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Genetic diversity and antimicrobial susceptibility of motile aquatic aeromonads

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***Abstract*—A total of 10 motile *Aeromonas* strains were detected in water samples. These strains were identified by conventional microbiological techniques as Aeromonas hydrophila. Genetic diversity by PCR-RFLP analysis with Universal 16s rRNA primers and plasmid profiles were carried out on ten *A. hydrophila* isolates obtained from water samples. Antimicrobial sensitivity patterns of the Aeromonas isolates revealed that 100% were sensitive to gentamicin, 80% to sulphamethoxazole–trimethoprim, 70% to chloramphincol, 50% to ciprofloxacin, 40% to neomycin, (30% to tetracycline, 20% to streptomycin and 10% to erythromycin. all were resistant to novobiocin and bacitracin.**

***Index Terms*— *Aeromonas hydrophila*, aquatic environment, PCR-RFLP analysis of 16s rRNA, plasmid profiles.**

I. Introduction

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 (18). 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). The health consequences of the presence of motile species of the genus *Aeromonas* in drinking water are the subject of much debate (15, 19 & 45). 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 (16, 20, 23, 34 & 46). *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 (38). 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 (13 & 21). Only five species of *Aeromonas* were recognized 15 years ago (17), 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 (29). 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.

II. 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 (25). 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 & 36). 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

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were considered as *A. hydrophila.* 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 (37).

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 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 (30).

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 per 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. 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 and by the criteria of the National Committee for Clinical Laboratory Standards (32).

Haemolysis assay: Haemolysis was assayed on tryptone soy agar (Oxoid) plates with 5% whole sheep blood (24). 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.

III. Results

Characterization and Identification of *A. hydrophila* strains isolated: All bacterial isolates from water samples are are gram-negative, oxidase-positive, rod-shaped bacteria resistant to ampicillin, and so are grouped within ten bacterial strains. Mucoid yellow rounded, 2-3 mm in diameter, colonies resembling *Aeromonas* were subcultured on trypticase soy agar, Mac-conkey 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. Typically as the findings obtained yellow colonies of *A. hydrophila* on RS Media (39) indicating maltose fermentation, while were white to pale pink, round and covex colonies 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, 26, 28, 31, 33, 40 & 41).

Based on the obtained biochemical characterization of the isolates, both the positive and negative results were agreed with other reports including (5, 7, 26, 28, 31, 33, 40 & 41). 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* (36).

PCR amplification and RFLP profile of the 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 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 (a&b).

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. 1a, lane 1,2,3,4,5). Digestion of the amplified PCR product from Clade II including four strains of *A. hydrophila* (Fig 1a, lane 6, 7, 8 & 10). Clade III includes only one strain of *A. hydrophila* that comprised with unique banding pattern (Fig. 1a, lane 9).

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	+	+	+	+	+	+	+	+	+	+	+

As shown in figure 2(a&b), the banding pattern resulted from the HaeIII 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.2a, lane 1, 2, 3, 4 & 5) had a distinct DNA fragments, whereas four strains of *A. hydrophila* comprising clade II (Fig. 2a, 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). 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.

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
HaeIII	317	220	204	195	171	169	149	59	39

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 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.

M 1 2 3 4 5 6 7 8 9 10

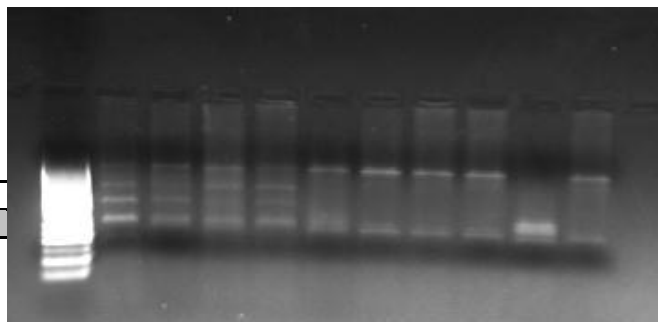

Fig. 1a: *Hinf* I RFLP profile of the 16S rRNA amplified from representative isolates of *Aeromonas hydrophila* . Lanes: 1 and 10, lane M, 100-bp DNA ladder used as a molecular size standard.

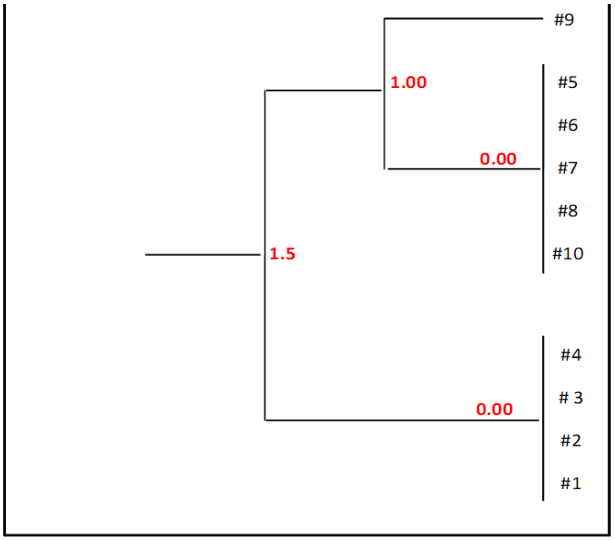

Fig. 1b: phylogenetic relation ship of Hinf I RFLP profile of the 16S rRNA amplified from representative isolates of *Aeromonas hydrophila* . .

