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Phenotypic and Genotypic Discrimination of Multifactorial Virulent *Aeromonas hydrophila* in Clinical and Environmental Samples

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Abstract: The prevalence of *Aeromonas hydrophila* from various samples (clinical and environmental) were analysed and confirming the percentage incidence of the organism. 16S rRNA–PCR analysis assists the identification of species of *Aeromonas* and also posing their epidemiology. All strains were showed the multi-drug resistance and MAR index showed that all the isolates may be originated from high risk sources. Virulent assays like haemolytic activity, serum susceptibility, and cytotoxicity confirming the frequency of virulence. Cytotoxic activity showed the effect of toxins on cancer cell lines (MCF-7). In the investigation, we determined different virulent genes like *hae*, *hly* A, *aer* A and *asc* U provides evidence for mutifactorial activities, which is encoded by the virulence factors like haemolysin, aerolysin and type three secretion systems which are potentially pathogenic. The determination of different virulent factor is a key component for the strong pathogenecity. RAPD-PCR could be highly reproducible, reliable and exactitude. On this basis, we came to a conclusion that presence of virulent *A. hydrophila* posed highly health risks towards human and other animals.

Keywords: *Aeromonas hydrophila*, MAR index, virulence genes, cytotoxicity, RAPD - PCR

1. Introduction

Aeromonads are known to be non-pathogenic for the past 100 of years. But nowadays, it is one of the major threats to the public health. Special attention was paid to *A. hydrophila* due to its association with a wide range of human illness. It also cause severe infections in humans such as bacteraemia, cellulitis, meningitis, endocarditis, peritonitis, endophthalmitis, corneal ulcer, septic arthritis, wound infections, osteomyelitis, suppurative arthritis, intra abnormal abscess, urinary tract infections, evolving into pneumonia, lung abscess, colicitis and soft tissue infections [1].

Species of Aeromonads are facultative anaerobic bacteria that occur ubiquitously in aquatic environments. *Aeromonas hydrophila* is receiving increasing attention because as its association with human disease and food born infections [2, 3, 4]. The pathogenesis of *Aeromonas* infections remain poorly understood, the mesophilic *A. hydrophila* can express a range of virulence factors [5], including attachment mechanism and production of a number of toxins such as haemolysin, cytotoxin, enterotoxin, protease, elastase, acetylcholine esterase, lipases, DNases, adhesins, aerolysin and haemagglutinins. The detection of the presence of such virulence factors is a better indicator of the potential health risk. The type three secretion systems (TTSS) is the major virulence mechanism that contributes to the pathogenesis and plays a crucial role in host pathogen interaction [6]. Hemolysin is another important exotoxin protein produced by bacteria and the lytic activities of hemolysins on red blood cells are reported to be important for nutrient acquisition or for causing certain conditions such as anemia.

This study may demonstrate the complexity of infection caused by the *A. hydrophila* and also it helps to understand the presence of functional TTSS and hemolytic gene in the *A. hydrophila* which is an important discovery for unlocking the pathogenesis of this bacterium. This will allow us to understand the intimate host-bacterium interactions in order to develop suitable strategy to disease caused by *A. hydrophila.* Majority of the studies showed the occurrences of the bacteria in different environment. But they were not studied in the molecular aspects of the same field. It reflects the lack of epidemiological status in foods versus human or animal sources. Thus it is used to study the molecular characterization of *A. hydrophila* in samples.

2. Materials and methods

2.1 Sample collection and bacterial strains

A series of 123 samples collected from various sources like clinical, water, fish and milk from Coimbatore and Thrissur. These samples were enriched with alkaline peptone water (APW) and were streaked on Starch Ampicillin Agar and incubated at 37 ° C were primarily confirms the presence of *A. hydrophila*. Kaper's multitest medium [7] presumptively identified the culture as *A. hydrophila*. For routine

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preservation, the isolated cultures were then purified by repeated streaking on nutrient agar slants and stored at 4 °C.

2.2 Phenotypic identification

A.hydrophila strain were subjected to phenotypic characterization such as Gram staining, motility, Kovac's oxidase and catalase test, oxidation and fermentation, indole production, methyl red test, citrate utilization test, Voges proskauer test, reduction of nitrate to nitrite, hydrogen sulphide production, sugar fermentation test, lysine decarboxylase and arginine decarboxylase [8]. Standard strain of *A. hydrophila* MTCC 646 was used parallel (Microbial Type Culture Collection Center, Chandigarh, India).

