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Comparative Study of Antibiotic Resistance Profile of *Enterobacteriaceae* Bacterial Isolates from the Gut of Intestinal Air-Breathing Fish, *Lepidocephalichthys guntea* and its Habitat

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Abstract: ***Lepidocephalichthys guntea is an intestinal air breathing fish and belonging to the family Cobitidae. It is frequently found in the muddy, low dissolved oxygen, hypoxic and hypercarbic condition. So the comparative study of the antibiotic resistance profile of bacterial isolates from the gut of intestinal air-breathing fish, L guntea and its habitat- small river Lotchka, West Bengal, India was executed. The bacterial isolates belong to the family Enterobacteriaceae were isolated from fish gut and river water sample and identified by Biochemical and tuf gene PCR based procedures. Diverse antibiotic-resistance phenotypes of the heterotrophic strains isolated and purified from the specific river water (the habitat of the fish) were compared with antibiotic-resistance profile of the gut isolates to predict occurrence of horizontal transfer of resistance-traits in the gut-bacteria.***

Keywords: Intestinal air-breathing loach, antibiotic resistance bacteria, fish gut bacteria, *Enterobacteriaceae*, tuf gene

1. Introduction

Presence of *Enterobacteriaceae* is a sign of faecal contamination in areas polluted by urban, industrial and domestical wastes [1&2]. All the organisms belong to this family are potential pathogens of man capable of causing a variety of diseases. When the aquatic system is contaminated with pathogenic bacteria, these bacteria too become part of microflora of fish. In the case of being contaminated with pathogenic bacteria, fishes pose a serious threat to public health [3]. *Enterobacteraceae* in fish are considered as an indicator to sewage pollution and has been reported as an opportunistic pathogen in fish [4]. Antibiotic pollution has been a growing concern for its human and environmental health impacts.

Antibiotics once seemed like a miracle weapon against infections but as the weapon is beginning to lose its force, physicians are struggling against a shrinking number of still-effective drugs. Antibiotic pollution is increasingly being recognized as a promoter of antibiotic resistance that may add to over-prescription and misuse of antibiotics [5]. Spread of antibiotic resistant bacteria in river water may be due to direct or indirect human and animal interference in river system. In this perspective, cells have been made for antibiotic resistance is to be considered when establishing bacteriological water quality criteria. All the known antibiotic resistance mechanism, acquired by opportunistic and pathogenic bacteria, evolve by means of mutations occurring in pre-existing genes of the bacterial chromosome positively selected by environmental forces [6&7].
Lepidocephalichthys guntea is belonging to the family Cobitidae and small body size having longitudinal black strips. It show signs of unique adaptive characters and has ability to survive in the muddy, low dissolved oxygen, hypoxic and hypercarbic condition habitat [8]. For gulp atmospheric air it come to upper water surface with swift movement and again returns to its previous position. As a result of its strange habit to encounter oxygen stress under depleted dissolved oxygen and drive to develop accessory respiratory organs among some teleosts. However it is also believed that aerial respiration as exhibited by few teleosts inhabiting the torrential mountain streams is an adaptation for drought only, since these streams carry well oxygenated water only during the wet season [9]. The loach, *Lepidocephalichthys guntea*, has been observed to use intestine as an accessory respiratory organ in addition to the original function in digestion [10]. Because of the peculiar adaptive characteristic of fish *L. guntea*, investigation of antibiotic resistance profile of bacterial flora in fish gut and fish habitat was much needed. In this study, so we are reporting about comparison study between the antibiotic resistance profile of bacterial isolates from the gut of *L. guntea* and its habitat.

2. Materials and Methods

2.1 Sample Collection and Bacteriological Study

During each sampling, freshly caught fishes from the river Lotchka were brought to the laboratory in sterile plastic bags containing the river water and processed within two hours of their collection. Each of the fishes was washed several times in sterile distilled water followed by surface sterilization with 70% EtOH, and made dry with sterile tissue paper before being dissected, through dissection, gut samples of each fish

were removed aseptically and collected in sterile containers. The organs were then homogenized in a sterile glass tissue homogenizer (1:10 w/v).

Water samples from different points of river Lotchka where fishes caught were also collected in sterile glass bottles for bacteriological analysis. Serial dilutions of both homogenized organs and water samples were prepared in sterile physiological saline and plated onto Nutrient Agar and Mac-Conkey Agar manufactured by Hi-Media Laboratories Ltd., Mumbai, India.

2.2 Numerical Taxonomy of the Purified Strains

Purified single colonies were obtained by the dilution streaking on selective media. The single-colony cultures were then used for observation of cell morphology followed by the Gram Staining. Physiological and biochemical tests included IMvic, H_2S production, Urease, Oxidase, Citrate, Arginine utilization, and Acid and Gas production from different carbohydrates [11, 12 & 13].

