Characterization of *Aeromonas hydrophila* Isolated from Aquatic Environments Using Phenotypic and Genotyping Methods

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Abstract: Ten strains of *Aeromonas hydrophila* isolated from water samples were characterized by various methods including biochemical and physiological tests, as well as PCR-RFLP analysis with Universal 16s rRNA primers. Biochemical characterization and PCR-RFLP analysis proved to be useful, rapid and reliable method for identifying widely distributed Aeromonas spp. from environmental samples. The significance and impact of the current study: monitoring the levels and species composition of Aeromonas in water samples is advisable to minimize human health risk.

Key words: *Aeromonas hydrophila*, aquatic environment, phenotypic and genotypic characters.

INTRODUCTION

The genus Aeromonas has undergone a number of taxonomic and nomenclature revisions over the past 15 years. Although originally placed in the family Vibrionaceae[54]. The subsequent phylogenetic investigations indicated that the genus Aeromonas is not closely related to vibrios and necessitated the removal of Aeromonas from the family Vibrionaceae and transfer to a new family, the Aeromonadaceae[9].

Aeromonas hydrophila and other motile aeromonads are among the most common bacteria in freshwater habitats throughout the world, and have been recognized as occasional pathogens of cultured and feral fishes. The genus *Aeromonas* comprises important human pathogens causing primary and secondary septicemia in immunocompromised persons, serious wound infections in healthy individuals and in patients undergoing medicinal leech therapy, and a number of less well described illnesses such as peritonitis, meningitis, and infections of the eye, joints, and bones[23]. They have been implicated in the etiology of human gastroenteritis; both clinical and laboratory investigations have suggested that the species is a significant enteric pathogen[7,15,18]. The health consequences of the presence of motile species of the genus *Aeromonas* in drinking water are the subject of much debate[19,24,46,55]. Recent studies have demonstrated that the presence of *Aeromonas* spp. in drinking water is a potential risk, since these microorganisms can produce a wide range of virulence factors[21,25,28,40,46,56]. *Aeromonas hydrophila* secretes many extracellular proteins associated with pathogenicity and environmental adaptability.

Most studies involving the ecology of A. hydrophila gastroenteritis have concentrated on its transmission in contaminated water supplies[44,47]. However, Buchanan and Palumbo[6] implicated *Aeromonas* sp. as potential food-poisoning agents. *A. hydrophila* is psychrotrophic and has been associated with the spoilage of refrigerated (5°C) animal products including chicken, beef, pork, lamb, fish, oysters, crab, and milk[10,12,16,26,52].

Only five species of *Aeromonas* were recognized 15 years ago[22], three of which (*A. hydrophila, A. sobria*, and *A. caviae*) existed as phenospecies, that is, a named species containing multiple DNA groups, the members of which could not be distinguished from one another by simple biochemical characteristics. Phenotypic characters that have been claimed to be related to virulence such as haemolysis and the Voges-Proskauer reaction were detected mostly in *A. hydrophila* and *A. sobria.* The distribution of the species was significantly related to levels of faecal pollution in waters. *Aeromonas caviae* predominated in sewage and waters with a high degree of faecal pollution. In less polluted waters, either fresh or marine, A. *caviae* and *A. hydrophila* were almost equally distributed. In waters with low or no faecal pollution, the proportion of *A. sobria* to other species increased considerably.

Aeromonas hydrophila is distributed widely in fresh and salt water, and can be found also in food, treated drinking or domestic water, and hospital water supply systems. Since the wide distribution of *A. hydrophila* is probably a consequence of its high capacity to adapt to different environments, it would seem that the genetic and phenotypic diversity of *A. hydrophila* is a natural result[34].

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In this work, several strains of *A. hyrophila* were isolated from water samples and characterized using various methods including biochemical/ physiological tests, RFLP PCR of 16S rRNA, Investigating haemolytic activity and plasmid profiling; and test for multiple antibiotic resistance.

MATERIALS AND METHODS

Isolation and Identification Procedure: Water samples were first filtered through sterile vacuum filtration system (0.45 µm). The filters were posed on Petri dishes containing Pseudomonas-Aeromonas Agar (GSP agar, Merck, Germany), containing ampicillin (10 mg/L). All plates were incubated aerobically for 24 h at 28ºC. Mucoid yellow colonies (2-3 nm diameter) were considered presumptive aeromonas and picked up, restreaked onto GSP plates and then subcultured on trypticase soy agar (TSA) plates, Triple sugar iron agar (TSI) and MacConkey agar incubated at 28ºC for 24 h and were purified[30].