2.3 Genotypic identification

16S rRNA gene amplification was carried out for the genotypic identification of *A.hydrophila* using Aero16S F primer (5'-CAGAAGAAGCACCGGCTAAC-3') and Aero16S R primer (5'-TTACCTTGTTACGACTTCAC-3') (Promega, USA) set with the PCR conditions using PCR thermal cycler (Eppendorf, Germany). The expected amplicon size is 1050 bp.

2.4 Sequencing of 16S rRNA

Random dye termination sequencing method (ABI3130 Genetic Analyser, USA) used to sequence and identified the 16S rRNA PCR product. It was identified and compared by using the basic local alignment tool (BLAST).

2.5 Multiple antibiotic resistance

Multiple antibiotic resistance tests were carried out by the disk diffusion method [9] using 17 antibiotic disc along with the standard for antimicrobial disk susceptibility test CLSI vol. 27 No.1, Jan 2007(chart of Kirby Bauer sensitivity method modified in July 1966, Schering Corporation, U.S.A, and Bloomfield, New Jersey).

2.6 Haemolytic activity

Blood agar plate assay was performed for the determination of haemolytic activity of *A.hydrophila* and haemolytic activity was observed on the plates with a complete destruction of erythrocytes around the wells.

2.7 Serum susceptibility

Serum susceptibility of *A.hydrophila* was determined by using micro colorimetric assay. Group "O" blood was obtained by vein puncture from healthy individuals with no history of infection with *Aeromonas* and serum resistance was assayed by visible colour change from green (inhibition) to yellow (growth).

2.8 Cytotoxic activity

Cytotoxic activity of *A. hydrophila* was performed by MTT assay [10] and the effect of toxin was measured by a micro plate ELISA reader (Bio-Rad Lab systems, USA).The Breast cancer (MCF-7) cell line was used in this study.

2.9 Identification of virulent genes

Amplification of virulence genes *hly*, *aer* A, *hae* and *asc* U was performed with DNA thermal cyclers (Eppendorf, Germany) using specific primers.

Table 1: Primer details

Primers	Sequences	bp
hly A	F5'-GGCCGGTGGCCCGAAGATGCAGG-3' R5'-GGCGGCGCCGGACGAGACGGG-3'	597
aer A	F5'-GCC TGA GCG AGA AGGT-3' R5'-CAG TCC ACA CCA CTTC-3'	416
hae	F5'-TGCCGATGTCTGGCTCAAGA–3' R5'-GAGATCTCGCGATGCTCGTA– 3'	779
*asc*U	F5'-TGGTGATCGCCATCGCCGA-3' R5' GACGGCGCTTGCTCTTGAT-3'	233

2.10 Molecular typing of *A. hydrophila* by RAPD-PCR

RAPD-PCR method determined the genetic diversity of *A. hydrophila* using specific primers 5'–TCGCGAGCTG–3'. The polymerase chain reaction, DNA amplification protocol was a modification of that of proposed by [11].

3. Results and Discussion

One of the major challenges in the gastrointestinal diseases is the recent increase in the number of probable aetiological agents. *Aeromonas* sp. is one of the main aetiological agents. In the present study, *A. hydrophila* isolated from various sources for the determination of haemolytic enterotoxin and functional TTSS. Among 123 samples processed, 28 isolates of *Aeromonas* sp. were confirmed based on biochemical profiling and 16S rRNA identification methods. Based on presumptive screening and conformation, the test-strains were selected for further studies.

3.1 Incidence of *A. hydrophila*

About 123 samples were collected and processed accordingly. Among 78 water samples processed, 23.07% of showed the presence of *A. hydrophila* was confirmed by Starch Ampicillin Agar (SAA) and Kaper's Multi-test Medium (KMM). Prevalence of A. hydrophila in various fish was analyzed and the results revealed that the significant level was recorded in all the sampling areas. About the 13 samples collected, 30.8 % of samples found to be contaminated with *Aeromonas* sp. Fishery products are of great importance for human nutrition worldwide and provide clear health benefits [12] can also act as source of food borne pathogens overall. The clinical distribution of *A. hydrophila* was also found and 22.2 % showed incidence of *Aeromonas* sp. from 27 clinical samples (Table 2). In the milk samples, the incidence of the *Aeromonas* sp. was found to be nil. To our knowledge this report was significantly threat to the public health, which indicates such a high level of incidence of *A. hydrophila* in various sources.

