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Bacterial diversity and antibiotic resistance in a wetland of Lakhimpur-Kheri, Uttar Pradesh, India

Authors Info

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Abstract

Aim : Knowledge about the structure of microbial community is essential to assess the risks of exposure of farmed fish to pathogens and studying the antibiotic resistance. The present study was carried out to assess the bacterial diversity in a freshwater ecosystem.

Methodology: In the present study, microbial composition of a wetland in Lakhimpur-Kheri (N 27° 30.25' and E 80° 79.80'), Uttar Pradesh was assessed employing amplified rDNA restriction analysis (ARDRA), using *Hae*III enzyme as well as 16S rDNA gene sequencing. Furthermore, antibiotic resistance of the bacterial isolates was determined on the basis of multiple antibiotic resistance (MAR) index.

Results : A total of one hundred and thirty five bacteria were isolated and the restriction analysis of amplified 16S rDNA gene of the above isolates, using *Hae*III enzyme, indicated that isolated bacteria belonged to 33 single member phylotypes. Molecular characterization of the isolates revealed that these phylotypes belonged to 20 genera under 6 classes viz., Gammaproteobacteria (72.59%), Betaproteobacteria (4.44%), Flavobacteria (9.63%), Bacilli (7.41%) Actinobacteria (3.70%) and Sphingobacteria (2.22%). In the present study, four species i.e. *Sphingobacterium kitahiroshimense*, *Yersinia nurellii*, *Pseudomonas koreensis* and *Vogesella alkaliphila* have been reported for the first time from a wetland. The antibiotic sensitivity test indicated that most of the isolates were sensitive to norfloxacin followed by ofloxacin, whereas, majority of these bacteria were resistant to penicillin G followed by polymyxin B. Moreover, MAR indexing revealed that amongst the 33 single member phylotypes, 17 were multiple antibiotic resistant.

Interpretation : The bacterial diversity analysis of the wetland indicated bacterial population of aquatic, soil, animal and plant origin. Moreover, the study indicated that wetland can be a reservoir for multidrug resistant bacteria even when there is no application of antibiotics for aquaculture purposes. It can be assumed that the occurrence of antibiotic resistance and MAR among bacteria isolated from wetland could be due to flow of runoff from mainland. These results would help in strengthening our knowledge of bacterial ecology of the wetland ecosystem.

Introduction

Over the last two decades, Indian aquaculture has shown phenomenal growth of 6-7% per year with carps in freshwater and shrimps in brackishwater as major contributors. Although aquaculture has been identified as sunrise sector for meeting the growing demand of fish in years to come, several disease outbreaks are posing severe threat to the growth of this sector. Irrespective of type of aquaculture and species, bacterial diseases rank number one amongst the fish diseases and pose a significant challenge (Bondad-Reantaso *et al.*, 2005).

Microbial communities are closely associated with plants and animals, and play an important role in making necessary nutrients, metals and vitamins available to their hosts. To understand the role of microbes in the ecosystems, it is essential to know the structure of microbial community, which can not only provide an insight into large-scale ecological processes in a freshwater environment but also help in assessing the risk of exposure of farmed fish to pathogens (Zeng *et al.*, 2010). To characterize microbial ecology, routinely employed culture techniques involve isolation and characterization of microorganisms using growth media which can be nonselective or selective. Nonselective media allow a wide variety of bacteria to grow, whereas, selective media allow only certain organisms to grow because they have specific inhibitors added to the media. However, most of these techniques are manual and require significant effort and time to be performed. To overcome these shortcomings, during the recent times, a large number of molecular techniques especially 16S rDNA sequencing and amplified rDNA restriction analysis (ARDRA) have been developed to explore the diversity of microbial communities (Baik *et al.*, 2008). These techniques are rapid and allow accurate identification of bacteria up to the species level (Urakawa *et al.*, 1999; Yoon *et al.*, 2003).

The development of antibiotic resistance among pathogens has been recognized as a serious global health threat (Nguyen *et al.*, 2014). There are many reports that have established a link between antibiotic use in food producing animals and emergence of antibiotic resistance in bacteria, and transfer of these resistant organisms to humans (or their resistance genes to human pathogens) via food chain (Barton, 2000; Stobberingh and van den Bogaard, 2000; Angulo *et al.*, 2004; Akinbowale *et al.*, 2007). Considering apparent increase in the occurrence of antibiotic resistance among bacteria during the past years, and its possible implications for public health, many countries have initiated intensified surveillance for bacterial drug resistance (Schmidt *et al.*, 2000; Ash *et al.*, 2002; Lim *et al.*, 2013; Nguyen *et al.*, 2014). However, there is meagre information regarding bacterial diversity and antibiotic resistance associated with freshwater ecosystem in the country. Therefore, in the present study, a preliminary assessment of the microbial composition in a freshwater environment was carried out to

examine antibiotic resistance of the isolated bacteria. Amplified rDNA restriction analysis (ARDRA) and 16S rDNA sequencing were used for rapid and accurate identification of bacteria up to species level.

