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OCCURRENCE AND ANTIBIOTIC SUSCEPTIBILITY AMONG COLIFORM BACTERIA ISOLATED FROM SEWAGE EXPOSED FISH.

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Abstract

Bacteriological contamination of muscles and digestive tract contents of *Oreochromis* sp. and *Labeo* sp. reared in a pond supplied with domestic sewage was enumerated followed by determination of resistance of thermotolerent coliforms for antibiotics. Numbers of bacteria in muscles and digestive tract contents of fish reflected their densities in water. Muscles of both the fish species contained high numbers of total coliforms (TC) and fecal coliforms (FC). *Escherichia Coli* (*E. coli*) were never recovered from the muscles but from the digestive tract contents of the fish. Ranking of the total and fecal coliform contamination levels showed a decrease in the order digestive tract contents> muscles ($p<.05$). The random thermotolerent coliform isolates from these fish organs showed resistance in decreasing order for tetracycline (82%) and ampicillin (65%). 58.11% (68 isolates out of 117) of the total isolates were resistant to both ampicillin and tetracycline. This observation indicates significant occurrence of bacterial population in organs of sewage fed fish with high incidence of antibiotic resistance which may pose risk to fish fauna and public health.

Keywords: Domestic sewage; Fish; Fecal coliforms; *Escherichia coli*; Water quality; Antibacterial resistance; Public health

Introduction

All around the world, people both in rural and urban areas have been using domestic wastes to fertilize fish ponds (Strauss *et al.* 2000). In the majority of cases, domestic sewages are applied untreated or only partially treated through storage (Strauss 2000). Domestic wastewater, rich in nutrients, used in aquaculture supports the growth of plankton and other microorganisms which are consumed by the fish with little intake of other supplemented feed. Recycling of domestic sewage through aquaculture is an effective form of pollution control, which contributes to cost recovery and provides a source of low cost animal protein production.

Domestic sewage transports a variety of human pathogenic microorganisms which may contaminate fish flesh when fish is grown in ponds receiving waste water (Niewolak and Tucholski 2000). Besides, municipal wastewater consists of huge amounts of incompletely metabolized antimicrobial drugs which can lead to the development of antibiotic resistant bacteria as well as resistant plasmids (Wiggins *et al.* 1999). The prominently affected bacteria are members of enterobacteriaceae and related gram negative rods (Kelch and Lee 1978). One of the important concern of wastewater fisheries is the contamination of fishes by fecal coliforms (Fapohunda, MacMillan, Marshall and Waites 1994). Their presence in fish intended for human consumption may constitute a potential danger not only by causing disease but also because of the possible transfer of antibiotic resistance from aquatic bacteria to human-infecting bacteria from nonaquatic sources (Olayemi, Adedayo and Ojo 1991). Therefore, periodic and comprehensive sanitary survey of wastewater fishery is required. For years, the group of fecal (also called thermotolerant) coliforms (FC) has been the most widely used as fecal contamination as their excreted load is similar or larger than that of pathogenic organisms, and their survival time in the environment longer than that of excreted bacteria and viruses (Strauss 1997).

In the present study, an attempt has been made to determine the bacteriological contamination of muscles and digestive tract contents of *Oreochromis* sp. and *Labeo* sp. reared in wastewater fed pond. Resistance to two very common antibiotics for random thermotolerent coliform isolates from muscle and digestive tract contents of both the fish was also determined.

Materials and Methods

Study Site : A sewage fed pond of Bandipur, Rahara, North 24 Parganas, (22°44'N Latitude and

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88º24'E Longitude) was taken into consideration for this study and to examine bacterial load of water and fish. Raw sewage was entirely of domestic origin, coming from Titagarh town of North 24 Parganas, West Bengal.

Sampling and Dissection

Fish samples were caught with a net and were immediately transferred to the laboratory in containers with pond water. They 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 sodium chloride (NaCl) physiological solution (10 ml of the solution for each 1 g of the muscle or digestive tract content). The suspensions were homogenized using Universal Laboratory Aid Type MPW-309 homogenizer, at 1000 rpm, for 10 minutes. The homogenates were then serially diluted (10^{-1} to 10^{-6} for muscles and 10^{-1} to 10^{-7} for digestive tract contents) and inoculated into culture media. Time lag from fish collection to the analyses did not exceed 6 hours. Water from sewage-supplied pond was sampled and analysed simultaneously with fish sampling. Samples were collected monthly from July 2009 to September 2009.

Microbiological Analyses

Total Coliforms

Lauryl Tryptose (LT) Broth at 35^0C for 48 hr was used for three-tube most-probable-number (MPN) presumptive determinations of coliforms (APHA 1998). From all positive presumptive tubes, total coliforms were confirmed by the formation of gas in any amount in the Durham fermentation tubes of brilliant green lactose bile broth (BGLB) for 48 hr at 35^0C.

