ISSN: 2394-1391

Indian Journal of Biology
Volume 2 Number 2, July - December 2015
DOI: https://dx.doi.org/10.21088/ijb.2394.1391.2215.1

Original Article

Isolation of Antibiotic and Heavy Metal Resistance Bacteria from Organs of Sewage Fed Farm Fish

Biplab Mandal*, Indranil Bhattacharjee, Sayantan Mukherjee**, Soroj Kumar Chatterjee***, Partha Sarathi Roy****

Abstract

Bacterial populations from organs (viz., liver, spleen, kidney and gill) of *Labeo rohita* of the sewage fed fish farm at Kalajharia, Asansol, West Bengal, India, were enumerated, followed by determination of resistance for antibiotics and heavy metals. The total viable counts of bacteria in these organs, observed, were 5.62×10^4, 4.12×10^4, 2.30×10^4 and 1.76×10^4 colony-forming units/mL, respectively. The random bacterial isolates from these fish organs showed resistance in decreasing order for ampicillin (95%), tetracylin (75%), amoxycillin (70%), amikacin (65%), chloramphenicol (50%), sparfloxacin (40%), gentamycin (30%), levofloxacin (25%), streptomycin (10%), and ciprofloxacin (05%). Most of the isolates exhibited an increasing order of tolerance for the metals (µg/mL) copper (200), cadmium (200), iron (400) and chromium (400), with minimum inhibitory concentration (MIC) ranging from <50 to 1600 µg/mL. A total of 40 bacteria have been successfully isolated from internal organs of *Labeo rohita* (8 isolates of *Aeromonas* spp., 21 of *Edwardsiella* spp., 6 of *Flavobacterium* spp. and 5 of *Vibrio* spp.). In terms of antibiotic susceptibility testing, each isolate was tested against 21 antibiotics, resulting in 482 (57.3%) cases of sensitivity and 61 (7.3%) cases of partial sensitivity. Meanwhile, 297 (35.4%) bacterial isolates were registered as resistant. The multiple antibiotic resistance (MAR) index of each bacterial species indicated that bacteria from raised bullfrogs have been exposed to tested antibiotics with results ranging from 0.27 to 0.39. These observations indicate that the significant occurrence of bacterial population in organs of fish with high incidence of resistance for antibiotics and heavy metals may pose risk to fish fauna and public health.

Keywords: *Labeo rohita*; Antibiogram; Heavy Metal Resistance; Multiple Antibiotic Resistance.

Introduction

Labeo rohita constitute a significant part of food for the global population and play an important role in the aquatic environment due to high value and huge demand from local and abroad. Heavy metals are ubiquitous and persist as environmental pollutants that are introduced into the environment through anthropogenic activities, such as mining and smelting, as well as through irrigation and other sources of industrial waste. However, untreated or partially treated wastewaters introduce a huge amount of contaminates particularly heavy metals into agricultural lands (Wang and Tao, 1998). The existence of heavy metals in the environment represents a significant and long-term environmental hazard since they are not biodegradable and tend to accumulate in living organisms (Kobya et al., 2005; Liao et al., 2008).

Author's Affiliation: *Department of Zoology, Vidyasagar University, Midnapore-721102, West Bengal, India. **Department of Zoology, Dr. Bhupendra Nath Dutta Smriti Mahavidyalaya, Hatgobindapur, Burdwan-713407, West Bengal, India. ***Mosquito and Microbiology Research Units, Department of Zoology, The University of Burdwan, Burdwan-713104, West Bengal, India.

Reprint's Request: Biplab Mandal, Department of Zoology, Vidyasagar University, Midnapore, West Bengal, 721102, India.
E-mail: biplab16_zoology@yahoo.com

Indiscriminate use of different antibiotics has caused development of resistance for various antimicrobials and chemotherapeutic agents among the gut flora of homeotherms. Use of antibiotics will exert more selective pressure and resistant pathogens will be encountered more frequently (MacMillan, 2001). Resistance to antibiotics and metals occurs simultaneously when the genes specifying resistant phenotypes are located together on the same genetic element such as a plasmid, transposon, or integron (Chapman 2003; Frost et al., 2005; Venner et al., 2009).

Therefore, the present study was conducted to evaluate the antibiotic and heavy metal tolerance of these microorganisms obtained from internal organs of sewage fed farmed *Labeo rohita*.