Materials and Methods

Sample collection : A wetland (N 27° 30.25' and E 80° 39.80') located in Lakhimpur-Kheri district of Uttar Pradesh, India, with extensive aquaculture practice and no evidence on the use of antibiotics, was selected for the present study. The area of water body was approximately 17 ha with average water depth of 1.5 m. Sampling was carried out in the month of January (winter season) when there was a maximum likelihood of disease occurrence. Water samples were collected by submerging sterile glass bottles (250 ml) at six representative areas of water body and transported aseptically to laboratory on ice for further processing. Furthermore, water quality parameters viz. dissolved oxygen, temperature and pH were also recorded by a Universal Pocket Meter (Thermo Scientific, Waltham, Massachusetts, United States).

Bacteriological analysis : All the six water samples were pooled in a single bottle aseptically and one ml of pooled sample was diluted ten-fold, up to 10⁻⁵ in 0.85% normal saline solution. Aliquots of 0.1 ml of serial dilutions were inoculated in duplicate on nutrient agar, as well as selective and differential media viz., *Aeromonas* specific agar, *Salmonella shigella* agar, *Yersinia* agar, MacConkey agar and *Cytophaga* agar (Hi-Media, India), using traditional spread plate method. The samples were processed aseptically and plated within 5 hrs of collection. All the inoculated plates were incubated at 25°C for 48 hrs. The colony types differing in terms of size, color, shape and morphology on different media were selected for further analysis. For characterization, all the purified isolates were stored at -80°C in nutrient broth supplemented with 15% glycerol. Representative purified isolates were subjected to Gram staining, motility, oxidase and catalase tests. The isolates were also subjected to biochemical tests employing Bacterial identification kit KB003 Hi25™ (HiMedia, India).

Bacterial genomic DNA extraction and 16S rDNA PCR amplification : DNA extraction was performed by CTAB method (Wilson, 1994). A single colony of purified isolates was inoculated in 2 ml nutrient broth, incubated overnight at 25°C, and the culture was used for DNA extraction. DNA was suspended in 50 µl of TE buffer (pH 7.8) and stored at -20°C for further study. The 16S rDNA gene was amplified using universal primers and PCR conditions as per Weisburg *et al.* (1991). The PCR products were visualized on 1.2% agarose gel. The PCR products were excised and purified using QIAquick gel extraction kit (Qiagen, Venlo, Netherlands).

Amplified rDNA restriction analysis (ARDRA) : About 10 µl (200 ng) of amplified 16S rDNA gene from different isolates was

digested using *HaeIII* restriction enzyme (Fermentas, Vilnius, Lithuania) as per manufacturer's instructions. The resulting fragments were resolved in 3% agarose gels which were stained with ethidium bromide and photographed with UV transilluminator (Bio-Rad, Hercules, California, United States). As per gel analysis, presence or absence of restriction site were either indicated as 1 or 0. These data were then used to generate a dendrogram (NTSYSpc 2.02e) to analyze the relationship among the isolates. Cluster analysis was performed on similarity matrix with sequential, agglomerative, hierarchical and nested clustering methods (SAHN) using unweighted pair-group method with arithmetic mean (UPGMA) and a dendrogram of the outcome was generated.

Sequencing and data analysis : The amplified and purified 16S rDNA gene products of representative isolates from each identified group in ARDRA were sequenced using forward primer 27F, from a commercial sequencing facility (Xcelris Bio-lab,

Hyderabad, India). Partial 16S rDNA sequences of all the bacterial isolates were submitted to NCBI GenBank nucleotide sequence database (Table 1). The homology of their 16S rDNA gene sequences was analyzed in the EzTaxon server (<http://www.ezbiocloud.net/eztaxon>) (Kim *et al.*, 2012). The sequences were aligned independently using CLUSTALX (Thompson *et al.*, 1997) and construction of phylogenetic tree by neighbor-joining method was carried out using MEGA6 program (Tamura *et al.*, 2013). The topology of phylogenetic tree was evaluated by bootstrap re-sampling method with 1000 replicates.

Antimicrobial sensitivity assay : Isolates stored at -80°C were streaked on nutrient agar plates and processed for antimicrobial sensitivity test using the disc diffusion method (Finogold and Martin, 1982). Twelve antimicrobial agents (Himedia, India) were selected as representative of different classes of antibiotics: Penicillin-G, Gentamicin, Norfloxacin, Tobramycin, Streptomycin, Kanamycin, Tetracycline, Chloramphenicol,

Table 1 : Total number of isolates (n) from wetland and similarity values of 16S rDNA gene sequences of 33 single member phylotype of bacterial isolates, showing similarity to nearest relative and taxonomic class of the strains