Fecal Coliforms and *E. coli*

All positive Lauryl Tryptose (LT) MPN tubes to tubes of *Escherichia coli* (EC) Broth followed by incubation at 44.5^0C for 48 hr constitute a positive fecal coliform test. The growth from positive EC tubes was then streaked onto Levine Eosin Methylene Blue (EMB) Agar plates and incubated at 35°C for 18 to 24 h. Colonies from EMB Agar plates typical of *E. coli* were transferred to Nutrient agar (NA) slants from which GIMViC tests were performed where "G"-medium is the secondary EC broth, "I" -medium is Tryptone broth, "M"- and "V"-medium is Buffered Glucose broth, and "C"-medium is Simmon's Citrate agar. MPN of *E. coli* was then computed based on the number of tubes found to contain isolates that produce GIMViC reaction patterns characteristic of *E. coli* (APHA 2001).

Representatives of typical thermotolerent coliform isolates from fish samples were selected randomly by colony morphology on Eosin methylene blue agar and were streaked aseptically several times on freshly prepared nutrient agar plates to obtain pure isolates (Ogbonna, Sokari and Amaku 2008). Nutrient agar plates were then supplemented with ampicillin (50µg ml^{-1}) and tetracycline (25µgml^{-1}) and were used to evaluate antibiotic susceptibility patterns of 117 pure isolates (Miranda and Zemelman 2001). 32 isolates from muscles and 24 isolates from digestive tract contents of *Oreochromis* sp. and 39 strains from muscles and 22 isolates from digestive tract contents of *Labeo* sp. were subjected to antibiotics sensitivity test.

Statistical Analyses

Means and standard errors (SE) were calculated. T test was performed between bacterial concentration of muscles and digestive tract contents of both the fish. A significance level of 5% was considered (Zar 2007).

Results

Table 1: Fecal indicator bacterial load (mean $\pm$ SE, n =3) in water of waste fed fish pond

Total coliform **(MPN* 100 ml^{-1})**	Fecal coliform **(MPN* 100 ml^{-1})**	*E.coli* **(MPN* 100 ml^{-1})**
$4.7\pm4.53\times10^7$	$3.86\pm3.63\times10^5$	$1.34\pm.95\times10^4$

*MPN =Most Probable Number

Bacterial loads in muscles and digestive tract contents of *Oreochromis* sp. and *Labeo* sp. were exceptionally high. Total coliforms and fecal coliforms were commonly found in all analysed fish tissues. *Escherichia Coli* were not found in the muscles of either fish. Additionally, however, thermotolerant *Escherichia coli* were present in the digestive tract contents of both the fish (Table 2). Bacterial loads in the fish were significantly higher ($p<0.05$) in the digestive tract contents than in edible muscles.

Table 2: Fecal indicator bacterial load (mean + SE, n =15, values having same superscript are significantly different at 5% level) in muscles and digestive tract contents of fish collected immediately after harvest from wastewater-fed fish pond

Type of Tissue	Fish species	Total coliform **(MPN 100gm-1)**	Faecal coliform (**MPN 100gm-1**)	*E.coli* (**MPN 100gm-1**)
Muscles	*Labeo sp.*	$0.80 \pm .32 \times 10^{7a}$	$2.68 \pm 1.45 \times 10^{5c}$	<3
	Oreochromis sp.	$0.13 \pm 0.06 \times 10^{7b}$	$1.74 \pm 0.81 \times 10^{5d}$	<3
Digestive tract contents	*Labeo sp.*	$2.92 \pm 0.87 \times 10^{8a}$	$4.63 \pm 1.47 \times 10^{6c}$	$2.3 \pm 0 \times 10^{5}$
	Oreochromis sp.	$2.07 \pm 0.76 \times 10^{8b}$	$4.12 \pm 1.22 \times 10^{6d}$	$0.64 \pm 0.33 \times 10^{5}$

Figure 1: Antibacterial resistance of thermotolerent coliforms isolated from fish of sewage-fed pond.