2.3 DNA Extraction, Primer Selection and PCR Amplification

Genomic DNA was extracted from each bacterial isolates by the standard method [14]. *Enterobacteriaceae* specific primers (Table 1) based on the transcription factor (tuf 1 and 2) gene were selected for Polymerase Chain Reaction (PCR) identification. PCR was performed with a thermal cycler (PeQ Lab, Germany). The PCR mixture consisted of 2.5 µl of 10X buffer (with $MgCl_2$), 3 µl of dNTP mixture (2.5 µM each), 1 µl of each primers and 0.5 µl of Taq DNA polymerase (3 U/µl) (Genei, Bangalore), in a final volume of 25 µl. Finally, 50 ng DNA template were used in each mixture [15].

Table 1: Genus specific tuf gene based primer(s)

Primer (s)	*Sequence (5'-3')*	*Length*	*References*
T1	AAYATGATIACIGGIGCIGC ICARATGGA	884bp	[15]
T2	CCIACIGTICKICCRCCYTCR CG		

2.4 Antibiotic Susceptibility Test

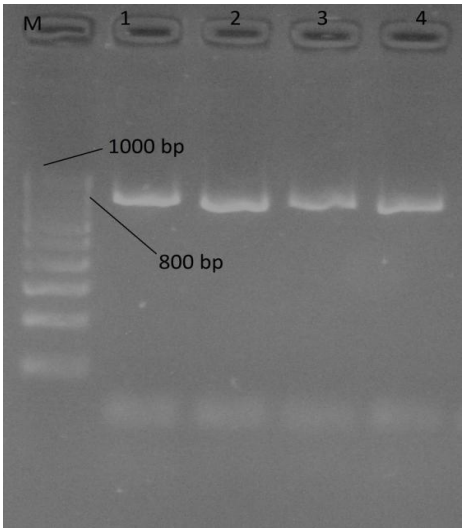
Fresh cultures of the isolates in nutrient broth were spread onto Mueller-Hinton Agar (HiMedia Laboratories Ltd, Mumbai, India) plates for obtaining the antibiotic resistance profile by the disc diffusion method [16]. A total of ten antibiotics (µg/ml) used were- penicillin (10), ampicillin (10), streptomycin (10), gentamycin (10), tetracycline (30), kanamycin (30), ciprofloxacin (5), moxifloxacin (5), erythromycin (15), and cephalothin (30) (HiMedia Laboratories Ltd, Mumbai, India). Antibiotic discs were placed on agar plates and incubated at 37°C for 12-18 hours. Susceptibility/resistance was determined by measuring the inhibition zone following instruction given by the manufacturer.

3. Results and Discussion

3.1 Identification of bacterial strains belonging to *Enterobacteriaceae* family isolated separately from river water and gut of *L. guntea*

Isolates that appeared as red and pink colonies on MacConkey agar medium were selected. All isolates were gram negative, non-spore forming, utilized citrate and gave positive results for VP test; but for MR and Indole test they were all negative. Acid and gas was formed against glucose fermentation. In order to confirm the identities of the isolates to *Enterobacteriaceae*, Polymerase Chain Reaction (PCR) was performed. Near about 90% of the total isolates those are identified by biochemical characterization were amplified with 'tuf' gene specific primer. The visualization of amplicons, approximately 884bp, on agarose gel (1.5%) electrophoresis containing ethidium bromide, confirmed *Enterobacteriaceae* (Fig. 1).

For the detection of *Enterobacteriaceae* in river water and fish gut samples genus specific 'tuf' gene based primer used in PCR. 'tuf' is the gene encoding elongation factor Tu. Elongation factor of Tu is involved in peptide chain formation [17]. The two copies of the tuf gene (tufA and tufB) found in *Enterobacteria* [18] share high levels of identity (99%) in *Salmonella typhimurium* and in *Escherichia coli*. A recombination phenomenon could explain sequence homogenization between the two copies [19 & 20]. Elongation factor Tu has been highly conserved throughout evolution and show functional constancy [21]. Phylogenies based on protein sequences from elongation factor Tu have shown good agreement with each other and with the rRNA gene sequence data [22].



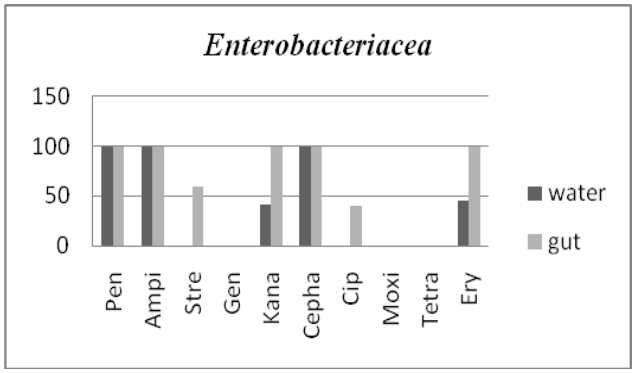
Lane M: 100 bp molecular weight marker, Lane 1- 4: amplicons

Figure 1: Positive amplicons of *Enterobacteriaceae* specific 'tuf' gene PCR amplification