Strain I.D.	Medium for primary isolation	GenBank accession no.	Closest relative species in the Ez-Taxon database and Ez-Taxon accession no.	Similarity (%)	Class
FHMDC2F3-1 (n=5)	SSA, NA	KM069460.1	<i>Citrobacter freundii</i> (ANAV01000046)	99.72	Gammaproteobacteria
FHMDA1P1-2 (n=12)	SSA, NA	KM069461.1	<i>Aeromonas punctata</i> subsp. <i>punctata</i> (X60408)	100	Gammaproteobacteria
FHMDA1A1-4 (n=5)	YA, NA, MCA	KM069462.1	<i>Aeromonas allosaccharophila</i> (S39232)	99.75	Gammaproteobacteria
FHMDC3P1-12 (n=2)	NA	KM069463.1	<i>Curtobacterium plantarum</i> (JN175348)	99.83	Gammaproteobacteria
FHMDE1B1-19 (n=1)	ASA	KJ997754.1	<i>Erwinia billingiae</i> (AM055711)	98.86	Gammaproteobacteria
FHMDP2A1-23 (n=2)	YA, NA	KJ997757.1	<i>Providencia alcalifaciens</i> (ABXW01000071)	99.55	Gammaproteobacteria
FHMDA1C1-24 (n=8)	YA, NA, MCA	KM069464.1	<i>Aeromonas punctata</i> subsp. <i>caviae</i> (X74674)	100	Gammaproteobacteria
FHMDS1F1-28 (n=2)	MCA	KJ997759.1	<i>Shigella flexneri</i> (X96963)	99.68	Gammaproteobacteria
FHMDA1V1-32 (n=6)	CA, NA, MCA	KJ997746.1	<i>Aeromonas veronii</i> (X60414)	100	Gammaproteobacteria
FHMDA3O1-34 (n=5)	NA, MCA	KJ997748.1	<i>Acinetobacter oryzae</i> (GU954428)	99.03	Gammaproteobacteria
FHMDE2C1-41 (n=4)	MCA, NA	KJ997755.1	<i>Enterobacter cloacae</i> subsp. <i>dissolvens</i> (Z96079)	100	Gammaproteobacteria
FHMDP1K1-42 (n=5)	SSA, NA, MCA	KJ997756.1	<i>Pseudomonas koreensis</i> (AF468452)	99.63	Gammaproteobacteria
FHMDR1A1-48 (n=2)	NA, MCA	KJ997758.1	<i>Rahnella aquatilis</i> (CP003244)	98.51	Gammaproteobacteria
FHMDE2M1-55 (n=2)	ASA, NA	KM069467.1	<i>Enterobacter mori</i> (GL890774)	99.02	Gammaproteobacteria
FHMDP1C1-59 (n=3)	CA, NA	KM069469.1	<i>Pseudomonas chengduensis</i> (EU307111)	99.40	Gammaproteobacteria
FHM DY1N4-60 (n=6)	NA, NA	KM069470.1	<i>Yersinia nurmii</i> (FJ171338)	98.59	Gammaproteobacteria
FHM DA1AUS1-66 (3)	NA, MCA	KM069471.1	<i>Aeromonas australiensis</i> (HE611955)	99.79	Gammaproteobacteria
FHM DR2C1-72 (n=2)	CA, NA	KM069473.1	<i>Rheinheimera chironomi</i> (DQ298025)	99.79	Gammaproteobacteria
FHM DP1P1-86 (n=6)	ASA, NA, MCA	KM014557.1	<i>Pseudomonas psychrophila</i> (AB041885)	100	Gammaproteobacteria
FHM DA1M1-87 (n=4)	ASA, NA, MCA	KM069477.1	<i>Aeromonas media</i> (X74679)	100	Gammaproteobacteria
FHM DE2L1-103 (n=1)	SSA	KM014555.1	<i>Enterobacter ludwigii</i> (AJ853891)	99.89	Gammaproteobacteria
FHM DK1I1-113 (n=2)	SSA, NA	KM014556.1	<i>Kluyvera intermedia</i> (AF310217)	100	Gammaproteobacteria
FHM DA1MOL1-114 (n=10)	SSA, NA, MCA	KM069478.1	<i>Aeromonas molluscorum</i> (AY532690)	99.65	Gammaproteobacteria
FHM DV1A1-107 (n=5)	NA, NA, MCA	KM014558.1	<i>Vogesella alkaliphila</i> (HE819389)	100	Betaproteobacteria
FHM DJ1L1-46 (n=1)	NA	KM069465.1	<i>Janthinobacterium lividum</i> (Y08846)	99.69	Betaproteobacteria
FHM DA2N1-29 (n=3)	CA, NA	KJ997747.1	<i>Arthrobacter nicotianae</i> (X80739)	97.87	Actinobacteria
FHM DM1L1-80 (n=2)	CA, NA	KM069476.1	<i>Micrococcus luteus</i> (CP001628)	100	Actinobacteria
FHM DC1N1-33 (n=7)	NA, CA, MCA	KJ997751.1	<i>Chryseobacterium nakagawai</i> (JX100822)	98.27	Flavobacteria
FHM DC1S1-47 (n=2)	NA, CA	KM069466.1	<i>Chryseobacterium scophthalmum</i> (AJ271009)	100	Flavobacteria
FHM DC1H1-78 (n=4)	CA, NA, MCA	KJ997750.1	<i>Chryseobacterium haifense</i> (EF204450)	97.32	Flavobacteria
FHM DB1M1-69 (n=6)	CA, NA, MCA	KJ997749.1	<i>Bacillus megaterium</i> (D16273)	99.65	Bacilli
FHM DB2C1-98 (n=4)	CA, NA, MCA	KR363106.1	<i>Bacillus cereus</i> (AE016877)	100	Bacilli
FHM DS2K1-74 (n=3)	NA, MCA	KM069475.1	<i>Sphingobacterium kitahiroshimense</i> (AB361248)	99.80	Sphingobacteria

Fig. 1: Dendrogram constructed from the ARDRA patterns and distance matrix calculated by the UPGMA method using NTSYSPC 2.02e. ARDRA band patterns were obtained by digestion of the amplified 16S rDNA gene with *Hae*III endonuclease

Fig. 2 : Phylogenetic relationship of 16S rDNA sequences (n=33) to show their relationship with their nearest relative taxon selected from Ez-Taxon database. Tree was constructed by using neighbor-joining analysis based on 16S rDNA sequences

