ORIGINAL ARTICLE

Isolation, Identification, and Antibiotics Resistance of *Aeromonas* spp. from Lakes of Udaipur (Rajasthan), India

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Abstract

Aim: *Aeromonas* spp. are commonly found in freshwater ecosystems. *Aeromonas* is Gram-negative, facultative anaerobes which causes aeromonasis in humans and are also pathogenic for aquatic and terrestrial animals. In this study, an attempt has been made to isolate *Aeromonas* spp. from lakes of Udaipur, namely, Fateh Sagar and Pichhola and to test their antibiotic resistance. **Materials and Methods:** The isolates were recovered on nutrient agar medium and screened for their growth on *Aeromonas* selective agar medium. Selected isolates were subjected to biochemical characterization. For molecular characterization, genomic DNA of all the isolates was amplified using universal primers 27 F and 1492 R, and amplified products were subjected for sequencing to confirm their identification. The isolates were tested for antibiotic resistance against 15 commonly used antibiotics, i.e., gentamycin, kanamycin, tetracycline, erythromycin, ampicillin, penicillin, polymyxin B, amikacin, ciprofloxacin, vancomycin, rifampicin, chloramphenicol, streptomycin, cefixime, and trimethoprim by disc diffusion method. **Results and Discussion:** Out of 116 isolates, a total of 14 strains able to grow on *Aeromonas* selective agar medium were selected. They were identified and grouped into 2 species as *Aeromonas veronii* and *Aeromonas hydrophila* according to their biochemical and molecular characteristics. All the strains, which were identified as *A. veronii,* were found resistant to 8 antibiotics, namely, AMP, penicillin, VA, kanamycin, polymyxin B, rifampicin, erythromycin, and streptomycin. All the strains, which were identified as *A. hydrophila,* were found resistant to 10 antibiotics, namely, ampicillin, penicillin, vancomycin, kanamycin, polymyxin B, rifampicin, erythromycin, streptomycin amikacin, and trimethoprim out of 15 antibiotics used in the study. **Conclusion:** The results indicated that the water of both the lakes was contaminated with multi-antibiotic-resistant enteric pathogenic bacteria. This study thus provides valuable information for making policy decisions aimed at reducing microbial contamination of lake water and the indiscriminate use of antibiotics.

Key words: *Aeromonas hydrophila, Aeromonas veronii,* antibiotic resistance, lake, Udaipur

INTRODUCTION

Resistant bacteria are becoming common place in health-care institutions. Bacterial resistance often results in treatment failure, which can have serious consequences, especially in critically ill patients. The surfacing of antibiotic resistance usually results from the misuse of antibiotics as growth-promoters in animal production, for therapy and prophylaxis.[1] Because humans consume these animal products, there is a probability of the spread of resistant strains from animals to humans, and thus, healthy individuals can become carrier hosts for multiple antibiotic-resistant bacteria.[2] Nutrient-rich environments such as sewage and wastewater create optimal conditions to promote horizontal gene transfer processes.[3] Studies have documented the detection of antimicrobial resistance in wastewater and drinking water.[4] Therefore, ubiquitous bacteria, which are capable of colonizing different water types, are of particular interest to assessing potential forms of antimicrobial resistance dissemination. The genus *Aeromonas* comprises ubiquitous bacteria, considered indigenous to aquatic environments.[5] *Aeromonas* spp. are human opportunistic pathogens with ability to cause various types of diseases, which include intestinal, blood, skin and soft tissue, and

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trauma-related infections.[6,7] Given their ubiquity in water environment and patterns of acquired antimicrobial resistance, members of the genus *Aeromonas* are good examples of such bacteria. Therefore, in this study, an attempt has been made to detect the antibiotic resistance in the *Aeromonas* spp. isolated from water of lakes of Udaipur.

MATERIALS AND METHODS

Isolation and preliminary characterization of *Aeromonas* spp.

Bacterial strains were isolated from water samples of Lake Fateh Sagar and Lake Pichhola on nutrient agar medium. Isolated strains were tested for their growth on *Aeromonas* selective agar medium after incubation at 37°C for 24 h.

Biochemical characterization

Selected isolates which were able to grow on *Aeromonas* selective agar medium were subjected to biochemical characterization. The tests include Gram-staining, motility, catalase test, oxidase test, citrate utilization, urea hydrolysis, methyl red (MR) test, Voges-Proskauer (VP) test, indole production, nitrate reduction, and carbohydrate fermentation. These results will be further matched to the criteria provided in Bergey's Manual of Systematic Bacteriology.