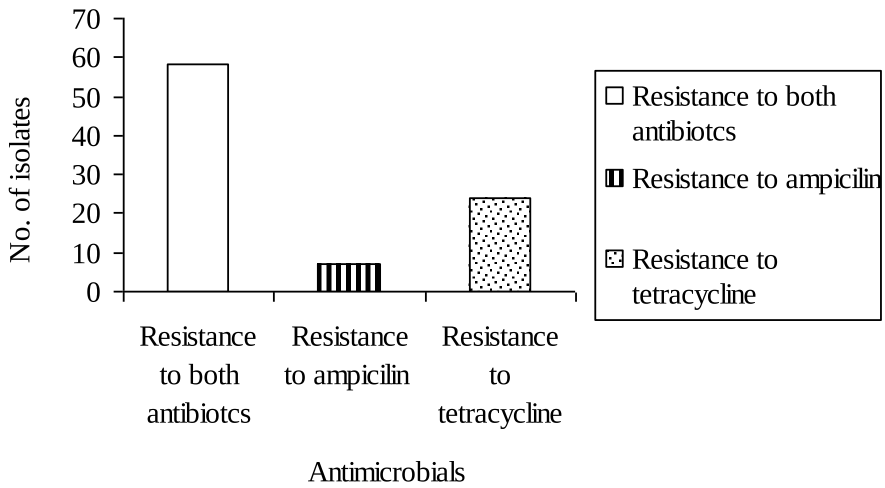
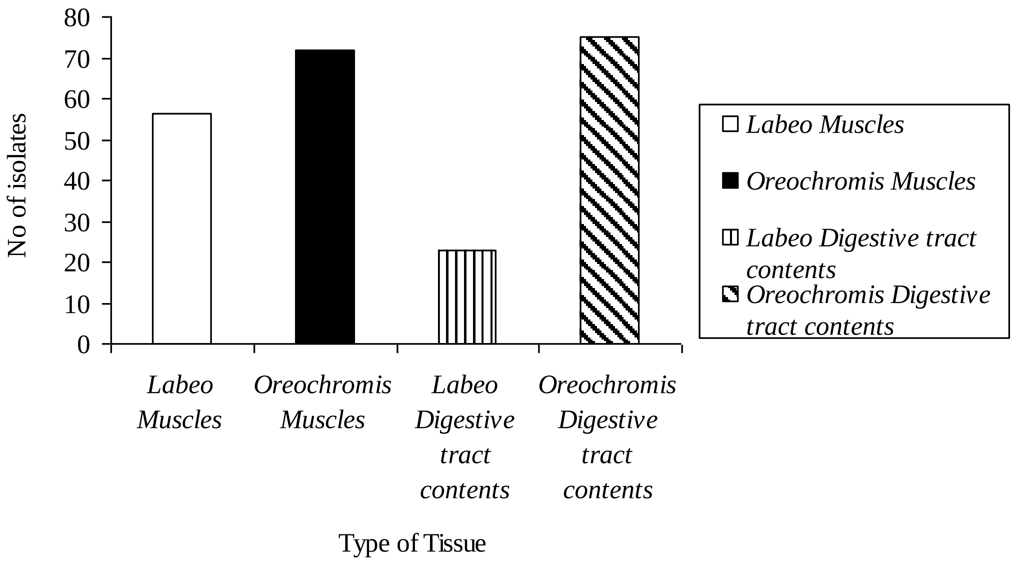


Figure 2: Pattern of resistance of thermotolerent coliforms to both ampicillin and tetracycline isolated from muscles and digestive tract contents of *Labeo* sp. and *Oreochromis* sp.



Antibiotic resistance pattern

Of the 117 thermotolerent coliform isolates examined for antibiotic sensitivity, 82% (96 isolates out of 117) were tetracycline resistant and 65% (76 isolates out of 117) were ampicillin resistant. 58.11% (68 isolates out of 117) of the total isolates were resistant to both antibiotics where as 31% (36 isolates out of 117) were resistant to single antibiotic. From the single antibiotic resistant isolates, 24% were tetracycline resistant and 7% were ampicillin resistant (Figure 1). 56.41% isolates (22 isolates out of 39) from flesh and 22.72% (5 isolates out of 22) from digestive tract contents of *Labeo* sp. showed resistance to both antibiotics whereas 71.87% isolates from flesh (23 isolates out of 32) and 75% (18 isolates out of 24) from digestive tract contents of *Oreochromis* sp. showed resistance to both ampicillin and tetracycline (Figure 2).

Discussion

Comparison of fecal coliform counts of water of Bandipur sewage fed fish pond with WHO (World Health Organization) water quality criteria (WHO 1989) suggests considerable contamination of the first. Bacterial flora of fish reflects the bacteriological quality of the water from where the fish harvested (Geldrich and Clarke 1966). Strong correlation between the bacterial species present in the pond water and the fish regardless of the type of fish were also reported by Buras *et al.* 1987; Ogbondeminu 1993; Apun, Yusofand and Jugang 1999. Thus, in our study, total coliforms, fecal coliforms and *E.coli* recovered from muscles and digestive tract contents of *Oreochromis* sp. and *Labeo* sp. may reflect bacteriological water quality of the Bandipur sewage fed pond.