Supplementary Table 1: Biochemical tests of representative bacterial strains isolated from a wetland of Lakhimpur-Kheri, U.P., India

Biochemical tests	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	
Gram staining	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	
Shape	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod	
Motility	+	+	+	+	-	-	+	-	+	-	-	-	+	+	-	-	
Oxidase	+	+	+	-	-	-	+	-	-	+	-	-	-	+	-	+	
Catalase	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	
ONPG	+	+	+	-	+	-	+	+	-	-	-	-	+	-	-	-	
Lysine utilization	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
Ornithine utilization	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
Urease	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
Phenylalanine deamination	-	-	-	+	+	-	-	+	+	+	-	-	-	-	-	-	
Nitrate reduction	+	+	+	+	+	+	+	+	-	+	-	-	+	-	-	+	
H ₂ S production	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
Citrate utilization	+	-	-	-	-	-	+	-	-	+	-	+	+	+	-	-	
Voges proskauer's	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
Methyl red	+	+	+	+	+	+	+	+	-	+	+	-	+	-	-	+	
Indole	-	+	-	-	-	-	+	+	-	-	-	-	-	-	-	-	
Malonate utilization	-	-	-	+	-	-	-	-	+	+	-	+	+	+	-	-	
Esculin hydrolysis	-	+	+	+	+	-	+	+	-	-	+	-	+	-	+	+	
Arabinose	+	+	-	+	+	-	+	+	-	+	-	-	+	+	+	-	
Xylose	+	-	-	+	+	-	-	+	-	+	-	-	+	+	+	-	
Adonitol	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	
Rhamnose	-	-	-	+	+	-	-	+	-	-	-	-	-	-	-	-	
Cellobiose	+	-	+	-	-	-	-	-	-	-	-	-	+	-	-	-	
Melibiose	-	-	-	-	+	-	-	+	-	+	-	-	+	+	-	-	
Saccharose	+	+	+	+	+	-	+	-	+	+	-	-	+	-	-	+	
Raffinose	-	-	-	-	+	-	-	-	-	-	-	-	+	-	-	-	
Trehalose	+	+	+	+	+	-	+	+	-	+	-	-	+	-	-	-	
Glucose	+	+	+	+	+	-	+	+	+	+	-	-	+	+	-	+	
Lactose	-	+	+	-	+	-	+	+	-	-	-	-	-	-	-	-	
Biochemical tests	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33
Gram staining	-	-	-	-	-	+	-	-	-	+	-	-	+	-	-	-	-
Shape	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod	cocii	Rod	Rod	Rod	Rod	Rod	Rod	Rod
Motility	-	-	+	+	+	+	+	-	-	-	+	+	-	+	-	+	+
Oxidase	-	+	-	-	+	-	+	+	+	-	+	+	+	-	+	+	+
Catalase	+	-	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+
ONPG	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	+	-
Lysine utilization	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-
Ornithine utilization	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-
Urease	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Phenylalanine deamination	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	+
Nitrate reduction	-	+	+	+	+	+	+	-	+	-	-	+	+	+	+	+	+
H ₂ S production	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Citrate utilization	+	+	+	+	+	+	-	+	-	+	-	+	-	+	+	+	-
Voges proskauer's	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Methyl red	-	+	+	+	-	-	-	-	+	-	+	+	+	-	-	-	-
Indole	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-
Malonate utilization	-	-	+	-	-	-	-	-	-	-	-	+	-	-	-	+	-
Esculin hydrolysis	+	+	+	+	+	+	-	+	+	-	-	-	+	-	-	-	-
Arabinose	+	+	+	+	+	+	-	-	-	+	-	+	-	+	+	+	+
Xylose	+	+	+	+	-	+	-	-	-	+	-	+	-	+	+	-	-
Adonitol	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-	+	-
Rhamnose	+	+	-	+	-	+	-	-	-	-	-	-	-	-	-	+	-
Cellobiose	-	+	-	+	-	-	+	-	-	+	+	-	-	+	-	-	+
Melibiose	-	+	-	+	-	-	+	-	-	+	+	+	-	+	-	+	-

Saccharose	-	-	-	-	+	-	-	-	+	-	-	+	+	+	+	+	+
Raffinose	-	-	-	+	-	-	-	-	+	-	-	-	+	+	+	+	-
Trehalose	+	+	+	+	+	+	-	-	-	-	-	+	-	+	-	+	+
Glucose	+	+	+	+	+	+	-	+	+	-	+	+	+	+	+	+	+
Lactose	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-

1: *C. freundii*, 2: *A. punctata* subsp. *punctata*, 3: *A. allosaccharophila*, 4: *C. plantarum*, 5: *E. billingiae*, 6: *P. alcalifaciens*, 7: *A. punctata* subsp. *caviae*, 8: *S. flexneri*, 9: *A. nicotianae*, 10: *A. veronii*, 11: *C. nakagawai*, 12: *A. oryzae*, 13: *E. cloacae* subsp. *dissolvens*, 14: *P. koreensis*, 15: *J. lividum*, 16: *C. scophthalmum*, 17: *R. aquatilis*, 18: *E. mori*, 19: *P. chengduensis*, 20: *Y. nirmii*, 21: *A. australiensis*, 22: *B. megaterium*, 23: *R. chironomi*, 24: *S. kitahiroshimense*, 25: *C. haifense*, 26: *M. luteus*, 27: *P. psychrophila*, 28: *A. media*, 29: *B. cereus*, 30: *E. ludwigii*, 31: *V. alkaliphila*, 32: *K. intermedia*, 33: *A. molluscorum*