Molecular characterization

DNA isolation

The genomic DNA from bacterial cultures of tentatively identified *Aeromonas* strains grown overnight in nutrient broth was extracted according to the method given by Pospiech and Neumann.[8]

Primers

For DNA sequencing bacterial 16S rRNA gene-based universal primers, namely, 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'CGGTTACCTTGTTACGACTT-3') designed by Weisburg *et al*.,[9] were used.

Polymerase chain reaction (PCR) amplification

The reaction mixture (20 µl) contained 10 pmol of each primer, 0.2 mM of each dNTP ($MgCl_2$), 1X PCR buffer, 2 µl of DNA solution, and 1 U/µl of Taq DNA polymerase (Bengaluru Genei). Amplification was carried out in a thermal cycler as follows: Initial denaturation at 95°C for 5 min, then 35 cycles consisting of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, extension at 72°C for 2 min, and a final extension for 10 min at 72°C. The final hold of amplified PCR products was at 4°C. Amplified products were visualized on a 2.0% agarose gel along with 500 bp DNA ladders. The PCR products amplified with universal primers were submitted to Bengaluru Genei Pvt. Ltd., Bengaluru, India for sequencing. The sequences of the 16S rRNA of the isolates were compared with available standard sequences of bacterial lineages in the National Center for Biotechnology Information (NCBI) Genebank using nBLAST. The obtained sequences were submitted to the NCBI Genebank.

Antibiotic susceptibility testing

Antibiotic susceptibility test of *Aeromonas* strains was determined according to the disc diffusion method of Kirby–Bauer[10] on Mueller-Hinton agar plates. A total of 15 antibiotics, namely, ampicillin (AMP 10 µg/disc), amikacin (AK 30 µg/disc), cefixime (CFM 5 µg/disc), ciprofloxacin (CIP 5 µg/disc), chloramphenicol (C 30 µg/disc), erythromycin (E 15 µg/disc), gentamicin (GEN 30 µg/disc), kanamycin (K 30 µg/disc), penicillin (P 10 µg/disc), polymyxin (PB 300 µg/disc), rifampicin (RIF 30 µg/disc), streptomycin (S 25 µg/disc), tetracycline (TE 30 µg/disc), trimethoprim (TR 5 µg/disc), and vancomycin (VA 30 µg/disc) were used. Results were interpreted according to the guidelines of the Clinical and Laboratory Standards Institute.[11]

RESULTS AND DISCUSSION

A total of 116 isolates (56 from Lake Fateh Sagar and 60 from Lake Pichhola) were isolated on nutrient agar medium after incubation at 37°C for 24 h. Out of them, a total of 14 isolates (6 from Lake Fateh Sagar and 8 from Lake Pichhola) were found to be able to grow on *Aeromonas* selective agar medium. They gave characteristic luxuriant green opaque colonies on this medium. These 14 isolates were preliminary characterized as *Aeromonas* spp. and subjected to biochemical characterization. The results for the same are presented in Table 1.

All these 14 isolates were found Gram-negative, motile, and rod-shaped. All the isolates were found positive for catalase test, oxidase test, indole production, VP test, nitrate reduction, arginine hydrolysis, and gelatin liquefaction. All these 14 isolates were found negative for MR test, citrate utilization, H_2S production, urea hydrolysis, starch hydrolysis, and casein hydrolysis. All these 14 isolates were found to be able to ferment maltose, dextrose, sucrose, fructose, and mannitol. They were found unable to ferment lactose, raffinose, rhamnose, and cellobiose. Out of these 14 strains, a total of 8 strains (FS 9, FM 38, FW 54, PS 68, PS 71, PM 85, PM 92, and PW 104) were found to be able to ferment arabinose and remaining 6 isolates (FS 5, FM 41, PM 79, PM 88, PM 98, and PW 111) were not found to be able to ferment arabinose. These results were further matched with Bergey's Manual of Systematic Bacteriology. A total of 8 strains (FS 9, FM 38, FW 54, PS 68, PS 71, PM 85, PM 92, and PW 104) were tentatively identified as *Aeromonas hydrophila* and remaining

