Isolation of motile aeromonads from aquatic environment

Article *in* Indian Journal of Fisheries · January 2005

5 authors, including:

Gaurav Rathore
National Bureau of Fish Genetic Resources
77 PUBLICATIONS **396** CITATIONS
SEE PROFILE

Raja Swaminathan
National Bureau of Fish Genetic Resources
58 PUBLICATIONS **297** CITATIONS
SEE PROFILE

Rehana Abidi
National Bureau of Fish Genetic Resources
30 PUBLICATIONS **79** CITATIONS
SEE PROFILE

Indian J. Fish., **52**(2) : 241-248, Apr.-June, 2005

Note

Isolation and characterization of motile aeromonads from aquatic environment

GAURAV RATHORE, T. RAJA SWAMINATHAN, REHANA ABIDI, P.C. MAHANTA AND D. KAPOOR

National Bureau of Fish Genetic Resources, Lucknow-226002.

ABSTRACT

Isolation of motile aeromonads was done on Starch Ampicillin Agar and modified Rimler-Shotts Agar from 13 water samples and 7 fish samples. A total of 22 isolates of *Aeromonas* were identified. Among the 22 isolates of *Aeromonas,* 9 were *A. hydrophila* (43%), 3 were *A. sobria* (13%), 3 were *A. veronii* (13%), 2 were *A. schubertii* (9%) and 2 belonged to *A. caviae* (9%). All the isolates were sensitive to gentamicin and ciprofloxacin, and resistant to erythromycin, furazolidone and penicillin. Oxytetracycline, commonly used antibacterial agent was found to be effective against only 60% of isolates.

Emergence of *Aeromonas* sp. as an important human pathogen has led to considerable interest in the organism in last two decades. Aeromonads are ubiquitous in nature and isolated from wide variety of sources. They are part of the normal microbial flora of aquatic and terrestrial animals as well as etiological agents of diseases in numerous cold-blooded and warm-blooded animals. The aquatic environment is considered to be the principal reservoir of *Aeromonas* sp. (Wadstrom and Ljungh, 1991) and the organism is isolated from different water sources including chlorinated drinking water (Kersters *et al.,* 1995). Aeromonads are also common contaminants in foods such as fish and other seafoods, raw and cooked meat, poultry, vegetables, milk and milk products. In India, the organism has been isolated from a variety of foods including fish, meat, milk, eggs, tortoise and snails (Agarwal, 1997).

Motile aeromonads are considered to be the main cause of bacterial hemorrhagic septicemia in fresh water fish and have been reported in association with various ulcerative syndromes and red spot disease (Frerichs, 1989). These infections can cause high mortalities in fish hatcheries and in natural freshwater fish population. In 1973, around 37500 fishes died over 13 days period in North Carolina lake due to *A. hydrophila* infection (Miller *et al.,* 1976).

Motile aeromonads are resistant to antibiotics; thereby infections are difficult to control due to indiscriminate antibiotic therapy. Krovacek *et al.* (1997) tested the antibiotic sensitivity pattern of *A. hydrophila* isolated from a gray seal. He found that the isolates were sensitive to enrofloxacin, neomycin, streptomycin,

gentamicin, oxytetracycline and nitrofurantoin.

The present study was therefore, carried out with the objective to isolate the motile aeromonads from aquatic fresh water environments, to identify and characterize the isolated aeromonads from fish and water samples and to test isolates for their antibiotic sensitivity pattern. Water samples were collected from aquaria and fishponds located in National Bureau of Fish Genetic Resources (NBFGR) campus, Lucknow. Water samples were also collected from local ponds and a canal passing through Telibagh area of Lucknow. In addition to the water samples, apparently healthy and diseased fish were also collected from Telibagh fish market, Lucknow for the isolation of *Aeromonas* species. The fish were brought to the laboratory in polythene bags and kept in a tub, while the water samples were brought in sterile 30 ml vials. The samples were processed for the bacterial isolations, within two hours of collection. Altogether, 13 water samples and 7 fish samples were used for the screening of *Aeromonas* species.

Selective media for isolation of Aeromonas sp.