Table 2: Percentage incidence of *Aeromonas* sp.

S. No.	Sample	No. of sample	Percentage incidence
1.	Water	78	23.07
2.	Fish	13	30.76
3.	Clinical	27	22.20
4.	Milk	05	0.00
	TOTAL	123	76.03

3.2 Identification of *A. hydrophila* by 16S rRNA-PCR method

The PCR method was developed in this study to amplify the 16S rRNA gene and *A. hydrophila* specific virulent gene was found to be useful for direct detection of pathogenic strains of *A. hydrophila*. Biochemically identified *A. hydrophila* strains were further identified by presence of 16S rRNA gene. An intact band appeared in most of the wells and it was showed that 16S rRNA gene positioned about 1050bp.

3.3 16S rRNA sequence analysis

The partial sequence was analyzed and submitted to NCBI (National Centre for Biotechnology Information).The sequence showed the size of 16s rRNA about 303bp (partial sequence). The Gen Bank number is JX888463.

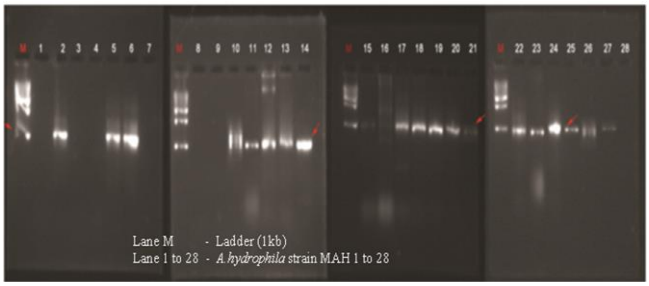

Figure 1: Identification of *A. hydrophila* by 16S rRNA-PCR method

3.4 Antibiotic resistance

We reviewed susceptibility patterns of *A. hydrophila* isolated from various sources. The study showed the existence of multi drug resistant strains. MAR index showing that all the isolates may be originated from high risk sources (Table 3). We found substantial hospital-to-hospital variability in proportional resistance to antibiotics in multiple organisms and significant increased antibiotic resistant diarrhoeagenic bacteria. A strict attention to maintain and control of the environment and of the antimicrobial use, appears the measures most likely to control the spread of this organism in hospitals [13].

3.5 Haemolytic activity

The haemolytic activity of all the strain was varying in their ability of the different haemolysins (α, β and γ) productions. About 57 % of the isolates showed γ haemolysis, 25 % of isolates showed β haemolysis and 18 % of isolates showed α haemolytic activity. The production of hemolytic toxins has been regarded as strong evidence for pathogenic potential in *Aeromonads*. In our study, we observed strong haemolysin activity against blood agar plates.

3.6 Serum susceptibility

The serum susceptibility of *A. hydrophila* strains were observed by the colour change from green to yellow. Only 25 % of the isolates showed the susceptibility towards the normal human serum (O^+ blood group) and remaining 75 % of the isolates showed the resistance towards the normal human serum (Table 4). In the present study, it was observed that higher degree of resistance showed in all the tested strains. It significantly induces the virulence mechanism.

3.7 Cytotoxic activity

In the present investigation, the haemolysin toxin was isolated from two strains (MAH1 and MAH5). Observed the effect of toxin on cancer cell line and optical density was measured by ELISA reader reported that cytotoxin production was the most common virulence factor compared to adhesive and invasive ability (Table 5).