Materials and Methods

Labeo rohita were collected from the sewage fed fish farms at Kalajharia Asansol, West Bengal, India, receiving municipal sewage and industrial effluent from Asansol town and its adjoining areas. Fishes were sacrificed by stunning to dissect out aseptically the liver, spleen, kidney and gills (Pathak and Gopal, 2005) by using sterile cotton bud; subsequently, spread-plate technique was employed with several selective agars such as cytophaga agar (CA), glutamate starch pseudomonas (GSP), thiosulfate citrate bile salt (TCBS) and xylose lysine deoxycholate agar (XLD) (Oxoid, England). Plates were incubated at room temperature (28-30°C)– since this the optimal growth temperature for mesophilic bacteria – for 24 to 48 hours. Colonies were then selected and kept in TSA deep tube for further studies.

Antibiotic susceptibility testing was conducted according to Kirby and Bauer disk diffusion method (Bauer et al., 1966) by employing Müeller-Hinton agar (Oxoid, England). 10 antibiotics namely 30 µg of amikacin, 25 µg of amoxicillin, 10 µg of ampicillin, 30 µg of chloramphenicol , 5 µg of ciprofloxacin, 10 µg of gentamycin, 5 µg of levofloxacin, 10 µg of sparfloxacin, 25 µg of streptomycin and 30 µg of tetracyline were used for studying antibiotic resistance (%) among bacterial isolates from fish organs.

Minimum inhibitory concentrations (MIC) of four heavy metals for the present bacterial isolates were determined by two-fold agar dilution method (Miranda and Castillo, 1998). $CdCl_2$, $K_2Cr_2O_7$, $CuSO_4.5H_2O$ and $FeCl_3$ were added to tryptic soy agar (TSA) (Merck, Germany). The MIC of these metals was observed after incubation overnight at 37±1°C.

The multiple antibiotic resistance (MAR) index of isolates were tested against 15 µg of erythromycin (E); 100 µg of spiramycin (SP); 30 µg of oxytetracycline (OT); 15 µg of furazolidone (FR); 30 µg of kanamycin (K); 30 µg of nalidixic acid (NA); 30 µg of chloramphenicol (C); 10 µg of ampicillin (AMP); 25 µg of sulfamethoxazole (RL); 25 µg of amoxicillin (AML); 25 µg of colistin sulfate (CT); 30 µg of doxycycline (DO); 30 µg of florfenicol (FFC); 30 µg of flumequine (UB); 50 µg of fosfomycin (FOS); 15 µg of lincomycin (MY); 50 µg of nitrofurantoin (F); 30 µg of novobiocin (NV); 15 µg of oleandomycin (OL); 2 µg of oxolinic acid (OA) and 100 µg of spiramycin (SP) antibiotics. The MAR index of isolates against tested antibiotics was calculated based on the following formula (Krumperman, 1983):

MAR index = X / (Y x Z)

X: total of bacteria resistant to antibiotics;

Y: total of antibiotics used in the study;

Z: total of isolates.

A MAR index value equal to or less than 0.2 indicated antibiotics that had been seldom or never used, in terms of treatment, whereas a value greater than 0.2 suggested that the animal had been highly exposed to those antibiotics.

Results and Discussion

Contamination of river water with municipal sewage and industrial effluent results in the occurrence of pathogenic microorganisms, particularly fecal bacteria and toxic metals, above their maximum permissible limits (Chatterjee et al., 2010). Fish in such water are exposed to these bacteria and metals, which bioconcentrate in different organs of fish. The bacterial load, i.e., total viable count, was found to be 5.62×10^4, 4.12×10^4, 2.30×10^4 and 1.76×10^4 c.f.u. / mL in liver, spleen, kidney and gill of the experimental fish, respectively. It has been observed to be maximum in liver and minimum in gills. Thus, it appears from these findings that soft tissues in massive organs are more prone to bioconcentration of bacteria, leading to incidence of infectious diseases among the aquatic fauna. This may be due to availability of more nutrients and lack of exposure to the surroundings. Bioconcentration of aquatic bacteria such as coliforms, streptococci, and aeromonads in gut, liver, and muscles of tilapia fish grown in a sewage-contaminated pond has also been noticed (Fattal et al., 1993).