In the present study, Rimler-Shotts Agar (RSA) (Austin and Austin, 1993) and Starch Ampicillin Agar (SAA) (Palumbo *et al.,* 1985) were used as selective media for the isolation of the *Aeromonas* sp. Ampicillin was used as a selective agent in the modified RSA instead of novobiocin, to inhibit the growth of other competing microorganisms. Ampicillin @ 10mg/1 was found to be effective as inhibitory agent.

Processing of samples

Ten fold dilutions of water samples were made in sterile normal saline. Fish muscle was used for the isolation of *Aeromonas* species. The visceral organs were excluded during processing of samples so as to restrict the isolation of members of Enterobacteriaceae. Ten gm of fish muscle was triturated in 90 ml sterile normal saline. Thereafter ten fold dilutions of the triturated sample were made in sterile normal saline. Spread plating was done on selective media plates for the bacterial isolation. A total of 100 µl of inoculum from each dilution was spread on the SAA and modified RSA and spread uniformly with the help of a sterile glass rod spreader. The plates were then incubated at 28^{0}C for 24 hours and examined for colony characters. On modified RSA, the colonies of *Aeromonas* sp. were small, round and raised with black center. The colour of the medium turned green after 12 hours of incubation and yellow after 24 hours. The colonies of *Aeromonas* on SAA plates showing a colony number between 50-300 were subjected to the amylase test (Palumbo *et al.,* 1985). Few crystals of iodine were put on the pre-warmed lid of the selected petri-plate which was inverted and left for 5 minutes. The clearance of the zone around the typical *Aeromonas* colonies was observed in presence of iodine vapours, indicating the amylase activity of the organisms.

The typical colonies on RSA and Amylase positive colonies on SAA were picked and subjected to biochemical tests for the identification of *Aeromonas* species. The confirmation of isolates as *Aeromonas* sp. was done as per the procedure described by Lee and Donovan (1985) (Table 1). Biochemical testing of isolates was done as per Barrow and Feltham (1992).

Species identification

Identification of *A. hydrophila, A. caviae* and *A. sobria* was achieved

TABLE 1. *Identifying characteristics of* Aeromonas sp.

Characteristics	Result
Gram's reaction	-
Motility	+
Morphology	Coco-bacilli
Arginine decarboxylase	+
Ornithine decarboxylase	-
Acid from mannitol	+

+ = Positive, - = Negative

following modified Aero-key (Fig.1), (Agarwal *et al.,* 1998). This key is based on aesculin hydrolysis, production of acetoin from glucose (VP test), gas from glucose and acid from arabinose. Strains giving positive results for aesculin hydrolysis, gas from glucose and acid from arabinose were identified as *A. hydrophila,* whereas, isolates that hydrolyzed aesculin, but did not produce gas from glucose were taken as *A. caviae.* Strains that were negative for aesculin hydrolysis and positive for VP reaction were recognized as *A. sobria.* Strains that did not hydrolyze aesculin and negative for indole test were identified as *A. schubertii.* In addition, the strains that were also VP negative were identified as *A. trota.* Isolates that produced gas from glucose but no acid from arabinose were considered as *A. veronii* (Joseph and Carnahan, 1994).

The isolates of *Aeromonas* from different sources are given in Table 2. The details of the biochemical tests undertaken for genus and species identification are listed in the Table 3. The biochemical characters of isolates on RSA and SAA were identical. Hence, either of these two media can be used for isolation of motile aeromonads. A total of 22 isolates of *Aeromonas* were identified from 13 water samples and 7 fish samples. Among the 22 isolates of *Aeromonas,* 9 were *A. hydrophila,* 3 each were *A. sobria, A. trota and A. veronii,* 2 were *A. schubertii* and 2 belonged to *A. caviae.* These results indicate diversity of motile aeromonads in fresh water aquatic environment of this region, with predominance of *A. hydrophila.* This can be a potential threat to fish population in stress conditions. *A. hydrophila,* principal cause of bacterial haemorrghic

TABLE 2. *Source and type of sample collected for isolation of* Aeromonas sp.