3.2 Antibiotic Resistance Profile (ARP) of *enterobacterial* Isolates from (i) River Water and (ii) Fish Gut

All *Enterobacteriaceae* strains from either river water or fish gut exhibited resistance against ampicillin, penicillin and cephalothin. All *Enterobacteriaceae* strains isolated from

fish gut showed resistance to erythromycin and kanamycin whereas from amongst the strains isolated from river water, only 40 and 45% were resistant to erythromycin and kanamycin respectively. All strains, irrespective of its origin of isolation, river water or gut, were found sensitive to moxifloxacin, tetracycline and gentamicin. On comparing ARP profiles of the two groups of similar isolates, one from the habitat of the fish and the other from the gut of the fish, the difference was significant (Fig 2). All isolates exhibited resistance to ampicillin, penicillin-G, and cephalothin (belonging to the β- lactam class). It was reported that bacteria of *Enterobacteriaceae* family are capable of producing chromosomal β- lactamases enzyme [23]. All isolates were sensitive to gentamicin, tetracycline, ciprofloxacin and moxifloxacin, which are generally effective against gram negative bacteria. Tetracycline traverse the outer membrane of gram-negative enteric bacteria through the OmpF and OmpC porin channels, as positively charged cation (probably magnesium)-tetracycline coordination complexes [24]. Ciprofloxacin and Moxifloxacin belong to the class of quinolone which are effective against gram negative bacteria including *Enterobacteriaceae* [25]. But the fish-gut isolates showed higher incidence of resistance (60-100% resistance) against streptomycin, kanamycin and erythromycin than isolates (0-45% resistance) of river water (Fig. 2). In our previous study, it was shown that the isolates belonging to the *Salmonella* spp. in fish gut rendered maximum resistance ability against streptomycin, kanamycin, erythromycin and gentamicin than the river water isolates. Again isolates belonging to *Aeromonas* spp. of fish gut have shown higher incidence of resistance against erythromycin and gentamicin than *Aeromonas* spp. of the same river water [26]. All the three antibiotics, streptomycin, kanamycin and gentamicin, belong to the class of aminoglycoside. Aminoglycosides are highly potent, broad-spectrum antibiotics with many desirable properties for the treatment of life threatening infections [27]. Aminoglycoside-modifying enzymes are often plasmid encoded but are also associated with transposable elements. Plasmid exchange and dissemination of transposons facilitate the rapid acquisition of a drug resistance phenotype not only within a given species but among a large variety of bacterial species [28]. It was also reported that class 1 integron contain genes that modify aminoglycoside-modifying enzymes and isolates of Mahanada river (principal river of the region where the present study is conducted) also carry gene cassettes responsible for resistance to aminoglycosides [29]. Antibiotic resistance pattern of isolates (belong to the *Enterobacteriaceae*, *Salmonella* and *Aeromonas* spp.) of fish gut differed from the river water isolates with an exception. Isolates putatively assigned to the genus *Pseudomonas* showed similar kind of antibiotic resistance pattern in gut of the fish and river water sample [30]. *Enterobacteriaceae* bacterial family are present in the intestinal tract where they frequently exposed to different antimicrobials and creating the potential to disseminate genes of resistance to antimicrobials [31]. Development of antibiotic resistance ability in bacteria is one of the major parts of natural selection. Also, bacteria are able to transfer resistant genes to one another via vertical and horizontal gene transfer which aids in their ability to adapt to their environment [32&33]. Some researchers observed that high percentage of antibiotic resistance gene can horizontally transferred via conjugation within members of *Enterobacteriaceae* family [34&35]. Since some members of the genus *Aeromonas* spp. were shown to transfer antibiotic-resistance genes [36] and also such multiple-antibiotic-resistant strains resides in the gut of *L. guntea*, the possibility of gene transfer to the members of *Enterobacteriaceae* in the gut cannot be ruled out. Hence, this phenomenon of gene-transfer may be accorded as a plausible reason why fish-gut isolates showed higher incidence of resistance (60-100% resistance) against streptomycin, kanamycin and erythromycin than isolates (0-45% resistance) of river water.



Pen: Penicillin, Amp: Ampicillin, Stre: Streptomycin, Gen: Gentamycin, Kana: Kanamycin, Cepha: Cephalothin, Cip: Ciprofloxacin, Moxi: Moxifloxacin, Tetra: Tetracycline, and Ery: Erythromycin

Figure 2: Percentage incidence of antibiotic-resistant strains among the members of *Enterobacteriaceae* isolated from river water and fish gut samples.

4. Conclusion

Antibiotic resistant bacteria are present in both fish gut and river water sample indicating that fish gut bacteria may influenced by its surrounding river water but diverse antibiotic-resistance phenotypes of the bacterial strains isolated and purified from the specific river water (the habitat of the fish) were compared with antibiotic-resistance profile of the gut isolates to predict occurrence of horizontal transfer of resistance-traits in the gut-bacteria.

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