Preliminary aeromonads were identified to genus and species after checking the morphological characteristics of the culture, Gram staining, and biochemical characteristics based on Aerokey II group of tests for the identification of *Aeromonas*[8,43]. The colonies that positive for Catalse test, oxidase test, Fermentative reaction in O/F test and typical growth reaction on Triple sugar iron agar medium were considered as *A. hydrophila.* Each isolate was further examined for the production of diffusible pigments on TSA; MR test; VP test; indole production; esculin hydrolysis; growth on KCN; acid production from sucrose, arabinose, mannitol; gas production from glucose; production of ornithine decarboxylase and lysine decarboxylase. All isolates were stored in Luria-Bertani (LB) broth containing 20% glycerol at-80°C until further analyses were carried out.

Computer Analysis of *Aeromonas* 16S rDNA Gene Sequences: A database containing 16S rRNA gene sequences of all validly published *Aeromonas hydrophila* was compiled from GenBank (http://www.ncbi.nlm.nih.gov). All sequences used were longer than 1532 bp. For restriction endonuclease selection and species discrimination, the amplified regions within published 16S rDNA gene sequences were analysed using NEB cutter V2.1 software program (http://tools.neb.com/NEBcutter2/) . An analysis of the theoretical banding patterns for various restriction enzymes was performed, and *HinfI* and *HaeIII* were selected for species discrimination.

DNA Manipulation: Genomic DNA of all *Aeromonas* strains was isolated by a Wizard Genomic DNA purification kit (Promega, Madison, Wis., USA). Isolation of plasmids and digestion of DNA with restriction endonucleses were carried out by standard procedures[45].

PCR Amplification of 16S rRNA from *Aeromonas* spp: The following oligonucleotides were used to amplify the 16S rRNA gene in a PCR: oligonucleotide F1 (5'-AGAGTTTGATCATGGTCAG-3') and oligonucleotide F2 (5'-GGTTACCTTGTTACGACTT-3'). The primers were were purchased from Amershambioscience. The PCR amplification reactions were carried out in a GeneAmp PCR System 9700 (Applied Biosystems, Forrest City, CA). The reaction mixture contained 47 µl of QIAGEN PCR mixture, 2 µl of the PCR primer mix, and 1 µl of the genomic DNA. PCR was performed under the following conditions: denaturation at 93°C for 3 min, followed by 35 cycles at 94°C for 1 min, 56°C for 1 min, and 72°C for 2 min. After the final cycle, an extension at 72°C was allowed for 10 min. The gels were electrophoresed, stained with ethidium bromide, and photographed[36].

RFLP of 16S rRNA from Aeromonads: The PCR amplicon was purified with a QIAquick PCR purification kit (QIAGEN, Valencia, CA). Restriction digestions were performed by incubating 10 µl of the amplified PCR product with 5 U of each restriction enzyme (HinfI and HaeIII; New England Biolabs, Beverly, MA) as the manufacturer's instructions[6,13]. Digestion was performed at 37°C for 4 h. The digested samples were electrophoresed on 2.0% agarose gel, stained, and photographed.

Antibiotic Susceptibility Testing: The antibiotic susceptibility of each aeromonad was determined by the disk diffusion method[2]. Aeromonad strains were streaked on Mueller-Hinton agar plates, and the various antibiotic disks were applied on the streaked cultures with a Dispens-O-Disc dispenser (Difco Laboratories, Detroit, MI). Disks of bacitracin (10 µg), neomycin (30 µg), erythromycin (15 µg), gentamicin (10 µg), trimethoprim-sulfamethoxazole (23:75; 1.25 µg), tetracycline (30 µg), chloramphenicol (30 µg), streptomycin (10 µg), novobiocin (30 µg), and ciprofloxacin (5 µg) were used. After 18 h of incubation at 37°C, the zones of inhibition were measured and compared to the manufacturer's instruction and by the criteria of the National Committee for Clinical Laboratory Standards[35,38]. Characterization of strains as sensitive, intermediate or resistant was based on the size of inhibition zones around each disc according to the manufacturer's recommendations.