Sample Source	Type of sample	No. of Samples	Isolate number
NBFGR fish pond 1 and 2	Water	2	1,2
Canal	Water	2	3,4,5,6,7
Devekhera fish pond	Water	2	8,9
NBFGR	Water	4	10,11,12,13,14,15
Diseased *Clarias batrachus*	Skin, kidney	1	16,17
Fish aquaria at NBFGR	Water	3	18,19,20
Clarias batrachus	Skin	6	21,22

TABLE 3**:** *Biochemical test results of the bacterial isolates.*

A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U
1	-	Rod	+	+	+	+/+	f	+	-	+	+	-	-	A/A	+	+	+	+	+	*A. hydrophila*
2	-	Rod	+	+	+	+/+	f	-	-	-	+	-	+	K/A	+	+	+	+	-	*A. schubertii*
3	-	Rod	+	+	+	+/+	f	+	+	-	+	-	+	A/A	+	+	+	+	-	*A. sobria*
4	-	Rod	+	+	+	+/+	f	+	-	-	+	-	-	A/A	+	+	+	+	+	*A. caviae*
5	-	Rod	+	+	+	+/+	f	+	-	-	+	-	-	A/A	-	-	+	+	-	*A. trota*
6	-	Rod	+	+	+	+/+	f	+	+	+	+	-	-	A/A	+	+	+	+	+	*A. hydrophila*
7	-	Rod	+	+	+	+/+	f	-	-	-	+	-	-	K/A	-	-	+	-	-	*A. schubertii*
8	-	Rod	+	+	+	+/+	f	+	+	+	+	-	-	A/A	+	+	+	+	+	*A. hydrophila*
9	-	Rod	+	+	+	+/+	f	+	-	-	-	-	-	A/A	-	-	+	+	+	*A. caviae*
10	-	Rod	+	+	+	+/+	f	+	-	+	+	-	+	A/A	+	-	+	+	+	*A. hydrophila*
11	-	Rod	+	+	+	+/+	f	+	+	-	+	-	+	A/A	+	+	+	+	-	*A. trota*
12	-	Rod	+	+	+	+/+	f	+	-	-	+	-	+	A/A	-	-	+	+	-	*A. sobria*
13	-	Rod	+	+	+	+/+	f	+	-	+	-	-	+	A/A	+	-	+	+	+	*A. hydrophila*
14	-	Rod	+	+	+	+/+	f	+	-	+	+	-	-	A/A	+	+	+	+	+	*A. hydrophila*
15	-	Rod	+	+	+	+/+	f	+	+	+	+	-	+	A/A	+	+	+	+	+	*A. hydrophila*
16	-	Rod	+	+	+	+/+	f	+	+	+	+	-	-	A/A	-	-	+	+	+	*A. veronii*
17	-	Rod	+	+	+	+/+	f	+	-	-	-	-	+	K/A	+	-	+	-	-	*A. trota*
18	-	Rod	+	+	+	+/+	f	+	-	-	+	-	+	A/A	+	-	+	+	-	*A. sobria*
19	-	Rod	+	+	+	+/+	f	+	+	+	+	-	+	A/A	+	-	+	+	+	*A. hydrophila*
20	-	Rod	+	+	+	+/+	f	+	-	+	+	+	-	A/A	-	+	+	+	+	*A. veronii*
21	-	Rod	+	+	+	+/+	f	+	-	+	+	+	-	A/A	-	+	+	+	+	*A. veronii*
22	-	Rod	+	+	+	+/+	f	+	+	+	+	-	+	A/A	+	-	-	+	+	*A. hydrophila*

Note **A**- isolate number; **B**- Gram staining; **C**- shape; **D**-motility; **E**- catalase; **F**- oxidase; **G**- acid/gas from glucose; **H**- oxidation/fermentation; **I**- indole; **J**- methyl red; **K**- Voges proskauer; **L**- arginine decarboxylation; **M**- ornithin decarboxylation; **N**- lysine decarboxylation; **O**- triple sugar iron agar reaction; **P**- arabinose fermentation; **Q**- salicin fermentation; **R**- trehalose fermentation; **S**- mannitol fermentation; **T**- aesculin hydrolysis; **U**- isolates.

Note: + = positive; - = negative; f - fermentative; K/A - alkaline slant and acid butt; A/A - acid slant and butt.

