Antibiotic resistant *Aeromonas* strains were maintained on TSA slants supplemented with oxytetracycline (Pfizer, Bangalore) at 5 mg ml⁻¹.

Five strains of oxytetracycline sensitive *A. hydrophila* isolated from Indian major carp larvae were used for determining the mutation frequency to oxytetracycline following Barnes et al. (1991) on TSA plates with or without oxytetracycline (25 µg ml⁻¹). The seeded agar plates were incubated at 30±2°C for 2 - 7 days and the number of growing colonies on the plates was counted. Mutation frequency was calculated as Na/N, where Na = number of colonies on TSA with oxytetracycline (25 µg ml⁻¹) and N = number of colonies on oxytetracycline-free TSA.

Three oxytetracycline resistant *A. hydrophila* strains isolated from *Clarias gariepinus* (n=2) and *Catla catla* (n=1) larvae were selected as donors for the conjugal transfer assay. The recipient strain, *Escherichia coli* K12 (F⁺, Lac⁺, Nf⁺) was from Microbial Type Collection Center (MTCC - 1302), Chandigarh, India. Conjugal transfer experiments were carried out by mating in Lauria - Bartani medium as described by Gerhardt et al. (1994). The number of recipient cells was determined on spread plates with bromothymol blue lactose nutrient agar (BLNA). The number of donor cells was determined on spread plates with TSA agar. The mating flasks were incubated at 30±2°C for 5 h without agitation. The transconjugants that appeared yellow on to BLNA with oxytetracycline after 3 - 4 days of incubation were purified twice on a similar medium.

Transconjugants were confirmed as *E. coli* clones based on their ability to utilize and produce gas from lactose and Voges Proskauer negative reaction. Frequency of transfer was expressed in terms of number of transconjugants / number of donor cells. Transfer efficiency was expressed in terms of number of transconjugants / number of recipient cells. Mutation frequency of recipient *E. coli* K12 was determined by spreading 0.10 ml each of undiluted to 10^{-3} dilution on to BLNA containing oxytetracycline ($25 \mu\text{g ml}^{-1}$). *E. coli* clones that received transferable oxytetracycline resistant factors were examined for other resistant markers by agar disc diffusion assay (Bauer et al., 1966).

Plasmid DNA was extracted by alkaline lysis as described by Sambrook et al. (2001) and electrophoresed in a horizontal 1.0% agarose gel with pH 8.0 TBE buffer. The gels were subsequently stained with $0.5 \text{ mg}^{-1}\text{ml}$ ethidium bromide for 20 min. Plasmid sizes were measured by comparison with standard molecular markers of known sizes.

Results of the bacterial counts were processed by log transformation. Student t test was used to test the significance of differences in motile aeromonads among the hatchery samples using Microsoft Excel package. Chi-square test was followed to know the significance of difference in multiple antibiotic resistance.

Results and Discussion

The present study revealed presence of motile aeromonads in all the samples in varying levels (Fig. 1). The levels of motile aeromonads were significantly high in carp hatching pool water than in source water ($p < 0.001$) and carp larval rearing water ($p < 0.01$). The wide variation in motile aeromonads of source water ($\log \text{cfu } 2.09 \pm 1.11 \text{ ml}^{-1}$) was because of the use of varied source of water such as borewell, storage tanks and pond water without treatment. The high levels of motile aeromonads in hatching pool water ($\log \text{cfu } 4.35 \pm 0.12 \text{ ml}^{-1}$) could be attributed to the presence of high organic load derived from unfertilized eggs, dead eggs and eggshells settled on tank bottom. Earlier studies recorded motile aeromonads up to 10^5 cfu ml^{-1} of summer flounder *Paralichthys dentatus* hatchery water (Eddy & Jones, 2002) and to the tune of 30 - 85% of the total bacterial flora on carp fry (Kozinska, 2007). High levels of motile aeromonads ($3.43 - 5.87 \log \text{cfu g}^{-1}$ larvae) reportedly hampered the commercial production of halibut *Hippoglossus*

