http://www.pjbs.org

PJBS

ISSN 1028-8880

Pakistan Journal of Biological Sciences

ANSInet
Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

Pakistan Journal of Biological Sciences 16 (21): 1303-1310, 2013
ISSN 1028-8880 / DOI: 10.3923/pjbs.2013.1303.1310

A Study on Genetic Variability of Pathogenic *Aeromonas hydrophila* Strains and the Varied Responses of the Strains Towards Phyto-extracts

A. Balasundaram, P. Rathna Kumari, P. Kolanchinathan, V. Masilamani and George John
P.G. and Research Department of Zoology, Periyar E.V.R. College, Tiruchirappalli-620023, India

Abstract: The present study evaluated genetic variation in *Aeromonas hydrophila* strains using PCR-RAPD and their varied susceptibility to phyto-extract. Four strains of *Aeromonas hydrophila* isolated from skin infections of common freshwater fish, *Cyprinus carpio* were characterized by various biochemical methods, physiological tests and PCR- RAPD. Antimicrobial activity of the leaf extracts of three medicinal plants, *Ocimum sanctum*, *Adathoda vasica* and *Calendula officinalis* were tested against the four strains of *A. hydrophila* by disc diffusion (Kirby-Bauer) method. Antagonistic effects of leaf extracts against *A. hydrophila* strains were assessed by co-culture method. RAPD analysis showed that all the microbes isolated from skin infection belong to the same species but there was no 100% genetic similarity among them Dendrogram constructed by UPGMA clearly supported the PCR pattern of genetic variability among the strains. This study revealed that *Aeromonas hydophila* exhibits genetic variability and varied susceptibility towards phyto-extracts. Results indicated that phyto-extracts offers a promising alternative to the use of antibiotics in controlling *Aeromonas hydrophila.*

Key words: *Aeromonas hydrophila*, *Ocimum sanctum*, *Adathoda vasica*, *Calendula officinalis*, RAPD analysis, dendrogram

INTRODUCTION

Aeromonas hydrophila and *Pseudomonas* are common in freshwater and can survive and multiply in that environment provided there is enough organic matter and suitable growth temperature. Most bacteria in this group are not usually capable of multiplying or causing disease below 10-12°C, although some strains are pathogenic at temperatures as low as 5°C. They are capable of producing a variety of diseases, which predominate during summer months when water temperatures and organic loadings are high. Skin infections may appear resulting in bright red blotches around the vent, back and sides. Haemorrhages may appear in the internal organs and in advanced stages, the kidney appears liquefied (Hazen *et al*., 1978; Kaper *et al*., 1980; Van der Kooij, 1988). *Aeromonas* infections are probably the most common bacterial disease diagnosed in cultured warmwater fish. Usually, mortality rates are low (10% or less) and losses may occur over a period of time (2-3 weeks or longer). In these instances, some factor; usually stress, has caused the fish to become more susceptible to the bacteria. Common sources of stress are poor water quality, overcrowding, or rough handling. (Mathewson and Dupont, 1992; Larsen and Jensen, 1977). It was considered as a opportunistic pathogen in the past, but recent surveys have emphasized its emergence an primary pathogen, particularly in compromised hosts or in wound infections (Davis *et al*., 1978; Fraire, 1978, Salyers and Whitt, 1994).

Several diseased conditions such as tail rot, fin rot and haemorrhagic septicemia has been associated with *A. hydrophila* infection (Miyazaki and Kaige, 1985). Apart from infecting fish, it also causes food borne diseases in humans (Palumbo *et al*., 1989). Because of its high adaptability in different environments, it would seem that genetic and phenotypic diversity of *A. hydrophila* is a natural phenomenon.

As use of antibiotic to control microbial pathogens such as *Aeromonas* leads to multidrug resistance, antibiotic residues in environment, transmission of antibiotic in the food chain leads to several problems. So it is inevitable to probe for alternative methods of controlling pathogens (Rahim *et al*., 1984; Bonjar and Nik, 2004). One such alternative is the use of herbal medicinal plant extracts as feed supplement which not only enhance immunity but also increase the size of fishes. Hence, in this study antimicrobial activity of three selected

Corresponding Author: A. Balasundaram, P.G. and Research Department of Zoology, Periyar E.V.R. College, Tiruchirappalli-620023, India

medicinal plants *Ocimum sanctum*, *Adathoda vasica* and *Calendula officinalis* were used against *Aeromonas hydrophila*. The present study was also intended to establish a correlation between the antibiotic susceptibility patterns and genetic diversity of *Aeromonas hydrophila* strains using DNA-PCR (RAPD) analysis.