TABLE 4: *Antibiotic sensitivity of* Aeromonas *isolates (Diameter of Inhibition Zone in mm)*

Isolate No.	Different antibiotics along with their concentration (per disc) used (Range of sensitivity in mm)															
	G	**Cf**	**Cp**	**Nx**	**E**	**Nv**	**O**	**Co**	**K**	**Fr**	**A**	**C**	**N**	**Na**	**B**	**P**
	50 µg (15)	10 µg (21)	30 µg (22)	5 µg (19)	15 µg (23)	30 µg (22)	30 µg (19)	25 µg (19)	30 µg (18)	50 µg (25)	25 µg (17)	25 µg (18)	30 µg (17)	30 µg (19)	10 I.U (13)	5 I.U (15)
1	26	34	R	32	R	R	24	20	18	R	R	29	18	30	R	R
2	26	34	R	29	R	R	24	R	20	R	18	39	19	31	R	R
3	30	35	R	25	R	R	24	19	20	R	20	31	21	R	R	R
4	25	32	NA	25	R	R	R	R	22	R	R	34	19	R	R	R
5	24	35	22	28	R	R	23	20	18	R	R	30	18	30	R	R
6	25	32	R	25	R	R	20	R	18	R	R	28	R	21	R	R
7	30	35	R	26	R	R	R	R	20	R	R	R	22	R	R	R
8	26	40	23	35	R	R	NA	21	23	R	18	NA	20	33	R	R
9	24	24	R	R	R	R	NA	R	19	R	R	NA	18	R	R	R
10	27	32	R	29	R	R	22	22	20	R	R	NA	20	29	R	R
11	28	NA	22	32	R	R	R	28	22	R	R	NA	21	31	R	R
12	25	NA	23	28	R	R	R	R	20	R	R	NA	18	28	R	R
13	24	NA	R	26	R	R	R	R	18	R	R	NA	18	R	R	R
14	25	NA	R	25	R	R	23	R	18	R	R	NA	18	27	R	R
15	28	NA	24	32	R	R	23	23	23	R	R	NA	20	NA	R	R
16	27	NA	NA	21	R	R	R	R	20	R	R	NA	20	NA	R	R
17	26	NA	R	R	R	R	R	24	R	R	R	NA	22	NA	R	R
18	24	NA	R	28	R	24	24	27	R	R	R	NA	R	NA	R	R
19	27	NA	22	20	R	R	R	25	21	R	R	NA	21	NA	R	R
20	29	NA	NA	19	R	R	21	28	21	R	R	NA	18	NA	14	R
21	24	NA	R	28	R	R	24	R	20	R	R	NA	19	NA	R	R
22	29	NA	R	R	R	R	23	22	20	R	R	NA	20	NA	R	R

Note: G- gentamicin, Cf- ciprofloxacin, Cp- cephalexin, Nx- norfloxacin, E- erythromycin, Nv- novobiocin, O- oxytetracycline, Co- co- trimaxazole, K- kanamycin, Fr- furazolidone, A- ampicillin, C- chloramphenicol, N- neomycin, Na- nalidixic acid, B- bacitracin and P- penicillin.

Numerical in bold indicates the sensitivity.

NA - not done; R- resistant; I.U. - international units.