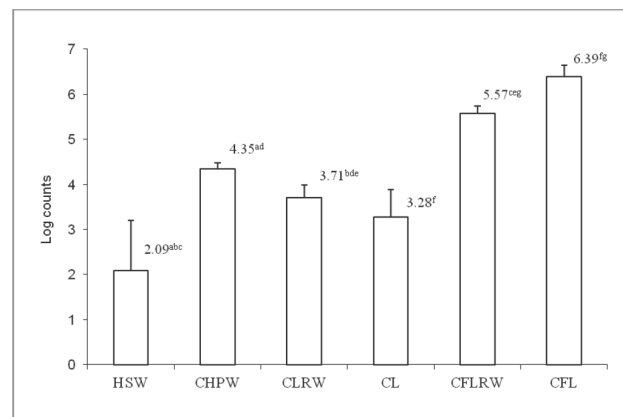


Fig. 1. Mean motile aeromonads count

HSW: carp hatchery source water (cfu ml^{-1}); CHPW: carp hatching pool water (cfu ml^{-1}); CLRW: carp larval rearing water (cfu ml^{-1}); CL: carp larvae (cfu larva^{-1}); CFLRW: catfish larval rearing water (cfu ml^{-1}); and CFL: catfish larvae (cfu g^{-1} larvae). a - g: Bars sharing common superscripts differed significantly ($p < 0.05$).

hippoglossus larvae by inducing mortalities during the early life stages (Verner-Jeffreys et al., 2003). Although the carp larval rearing water ($3.71 \pm 0.28 \log \text{cfu ml}^{-1}$) had high levels of motile aeromonads than the carp larvae ($3.28 \pm 0.60 \log \text{cfu larva}^{-1}$), the difference was not significant ($p > 0.05$). As the larvae had not started feeding and subsisted on egg yolk in first 24 - 48 h no feeding was done.

Mean counts of motile aeromonads were significantly ($p < 0.0001$) high, to the tune of about 2.48 log units, in catfish larval rearing water than in source water. Further, the levels of motile aeromonads were significantly ($p < 0.0002$) high in catfish larvae than in catfish larval rearing water. Catfish larvae had significantly ($p < 0.00001$) high levels of motile aeromonads than that of carp larvae possibly because of the age, size and the practice of feeding. The significantly high levels ($p < 0.0001$) of motile aeromonads in catfish larval rearing water ($5.57 \pm 0.17 \log \text{cfu ml}^{-1}$) than in carp larval rearing water ($3.71 \pm 0.28 \log \text{cfu ml}^{-1}$) reflected the type of feed and feed management practices followed in the system. Motile aeromonads were recovered from fish farm hatchery tanks at sites within the English Lake District, Cumbria, England (Rhodes et al., 2000) and catfish hatchery and farms in The Netherlands (Penders & Stobberingh, 2008). The results of the present study evidently showed that motile aeromonads are common in fish hatcheries, as they are ubiquitous in freshwater environment.

Aeromonas strains of the present study exhibited high resistance towards co-trimoxazole (54 - 73%) and oxytetracycline (36 - 69%) followed by nitrofurantoin (36 - 54%) possibly because of the antibiotic pressure in fish hatcheries. All the *A. caviae* strains from carp and catfish hatcheries were highly sensitive to gentamycin (Table 1) and this corroborates the findings of Son et al. (1997). Gentamycin resistant *A. hydrophila* and other bacteria were reported from the fish and aquatic environment (DePaola et al., 1995; Majumdar et al., 2006). The results of oxytetracycline (69%), co-trimoxazole (54%) and nitrofurantoin (54%) resistance among *A. hydrophila* strains corroborate the earlier observations (McPhearson et al., 1991; Schmidt et al., 2000). However, Rhodes et al. (2000) reported 100% oxytetracycline-resistance in mesophilic aeromonads from fish farm hatchery tanks in England. Only a few strains of *A. hydrophila* (15%) and *A. caviae* (9%) from fish hatcheries of the present study exhibited resistance to chloramphenicol so also in earlier studies from Malaysian freshwater fish farms (Son et al., 1997), Danish rainbow trout farms (Schmidt et al., 2000) and West Bengal aquaculture systems (Abraham et al., 2004). The differences in the frequency of resistance may be related to the source of the *Aeromonas* isolates as the prevalence of antimicrobial resistance among different pathogens was reportedly varied between and within countries (Vila & Pal, 2010).