Table 1: Biochemical characteristics of bacterial isolates

Biochemical characteristics	*A. veronii* (*n*=6)	*A. hydrophila* (*n*=8)
Gram's reaction	–	–
Shape	Rod	Rod
Motility at 37°C	+	+
Catalase activity	+	+
Oxidase activity	+	+
Indole production	+	+
MR reaction	–	–
VP reaction	+	+
Citrate utilization	–	–
Nitrate reduction	+	+
H_2S production	–	–
Arginine hydrolysis	+	+
Urea hydrolysis	–	–
Starch hydrolysis	–	–
Casein hydrolysis	–	–
Gelatin liquefaction	+	+
Carbohydrate fermentation		
Lactose	–	–
Maltose	+	+
Dextrose	+	+
Sucrose	+	+
Raffinose	–	–
Rhamnose	–	–
Arabinose	–	+
Cellobiose	–	–
Fructose	+	+
Mannitol	+	+

VP: Voges-Proskauer, MR: Methyl red, *A. veronii: Aeromonas veronii, A. hydrophila: Aeromonas hydrophila*

6 isolates (FS 5, FM 41, PM 79, PM 88, PM 98, and PW 111) were tentatively identified as *Aeromonas veronii.*

For molecular identification, PCR amplification of DNA isolated from all these 14 strains was done using universal primers 27F and 1492R. The amplified products gave 1500 bp product on 2% agarose gel [Figure 1]. On the basis of the sequence similarity of the partial 16S rRNA sequences of all the strains compared with available standard sequences of bacterial lineages in NCBI Genebank reference strains; 6 isolates, 2 from Lake Fateh Sagar (FS 5 and FM 41) and 4 isolates from Lake Pichhola (PM 79, PM 88, PM 98, and PW 111) were identified as *A. veronii.* A total of 8 isolates 3 from Lake Fateh Sagar (FS 9, FM 38, and FW 54) and 5 from Lake Pichhola (PS 68, PS 71, PM 85, PM 92, and PW 104) were identified as *A. hydrophila.*

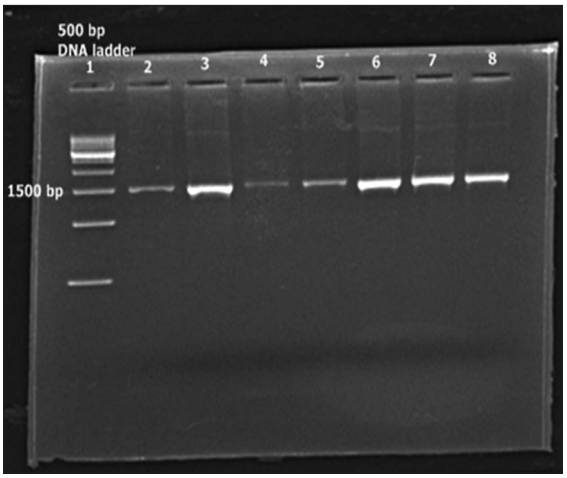

Figure 1: Identification of isolates using bacterial 16S rRNA specific universal primer 27 F and 1492 R in polymerase chain reaction, Lane 1 - 500 bp ladder, Lane 2 standard *Aeromonas hydrophila*, Lane 3-8 *Aeromonas veronii* FS 5, *A. hydrophila* FS 9, *A. veronii* FM 41, *A. hydrophila.* PS 68, *A. veronii* PM 88, and *A. hydrophila* PW 104

The nucleotide sequences of all these strains were deposited in NCBI gene bank under the allotted accession numbers [Table 2].

The antibiotic resistance of *A. veronii* and *A. hydrophila* isolated and identified in this study showed a full range of resistance (0-100%) for the 15 antibiotics [Table 3], which are commonly used in humans and aquaculture. The presence of *Aeromonas* spp. in drinking water is undesirable, as may have implications for user health, mainly via contact transmission.[12] Nevertheless, aeromonads have been detected in different types of drinking water, namely, tap, mineral bottled, and wells.[13,14]

In many countries, the release of pathogenic bacteria in feces dispersed into the aquatic environment can contaminate these waters. Once these bacteria are in the aquatic environment, plasmid exchange between the bacteria is readily facilitated and can result in a higher frequency of multiple antibiotic-resistant strains.[15]