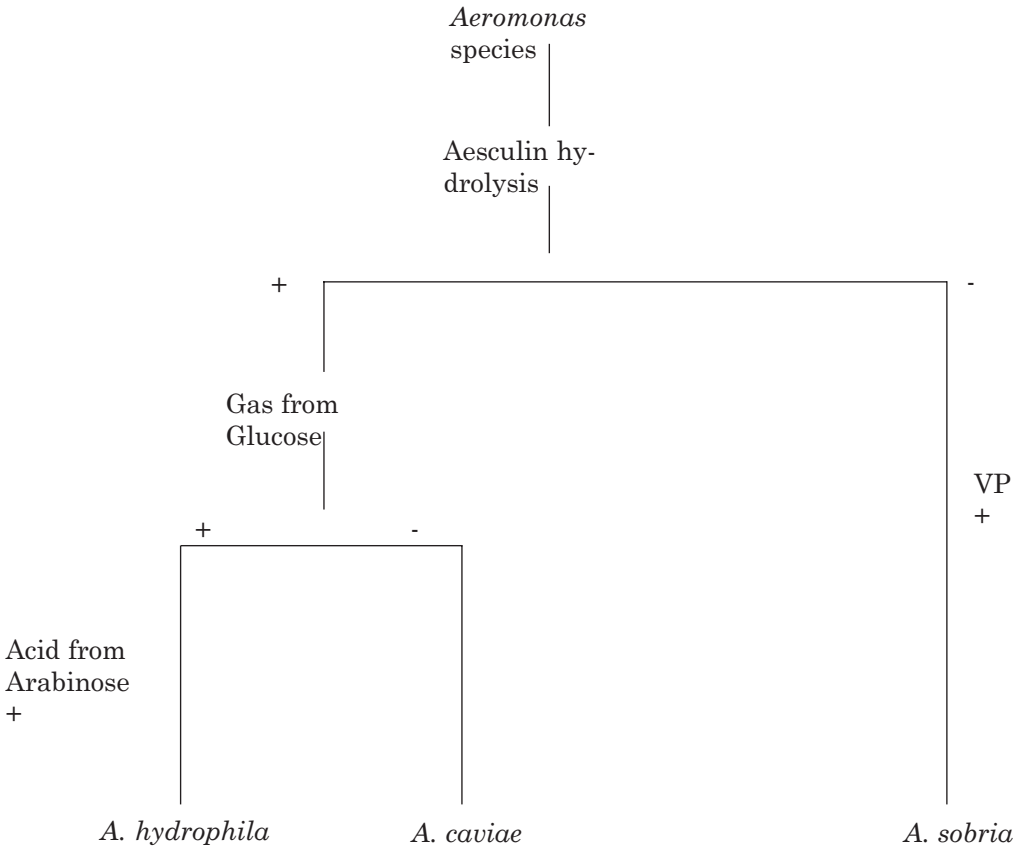

Fig. 1. Aero key (Modified) for identification of *Aeromonas* up to species level (Agarwal *et al.,* 1998)

septicaemia in fresh water fish, has also been reported in association with various ulcerative conditions including abdominal dropsy and ulcerative disease (Shome *et al.,* 1999).

Antibiotic sensitivity of Aeromonas isolates

The antibiotic sensitivity of all 22 isolates of *Aeromonas* was determined by disc diffusion technique of Bauer *et al.* (1966) using 16 antimicrobial agents (Table 4).

The antibiotic sensitivity pattern (number of sensitivity isolates/ number of tested isolates) of 22 isolates was as follows; gentamicin (22/22), ciprofloxacin (10/10), kanamycin (20/20), neomycin (20/22), norfloxacin (19/22), chloramphenicol (6/7), nalidixic acid (9/14), oxytetracyclin (12/20), co-trimaxazole (12/22), cephalexin (6/19), ampicillin (3/22), bacitracin (1/22), and novobiocin (1/22), whereas, all the isolates were resistant to erythromycin, furazolidone and penicillin. However, in the present study, three isolates were sensitive to ampicillin. The growth of these sensitive isolates on selective media could be due to the lower concentration of ampicillin in the media (10µg/ml) than the concentration used for antibiotic

sensitivity test (25µg/disc). Joseph and Carnahan (1994) reviewed that some species of *Aeromonas* sp. (*A. trota* and *A. caviae*) were ampicillin susceptible. Because of the widespread resistance of *Aeromonas* to ampicillin, it was most commonly used as selective agent for the isolation of this organism (Carlson *et al.*, 1983). Use of ampicillin (10mg/1) in modified RSA and SAA revealed that it could be used as selective agent for isolation of motile aeromonads.

The antibiotic sensitivity results showed that gentamicin, ciprofloxacin, chloramphenicol, neomycin and norfloxacin inhibited the growth of most of the isolates. Doukas *et al.* (1998) observed that oxytetracyline and flumequin were the effective antibiotics against *A. hydrophila* isolated from sea bass and *Puntazzo*. However, in the present study, only 60% of isolates were sensitive to oxytetracycline. In accordance with our study, Sahoo and Mukherjee (1997) also reported that *A. hydrophila* were sensitive to ciprofloxacin and norfloxacin.

Prevalence of motile aeromonads in aquatic environment is quite widespread. They have been isolated from brackish, fresh, estuarine, marine, chlorinated and non-chlorinated water. There are number of diseases associated with motile aeromonads. Therefore it is important to identify the motile aeromonads in order to determine the true etiology of the fish disease outbreaks. The present study will help in easy and selective isolation of motile aeromonads, their biochemical characterization for its classification and antibiotic sensitivity.