The antibiotic resistance among random bacterial isolates from all four organs has shown a full range of resistance (0–100%) for ten common antibiotics of therapeutic and prophylactic use among human beings and in fish aquaculture. Resistance was found to be maximum among the isolates from spleen, kidney, and liver, while it was minimum among those from gill. Maximum average resistance was exhibited for ampicillin (95%) and tetracycline (75%) and minimum for ciprofloxacin (05%) (Table 1). The resistance exhibited for ciprofloxacin, levofloxacin and gentamycin is a signal of the ineffectiveness of broad-spectrum antibiotics of the present generation. With these observations it appears that the source of the problem of antibiotic resistance in riverine ecosystems is fecally contaminated water and fish populations in them plays important role in creating resistance. Antibiotic resistance patterns in the bacterial population in an aquatic ecosystem have been found to be useful in identifying non point sources of fecal pollution (Wiggins et al., 1999). The occurrence of resistance for common antibiotics is, further, an indication of indiscriminate use of these antibiotics, leading to constraint in antimicro-bial therapy for infectious diseases. The loss of antibiotic susceptibility among the aquatic bacteria has been observed to be affected to a considerable extent by the physicochemical qualities of water and seasonal varia-tions (Pathak et al., 1993).

The maximum tolerance, in general, was observed for chromium and iron (400 µg/mL), while it was minimum for copper and cadmium (200 µg/mL). Likewise, the maximum MIC was observed for chromium and iron (400 µg/mL), while it was minimum for copper and cadmium (200 µg/mL) (Table 2). In addition to assessment of loss of antibiotic susceptibility, the test isolates were also found to be tolerant to different concentrations of various toxic heavy metals as evidenced by their MICs ranging from <50 to 1600 µg/mL (Table 3).Thus, the isolates from visceral organs, i.e., spleen, kidney, and liver, exhibited maximum resistance for ampicillin, tetracycline, and amoxicillin with highest tolerance for iron and chromium, while the isolates from gills showed minimum resistance for ampicillin and tetracycline with rather low tolerance for cadmium and copper (Tables 1 and 3). These observations indicate that visceral organs provide better conditions for bacterial growth and biological activity than the exposed organs such as gill. Therefore, this increase in the MIC of toxic metals as well as antibiotic resistance among aquatic bacterial populations is also an indication of risk to the safety of the aquatic ecosystem, fish fauna, and ultimately human health.

In the present study, a total of 40 bacterial isolates were successfully isolated comprising 8 isolates of *Aeromonas* spp., 21 of *Edwardsiella* spp., 6 of *Flavobacterium* spp. and 5 of *Vibrio* spp. A total of 482 cases (57.30%) were reported as sensitive, whereas 61 (7.30%) and 297 (35.40%) were, respectively, partially sensitive and resistant (Figure 1). MAR values were ranging from 0.27 to 0.39, *Vibrio* spp. presented the highest MAR value (0.39) while *Aeromonas* spp. revealed the lowest one (0.27) (Table 4). MAR indexes of the present work revealed that bacteria from locally raised fish may have been exposed to tested antibiotics. McPhearson et al., 1991 reported that the MAR index of bacteria from a catfish pond, near a river where antibiotic was commonly used as treatment, was as high as 0.76.

Table 1: Antibiotic resistance (%) among bacterial isolates from fish organs

Antibiotics (µg/mL)	Fish organs				Average
	Liver	Spleen	Kidney	Gill	resistance
Amikacin (30)	90	80	60	30	65
Amoxycillin (25)	90	80	70	40	70
Ampicillin (10)	100	100	90	90	95
Chloramphenicol (30)	80	50	50	20	50
Ciprofloxacin (5)	10	10	00	00	05
Gentamycin (10)	50	40	20	10	30
Levofloxacin (5)	50	40	10	00	25
Sparfloxacin (10)	60	50	30	20	40
Streptomycin (25)	20	10	10	00	10
Tetracyline (30)	100	80	70	50	75

Table 2: Heavy metal resistance (%) among bacterial isolates from fish organs

Heavy metals (µg/mL)	Fish organs				Average
	Liver	Spleen	Kidney	Gill	resistance
Copper (200)	10	00	00	00	2.5
Chromium(400)	100	100	80	70	87.5
Cadmium (200)	30	20	10	00	15
Iron (400)	90	100	80	50	80

Table 3: MIC values for different heavy metals among bacterial isolates from fish organs

Heavy metals	MIC values (µg/mL) for different Fish organs			
	Liver	Spleen	Kidney	Gill
Copper	< 50	-	-	-
Chromium	1600	1600	800	400
Cadmium	100	50	< 50	-
Iron	1600	800	800	400