Isolate No.	Resistance Pattern	MAR index
MAH1	A Pc Ao P R Ac Cb Ch Cef	0.58
MAH2	A E S Pc Ao P Va R Ac Cb N Ch Cef	0.76
MAH3	Pc Ao Ac Cb Ch	0.29
MAH4	A E Pc Ao P Va R Ac Cb Ch Cef	0.70
MAH 5	A E S G Pc Ao P Va R Ac Cb T Cf Ch Cef C	0.94
MAH6	A E S G Pc Ao P Va R Ac Cb T Ch Cef C	0.88
MAH7	A E S Pc Ao P Va R Ac Cb T N Ch Cef C	0.88
MAH8	A E S G Pc Ao P Va R Ac Cb T N Ch Cef C	0.94
MAH9	A E S Pc Ao P Va Ac Cb Ch Cef	0.64
MAH10	A E S Pc Ao P Va Ac Cb Ch	0.58
MAH11	A E S Pc Ao P Va R Ac Cb T Ch Cef	0.76
MAH12	A E S Pc Ao P Va R Ac Cb N Ch Cef C	0.82
MAH13	A E Pc Ao P Va R Ac Cb T N Ch Cef C	0.82
MAH14	A E S Pc Ao P Va R Ac Cb T Ch Cef C	0.82
MAH15	A E S G Pc Ao P Va R Ac Cb T N Cf Ch Cef C	1
MAH16	A E S G Pc Ao P Va R Ac Cb T N Cf Ch Cef C	1
MAH17	A E S Pc Ao P Va R Ac Cb T N Ch Cef	0.82
MAH18	A E S Pc Ao P Va R Ac Cb T Ch Cef C	0.82
MAH19	A E S Pc Ao P Va R Ac Cb T N Cf Ch Cef	0.94
MAH20	A E S G Pc Ao P Va R Ac Cb T N Cf Ch Cef C	1
MAH21	A E S G Pc Ao P Va R Ac Cb T N Cf Ch Cef C	1
MAH22	A E S G Pc Ao P Va R Ac Cb T N Cf Ch Cef C	1
MAH23	A E S G Pc Ao P Va R Ac Cb T N Cf Ch Cef C	1
MAH24	E S G Pc Va R Ac Cb N Cf Ch	0.64
MAH25	A E S G Pc Ao P Va R Ac Cb T N Cf Ch Cef	0.94
MAH26	A Ao Ac Ch	0.23
MAH27	G Pc Ao P Va Ac Ch	0.47
MAH28	A Pc Ao R Ac Ch Cef	0.47

Table 3: Resistance pattern and MAR index of the isolates

3.8 Identification of virulence gene

Haemolysin (*hly*) and aerolysin (*aer*) were present in the fish isolates of *A. hydrophila*. Most of the *A. hydrophila* found conserved region for *hly* and *aer* gene and the amplification of 597 and 416 bp fragments (Figure 2). The identification of extracellular haemolysin *(hae)* showed that all the isolates were intact band at the position of 779 bp with reference to DNA marker (100bp) (Figure 3). The nonspecific amplification noticed in all the lanes. The *asc*U gene located at position of 233 bp from the conserved sequence. The identification of TTSS in *A. hydrophila* is an important discovery for unlocking the pathogenesis of this bacterium (Figure 4).

Table 4: Serum susceptibility of *A. hydrophila*

Sl. No.	Strain	OD (490 nm)	Susceptibility
1	MAH01	0.303	-
2	MAH02	0.404	+
3	MAH03	0.452	+
4	MAH04	0.463	+
5	MAH05	0.533	+
6	MAH06	0.312	+
7	MAH07	0.303	+
8	MAH08	0.401	-
9	MAH09	0.405	-

10	MAH10	0.298	-
11	MAH11	0.403	-
12	MAH12	0.837	-
13	MAH13	0.275	-
14	MAH14	0.483	-
15	MAH15	0.452	-
16	MAH16	0.465	-
17	MAH17	0.923	-
18	MAH18	0.666	-
19	MAH19	0.770	+
20	MAH20	0.371	-
21	MAH21	0.470	-
22	MAH22	0.489	-
23	MAH23	0.213	-
24	MAH24	0.331	-
25	MAH25	0.292	-
26	MAH26	0.312	-
27	MAH27	0.337	-
28	MAH28	1.132	-

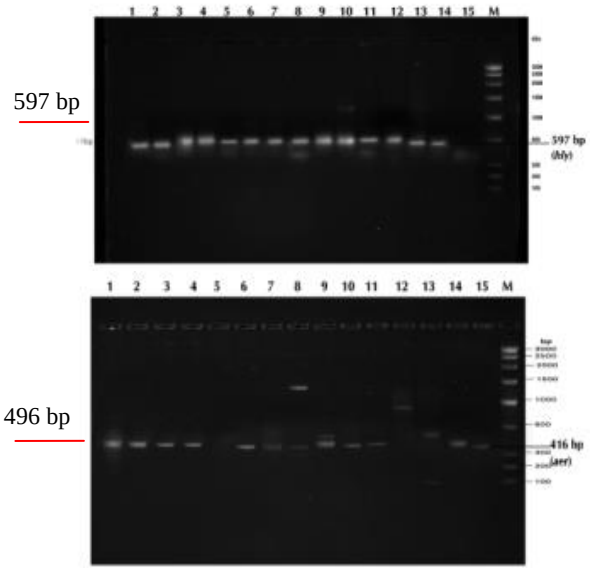

Figure 2: PCR Identification of *hly* and *aer* gene

Table 5: cytotoxicity of cell lines

Sl. No.	OD of cell lines	
	MAH01	MAH02
1	0.000	0.053
2	0.342	0.467
3	0.307	0.319
4	0.540	0.171
5	0.174	0.425
6	0.246	0.187
7	0.200	0.267
8	0.038	0.392