MATERIALS AND METHODS

Isolation and identification of *A. hydrophila*: Diseased common freshwater fish *Cyprinus carpio* were covered in plastic wrap and transported from the fish farm in an ice chamber to the laboratory. Four strains *A. hydrophila* were isolated from swab specimens of superficial skin ulcers. *A. hydrophila* was cultured on tryptone soya agar (Himedia) and harvested in tryptone soya broth (Himedia). The inoculated broth was incubated in shaker at 200 rpm for 12 h at 27°C and centrifuged at 10000 rpm for 20 min at 4°C. Supernatant was discarded and the bacterial pellet was used for further analysis.

Extract preparation: Leaves of *Adathoda vasica*, *Ocimum sanctum* and *Calendula officinalis* were collected from various areas. Collected leaves were dried in shade and ground into fine powder and stored in containers. The 125 g of powdered leaf was successfully extracted with cold butanol, using Soxhlet apparatus. Fractions were completely dried by evaporation at room temperature and were stored in sterile container.

Antibacterial assay: Petri plates containing 20 mL of tryptone soya agar medium were seeded with a 24 h old culture of the bacterial strain. The leaf extracts and fractions were dissolved in Dimethyl sulphoxide (DMSO) and filtered by using sartorius syringe filter (pore size of 0.22 mm). 40, 50 and 75 μL, of leaf extracts were impregnated into the sterile 6 mm diameter discs. Discs were dried in room temperature and dispensed on the solidified tryptone soya agar inoculated with test microorganisms. Incubation was made at 37°C for 24 h. Assessment of antibacterial activity was based on matching the diameter of the inhibition zone formed around the discs with interpretive criteria on Kirby-Bauer chart (NCCLS, 2002).

Co-culture of *A. hydrophila* strains with plant extract: The 0.25 and 0.50 mL of the plant extracts *Adathoda vasica* and *Ocimum sanctum* were added to the broth culture flasks of *A. hydrophila* and the growth of bacteria was assessed by checking the optical density, daily, for a period of seven days.

Biochemical tests: The bacterial strains used in this study were identified using standard morphological, physiological and biochemical tests (Holt *et al*., 1994).

Antibiotic susceptibility: Susceptibility of *A. hydrophila* to different standard antibiotics was tested by agar diffusion method using discs purchased from Himedia.

Extraction of genomic DNA for PCR-RAPD analysis: Bacterial samples were well ground and mixed with cTAb extraction buffer. This homogenate was incubated with 150 μg mL^{-1} of proteinase K at 50°C for 4-12 h. It was then extracted with equal volume of phenol:chloroform (1: 1). It was then centrifuged at 10,000 rpm at room temperature for 5 min. The upper aqueous phase was collected. To this equal volume of chloroform:isoamyl alcohol (24: 1) was added and mixed by gentle shaking. Contents were centrifuged for 5 min at room temperature. The upper aqueous phase was collected. DNA was precipitated with cold absolute ethanol. The contents were centrifuged at 5000 rpm for few minutes and the pellet was then dissolved in 400 μL of 1 N NaCl . To this 2 μL of RNAase was added and incubated at 37°C for 30 min. To this 1 mL of cold absolute ethanol was added and kept at -20°C for 30 min. The sample was centrifuged at 10,000 rpm for 10 min at 4°C. The supernatant was discarded, the pellet was washed with 70% ethanol and the centrifugation was repeated. The pellet was collected; the ethanol content was evaporated and dissolved in 1X TE buffer.

Amplification of DNA using random primer: PCR amplification was performed as described by Muyzer *et al*. (1993). Twenty RAPD primers (Kit A1-A20) were obtained from IDT (New Delhi) and were tested with DNA samples of *Aeromonas*.

Data analysis: The data analysis was performed using the Diversity database software (Bio-Rad) and similarities among isolates were estimated by means of dice co-efficient. The program calculated all the Pearson Correlation Coefficients between pairs of variables, transformed these coefficients into distances and made a clustering using Unweighted Pair Group Method with Arithmetic Mean (UPGMA). Dendrograms were produced based on the UPGMA clustering (Garcia-Vallve *et al*., 1999).

RESULTS

Biochemical and physiological characterization: Four strains of *A. hydrophila* were isolated and characterized using standard biochemical tests. All the four strains were

gram negative, oxidase positive and fermenting glucose. But there was slight variation in the degree of fermentation. All the four strains could utilize sucrose and arabinose. *Aeromonas* strain 1-3 were able to utilize sorbitol, strain 2 and 3 could utilize xylase and only strain III was able to utilize lactose (Table 1).