Haemolysis Assay: Haemolysis was assayed on tryptone soy agar (Oxoid) plates with 5% whole sheep blood[29]. Each isolate was streaked on a tryptic soy agar (TSA) containing 5% sheep RBCs. Plates were incubated at 30°C and were checked for the type (*a* or *b*) of haemolytic activity after 24 h by detecting a clear zone was formed around a colony.

RESULTS AND DISCUSSIONS

Characterization and Identification of *A.hydrophila* Strains Isolated: All bacterial isolates from water samples are exhibiting the typical aeromonad morphological characteristics, such as mucoid yellow colored colonies on GSP plates and buffcolored colonies on Trypticase soy agar plates. All are gram-negative, oxidase-positive, rod-shaped bacteria resistant to ampicillin, and so are grouped within ten bacterial strains. Results from the culture characteristics indicated that colonies were rounded, 2-3 mm in diameter, and mucoid yellow appearance on the GSP plates. Mucoid yellow colonies resembling *Aeromonas* were subcultured on trypticase soy agar, Macconkey agar and Triple sugar iron (TSI) agar and incubated at 28ºC for 24 h. All isolates showed typical reaction on Triple sugar iron (TSI) agar and produce buffocolored on Tryotic soya agar confirmed that they belongs to *Aeromonas hydrophila*. The results recorded in Table (1) revealed that all the culture and biochemical characteristics of the isolated bacteria.

The characteristic type of colony appearance obtained in the present work when each *A. hydrophila* was separately inoculated on GSP plates as a result of amylolytic activity of the isolated bacteria and subsequent fermentation of the resulted maltose as indicated by the yellow zone around the colonies. This agrees with the findings of many reports including Rimler and Shotts[48] who obtained yellow colonies when was inoculated on to RS Media and these type of colonies indicating maltose fermentation, and Hazen *et al*,[18] who stated that RS Media was 94% efficient for isolation of *Aeromonas hydrophila* and Hsu *et al*[20] who noted that all 127 strains of *A. hydrophila* tested produced yellow colonies on RS Media, while were. White to pale pink, round and covex colonies appear on nutrient agar.

Gram negative, oxidase positive, motile organisms were further tested for the following characteristics: oxidation and fermentation of glucose *(OF),* fermentation of mannitol and salicin, utilization of arabinose, Aesculin hydrolysis, gas production from glucose, growth in KCN broth (Table 1).

Based on the obtained biochemical characterization of the isolates, both the positive and negative results were agreed with other reports including[5,7,20,31,33,37,39,42,48,49,51]. The biochemical reactions of the isolates showed that typical reaction of majority of the biochemical tests with that of the reference strain. Although ten isolates were found to be negative lysine decarboxylase, all had the ability to utilize L-arginine (Table 1), which is the characteristic of *A. hydrophila* and thus differentiates them from *A. sorbia* and *A. caviae*[43].

PCR amplification and RFLP profile of 16s rRNA gene: One pair of synthetic 16S rRNA-specific oligonucleotide primers, targeting a 1.5-kb region of the 16S rRNA, was used in the PCR assay. The protocol amplified the 1.5-kb gene from the genomic DNA obtained from all ten aeromonads. The purified PCR amplicon was digested with HinfI and HaeIII, and the digests were separated on a 2% agarose gel. The resulted RFLP patterns from the two restriction enzymes classifies the ten strains of *Aeromonas hydrophila* into three clades as shown in figures 1 & 2.

Digestion by HinfI restriction enzyme grouped the ten strains of *A,hydrophila* into three caldes based on the resultant three different restriction patterns. Digestion of the 16S rRNA PCR amplicon by HinfI enzymes from clade I including 5 strains of *A. hydrophila* yielded 5 restriction fragments measuring 60 to 350 bp (Fig. 1, lane 1,2,3,4,5). Digestion of the amplified PCR product from Clade II including four strains of *A. hydrophila* (Fig 1, lane 6, 7, 8 & 10). Clade III includes only one strain of *A. hydrophila* that comprised with unique banding pattern (Fig. 1, lane 9).