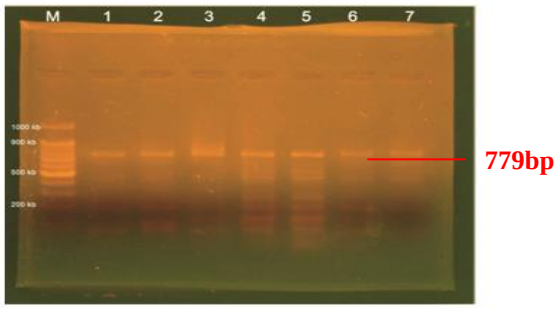

Figure3: Identification of *hae gene* by PCR method

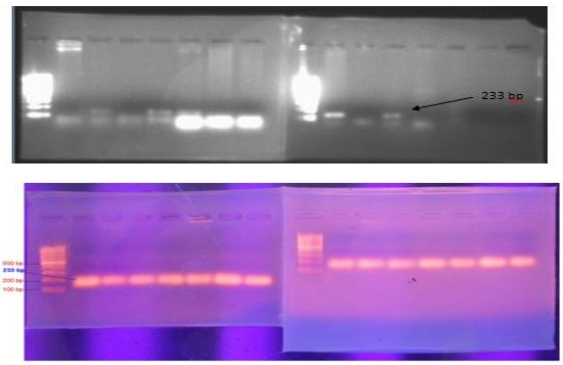

Figure 4: Identification of *asc U gene* by PCR method

3.9 RAPD-PCR profiles of *A. hydrophila*

All the *A. hydrophila* strains showed (Figure. 5) different RAPD profile have produced bands with different molecular weights and it ranged from 0.25 to 1.3 kbp and the molecular weight was determined by comparing with 100 bp DNA ladder (Genei, Bangalore). RAPD has been used widely for epidemiological investigation of numerous bacterial species [14], [15]. In recent reports [16], [17],[18],[19] proved that RAPD are powerful tools for differentiating the bacterial strains, while [20] reported that RAPD have the same discriminatory power of the bacterial species.

On the basis of the previous reports and the present investigation, concluded that the coexistence of genetic diversified strains of isolated from various samples collected from different areas was well established. The role in assessing *Aeromonas* influences on adverse public health is warranted.

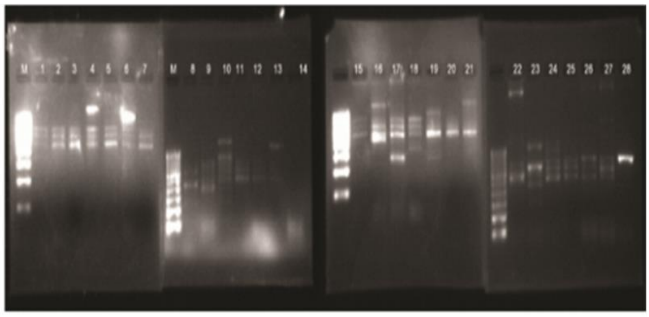

Figure 5: RAPD-PCR profiles of *A. hydrophila*
Lane M – DNA marker (100 bp) Lane 2-28 strains of *A. hydrophila*

Reference

[1] J. M Janda, and S.L. Abott, "Evolving concepts regarding the genus *Aeromonas:* an expanding Panorama of species, disease presentations and unanswered questions", *Clin. Infect. Dis*, 27: pp.332-344, 1998.

[2] M. Gracey, V.Burke. J, Robinson, "*Aeromonas*-associated gastroenteritis", *Lancet 2*, pp. 1304 – 1306, 1981.

[3] C. Pitarangsi, P. Echeverria, R. Whitmire, C. Tirapat, S. Formal, G. J Dammin and M.Tingtalapong,"Enteropathogenecity of *Aeromonas hydrophila* and *Plesiomonas shigellodes*: prevalence among individuals with and without diarrohea in Thailand", *Infect. Immun.,* 35: pp. 666-673, 1982.