Antibiotic sensitivity test: The antibiotic concentrations per disc were (in micrograms) as follows: Amphicillin 30; Amikacin 30; Cefuroxime 30; Chloramphenical 30; Ciprofloxacin 5; Ceffriaxone ; Cotrimaxazole ; Gentamycin 10; Levofloxacin 5; Imipenem 10; Nalidixic acid 30; Norfloxacin 10; Novobiocin 30; Ofloxacin 5; tetracycline 30; Trimethoprim 5. All the four strains showed varied susceptibility to standard antibiotics. Among 16 antibiotics tested Aeromonas-I was susceptible to 7 antibiotics and resistant to eight antibiotics. Strain 2 was highly multidrug resistant and showed resistance against 10 antibiotics. Strain 3 was susceptible to 11antibiotics and strain 4 showed resistance against 8 antibiotics. Thus except strain 3 other strains were highly multidrug resistant strains. All the four strains were susceptible to chloramphenical and Ceffriaxone. Resistance was observed against Novobiocin and Nalidixic acid in all the four strains (Table 2).

Disc diffusion method: Antibiotic sensitivity pattern assessed for all bacterial isolates revealed that except for Novobiocin and Nalidixic acid the bacterial isolates were sensitive to all other antibiotics. Their zones of inhibition were compared with standard zones as per Kirby Bauer Chart and the degree of inhibitions by the two antibiotics could serve as a benchmark for the antimicrobial activity of plant extracts.

Antimicrobial activity of butanolic extracts (40, 50 and 75 μg $disc^{-1}$) of *Adathoda vasica* against *Aeromonas* strains is presented in Fig. 2. There was not much variation between *A. hydrophila*-II and Aeromonas IV in the inhibitory activity. Maximum inhibitory activity was observed against Aeromonas-IV at 75 μL concentration. Minimum inhibitory zone were observed against *A. hydrophila*-I in all the three concentrations.

Butanolic extracts (40, 50 and 75 μg $disc^{-1}$) of *Ocimum sanctum* showed maximum activity against *A. hydrophila*-II and III with a zone of 18 and 17 mm dm^{-1}. Inhibitory zone observed against *A. hydrophila*-I and *Aeromonas*-IV were similar in all the three concentrations (Fig. 3).

Inhibitory zones could be observed against the microbes using butanolic extracts of *Calendula officinalis*. It showed significant inhibitory effect on all the four strains of Aeromonas, in which the maximum zone of inhibition was observed against *Aeromonas hydrophila*-II. (21 mm at 75 μL concentrations). Minimum inhibitory zone was observed against *Aeromonas*-IV (10 mm at 40 μL). Similar levels of inhibitory zones were observed for *Aeromonas* I and III (Fig. 4).

Butanolic leaf extract of all the three plants *Ocimum sanctum*, *Adathoda vasica* and *Calendula officinalis* thus showed antimicrobial activity against the strains of *Aeromonas*.

Co-culture technique: Inhibitory effect of plant extracts on *Aeromonas* strains in broth culture both the

Table 1: Identification of *Aeromonas hydrophilla* by biochemical characterization

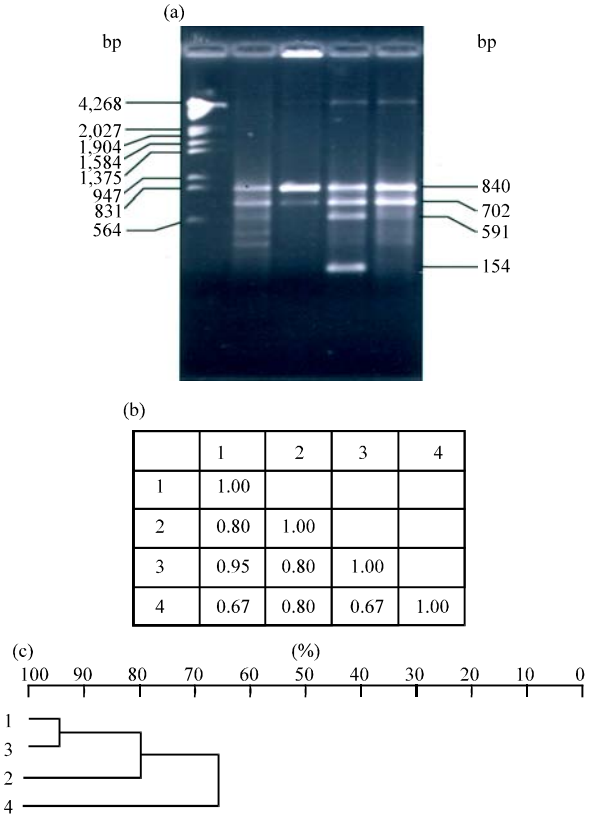
Tests	*Aeromonas hydrophila*
Mannitol	+
Indole	+
Methyl red	+
VP	+/-
Citrate	+/-
Urease	-
Lysine	-
Arginine	+
Ornithine	+
Phenyl alanine	-
Gas from glucose	A(G)
TSI	A/A
Motility	+/-
Gelatine	+
Lactose	+/-
Sucrose	+
Dulcitol	-
Salicine	+
Adonitol	-
Inositol	-
Sorbitol	V
Arabinose	+/-
Xylose	+
Malonate	-
Oxidase	+
Oxidase/Ferme	++
H_2S production	-