Table 2 : Antibiotic sensitivity test of the bacterial isolates from a wetland of Lakhimpur-Kheri, U.P., India

Strain I.D.	1	2	3	4	5	6	7	8	9	10	11	12
<i>Citrobacter freundii</i>	R	10 ^R	25 ^S	10 ^R	15 ^S	15 ^S	8 ^R	R	R	R	R	8 ^R
<i>Aeromonas punctata</i> subsp. <i>punctata</i>	R	20 ^S	35 ^S	15 ^S	22 ^S	23 ^S	25 ^S	30 ^S	R	25 ^S	20 ^I	12 ^S
<i>Aeromonas allosaccharophila</i>	R	15 ^S	20 ^S	15 ^S	15 ^S	15 ^S	20 ^S	15 ^S	15 ^S	15 ^S	15 ^S	15 ^S
<i>Curtobacterium plantarum</i>	15 ^S	15 ^S	20 ^S	15 ^S	15 ^S	15 ^S	15 ^S	15 ^S	15 ^S	15 ^S	15 ^S	10 ^R
<i>Erwinia billingiae</i>	R	20 ^S	35 ^S	18 ^S	25 ^S	25 ^S	30 ^S	30 ^S	30 ^S	30 ^S	15 ^S	12 ^S
<i>Providencia alcalifaciens</i>	R	15 ^S	20 ^S	15 ^S	15 ^S	15 ^S	15 ^S	15 ^S	15 ^S	15 ^S	10 ^R	10 ^R
<i>Aeromonas punctata</i> subsp. <i>caviae</i>	R	15 ^S	20 ^S	15 ^S	15 ^S	20 ^S	15 ^S	15 ^S	15 ^S	15 ^S	R	10 ^R
<i>Shigella flexneri</i>	R	13 ^I	R	12 ^R	14 ^I	13 ^R	8 ^R	15 ^I	11 ^R	R	8 ^R	8 ^R
<i>Arthrobacter nicotianae</i>	20 ^S	15 ^S	25 ^S	15 ^S	15 ^S	15 ^S	20 ^S	15 ^S	15 ^S	15 ^S	15 ^S	10 ^R
<i>Aeromonas veronii</i>	R	15 ^S	33 ^S	25 ^S	20 ^S	25 ^S	30 ^S	30 ^S	30 ^S	30 ^S	R	8 ^R
<i>Chryseobacterium nakagawai</i>	R	R	20 ^S	R	R	R	15 ^S	10 ^R	20 ^S	20 ^S	10 ^R	R
<i>Acinetobacter oryzae</i>	R	15 ^S	20 ^S	15 ^S	20 ^S	20 ^S	25 ^S	15 ^S	25 ^S	20 ^S	15 ^S	12 ^S
<i>Enterobacter cloacae</i> subsp. <i>dissolvens</i>	R	13 ^I	25 ^S	15 ^S	15 ^S	15 ^S	17 ^S	R	20 ^S	20 ^S	R	R
<i>Pseudomonas koreensis</i>	R	15 ^S	20 ^S	15 ^S	15 ^S	20 ^S	20 ^S	15 ^S	25 ^S	28 ^S	R	15 ^S
<i>Janthinobacterium lividum</i>	R	20 ^S	25 ^S	20 ^S	30 ^S	25 ^S	30 ^S	18 ^S	30 ^S	30 ^S	15 ^S	12 ^S
<i>Chryseobacterium scophthalmum</i>	R	15 ^S	50 ^S	R	20 ^S	R	30 ^S	20 ^S	50 ^S	30 ^S	12 ^R	12 ^S
<i>Rahnella aquatilis</i>	R	20 ^S	35 ^S	15 ^S	30 ^S	25 ^S	30 ^S	30 ^S	30 ^S	30 ^S	20 ^I	12 ^S
<i>Enterobacter mori</i>	R	15 ^S	30 ^S	15 ^S	20 ^S	20 ^S	16 ^S	16 ^S	20 ^S	20 ^S	14 ^S	8 ^R
<i>Pseudomonas chengduensis</i>	R	15 ^S	20 ^S	15 ^S	15 ^S	20 ^S	15 ^S	R	20 ^S	20 ^S	R	15 ^S
<i>Yersinia nirmii</i>	R	15 ^S	15 ^S	15 ^S	15 ^S	15 ^S	20 ^S	20 ^S	20 ^S	20 ^S	20 ^I	15 ^S
<i>Aeromonas australiensis</i>	R	15 ^S	20 ^S	15 ^S	15 ^S	15 ^S	15 ^S	15 ^S	20 ^S	20 ^S	10 ^R	10 ^R
<i>Bacillus megaterium</i>	R	20 ^S	20 ^S	20 ^S	20 ^S	20 ^S	20 ^S	15 ^S	20 ^S	20 ^S	R	15 ^S
<i>Rheinheimera chironomi</i>	R	20 ^S	25 ^S	20 ^S	20 ^S	20 ^S	20 ^S	20 ^S	20 ^S	20 ^S	20 ^I	R
<i>Sphingobacterium kitahiroshimense</i>	R	15 ^S	30 ^S	15 ^S	20 ^S	20 ^S	16 ^S	16 ^S	20 ^S	20 ^S	14 ^S	8 ^R
<i>Chryseobacterium haifense</i>	R	15 ^S	50 ^S	R	20 ^S	R	30 ^S	20 ^S	50 ^S	30 ^S	12 ^R	12 ^S
<i>Micrococcus luteus</i>	25 ^S	20 ^S	20 ^S	10 ^R	15 ^S	15 ^S	25 ^S	25 ^S	20 ^S	15 ^S	10 ^R	5 ^R
<i>Pseudomonas psychrophila</i>	R	10 ^R	20 ^S	15 ^S	10 ^R	15 ^S	20 ^S	10 ^R	15 ^S	15 ^S	10 ^R	5 ^R
<i>Aeromonas media</i>	R	15 ^S	33 ^S	25 ^S	20 ^S	25 ^S	30 ^S	30 ^S	30 ^S	30 ^S	R	8 ^R
<i>Bacillus cereus</i>	R	15 ^S	15 ^S	15 ^S	15 ^S	15 ^S	20 ^S	20 ^S	20 ^S	20 ^S	20 ^I	15 ^S
<i>Enterobacter ludwigii</i>	R	10 ^R	20 ^S	10 ^R	15 ^S	15 ^S	17 ^S	15 ^S	15 ^S	15 ^S	10 ^R	10 ^R
<i>Vogesella alkaliphila</i>	R	20 ^S	22 ^S	15 ^S	15 ^S	20 ^S	18 ^S	20 ^S	20 ^S	18 ^S	20 ^I	15 ^S
<i>Kluyvera intermedia</i>	R	5 ^R	20 ^S	10 ^R	20 ^S	10 ^R	15 ^S	20 ^S	20 ^S	20 ^S	R	R
<i>Aeromonas molluscorum</i>	R	10 ^R	20 ^S	10 ^R	15 ^S	15 ^S	20 ^S	20 ^S	20 ^S	15 ^S	20 ^I	5 ^R