As shown in figure 2, the banding pattern resulted from the digestion of 16 s rRNA amplicon of ten isolates of *A. hydrophila* yielded also three clades. The RFLP of clade I includes five strains of *A. hydrophila* (Fig.2, lane 1, 2, 3, 4 & 5) had a distinct DNA fragments, whereas four strains of *A. hydrophila* comprising clade II (Fig. 2, lane 6, 7, 8 & 10).

The size and number of restriction fragments from these isolates were identical to the size and number of restriction fragments deduced from analysis using NEB cutter V2.1 software program (Table 2). The obtianed results, based on the PCR- RFLP pattern of the 16S rRNA gene from the ten aeromonads isolated from aquatic environments, indicated that all are strains of *A. hydrophila* and confirmed the biochemical and physiological tests.

Antibiotic Resistance Profiles of *Aeromonas* Isolates: Antimicrobial susceptibility tests of ten *A. hydrophila* strains identified from water samples were carried out by the agar disc diffusion method on Muller–Hinton agar (Merck). Antimicrobial sensitivity patterns of the

Table 1: Biochemical characteristics of the isolated *Aeromonas* spp

	Reference Strain	1	2	3	4	5	6	7	8	9	10
Gram stain	-	-	-	-	-	-	-	-	-	-	-
KOH test	+	+	+	+	+	+	+	+	+	+	+
Motility	+	+	+	+	+	+	+	+	+	+	+
Oxidase	+	+	+	+	+	+	+	+	+	+	+
Catalase	+	+	+	+	+	+	+	+	+	+	+
O/F of glucose	F	F	F	F	F	F	F	F	F	F	F
VP test	+	+	+	+	+	+	+	+	+	+	+
MR test	+	+	+	+	+	+	+	+	+	+	+
Gas from glucose	+	+	+	+	+	+	+	+	+	+	+
Acid from:											
Glucose	+	+	+	+	+	+	+	+	+	+	+
D- Mannitol	+	+	+	+	+	+	+	+	+	+	+
L-Arabinose	+	+	+	+	+	+	+	+	+	+	+
Sorbiotol	-	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Sucrose	-	+	+	-	+	-	+	-	+	-	+
Indole production	+	+	+	+	+	+	+	+	+	+	+
Citrate utilization	+	+	-	+	-	+	+	+	-	-	+
Growth on MacConkey agar	+	+	+	+	+	+	+	+	+	+	+
Growth on TSI	+	+	+	+	+	+	+	+	+	+	+
Growth on KCN	+	+	+	+	+	+	+	+	+	+	+
Ornithine decarboxylase	-	-	-	-	-	-	-	-	-	-	-
Lysine decarboxylase	+	+	+	-	+	+	-	-	+	+	+
Esculin hydrolysisn	+	+	+	+	-	+	-	+	+	+	+
Arginine utilization	+	+	+	+	+	+	+	+	+	+	+

ND= not detected

Table 2: DNA fragments (in bp) obtained from the 16S rRNA gene sequence analysis with endonuclease *HinfI* and HaeIII by using NEB cutter V2.1 software program (http://tools.neb.com/NEBcutter2/)

HinfI	349	332	317	245	137	61	32	31	15	13	
HaeIII	317	220	204	195	171	169	149	59	39	9	

Aeromonas isolates are shown in Table 3. Of the 10 motile Aeromonas isolates, all (100%) were sensitive to gentamicin, 8 (80%) to sulphamethoxazole-trimethoprim, 7 (70%) to chloramphincol, 5 (50%) to ciprofloxacin, 4 (40%) to neomycin, 3 (30%) to tetracycline, 2 (20%) to streptomycin and 1 (10%) to erythromycin; all were resistant to novobiocin and bacitracin.