+: Positive, -: Negative, V: Variable, A/A: Acid/Acid, A/Ak: Acid/Alkaline

Table 2: Antibiotic susceptibilities of *A.hydrophila* strains

	A.hydrophila strains			
Antibiotic	I	II	III	IV
Ampicillin-30 mcg-A	R	R	S	S
Amikacin-30 mcg-AK	MS	MS	S	S
Cefuroxime-30 mcg-CU	MS	MS	R	S
Chloramphenical-30 mcg-C	S	S	S	S
Ciprofloxacin-5 mcg-Cf	R	R	MS	R
Ceffriaxone-Ci	S	S	S	S
Cotrimaxazole-Co	S	R	S	R
Gentamycin-10 mcg-G	G	S	R	S
Imipenem-10 mcg-I	MS	R	S	S
Levofloxacin-5 mcg-Le	R	R	MS	R
Nalidixic acid-30 mcg-Na	R	R	R	R
Norfloxacin-10 mcg-Nx	R	MS	R	R
Novobiocin-30 mcg-Nv	R	R	R	R
Ofloxacin-5 mcg-Of	R	R	MS	S
Tetracycline-30 mcg-Tc	R	R	MS	R
Trimethoprim-5 mcg-Tr	S	R	S	R

R: Resistance, S: Susceptibility, MS: Mild susceptibility



	1	2	3	4
1	1.00			
2	0.80	1.00		
3	0.95	0.80	1.00	
4	0.67	0.80	0.67	1.00

Fig. 1: PCR pattern of genetic variability

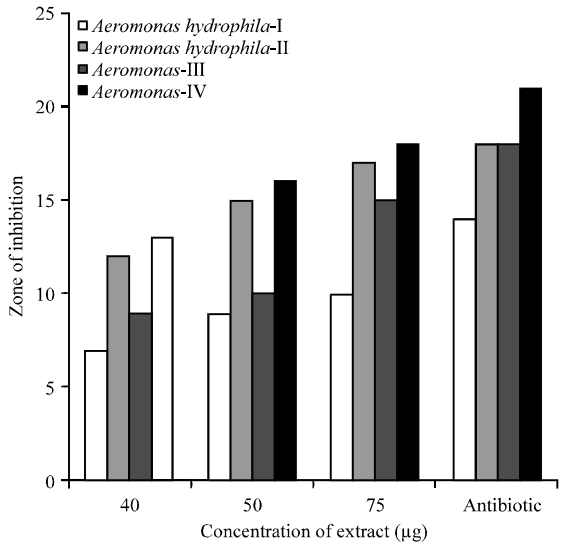


Fig. 2: Antimicrobial activity of butanolic extract of *Adathoda vasica*

concentrations (0.25 and 0.50/200 mL broth) of *Adathoda vasica* showed inhibitory activity against *Aeromonas*

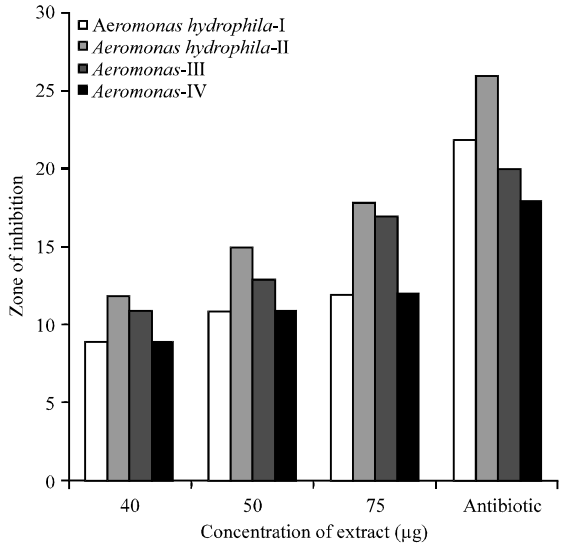


Fig. 3: Antimicrobial activity of butanolic extract of *Ocimum sanctum*

hydrophila-I at the later stage of culturing i.e., beyond the 5th day. 0.50 mL *A. vasica* group showed higher inhibition when compared with 0.25 mL concentration of *A. vasica* (Fig. 5).

Ocimum sanctum also showed, similar level of inhibitory action in both 0.25 and 0.50 mL concentrations. 0.50 mL concentration of *Ocimum sanctum* inhibited the growth of *A. hydrophila* in 5 days of culture.