Acknowledgement

The authors are grateful to Dr. Neeraj Sood, Scientist, NBFGR, Lucknow for critical evaluation of the manuscript and valuable suggestions.

References

Agarwal, R.K. 1997. *Characterization of virulence factors of aeromonads isolated from foods of animal origin*. Ph.D. Thesis, IVRI, Izatnagar, India.

Agarwal, R.K., K.N. Kapoor and V.N. Bachhil 1998. Evaluation of selective media for the isolation of aeromonads from foods. *Indian J. Vet. Res.,* **1**: 51-53.

Austin B and D.A. Austin 1993. Aeromonadaceae representatives (excluding *Aeromonas salmonicida*). In: *Bacterial Fish Pathogens. Diseases in Farmed and Wild Fish,* L.M. Liard (Ed.), p 171-187. Chichester: Ellis Horwood.

Barrow, G.I. and R.K.A. Feltham 1992. *Cowan and Steel's Manual for the identification of medical bacteria.* 3rd edition. Cambridge Univ. Press, Cambridge.

Bauer, A. W., W.M.M Kirby, J.C. Sherris and M. Turck 1966. Antibiotic susceptibility testing by a standardized single disk method. *Amer. J. Clin. Pathol.,* **45**: 493-496.

Carlson, J.R., S.A Thornton, H.L Dupont, A.H. West and J.J. Methewson 1983. Comparative *in vitro* activities of ten antimicrobial agents against bacterial enteropathogens. *Antimicrob. Agents Chemotherap.,* **24**: 509-513.

Doukas, V., F. Athnassopoulou, E. Karagouni and E. Dotsika 1998. *Aeromonas hydrophila* infection in cultured sea bass, *Dicentrarchus labrax* (L.) and *Puntazzo puntazzo* (Cuvier) from the Aegean Sea. *J. Fish Dis.,* **21**: 317-320.

Frerichs G.N. 1989. Bacterial diseases of marine fish. *The Vet. Rec.,* **125**: 315-318.

Joseph, S.W. and A. Carnahan 1994. The isolation, identification and systematics of motile *Aeromonas* species. *Ann. Rev. Fish Dis.,* **4**: 315-343.

Kersters, I., L. van Vooren, G. Huys, P. Janssen, K. Kersters and W. Verstraete

1995. Influence of temperature and process technology on the occurrance of *Aeromonas* species and hygienic indicator organisms in drinking water production plants. *Microbiol. Ecol.,* **30**: 203-218.

Krovacek, K., K. Huang, S. Sternberg and S.B. Svenson 1997. *Aeromonas hydrophila* septicaemia in a grey seal (*Halichoerus grypus*) from the Baltic Sea: a case study. *Comp. Immunol. Microbiol. Infect. Dis.,* **21**: 43-49.

Lee, J.V. and T.J. Donovan 1985. *Vibrio, Aeromonas* and *Plesiomonas*. In: *Isolation and identification of microorganisms of medical and veterinary importance.* C.H. Collins and J.M. George (Ed.), Academic Press, London, p. 13-33.

Miller, R. M. and W. R. Chapman 1976. *Epistyles* sp. and *Aeromonas hydrophila* infections in fishes from North California reservoirs. *Prog. Fish. Cult.,* **38**: 165-168.

Palumbo, S.A., F. Maxino, A.C. Williams, R.L. Buchanan and D.W. Thayer 1985. Starch Ampicillin Agar for qualitative detection of *Aeromonas hydrophila. Appl. Environ. Microbiol.,* **50**: 1027-1030.

Sahoo, P. K. and S. C. Mukherjee 1997. *In-vitro* susceptibility of three bacterial pathogens of catfish to 23 antimicrobial agents. *Indian J. Fish.,* **44**: 393-397.

Shome, R., B. R. Shome and N. Ram 1999. Study of virulence factors of *Aeromonas hydrophila* isolates causing acute abdominal dropsy and ulcerative diseases in Indian major carps. *Indian J. Fish.,* **46**: 133-140.

Wadstrom, T. and A. Ljungh 1991. *Aeromonas* and *Plesiomonas* as food and waterborne pathogens. *Int. J. Food Microbiol.,* **12**: 303-312.

Date of Receipt : 24-08-2002
Date of Acceptance : 06-06-2005