The prevalence of multiple antibiotic resistance (MAR) was more in *A. hydrophila* (69%) than in *A. caviae* (55%) and the difference was not significant ($p>0.05$). The abuse of antibiotics in catfish hatcheries has lead to significantly high incidence (95%) of MAR bacteria ($p<0.05$) than in carp hatcheries (41%).

Table 1. Antibiotic resistance (%) among the *Aeromonas* strains from fish hatcheries

Antibiotic	<i>Aeromonas hydrophila</i> (n = 26)	<i>Aeromonas caviae</i> (n = 22)
Chloramphenicol, 30 µg	15.39	9.09
Ciprofloxacin, 5 µg	23.08	9.09
Co-trimoxazole, 25 µg	53.85	72.73
Gentamycin, 10 µg	7.69	0.00
Nitrofurantoin, 300 µg	53.85	36.36
Oxytetracycline, 30 µg	69.23	36.36

These MAR data, as shown in Table 2, are higher than the MAR (21%) reported from diseased freshwater fishes of West Bengal (Abraham et al., 2004). These results point out that when antibiotics are used more often in an environment, as in catfish hatcheries, the higher will be the occurrence of resistant bacteria at that site. The variations in resistance to different antibiotics observed among the motile aeromonads of carp and catfish hatcheries may be attributed to the varied management measures followed in these systems like addition of antibiotics in the hatching pool of catfish, direct drawal of water without treatment from storage tanks and ponds and use of boiled eggs, slaughter house and poultry wastes as feed for catfish larvae. Similarly, the MAR has been reported in bacteria from aquaculture environments associated with the variety of drugs or an uncertain antibiotic usage (Mukherjee et al., 2005). The results further suggested that the catfish hatcheries are the reservoirs of MAR bacteria. Probably, the high prevalence of MAR bacteria may be responsible for the repeated outbreak of diseases and high mortalities in certain catfish hatcheries of the present study.

Mutation frequency of *A. hydrophila* strains to oxytetracycline (25 µg ml⁻¹) was in the range of 6.71×10^{-9} - 1.60×10^{-6} (Table 3). Generally, in bacteria spontaneous mutation occurs at a rate of 10^{-9} - 10^{-10} (Miller, 1998). The present study also demonstrated the presence of two resistance factors (plasmids) of size 20.13 and 25.41 kb in *A. hydrophila* strains of

Table 2. Antibiotic resistance pattern of *Aeromonas* strains isolated from fish hatcheries.

Particulars	Number of strains sensitive to all antibiotics tested	Multiple antibiotic resistance* (%)
Species		
<i>Aeromonas hydrophila</i> (n = 26)	6	69.23
<i>Aeromonas caviae</i> (n = 22)	6	54.55
Source		
Carp hatchery (n = 29)	11	41.38 ^a
Catfish hatchery (n = 19)	1	94.74 ^a

* Resistant to at least two broad - spectrum antibiotics. Values sharing common superscript within the column differed significantly (a: $\chi^2=5.03$; $p<0.05$).