Keys: R = Resistant, I = Intermediately sensitive, S = Sensitive 1: Penicillin-G 10 U disc⁻¹, 2: Gentamicin 10 mcg disc⁻¹, 3: Norfloxacin 10 mcg disc⁻¹, 4: Tobramycin 10 mcg disc⁻¹, 5: Streptomycin 10 mcg disc⁻¹, 6: Kanamycin 30 mcg disc⁻¹, 7: Tetracycline 30 mcg disc⁻¹, 8: Chloramphenicol 30 mcg disc⁻¹, 9: Levofloxacin 5 mcg disc⁻¹, 10: Ofloxacin 5 mcg disc⁻¹, 11: Erythromycin 15 mcg disc⁻¹, 12: Polymyxin-B 300 U disc⁻¹

Levofloxacin, Ofloxacin, Erythromycin and Polymyxin-B. Overnight grown bacterial suspension was spread on Mueller-Hinton Agar and each antimicrobial agent (antibiotic disc) was placed uniformly on medium and incubated at 25°C. The clearance zone for each isolate was recorded after 24 hr of

incubation. The isolates were classified as sensitive, intermediately sensitive and resistant according to the zone diameter (NCCLS, 2002). For all the isolates, the multiple antibiotic resistance (MAR) index values (a/b, where a represents the number of antibiotics the isolate was resistant to and b

Fig. 3: Distribution of taxonomical classes of bacteria isolated from a wetland of Lakhimpur-Kheri, U.P., India

represents the total number of antibiotics the isolate was tested against) were calculated (Krumperman, 1983).

Results and Discussion

The present study was undertaken during winter season when the physico-chemical parameters viz., temperature, pH and dissolved oxygen of the water body were $14 \pm 1.8^\circ\text{C}$, 7.4 ± 0.2 and 5.68 ± 0.54 ppm, respectively. In the present study, a total of 135 bacterial strains were isolated by the standard agar plating method (Table 1). From the total isolates, 33 single member phylotypes were produced by ARDRA, using *HaeIII* endonuclease (Fig. 1) and also characterized biochemically (Supplementary Table 1). All the phylotypes were subjected to 16S rDNA sequencing and the above sequences were examined for similarity with reference sequences available in Ez-Taxon server, to establish their genotypic identification. Similarity

between selected reference strains and the phylotypes ranged from 97.32-100%, and these sequences were taken for phylogenetic analysis (Fig. 2).