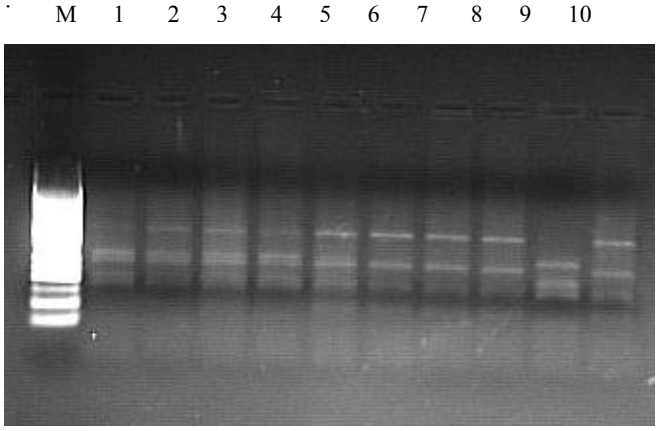

Fig. 2a: *HaeIII* RFLP profile of the 16S rRNA amplified from representative isolates of *Aeromonas hydrophila*p. Lanes: 1 and 10, lane M, 100-bp DNA ladder used as a molecular size standard.

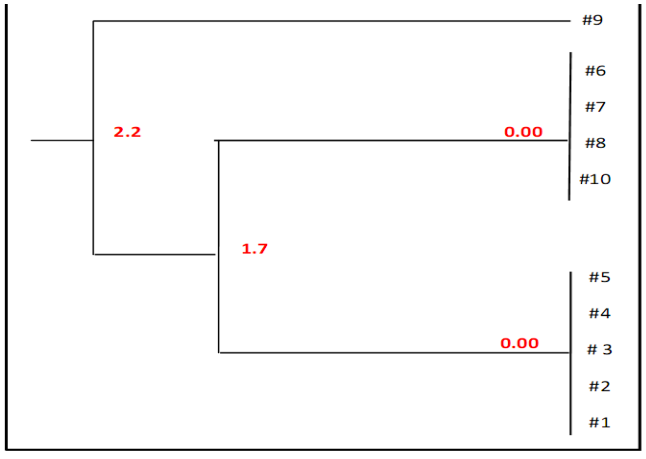

Fig. 2b:Phylogentic tree of *Hea* III RFLP profile of the 16S rRNA amplified from representative isolates of *Aeromonas hydrophila*p

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 (1, 12 & 27) and high resistance to novobiocin (50). However, least resistance to gentamicin has been described in the range of 1–10% (12). Whereas high sensibilities to sulfamethoxazole-trimethoprim (1, 10 & 27) 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: Plasmids, varied in sizes ranging from 1.5 kb to 16.0 kb, were isolated from the ten strains of *Aeromonas hydrophila* (Fig 3). One strain was 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 (14). Plasmids of similar size have been observed by Vadielu *et al.* (43) 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 (7) and their ability to cause a wide range of infections in humans and animals involves protein toxins (35). 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 & 22) on clincial and environmental strains. The haemolytic activity is strongly associated with enterotoxin production in members of the genus *Aeromonus* (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).

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
Sulphamethox-asoletrimethoprim	S	S	S	S	S	S	I	S	I	S

R: resistant; I: intermediate; S: sensitive

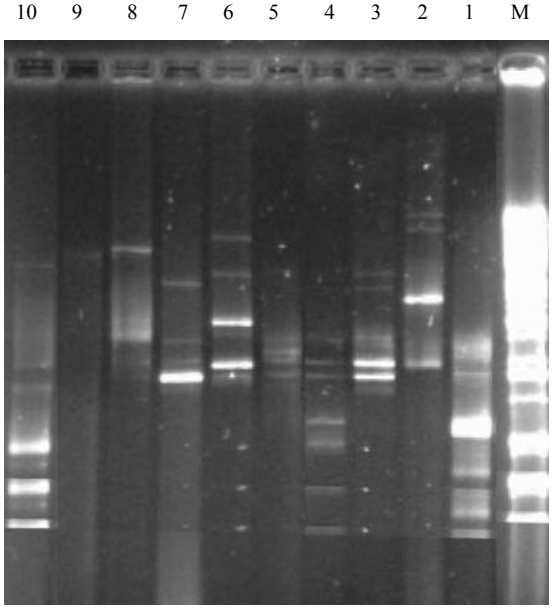

Fig. 3: Plasmid profiles Obtained by alkaline-lysis Method of *A. hydrophila* separated by Agarose Gel electrophoresis. (M: Lkb Molecular DNA marker Kd; 1-10: the code number of the aeromonas isolates)

IV. CONCLUSION

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 (33). 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). Plasmids play a major role in bacterial adaptation to environmental or man-made stress. The rapid dissemination of antibiotic resistance genes in bacterial populations as a consequence of the intensive use of antibiotics in medicine and agriculture (including, aquaculture) can be partly attributed to plasmid-mediated horizontal transfer. Plasmids capable of being transferred and stably maintained in a wide range of bacteria, the so-called broad-host-range plasmids, are of special interest with respect to interspecies gene exchange.

Motile aeromonad septicemias are generally mediated by stress. Elevated water temperature , a decrease in dissolved oxygen concentration, or increases in ammonia and carbon dioxide concentrations have been shown to promote stress in fish and trigger motile aeromonad infections. The monitoring of environmental variables can therefore enable one to forecast stressful situations and possibly avoid problems before they arise.

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