These results indicated that two plant extracts inhibited the growth of *A. hydrophila*-I in similar manner. Extracts of *Adathoda vasica* and *Ocimum sanctum* had no apparent effect on *A. hydrophila*-II as evidenced by the progressive O.D values observed with the progression of the experiment. This indicates that *A. hydrophila*-II strain could not be inhibited by the two plants extracts (Fig. 6).

PCR-RAPD studies: The RAPD procedure was used in the present study because of its simplicity and speed in identifying the genetic polymorphisms within the species level. DNA samples of *Aeromonas* were screened by using the arbitrary 10-mer primers.

From the 20 random primers tested the primer A 03 gave reproducible, consistent and gave scorable fragments. Following is the sequence of the primer A 03 which gave results 5"AGT CAG CCAC 3'.

The pattern of RAPD profile for *Aeromonas* in this study revealed characteristics of genetic variability of each population. PCR pattern of genetic variability is shown in Fig. 1a.

Similarity indices and dendrogram were computed and presented in Fig. 1b and c. Amplified fragments

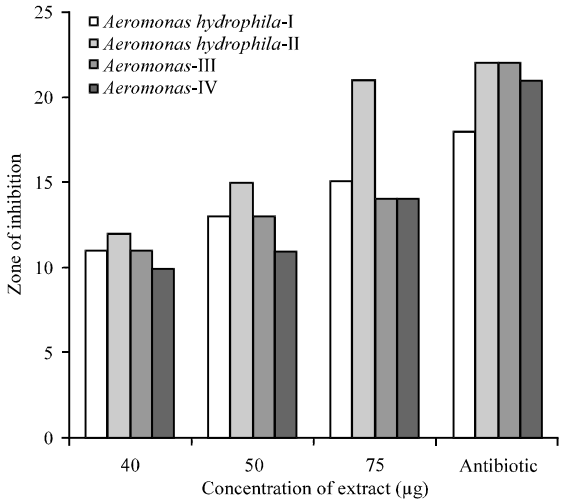


Fig. 4: Antimicrobial activity of butanolic extract of *Calendula officinalis*

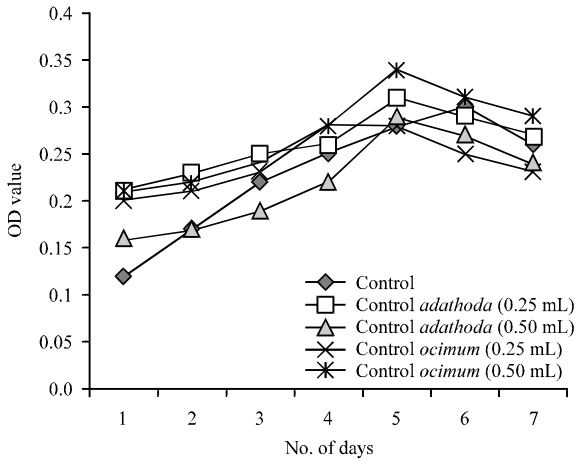


Fig. 5: Effect of plant extracts on *Aeromonas hydrophila*-I

ranged from 840 to 154 bp. Species specific fragments were identified at 702 bp (Fig. 1a). An interesting finding of this study was that 100% similarity did not exist among the four populations studied (Fig. 1b). The similarity indices and the dendrogram constructed by UPGMA, clearly supported PCR pattern of genetic variability in the populations. A maximum similarity index was exhibited among isolates 1 and 3 *Aeromonas* followed by isolates 1 and 2 and between 2 and 3. The UPGMA based dendrogram analysis grouped isolates 1 and 3. The first cluster was further sub grouped with isolate 2 and then with isolate 4. As *Aeromonas* species isolates were grouped under different clusters, in general one could assume that distinct genetic variations existed in these microbial populations.