The misuse of antimicrobial agents leads to the high incidences of multidrug resistance and development of resistant strains from drug sensitive microorganisms from the antibiotic saturated environment.[16] All the strains, which were tentatively identified as *A. veronii,* were found resistant to 8 antibiotics, namely, ampicillin (100%), penicillin (100%), vancomycin (100%), kanamycin (66.6%), polymyxin B (66.6), rifampicin (50%), erythromycin (50%), and streptomycin (50%). All the strains, which were tentatively identified as *A. hydrophila,* were found strongly resistant to 10 antibiotics ampicillin (100%), penicillin (100%), vancomycin (100%), kanamycin (100%), rifampicin (75%), polymyxin B (75%) erythromycin (75%), streptomycin (87.5%), amikacin (37.5%), and trimethoprim (37.5%) out of 15 antibiotics

Table 2: Molecular identification of isolates using 16S rRNA sequencing

Name of isolate	Identification	% Similarity	Accession no
FS 5	*A. veronii*	99	KP665258
FS 9	*A. hydrophila*	99	KP665263
FM 38	*A. hydrophila*	99	KP665264
FM 41	*A. veronii*	99	KP665265
FW 54	*A. hydrophila*	99	KP665266
PS 68	*A. hydrophila*	98	KR063150
PS 71	*A. hydrophila*	100	KR063151
PM 79	*A. veronii*	99	KJ729112
PM 85	*A. hydrophila*	99	KR063152
PM 88	*A. veronii*	99	KP969061
PM 92	*A. hydrophila*	99	KM507165
PM 98	*A. veronii*	100	KP969062
PW 104	*A. hydrophila*	99	KR063153
PW 111	*A. veronii*	99	KP969063

A. veronii: Aeromonas veronii, A. hydrophila: Aeromonas hydrophila

Table 3: Antibiotic resistance among *A. veronii* and *A. hydrophila* isolated from Fateh Sagar and Pichhola Lakes of Udaipur

Antibiotics (µg/disc)	% of resistant strains	
	***A. veronii* (*n*=6)**	***A. hydrophila* (*n*=8)**
Ampicillin (10)	100	100
Amikacin (30)	0	37.5
Cefixime (5)	0	0
Ciprofloxacin (5)	0	0
Chloramphenicol (30)	0	0
Erythromycin (15)	50	75
Gentamicin (30)	0	0
Kanamycin (30)	66.6	100
Penicillin (10)	100	100
Polymyxin (300)	66.6	75
Rifampicin (30)	50	75
Streptomycin (25)	50	87.5
Tetracycline (30)	0	0
Trimethoprim (5)	0	37.5
Vancomycin (30)	100	100

A. hydrophila: Aeromonas hydrophila, A. veronii: Aeromonas veronii

used in the study. Roy *et al.*,[17] also isolated, identified, and detected antibiotic resistance patterns of *Aeromonas* spp. from water of Terai river Lotchka, West Bengal, India. They found maximum resistance in *Aeromonas* spp. against penicillin and ampicillin and no resistance in *Aeromonas* spp. against ciprofloxacin. In this way, their results were in accordance with our results regarding these three antibiotics.

Igbinosa and Okoh[3] isolated tetracycline resistant *Aeromonas* species from wastewater treatment plant in the Eastern Cape Province of South Africa. Jacobs and Chenia[16] also observed high resistance against tetracycline in *Aeromonas* species from aquaculture system in South Africa. These results were in contradiction with the results of this study as none of the isolates of this study showed resistance against tetracycline.

None of the *Aeromonas* strains encountered in this study were found resistant to gentamicin, cefixime, ciprofloxacin, and chloramphenicol. The resistance levels to streptomycin when compared to the findings of Hatha *et al.*,[15] it was found that the resistance level was higher in this study against *Aeromonas* species.

CONCLUSION

In this study, high incidence of multiple antibiotic resistance among *Aeromonas* species was observed suggesting these lakes as a reservoir of antibiotic resistance determinants in the study communities. The high antibiotic resistance also indicates a negative impact on therapy with these classes of antibiotics. Strict quality control measures should be put in place to ensure proper treatment of lake water. This would ensure the discharge of properly treated wastewater into the lake to prevent the occurrence and spread of water- and food-borne diseases and to remove such pathogens as *Aeromonas* species is here advocated to prevent the dissemination of multidrug-resistant determinants into the receiving waterbodies.

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