both Indian major carp and African catfish larvae (Fig. 2). Both donor strains were resistant to oxytetracycline and co-trimoxazole. The transconjugants acquired the resistance factors of same size and resistance to oxytetracycline and co-trimoxazole (Fig. 2, Tables 4 and 5). During horizontal gene transfer, the transconjugants acquired the same size plasmids, thus implying that the observed resistance mechanism to co-trimoxazole and oxytetracycline of the present study is plasmid mediated. The transfer frequency of resistance factors from *A. hydrophila* to *E. coli* K12 to the tune of 2.70×10^{-6} - 5.35×10^{-3} corroborates the findings of Aoki et al. (1971), who observed the frequency of transfer of the R - factors in *A. salmonicida* from trout hatchery in the range of 3.10×10^{-6} - 2.00×10^{-3} . The results of the present study corroborate the findings of Majumdar et al. (2006), who related the presence of 21 kb plasmid in *A. hydrophila* as a responsible factor for multiple antibiotic resistance. Another significant observation of the present study was that the factors responsible for resistance to oxytetracycline, nitrofurantoin, co-trimoxazole and chloramphenicol could not be transferred to recipient *E. coli* K12 from donor *A. hydrophila* O8 isolated from *Clarias gariepinus* larva (Tables 4 and 5) possibly due to high molecular weight of the resistance factors. Unidentified resistant determinants (150 kb) in *A. hydrophila* isolates collected from rainbow trout and water samples could not be transferred horizontally (Agero et al., 2007).

The present study confirmed the presence of oxytetracycline and co-trimoxazole resistance factors (R - plasmids) in *A. hydrophila* strains from fish hatcheries, which are capable of interspecies transfer to *E. coli* K12. It can be inferred from these results that the bacteria carrying resistant factors may be the

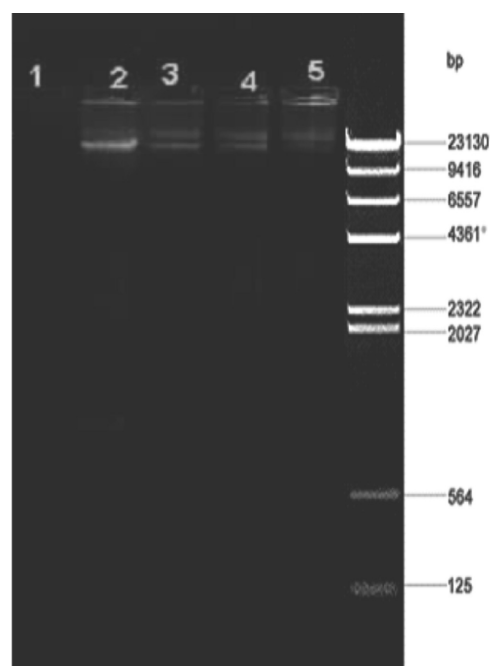


Fig. 2. Plasmid DNA profile of *Aeromonas hydrophila* strains from Indian major carp and catfish larvae, and transconjugant *Escherichia coli* strains

1. Recipient: *Escherichia coli* K12 or MTCC - 1302 (F⁺, Lac⁺, Nf⁺)
2. Donor: *Aeromonas hydrophila* O4 from *Clarias gariepinus* larva
3. Transconjugant: *Escherichia coli* K12 - O4
4. Donor: *Aeromonas hydrophila* H5 from *Catla catla* larva
5. Transconjugant: *Escherichia coli* K12 - H5

source of spreading oxytetracycline and co-trimoxazole resistance to other environmental and pathogenic bacteria, which share the same aquatic environment. Restriction on the use of antibiotics in

Table 3. Frequency of mutant oxytetracycline resistant *Aeromonas hydrophila* from Indian major carp larvae

Bacterial strain	Source	Antibiotyping	Mutation frequency
<i>Aeromonas hydrophila</i> A4	<i>Catla catla</i>	C ^S , Cf ^S , Co ^R , G ^S , N ^S , O ^S	5.60×10^{-6}
<i>A. hydrophila</i> A1	<i>Catla catla</i>	C ^S , Cf ^S , Co ^S , G ^S , N ^S , O ^S	1.60×10^{-6}
<i>A. hydrophila</i> N15	<i>Labeo rohita</i>	C ^S , Cf ^S , Co ^R , G ^S , N ^S , O ^S	4.30×10^{-6}
<i>A. hydrophila</i> S13	<i>Labeo rohita</i>	C ^S , Cf ^S , Co ^R , G ^S , N ^S , O ^S	4.50×10^{-6}
<i>A. hydrophila</i> S14	<i>Cirrhinus mrigala</i>	C ^S , Cf ^S , Co ^S , G ^S , N ^S , O ^S	6.71×10^{-9}