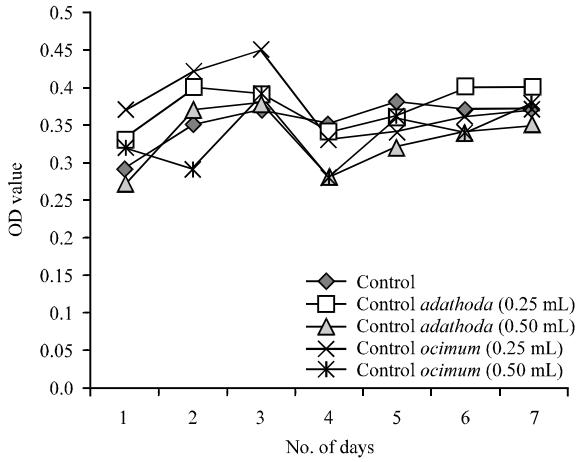


Fig. 6: Effect of plant extracts on *Aeromonas hydrophila*-II

DISCUSSION

Because of the side effects and the development of resistance by microbes against conventional antibiotics, much attention has been paid recently to medicinal plants, as a source of safe compounds to be used as drugs for human beings and also in aquaculture environments. Observation by Samy and Ignacimuthu (2000) on the folklore medicines of Western Ghats (South India) has highlighted the potential of plant based products in tackling many of the modern day diseases. Screening of 165 medicinal plants by Pereira *et al.* (2004) revealed that medicinal property in plants are not universal and hence specific compounds have to be tested against each bacterial strain, so that suitable plant source may be identified and scientific validation of those compounds may be carried out. While testing against *Pseudomonas* and *Aeromonads*, they could identify only 13 species of plants coming under 12 families which had the relevant antimicrobial potential.

In the present study, based on their traditional medicinal value, three plants *Adathoda vasica*, *Ocimum sanctum* and *Calendula officinalis* were selected and tested against *A. hydrophila* strains an opportunistic pathogen affecting cultivable fish species. Antimicrobial activity of plant extracts could be established effectively in the present study (Graph 1-3). Anti *Aeromonas* effect of *Begonia malabarica* leaf extract could be established by Ramesh *et al.* (2002). Disease resistance and immunostimulatory effect could be observed in *A. hydrophila* challenged *Oreochromis mosambicus* administered with leaf extract of *Solanum trilobatum* (Divyagnaneswari *et al.*, 2007). Resistance against *A. hydrophila* could also be observed in the leaf extracts

of many plants like *Eclipta alba*, *Achyranthes aspera*, *Zingiber officinalis* (Rao *et al*., 2006; Christybapita *et al*., 2007). Enhancement of disease resistance could be observed achieved by using the leaf extract of *Ocimum sanctum* against *A. hydrophila* in *Orechromis mosambicus* by Logambal *et al*. (2000). All the above works reveal the susceptibility of *A. hydrophila* towards medicinal plant extracts, as observed in the present study. Although bacterial infection can be controlled by antibiotic treatment, the use of antibiotics leads to environmental hazards and the development of antibiotic resistant genes in the bacteria. Several strains of microbes have no effective therapeutic measures at all (Itami *et al*., 1998). In this context search for nutraceuticals gain much importance in improving disease resistance in animals (Gerin, 1999). From the preceding reports, it can be inferred that all the works were pertaining to a single strain of the bacterium in contention and as one has taken into account their differences in function and resistance development based on their genetic variability, which should be at high frequency taking into considerably their low generation time and higher rate of mutations. Hence in the present study genetic variability was given due importance while studying their susceptibility to plant extracts as well as antibiotics.

Susceptibility of *A. hydrophila* to different antibiotics was estimated using disc diffusion method (Fig. 1-3). Among sixteen antibiotics used, susceptibility pattern of the four strains of *A. hydrophila*, varied from the observation by earlier workers like McNicol *et al*. (1980), Fass and Barnishan (1981) and Fainstein *et al*. (1982). The four strains of *A. hydrophila* exhibited susceptibility to chloramphenicol; three strains exhibited resistance to tetracycline and two strains exhibited resistance towards trimethoprim, which was in accordance with the result observed with McNicol *et al*. (1980). In contrast to the result obtained by Rahim *et al*. (1984), these four strains of *A. hydrophila* showed complete resistance for novobiocin and nalidixic acid. It was observed by many workers that almost all strains of *A. hydrophila* are resistant to ampicillin, hence it was recommended for the selective medium in 30 mcg mL^{-1} concentration for isolation of *A. hydrophila* (Rogol *et al*., 1979). Recovery of multidrug resistant strains in cultured fish and water was reported by Hayashi *et al*. (1982) and McNicol *et al*. (1980). Three strains of multidrug resistant *Pseudomonas* spp. were isolated from *Channas gachua*, which exhibited resistance against eleven standard antibiotics and sensitivity towards chloramphenical and gentamycin susceptibility of *A. hydrophila* to three plant extract was also strain dependent (Fig. 1-3).

Genetic variations between bacterial species and genomic polymorphism between bacterial isolates can be identified by the variations in sizes and number of fragments. Randomly amplified polymorphic DNA (RAPD) is the simplest and easily reproducible DNA fingerprinting method (Williams *et al*., 1990). RAPD and PCR are established methods for generating DNA fingerprints and find discrimination among strains of *A. hydrophila* (Szczuka and Kaznowski, 2004, 2007; Aguilera-Arreola *et al*., 2005). Genetic heterogeneity among the strains of *A. salmonicida* isolated from different species of fish was reported by Garcia *et al*. (2000). The scattered RAPD profiles observed in the present study resembles to an extent the genetic variations observed in *A. hydrophila* isolated from Rainbow trout by Miyata *et al*. (1995) and Lee *et al*. (2000). The results of the present study confirm the fact, while testing antimicrobial compounds, due importance should be given to the variety of strains available further studies of their genomic identity.