In the current study, all Aeromonas isolates displayed high sensibility to gentamicin (100%), sulphamethoxazole-trimethoprim (80%) and ciprofloxacin (50%). On the other hand high resistance were displayed to novobiocin and bacitracin (100%), erythromycin (90%), streptomycin (80%), tetracycline (70%) and neomycin (60%). The present findings agreed with previous reports that showed high sensitivity of Aeromonas spp. to gentamicin[32,14,1] and high resistance to novobiocin[50]. However, least resistance to gentamicin has been described in the range of 1–10%[14]. Whereas high sensibilities to
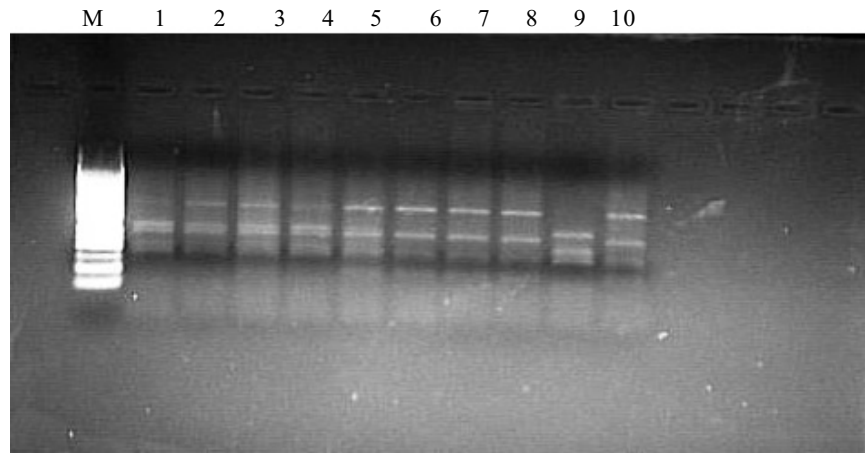
Fig. 1: *Hinf* I RFLP profile of the 16S rRNA amplified from representative isolates of *Aeromonas hydrophila* . Lanes: 1 and 10, lane M, 100-bp DNA ladder used as a molecular size standard.
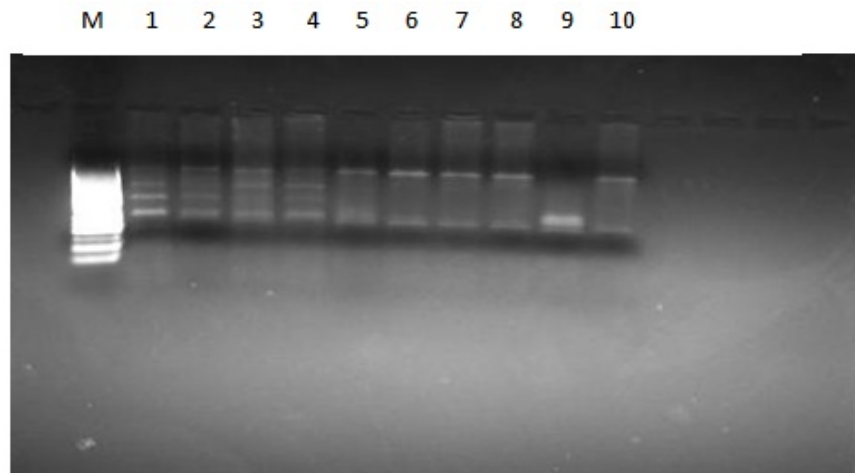

Fig. 2: *Hinf* I RFLP profile of the 16S rRNA amplified from representative isolates of *Aeromonas hydrophila*p. Lanes: 1 and 10, lane M, 100-bp DNA ladder used as a molecular size standard.

Table 3: Antimicrobial susceptibility of motile *Aeromonas* strains to various antimicrobials

| Antimicrobial agent	*Aeromonas hydrophila*									
	1	2	3	4	5	6	7	8	9	10
Erythromycin	R	R	R	R	R	R	I	S	I	I
Neomycin	S	S	S	I	I	R	S	I	R	R
Gentamicin	S	S	S	S	S	S	S	S	S	S
Novobiocin	R	R	I	R	R	R	I	I	R	R
Tetracycline	S	R	S	S	I	I	R	I	R	I
Streptomycin	R	R	R	I	R	R	I	R	S	S
Chloramphincol	S	S	S	I	S	I	I	S	S	S
Bacitacin	I	I	R	R	I	R	R	R	R	I
Ciprofloxacin	R	R	I	I	S	S	I	S	S	S
Sulphamethoxasoletrimethoprim	S	S	S	S	S	S	I	S	I	S