Amplified rDNA restriction and confirmation analysis was used for the analysis of mixed bacterial population from different environments (Martínez-Murcia *et al.*, 1995). Similarly, the 16S rDNA sequencing is considered to provide unambiguous data for isolates and is reported to yield results, which are reproducible (Drancourt *et al.*, 2000). The above molecular techniques helped in assigning bacterial isolates to 33 species belonging to 20 genera in 6 different classes namely, Gammaproteobacteria, Betaproteobacteria, Flavobacteria, Bacilli, Actinobacteria and Sphingobacteria. Majority of bacterial isolates belonged to class Gammaproteobacteria (72.59%; 98/135) followed by Flavobacteria (9.63%; 13/135), Bacilli (7.41%; 10/135), Betaproteobacteria (4.44%; 6/135), Actinobacteria (3.70%; 5/135) and Sphingobacteria (2.22%; 3/135) (Fig. 3). In the present study, out of one hundred thirty five isolates, *Aeromonas* spp. were predominant (35.55%; 48/135) and included *A. punctata* subsp. *punctata* (8.89%; 12/135), *A. punctata* subsp. *caviae* (5.93%; 8/135), *A. veronii* (4.44%; 6/135), *A. allosaccharophila* (3.70%; 5/135), *A. australiensis* (2.22%; 3/135), *A. media* (2.96%; 4/135) and *A. molluscorum* (7.41%; 10/135). In addition, three species of *Chryseobacterium* spp. viz. *C. nakagawai* (5.19%; 7/135); *C. haifense* (2.96%; 4/135); *C. scophthalmum* (1.48%; 2/135) and two species of *Bacillus* viz. *B. megaterium* (4.44%; 6/135) and *B. cereus* (2.96%; 4/135) were also recorded. In a previous study, 203 bacterial strains isolated from freshwater of Woopo wetland were grouped under 39 single member phylotypes using *HaeIII* endonuclease in ARDRA (Baik *et al.*, 2008). Similarly, 43 different bacterial species isolated from Gangetic river in Uttarakhand were grouped in 24 genera on the

Fig. 4 : Antibiotic sensitivity test of bacterial strains isolated from a wetland of Lakhimpur-Kheri, U.P., India.

Table 3 : Multiple antibiotic resistance pattern of bacterial phylotypes of a wetland of Lakhimpur-Kheri, U.P., India

Strain	Percentage (%) composition of bacteria	Sensitivity to antibiotics			MAR index
		R	I	S	
<i>Citrobacter freundii</i>	3.70	75	8.33	16.67	0.75
<i>Aeromonas punctata</i> subsp. <i>punctata</i>	8.89	16.67	8.33	75	0.17
<i>Aeromonas allosaccharophila</i>	3.70	8.33	41.67	50	0.08
<i>Curtobacterium plantarum</i>	1.48	8.33	41.67	50	0.08
<i>Erwinia billingiae</i>	0.74	8.33	8.33	83.34	0.08
<i>Providencia alcalifaciens</i>	1.48	25	33.33	41.67	0.25
<i>Aeromonas punctata</i> subsp. <i>caviae</i>	5.93	25	25	50	0.25
<i>Shigella flexneri</i>	1.48	75	25	0	0.75
<i>Arthrobacter nicotianae</i>	2.22	8.33	41.67	50	0.08
<i>Aeromonas veronii</i>	4.44	25	0	75	0.25
<i>Chryseobacterium nakagawai</i>	5.19	66.67	0	33.33	0.67
<i>Acinetobacter oryzae</i>	3.70	8.33	16.67	75	0.08
<i>Enterobacter cloacae</i> subsp. <i>dissolvens</i>	2.96	33.33	16.67	50	0.33
<i>Pseudomonas koreensis</i>	3.70	16.67	8.33	75	0.17
<i>Janthinobacterium lividum</i>	0.74	8.33	8.33	83.34	0.08
<i>Chryseobacterium scophthalmum</i>	1.48	33.33	0	66.67	0.33
<i>Rahnella aquatilis</i>	1.48	8.33	8.33	83.34	0.08
<i>Enterobacter mori</i>	1.48	16.67	8.33	75	0.17
<i>Pseudomonas chengduensis</i>	2.22	25	0	75	0.25
<i>Yersinia nurmii</i>	4.44	8.33	25	66.67	0.08
<i>Aeromonas australiensis</i>	2.22	25	8.33	66.67	0.25
<i>Bacillus megaterium</i>	4.44	16.67	8.33	75	0.17
<i>Rheinheimera chironomi</i>	1.48	16.67	8.33	75	0.17
<i>Sphingobacterium kitahiroshimense</i>	2.22	16.67	16.67	66.66	0.17
<i>Chryseobacterium haifense</i>	2.96	33.33	0	66.67	0.33
<i>Micrococcus luteus</i>	1.48	25	16.67	58.33	0.25
<i>Pseudomonas psychrophila</i>	4.44	50	25	25	0.50
<i>Aeromonas media</i>	2.96	25	0	75	0.25
<i>Bacillus cereus</i>	2.96	8.33	25	66.67	0.08
<i>Enterobacter ludwigii</i>	0.74	41.67	33.33	25	0.42
<i>Vogesella alkaliphila</i>	3.70	8.33	8.33	83.34	0.08
<i>Kluyvera intermedia</i>	1.48	50	0	50	0.50
<i>Aeromonas molluscorum</i>	7.41	33.33	25	41.67	0.33

basis of 16S rDNA sequencing (Sood *et al.*, 2010). In the present study, bacterial diversity analysis indicated dominance of Gram negative bacteria. The abundance of Gram negative bacteria at different sites may be attributed to increased addition of the excretory substances to the water. In addition, few Gram positive bacteria were also isolated in the present study and this is in agreement with findings of Torimiro *et al.* (2014) who also reported Gram negative, as well as Gram positive bacteria in fish pond water.