CONCLUSION

From the present study it was concluded that all the four strains of Aeromonas was susceptible to butanolic extracts of three medicinal plants namely *Calendula officinalis*, *Adathoda vasica* and *Ocimum* sanctum; eventhough they show genetic variation and varied responses towards standard antibiotics.

ACKNOWLEDGMENT

Authors are grateful to Ministry of Earth Sciences, Ocean Atmospheric Science Technology Cell (MOES-OASTC), UGC, Government of India for providing financial support.

REFERENCES

Aguilera-Arreola, M.G., C. Hernandez-Rodriguez, G. Zuniga, M.J. Figueras and G.C. Escarpulli, 2005. *Aeromonas hydrophila* clinical and environmental ecotypes as revealed by genetic diversity and virulence genes. FEMS Microbiol. Lett., 242: 231-240.

Bonjar, G.H.S. and A.K. Nik, 2004. Antibacterial activity of some medicinal plants of Iran against *Pseudomonas aeruginosa* and *P. fluorescens*. Asian J. Plant Sci., 3: 61-64.

Christybapita, D., M. Divyagnaneswari and R.D. Michael, 2007. Oral administration of *Eclipta alba* leaf aqueous extract enhances the non specific immune responses and disease resistance of *Oreochromis mossambicus*. Fish Shellfish Immunol., 23: 840-852.

Davis, W.A., J.G. Kane and V.F. Garagusi, 1978. Human *Aeromonas* infections: A review of the literature and a case report of endocarditis. Med. (Baltimore), 57: 267-277.

Divyagnaneswari, M., D. Christybapita and R.D. Michael, 2007. Enhancement of nonspecific immunity and disease resistance in oreochromis mossambicus by *Solanum trilobatum* leaf fractions. Fish Shellfish Immunol., 23: 249-259.

Fainstein, V., S. Weaver and G.P. Bodey, 1982. *In vitro* susceptibility of *Aeromonas hydrophila* against new antibiotics. Antimicrob. Agents. Chemother., 22: 513-514.

Fass, R.J. and J. Barnishan, 1981. *In vitro* susceptibility of *Aeromonas hydrophila* to 32 antimicrobial agents. Antimicrob. Agents Chemother., 19: 357-361.

Fraire, A.E., 1978. *Aeromonas hydrophila* infection. JAMA, 239: 192-192.

Garcia, J.A., J.L. Larsen, I. Dalsgaard and K. Pedersen, 2000. Pulsed-field gel electrophoresis analysis of *Aeromonas salmonicida* ssp. *Salmonicida*. FEMS Microbiol. Lett., 190: 163-166.

Garcia-Vallve, S., J. Paulu and A. Romeu, 1999. Horizontal gene transfer in glycosyl hydrolases inferred from codon usage in Escherichia coli and *Bacillus subtilis*. Mol. Biol. Evol., 16: 1125-1134.

Gerin, M., 1999. Feed additives and biotechnologies in aquafeeds: Moving towards sustainable development. Int. Aqua Feed Directory, 1999: 32-40.

Hayashi, F., Y. Araki, K. Harada, M. Inoue and S. Mitshuhashi, 1982. Epidemiological studies of drug resistant strains in cultured fish and water. Bull. Jap. Soc. Sci. Fish., 48: 1121-1127.

Hazen, T.C., C.B. Fliermans, R.P. Hirsch and G.W. Esch, 1978. Prevalence and distribution of *Aeromonas hydrophila* in United States. Applied Environ. Microbiol., 36: 731-738.

Holt, J.G., N.R. Kreig, P.H.A. Sneath and J.T. Staley, 1994. Facultatively Anaerobic Gram Negative Rods. In: Bergey's Manual of Determinative Bacteriology, Baltimore, M.D. (Ed.). 9th Edn., Wilkins and Wilkins, USA., pp: 255-273.

Itami, T., M. Asano, K. Tokushige, K. Kubano and A. Nakawaga *et al*., 1998. Enhancement of disease resistance of kuruma shrimp, *Penaeus japonicus*, after oral administration of peptidoglycan derived from *Bifidobacterium thermophilum*. Aquaculture, 164: 277-288.

Kaper, J.B., H. Lockman, R.R. Colwell and S.W. Joseph, 1980. *Aeromonas hydrophila*: Ecology and toxigenicity of isolates form an estuary. J. Applied Microbiol., 50: 359-377.