[4] Z.Rahim, S. C. Sanyal, K. M. S. Aziz, M. I. Hug and A. A. Chaudhury, "Isolation of enterotoxigenic, haemolytic and antibiotic resistant *Aeromonas hydrophila* strains from infected fish in Bangladesh", *Appl.Environ.Microbiol*., 48: pp.865-867, 1984.

[5] P. J Gosling, "Pathogenic mechanisms. *In: Austin* B *et al., Eds.* The genus *Aeromonas",* London, Wiley, pp.245-265, 1996.

[6] G. R Cornelis and F.Van Gijsege, "Assembly and function of the type III secretory systems." *Ann. Rev. Microbiol*., 54: pp. 735-774, 2000.

[7] J. Kaper, B, R. J. Seidler, H. Lockman and R. R. Colwell, "Medium for the presumptive identification of *Aeromomas hydrophila* and *Enterobacteriaceae". Appl. Environ. Microbiol.,* 38: pp.1023-1026, 1979.

[8] S.E Millership, and Austin B *et al*., eds. Identification. In: The genus *Aeromonas.* London, *Wiley*: 85–107, 1996.

[9] A.W Bauer, W.M. M. Kirby, J. C. Sherris and M. Turck, "Antibiotic susceptibility testing by a standard single disc method". *Am. J. Clin. Pathol*., 36: pp. 493-496, 1996.

[10] C.B.S Lau, C.Y. Ho, C.F. Kim, K.N. Leung, K.P. Fung, T.F. Tse, H.H.L. Chan and M.S.S. Chow, "Cytotoxic activities of *Coriolus versicolor* (Yunzhi) extract on human leukemia and lymphoma cells by induction of apoptosis", *Life Sci.,* 75: pp.797-808, 2004.

[11] A.P.L Delamare, L.O Artico, F.G Grazziotin, S. Echeverrigaray and S.O.P. Costa, "Total protein electrophoresis and RAPD finger print analysis for the identification of *Aeromonas* at the species level", *Brazl. J. Microbiol*., 33: pp. 358-362, 2002.

[12] L.G Darlington and T. W. Stone, "Antioxidants and fatty acids in the amelioration of rheumatoid arthritis and related disorders", *Br. J. Nutr*., 85: pp. 251-269, 2001.

[13] D. Jayarajan, R. Subashkumar, F. Sylviamary, S. Silvan and E. K.Elumalai, "Dissemination of serum resistant multiple antibiotic resistant *Acinetobacter* sp. in clinical settings - a New Threat", *European Journal of Academic Essays* 1(3): pp. 51-57, 2014.

[14] A.Van Belkum, R. Bax and G. Prevosl, "Comparison for genotyping assays for epidemiological study of methicillin resistant *Staphylococcus aureus". Eur. J. Clin. Microbiol., Infect. Dis.*, 13: 420-424, 1994.

[15] Y. Millemann, M. Lesage –Descauses, J. Lafont and E. Chaslus-Dancla, "Comparison of random amplified polymorphic DNA analysis and enterobacterial repetitive intergenic consensus- PCR for epidemiological studies of *Salmonella", FEMS Immunol. Med. Microbiol*., 14: pp.129-134, 1996.

[16] A. Davin-Regli, C. Bolet, E. Chamorey, V. C. D'istria and A. Cremieux, "A cluster of cases of infections due to *Aeromonas hydrophila* revealed by combined RAPD and ERIC-PCRs", *J. Med. Microbiol*., 47: PP. 499-504, 1998.

[17] Ma. Aguilera- Arreola, G.C. Fernandez-Rodriguez, G. Zuniga, M. J. Figueras and G. C. Escarpulli, "*Aeromonas hydrophila* –clinical and environmental ecotypes as revealed by genetic diversity and virulence genes", *FEMS Microbiol.Lett.,* 45:pp. 47-56, 2005.

[18] Tha Thayumanavan, "Molecular typing of *Aeromonas hydrophila* isolated from fresh and marketed fish and prawns of East and West coasts of South India". Ph. D., thesis, submitted to Bharathiar University, Coimbatore, India, 2005.

[19] R. Subashkumar, Tha Thayumanavan, C. Thilagavathy, G. Vivekanandhan, K. Savithamani and P. Lakshmanaperumalsamy, 'Typing of haemolytic and antibiotic resistant *Aeromonas hydrophila* isolated from raw milk of Coimbatore, South India", *Int. J. Dairy Sci*., 1(1): pp.70-83, 2006.

[20] E. Szczuka and A. Kaznowski, "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: pp. 220-228, 2004.