R: resisttant; I: intermediate; S: sensitive
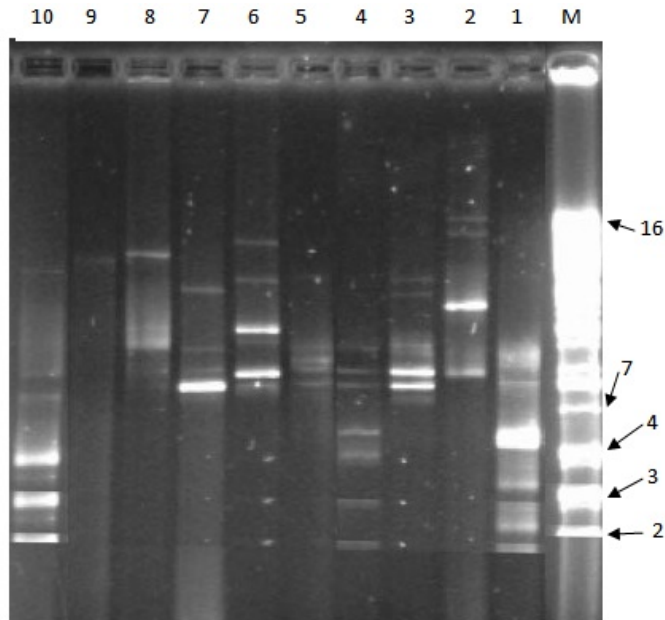
Fig. 3: Plasmid profiles Obtained by alkaline-lysis Method of *A. hydrophila* separated by Agarose Gel electrophoresis. (M: kb Molecular DNA marker; 1-10: the code number of the aeromonas isolates)

sulfamethoxazole- trimethoprim[32,11,1] were similar to the results of this study, high resistance (43%) was also reported. In contrast to the present findings, Akinbowale *et al.*,[1] reported low resistance (10.4%) to oxytetracycline.

Plasmid Profiling: Attempts were made to isolate plasmids from the ten strains of *Aeromonas hydrophila*. All of ten strains contained plasmids. These plasmids varied in sizes ranging from 1.5 kb to 16.0 kb (Fig 3). One strain were distinct from other plasmid-containing strains by containing just one plasmid as indicated by one band DNA fragment (Figure 3, Lane 9). Three strains appear to be have the same plasmid profiles as indicated in the banding patterns observed in figure 3 (lanes 1, 4 and 10). The remaining Aeromonas strains contained multiple plasmids measuring more than 7.0 kb (Fig 3, lanes 2, 3,5, 6, 7 & 8). Since *A. hydrophila* can be transferred from animals to humans and several bacterial phenotypic properties such as antimicrobial resistance or virulence factors have been demonstrated to be plasmid encoded, the presence of plasmids may present a potential public health hazard. Thus, the presence of plasmids in clinically important bacteria increases their virulence[17]. Plasmids of similar size have been observed by Vadielu *et al.*[53] and Borrego *et al.*[4]. The role of these plasmids may be identified by observing their various characteristics after curing them.

Hemolysin Activity: *Aeromonas* spp. are potential opportunistic agents of gastroenteritis, bacteraemias and other disease in man and animals[15] and their ability to cause a wide range of infections in humans and animals involves protein toxins[41]. Hemolysin as a putative virulence factors of *A. hydrophila* have been demonstrated in an effort to explain the process of pathogenicity. The majority of the strains had a high percentage (70%) of hemolytic activity, with a variable halo diameter between 0.5 and 2 mm. The higher concentration of haemolysins of *A. hydrophila* in our environmental strains agrees with the results obtained by other authors[7,27] on clincial and environmental strains. The haemolytic activity is strongly associated with enterotoxin production in members of the *Aeromonus* genus [7]. Other report showed that 87% of 30 *A. hydrophila* strains isolated from superficial swimming pool water were haemolytic and that haemolytic and cytotoxic activities were frequently associated[3].

Identification and characterization of aeromonas species, emergent pathogens for humans, has long been controversial due to their phenotypic and genomic heterogeneities. Since biochemical properties do not accurately reflect the genomic complexity of a given species and the diagnostic results may be influenced by physical parameters, such as pH, temperature, and growth substrate concentrations, unambiguous identification of the different members of the genus by biochemical reactions is impossible. Thus, molecular methods, such as PCR amplification and restriction digestion of the16S rRNA, are invaluable for the identification of these isolates[13,39]. In addition,

computer analysis of the published 16s rRNA gene is a good and rapid way of assessing the identities of all known species of aeromonas[5].

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