Larsen, J.L. and N.J. Jensen, 1977. An *Aeromonas* species implicated in ulcer-disease of the cod (*Gadus morhua*). Nord. Vet. Med., 29: 199-211.

Lee, S., S. Kim, Y. Oh and Y. Lee, 2000. Characterization of *Aeromonas hydrophila* isolated from rainbow trouts in Korea. J. Microbiol., 38: 1-7.

Logambal, S.M., S. Venkatalakshmi and R.D. Michael, 2000. Immunostimulatory effect of leaf extract of *Ocimum sanctum* Linn. *Oreochromis mossambicus* (Peters). Hydrobiologia, 430: 113-120.

Mathewson, J.J. and H.L. Dupont, 1992. *Aeromonas* Species: Role as Human Pathogens. In: Current Clinical Topics in Infectious Diseases, Remington, J.S. and M.N. Swartz (Eds.). Vol. 12e. Blackwell Scientific, Cambridge, pp: 26-36.

McNicol, L.A., K.M. Aziz, I. Huq, J.B. Kaper and H.A. Lockman *et al*., 1980. Isolation of drug-resistant *Aeromonas hydrophila* from aquatic environments. Antimicrob. Agents Chemother., 17: 477-483.

Miyata, M., T. Aoki, V. Inglis, T. Yoshida and M. Endo, 1995. RAPD analysis of *Aeromonas salmonicida* and *Aeromonas hydrophila*. J. Applied Bacteriol., 79: 181-185.

Miyazaki, T. and N. Kaige, 1985. A histopathological study on motile aeromonad disease in *Crucian carp*. Fish Pathol., 21: 181-185.

Muyzer, G., E.C. De Waal and A.G. Uitterlinden, 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Applied Environ. Microbiol., 59: 695-700.

NCCLS, 2002. Performance standards for antimicrobial susceptibility testing. 12th International Supplement, M100-S12.

Palumbo, S.A., M.M. Bencivengo, F. Del Corral, A.C. Williams and R.L. Buchanan, 1989. Characterization of the *Aeromonas hydrophila* group isolated from retail foods of animal origin. J. Clin. Microbiol., 27: 854-859.

Pereira, R.S., T.C. Sumita, M.R. Furlan, A.O. Jorge and M. Ueno, 2004. Antibacterial activity of essential oils on microorganisms isolated from urinary tract infection. Rev. Saude Publ., 38: 326-328.

Rahim, Z., S.C. Sanyal, K.M. Aziz, M.I. Huq and A.A. Chowdhury, 1984. Isolation of enterotoxigenic, hemolytic and antibiotic resistant *Aeromonas hydrophila* strains from infected fish in Bangladesh. Applied Environ. Microbiol., 48: 865-867.

Ramesh, N., M.B. Viswanathan, A. Sarasawathy, K. Balakrishna, P. Brindha and P. Lakshmanaperumalsamy, 2002. Phytochemical and antimicrobial studies of *Begonia malabarica*. J. Ethnopharmocol., 79: 129-132.

Rao, Y.V., B.K. Das, J. Pradhan and R. Chakrabarti, 2006. Effect of *Achyranthes aspera* on the immunity and survival of *Labeo rohita* infected with *Aeromonas hydrophila*. Fish Shellfish Immunol., 20: 263-273.

Rogol, M., I. Sechter, L. Grinber and C.B. Gerichter, 1979. Pril-xyzole ampicillin agar, a new selective medium for the isolation of *Aeromonas hydrophila*. J. Med. Microbiol., 12: 229-231.

Salyers, A.A. and D.D. Whitt, 1994. Bacterial Pathogenesis: A Molecular Approach. ASM Press, Washington, D.C., USA.

Samy, R.P. and S. Ignacimuthu, 2000. Antibacterial activity of some folklore medicinal plants used by tribals in Western Ghats of India. J. Ethnopharmacol., 69: 63-71.

Szczuka, E. and A. Kaznowski, 2007. Characterization of Aeromonas caviae and A. veronii by standardized cellular protein electrophoretic patterns. Folia Microbiol., 52: 65-69.

Szczuka, E. and A.Kaznowski, 2004. Typing of clinical and environmental *Aeromonas* sp. strains by random amplified polymorphic DNA PCR, repetitive extragenic palindromic PCR and enterobacterial repetitive intergenic consensus sequence PCR. J. Clin. Microbiol., 42: 220-228.

Van der Kooij, D., 1988. Properties of aeromonads and their occurrence and hygenic significance in drinking water. Zentralbl. Bakteriol. Mikrobiol. Hyg. B, 187: 1-17.

Williams, J.G., A.R. Kubelik, K.J. Livak, J.A. Rafalski and S.V. Tingey, 1990. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucl. Acids Res., 18: 6531-6535.