The bacterial diversity analysis of wetland also indicated bacterial population of aquatic, soil, animal and plant origin. As it is well known that microorganisms are widely distributed in nature, they can be transported to the wetland system through the rains, mainly with the particulate matter. In the present study, four bacterial species of plant origin viz. *C. plantarum*, *E. billingiae*, *Enterobacter mori* and *A. oryzae* were also identified. It can be assumed that the bacterial isolates related to plant habitat might

have been contributed by the native plant inhabitants. Similar to the present results, Baik *et al.* (2008) reported that bacterial community in the wetland has a relationship with plant and animal inhabitants. Presence of bacteria of animal and plant origin is also considered as a property of freshwater pond ecological niche (Baik *et al.*, 2008). Interestingly, in the present study, four species of bacteria viz. *Sphingobacterium kitahiroshimense*, *Yersinia nurmii*, *Pseudomonas koreensis*, and *Vogesella alkaliphila* are first report from freshwater habitat. Earlier, these four species have been reported from broiler meat, *Yersinia nurmii* (Murros-Kontinen *et al.*, 2011), soil, *Vogesella alkaliphila* (Subhash *et al.*, 2013), soil, *Sphingobacterium kitahiroshimense* (Matsuyama *et al.*, 2008), soil, *Pseudomonas koreensis* (Kwon *et al.*, 2003). In addition, some of the bacterial isolates belonging to class Proteobacteria (Alpha and Beta-proteobacteria), Bacteroidetes, and Actinobacteria were also isolated, which are restricted to only freshwater ecosystems and thus, represent native organisms (Newton *et al.*, 2011; Huang *et al.*, 2013). Some of the bacterial

species isolated in the present study have been associated with fish diseases viz., *C. freundii* (Karunasagar *et al.*, 1992), *Aeromonas* spp. (Janda and Abbott, 2010), *E. cloacae* (Sekar *et al.*, 2008) and *P. koreensis* (Shahi and Mallik, 2014).

All the identified phylotypes (33) were examined for their sensitivity to 12 antibiotics. The numerical sensitivity profiles are given in Table 2 and Fig. 4. The *in-vitro* antibiotic sensitivity test revealed great variation in sensitivity of the bacterial isolates from freshwater source. The bacterial isolates viz. *Erwinia billingiae*, *Janthomonas lividum*, *R. aquatilis* and *V. alkaliphila* were sensitive to maximum number of antibiotics (83.34%; 10/12). On the contrary, aeromonads viz. *A. punctata* subsp. *punctata*, *A. veronii* and *A. media* were sensitive against minimum number of antibiotics (25%; 3/12) followed by *A. australiensis* (33.33%; 4/12), *A. allosaccharophila*, *A. punctata* subsp. *caviae* (50%; 6/12) and *A. molluscorum* (58.33%; 7/12).

The isolates from different genera also showed variable range of resistance pattern to different antibiotics (Table 3). *Citrobacter freundii* showed resistance to maximum numbers of antibiotics (75%; 9/12) followed by *C. nakagawai* (66.67%; 8/12) and *Pseudomonas psychrophila* (50%; 6/12). Among the twelve antibiotics tested, maximum bacterial resistance was observed to Penicillin-G (90.9%; 30/33) followed by Polymyxin-B (57.5%; 19/33) and Erythromycin (54.5%; 18/33), and minimum resistance was observed to Norfloxacin (3.03%; 1/33) followed by Ofloxacin (6.06%; 2/33) and Levofloxacin (9.09%; 3/33). Among the 33 representative isolates, 51.5% of the isolates (17/33) showed multiple antibiotic resistance (Fig.4 and Table 3). Multiple antibiotic resistance levels of some of the common bacterial species viz., *Aeromonas*, *Chryseobacterium* and *Pseudomonas* were 22.62%, 44.44% and 30.55%, respectively. In addition, two important opportunistic bacteria *C. freundii* and *Shigella flexneri* were 100% resistant against nine antibiotics and the level of multiple resistance were 75% of each. The MAR index mean values for the isolates viz. *Aeromonas*, *Pseudomonas*, *Chryseobacterium*, *C. freundii* and *S. flexneri* were 0.226, 0.306, 0.443, 0.75 and 0.75, respectively. The results of the present study on antibiotic resistance and multiple resistance are consistent with earlier reports of fish pathogens and bacteria in aquatic environments (Verner-Jeffreys *et al.*, 2009; Matyar *et al.*, 2010; Nguyen *et al.*, 2014). Motile *Aeromonas* spp. and *Pseudomonas* spp., which are recognized as ubiquitous aquatic Gram-negative bacteria in freshwater environment have been reported to show high incidence of resistance rates of 25–30% and 89–91%, respectively (Nguyen *et al.*, 2014). Since, these genera are reported to develop single or multiple antibiotic resistance readily (Akinbowale *et al.*, 2007), it has been suggested to use these genera as indicators for antimicrobial resistance in the freshwater aquaculture environment (Petersen and Dalsgaard, 2003). Earlier, Sunder *et al.* (2006) reported that the phenomenon of even distribution of resistance in bacteria, may be due to common environment and exchange of genetic

material. It is noteworthy that in the present study, we had selected the wetland where antibiotics are not used for aquaculture purposes. However, the MAR index mean values indicated that the wetland water can be a reservoir for multidrug resistance bacteria. Nonetheless, similar or higher values of MAR index were found in other reports and attributed mainly to aquaculture practices (Matyar *et al.*, 2010; Nguyen *et al.*, 2014). Hence from the present study, it can be assumed that the occurrence of antibiotic resistance and MAR among bacteria isolated from wetland could be due to flow of runoff from mainland. These results will help in strengthening our knowledge of bacterial ecology of the wetland ecosystem.

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