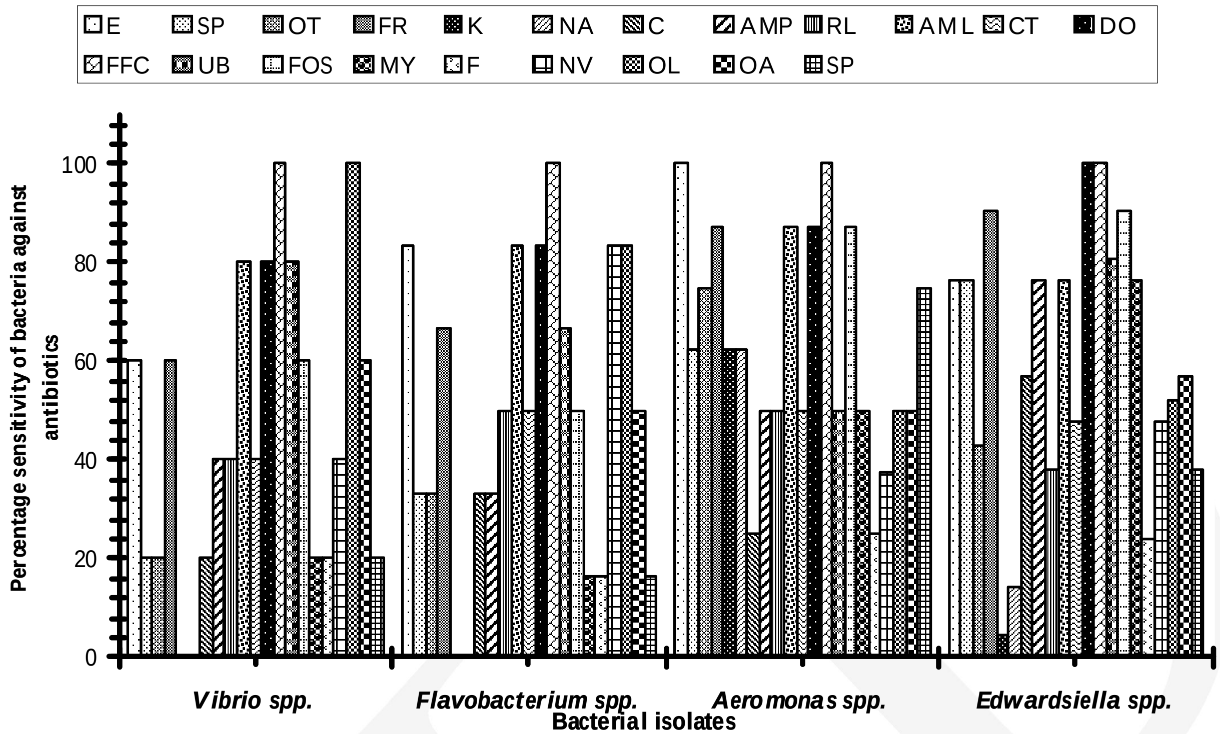

Fig. 1: Percentage sensitivity of *Vibrio* spp., *Flavobacterium* spp., *Aeromonas* spp. and *Edwardsiella* spp. against 21 antibiotics

In the current study, flumequine was found to be the most effective antibiotic for controlling bacterial disease in these fish farms. Other antibiotics that were also effective were oxolinic acid, florfenicol, nitrofurantoin, chloramphenicol and furazolidone. However, some of the antibiotics are banned for aquaculture use such oxolinic acid, nitrofurantoin, chloramphenicol and furazolidone. Therefore, based on published studies, farmers should use other drugs that are also effective for treatment and prophylactic purposes.

Currently, local fish farmers employ amoxicillin to treat fish diseases. The present results proven that amoxicillin is no longer effective, since not more than 27.5% of bacterial isolates were sensitive to it. These findings constitute an alert to antibiotic resistance of *Aeromonas* spp., *Edwardsiella* spp., *Flavobacterium* spp. and *Vibrio* spp.

It appears that the emergence of resistance is also influenced by the physicochemical characteristics of water and several environmental factors including hospital and aquaculture waste disposal (Rhodes et al., 2000) along with the form and bioavailability of metals in the ecosystem. Resistance may develop from a nonspecific mechanism with gene regulation of plasmids and chromosomes, which may be heritable or transfer-able due to the presence of a resistance factor (R-factor) among the aquatic bacterial population (Silver and Walderhaug, 1992). The infections caused by the pathogenic bacteria with R-plasmids may pose a risk of therapeutic problems to public health and fish population. Thus, the water bodies with antibiotic and metal resistant bacteria serve as an environmental reservoir and source for development of this trait among opportunistic pathogens and constitute a significant public concern. Therefore, such studies should be considered for selection of antibiotics in dealing with water-borne diseases, particularly among fishermen and fish consumers. These findings indicate that sewage and industrial pollution are responsible for the emergence of bacterial resistance and deterioration of water quality, along with risk to biodiversity of the hydrobionts and the human health.