R: Resistant; S: Sensitive;

C: Chloramphenicol, 30 µg; Cf: Ciprofloxacin, 5 µg; Co: Co-trimoxazole, 25 µg;

G: Gentamycin, 10 µg; N: Nitrofurantoin, 300 µg; O: Oxytetracycline, 30 µg

Table 4. Transfer frequency of oxytetracycline resistant factors from *Aeromonas hydrophila* strains to *Escherichia coli* K12

Donor strains	Source	Characteristics of donor			Plasmid profile
		Antibiotyping	Frequency of transfer	Transfer efficiency	
<i>Aeromonas hydrophila</i> O8	<i>Clarias gariepinus</i> larva - 30 days old	C ^R , Cf ^R , Co ^R , G ^S , N ^R , O ^R	$> 2.70 \times 10^{-6}$	$> 6.66 \times 10^{-6}$	ND
<i>Aeromonas hydrophila</i> O4	<i>Clarias gariepinus</i> larva - 30 days old	C ^S , Cf ^S , Co ^R , G ^S , N ^S , O ^R	5.35×10^{-3}	1.07×10^{-4}	20.13 kb, 25.41 kb
<i>Aeromonas hydrophila</i> H5	<i>Catla catla</i> larva - 2 days old	C ^S , Cf ^S , Co ^R , G ^S , N ^S , O ^R	1.23×10^{-5}	2.28×10^{-3}	20.13 kb, 25.41 kb
Recipient strain		Characteristics of recipient			
<i>Escherichia coli</i> K12 (F ⁺ , Lac ⁺ , Nf ⁺)	Microbial Type Collection Center, Chandigarh, India (MTCC - 1302)	C ^S , Cf ^S , Co ^S , G ^S , N ^R , O ^S	-	-	Nil

ND: Not done; - : No data; C: Chloramphenicol, 30 µg; Cf: Ciprofloxacin, 5 µg; Co: Co-trimoxazole, 25 µg; G: Gentamycin, 10 µg; N: Nitrofurantoin, 300 µg; O: Oxytetracycline, 30 µg; R: Resistant; S: Sensitive

Table 5. Characteristics of *Escherichia coli* K12 transconjugants and *E. coli* K12 mutant

Donor strains	Conjugal transfer	Transconjugants	Resistance phenotype	Plasmid profile
<i>Aeromonas hydrophila</i> O8	NT	-	-	-
<i>Aeromonas hydrophila</i> O4	+	<i>Escherichia coli</i> K12 - O4	Co ^R , O ^R , N ^R	20.13 kb, 25.41 kb
<i>Aeromonas hydrophila</i> H5	+	<i>Escherichia coli</i> K12 - H5	Co ^R , O ^R , N ^R	20.13 kb, 25.41 kb
Recipient strain		Mutant		
<i>Escherichia coli</i> K12 (F ⁺ , Lac ⁺ , Nf ⁺)	-	<i>Escherichia coli</i> K12-M	N ^R , O ^R	ND

Mutation frequency of *Escherichia coli* K12 to oxytetracycline (25 µg ml⁻¹) was in the range of 1.60×10^{-8} – 1.24×10^{-8} ;

NT: No transfer of resistance factors; +: Transfer of resistance factors; ND: Not done; - : No data;

Co: Co-trimoxazole, 25 µg; N: Nitrofurantoin, 300 µg; O: Oxytetracycline, 30 µg; R: Resistant.

hatcheries together with adoption of good hatchery hygiene, sanitation, water treatment and other health management measures will aid in minimizing the development and spread of antibody resistant bacteria.

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