Fecal coliforms in fish muscles were recovered when values of FC in water were $3.86 \pm 3.63 \times 10^5$ MPN 100 ml^{-1} which were much higher than those recommended by WHO (1989) in its health guidelines on wastewater use in aquaculture. Fecal coliforms in fish reflect the level of pollution of their environment, as the normal floras of fish do not include coliforms (Cohen and Shuval 1973). Presence of fecal coliforms indicates the presence of fecal material from warm-blooded animals. However, thermotolerent coliforms include the genera of fecal as well as non fecal origin. *E. coli* is a species of fecal coliform bacteria that is specific to fecal material from humans and other warm-blooded animals (Bhatia 2008). Environmental Protection Agency (1992) thus recommends *E. coli* as the best fecal indicator of health risk from water. No detectable penetration of *E. coli* in muscles of either fish was found at $1.34 \pm 0.95 \times 10^4$ MPN 100 ml^{-1} of *E. coli* concentration in water of sewage fed pond (Table 1). Thus, the fish flesh qualities at harvest were good on the basis of their *E. coli* counts. Safety precautions during fish processing are still needed to avoid cross-contamination due to high accumulation of microorganisms in the digestive tract of fish.

In this study significantly higher numbers of bacteria ($p<0.05$) were found in digestive tract contents than in muscles of both of the fishes which may cross contaminate fish fillet. Similar findings were reported by Ogbondeminu and Okoye 1992; Fatal *et al.* 1993. Bacterial (total coliforms, fecal coliforms and *E. coli*) loads in digestive tract contents of both *Oreochromis* sp. and *Labeo* sp. were found to be higher than surrounding water. Same findings were proposed by Ogbondeminu and Okoye (1992); Fatal *et al.* (1993). High concentrations of bacteria in digestive tract contents are mainly due to high concentrations of phagocytic cells localized in the intestines of these fish (Ellis, Munroe and Roberts 1976). These phagocytic cells constitute the first barrier to foreign organisms invading the fish.

A wide range of thermotolerent coliforms isolated from sewage fed fish showed resistance to both ampicillin and tetracycline. Multiple anitibiotic resistant faecal coliforms have been observed in wastewater across the world (Gallert *et al.* 2005). Antibiotic resistance among random bacterial isolates from different organs of fish captured from fecally contaminated water with a full range of resistance (00–100%) to different common antibiotics of therapeutic and prophylactic use among human beings and in various animal farms and fish farms was reported by several authors. (Rhodes *et al.* 2000; Miranda and Zemelman 2001; Pathak and Gopal 2005). Thus the source of the problem of antibiotic resistance bacteria in wastewater pond of Bandipur was fecally contaminated water.

Among thermotolerent coliforms recovered from fish, resistance to ampicillin and tetracycline was found in 65% and 82% of the isolates, respectively. Occurrence of thermotolerent coliforms with high resistance to ampicillin and tetracycline reflect human influence in the environment (Andersen and Sandaa 1994). Domestic sewage enters into the pond environment of the sewage fed farm with huge antibiotics which are used as medicines, as growth promoters or as preventative maintenance and may have established a selective pressure due to a slow degradation of antimicrobials favouring further growth of antibiotic-resistant bacteria (Petersen and Dalsgaard 2003). It may possible that these antibiotic resistant bacteria from wastewater may transfer their antibiotic resistant determinants to indigenous flora of fish, provoking their spread and prevalence in aquatic environment.

In the present study bacteria resistant to both ampicillin and tetracycline from digestive tract contents were higher in *Oreochromis* sp. than in *Labeo* sp. It may be related to detritus feeding habit of *Oreochromis* sp. by which it is more exposed to wastes as well as antimicrobials than *Labeo* sp. which is a column feeder. Similar findings were reported by Miranda and Zemelman (2001) with demersal and pelagic fish. Antibiotic resistant fecal bacteria form domestic sewage may change nutritionally beneficial intestinal microflora with unexpected consequences on fish health.

Our study indicates that fish flesh qualities were satisfactory in terms of *E.coli* counts. In spite of that flesh of both fish showed high numbers of antibiotic resistant thermotolerent coliforms which may include *Klebsiella* spp., *Citrobactor* spp. and *Enterobacter* spp. (non fecal origin) but till have 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 which may enter in human population through fish consumption. According to Walia *et al.* (2004) antibiotic resistance genes against ampicillin, streptomycin, and tetracycline are known to be transferable to other bacteria.

Thus, we can say that Wastewaters and fishes reside there are potent source of antibiotic-resistant bacteria, which in turn may transfer their resistance genes to nonresistant bacteria (Schwartz *et al.* 2003). Several studies indicate that the environmental conditions in wastewater may enhance the likelihood of gene transfer (Pote *et al.* 2003). Mach and Grimes (1982) demonstrated the high transfer frequencies of enteric bacteria in a wastewater. Additionally resistant bacteria may pose a risk of therapeutic problems to public health and fish population. So the study demands an elaborate investigation on the members of predominant multidrug resistant bacterial microflora associated with sewage fed fishery along with their plasmids profile as an evidence of conjugal transfer of antibiotic resistance genes in human and animal food chain through fish consumption.

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