References

1. Bauer, A.W., Kirby, W.M., Sherris, J.C., Turck, M. Antibiotic susceptibility testing by a standardized single disk method. Am. J. Clin. Pathol. 1966; 45(4): 493-496.
2. Chapman, J. S. Disinfectant resistance mechanisms, crossresistance, and co-resistance. Int. Biodeter. Biodegr. 2003; 51: 271–276.
3. Chatterjee, S.K., Bhattacharjee, I., Chandra, G. 2010. Water quality assessment near an industrial site of Damodar River, India. Environ. Monit. Assess. 2010; 161: 177-189, DOI 10.1007 / s10661-008-0736-1.

4. Fattal, B., Dotan, A., Parpari, L., Techorsh, Y., Cabelli, V.J., Microbiological purification of fish grown in fecally contaminated commercial fish pond. Water Sci. Technol. 1993; 27: 303-311.

5. Frost, L., Leplae, R., Summers, A., Toussaint, A. Mobile genetic elements: The agents of open source evolution. Nature Rev. Microbiol. 2005; 3: 722– 732.

6. Kobya, M., Demirbas, E., Senturk, E., Ince, M. 2005. Adsorption of heavy metal ions from aqueous solutions by sactivated carbon prepared from apricot stone. Bioresour. Technol. 2005; 96: 1518–1521.

7. Krumperman, P.H. Multiple antibiotic resistance indexing of *E. coli* to identify high-risk sources of fecal contamination of foods. Appl. Environ. Microbiol. 1983; 46(1): 165-170.

8. Liao, X. P., Tang, W., Zhou, R. Q., Shi, B. Adsorption of metal anions of vanadium(V) and chromium(VI) on Zr(IV)-impregnated collagen fiber. Adsorption, 2008; 14: 55–64.

9. MacMillan, J.R., Aquaculture and antibiotic resistance: a negligible public health risk?. World Aquacult. 2001; 32: 49-50.

10. McPhearson, R.M., DePaola, A., Zywno, S.R., Motes, M.L., Guarino, A.M. Antibiotic resistance in gram-negative bacteria from cultured catfish and aquaculture ponds. Aquaculture. 1991; 99: 203-211.

11. Miranda, C.D., Castillo, G. Resistance to antibiotic and heavy metals of motile aeromonads from Chilean freshwater. Sci. Total Environ. 1998; 224 (1-3): 167-176.

12. Pathak, S. P., Gopal, K. Occurrence of antibiotic and metal resistance in bacteria from organs of river fish. Environ. Res. 2005; 98: 100–103.

13. Pathak, S.P., Bhattacharjee, J.W., Ray, P.K. Seasonal variation in survival and antibiotic resistance among various bacterial populations in a tropical river. J. Gen. Appl. Microbiol. 1993; 39: 47-56.

14. Rhodes, G., Huys, G., Swings, J., McGann, P., Hiney, M., Pickup, R.W. Distribution of oxytetracycline resistance plasmids between aeromonads in hospital and aquaculture environments: Implication of xTn 1721 in dissemination of the tetracycline resistance determinant Tet A. Appl. Environ. Microbiol. 2000; 66: 3883-3890.

15. Silver, S., Walderhaug, M., Gene regulation of plasmid and chromosomes determined inorganic ion transport in bacteria. Microbiol. Rev. 1992; 5: 195-228.

16. Wang, X. J., Tao, S. Spatial structures and relations of heavy metal content in wastewater irrigated agricultural soil of Beijing's Eastern farming regions. B. Environ. Contam. Tox. 1998; 61: 261–268.

17. Venner, S., Feschotte, C., Biemont, C. Dynamics of transposable elements: Towards a community ecology of the genome. TIG, 2009; 25: 317–323.

18. Wiggins, B.A., Andrews, R.W., Conway, R.A., Dobratz, C.L., Dougherty, D.P., Eppard, J.R., Knupp, S.R., Limjoco, M.C., Sonsino, J., Torrijos, R., Zimmerman, M.E., Use of antibiotic resistance analysis to identify nonpoint sources of faecal pollution. Appl. Environ. Microbiol. 1999; 65: 3427-3432.