RAPD and ERIC – PCR typing of virulent *Aeromonas hydrophila* isolated from children with acute diarrhoea

Rathinasamy SUBASHKUMAR[1]*, Thagavelu THAYUMANAVAN[2], Perumalsamy LAKSHMANAPERUMALSAMY [3]

[1]PG and Research Department of Biotechnology, Kongunadu Arts and Science, Coimbatore – 641 029. TN, India
[2]School of Biotechnology, Dr. G. R. Damodaran College of Science, Coimbatore – 641 014, India.
[3]Environmental Sciences, Bharathiar University, Coimbatore – 641 046, India.
(*author for correspondence*; rsubashkumar@gmail.com)

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ABSTRACT
A total of 40 *A. hydrophila* strains were isolated from stool samples from children suffering from gastroenteritis. Haemolysin was considered as the major criteria for the selection of the strains for the molecular typing study. Eight diarrhoeal isolates were randomly selected and analysed for their genetic profiling. Non haemolytic diarrhoeal strains and environmental strains were also included. A greater heterogeneity among the clinical and environmental isolates of *A. hydrophila* has been demonstrated by RAPD and ERIC-PCR methods. The strains revealed a clear clonal structure. On this basis, we came to a conclusion that all the isolates of *A. hydrophila* having genetically heterogenous
Keywords: *Aeromonas hydrophila*, diarrhoea, heterogeneity, RAPD-PCR, ERIC-PCR

RAPD ve ERIC – Akut diyareli çocuklardan izole edilen virulan *Aeromonas hydrophila*'nın PZR ile tiplendirilmesi
Toplam 40 *A. hydrophila* suşu gastroenteritli çocuklardan alınan dışkı örneklerinden izole edildi. Suşların seçiminde hemolizin, moleküler tiplendirme çalışması için ana ölçüt olarak düşünüldü. 8 diyare izolatı rastgele seçildi ve genetik profillendirme için analiz edildi. Hemolitik olmayan diyare suşları ve çevresel örnekler de bu analize katıldı. *A. hydrophila*'nın klinik ve çevresel izolatları arasında daha büyük bir heterojenite olduğu RAPD ve ERIC-PCR metotlarıyla gösterilmiştir. Suşlar açık bir klonal yapıyı işaret etmektedir. Buna göre, bütün izolatların genetik olarak heterojen olabileceği sonucuna vardık.
Anahtar kelimeler: *Aeromonas hydrophila*, diyare, heterojenite, RAPD-PCR, ERIC-PCR.

Introduction

Aeromonas spp. comprises a complex group of ubiquitous bacteria that are widely distributed and often isolated form clinical, environmental and food samples (Carnahan and Altwegg, 1995; Sechi *et al.,* 2002). *Aeromonas hydrophila* has been recognized as an opportunistic pathogen and cause severe infections in human. They are implicated in a number of diarrhoeal and extraintestinal infections. Clinical and environmental *Aeromonas* isolates secrete many extracellular products, such as haemolysins, enterotoxins and proteases. *A. hydrophila* produce virulence factors more frequently than other species (Kuhn *et al*., 1997). *Aeromonas* might be a clonal property and only some clones may be responsible for disease. However, studies on the clonal structure and diversity within the strains are not made. Hence, this study was undertaken to investigate the clonal relatedness of strains from diarrhoeal stool specimens collected from children and compared with environmental (milk and fish) isolates by random amplified polymorphic DNA (RAPD) PCR and enterobacterial repetitive intergenic consensus (ERIC) PCR methods (Davin-Regli *et al.,* 1998; Szczuka and Kaznowski, 2004; Ramalivhana *et al.*, 2010).

Materials and Methods

Bacterial strains

The strains of *A. hydrophila* were collected from children with acute gastroenteritis (Subashkumar *et al.,* 2006a). Detection of virulence properties and multiple antibiotic resistant patterns of the strains were studied and published in Subashkumar *et al.* (2006a) Eight haemolytic and one non-haemolytic diarrhoeal strain were randomly selected for this study. One haemolytic strain of *A. hydrophila* from fish (Thayumanavan *et al.*, 2003) and one haemolytic strain of *A. hydrophila* from milk (Subashkumar et al., 2006b) and two reference *A. hydrophila* strains (ATCC 14715 and MTCC 646) were used in parallel.

Preparation of genomic DNA

Total DNA was prepared and purified from overnight culture by using GENTRA DNA extraction kit (Gentra System, Inc., USA) according to the manufacture's instructions. The extracted DNA was diluted into 100 ngμl^{-1} for RAPD and ERIC- PCR tests.

RAPD – PCR

One hundred ng of each DNA was individually used, and a final volume of 50 μl was used for PCR amplification. RAPD mixtures were prepared as described by Oakey *et al.* (1996). A typical RAPD-PCR reaction mix contained 5μl of 10X PCR buffer II (100 mM Tris-HCl. pH 8.3; 500mM KCl), 1 μl of dNTPs (10mM), 7 μl of primer, 0.4 μl of *Taq* DNA polymerase, 1 μl (100 ng/μl) of template DNA and 35.6 μl of DNA quality water and made upto a final volume of 50 μl DNA. Amplification was performed using a thermal cycler (MJ Research, Model PTC 100 Watertown, Mass., USA). The amplification conditions were as follows: 35 cycles of denaturation at 94 ºC for 10 sec, annealing at 37 ºC for 30 sec and extension at 72 ºC for 1 min. The primer used in this study was 5'–TCG CGA GCT G –3'.

ERIC – PCR

The ERIC-PCR method utilizes primers complementary to ERIC sequences of bacterial genomic DNA (Versalovic *et al.*, 1991). The primers ERIC 1 (5'–ATG TAA GCT CCT GGG GAT TCA C–3') and ERIC 2 (5'–AAG TAA GTG ACT GGG GTG AGC G–3') were used. The DNA (100ng) sample was used and final volume of 50 μl was used for PCR amplification. ERIC – PCR reaction mix contained 2.5μl of 10X PCR buffer II (100 mM Tris-HCl. pH 8.3; 500mM KCl), 0.2 μl of dNTPs (10mM), 1.25 μl of primer (ERIC I), 1.25 μl of primer (ERIC II), 0.2 μl of *Taq* DNA polymerase, 1 μl (100 ng) of template DNA and 43.6 μl of DNA quality water to make a final volume up to 50 μl. Amplification was performed using thermal cycler. Slight modification was made in ERIC–PCR cycle, which was described by Szczuka and Kaznowski (2004). The reaction mixtures were denatured at 94 ºC for 3 min and then subjected to 35 cycles of denaturation at 94 ºC for 30 sec, annealing at 45 ºC for 1 min and extension at 72 ºC for 5 min and final extension at 72 ºC for 5 min.

Visualization of PCR products

Amplified products were resolved by electrophoresis in 1.5 % (w/v) agarose gels containing ethidium bromide (1.6 mg ml^{-1}) at 11 V cm^{-1} for 90 min in TBE buffer (0.089 M Tris, 0.089 M boric acid, 0.002 M EDTA (pH 8)). Sizes of the amplified products were determined by comparison with 2 Log DNA ladder (New England Biolabs, UK).

### Phylogenetic analysis of RAPD and ERIC profiles by simple matching

The length of the both RAPD and ERIC-PCR gels were normalized and molecular size of the fragments were compared independently, but data for all molecular markers were combined in the final analysis. The similarity of DNA pattern was measured by simple matching and clustering was achieved by unweighed pair group method using average linkage (UPGMA). Dendrogram branches with bootstrap values higher than 50 % and relationships on the right side of vertical line were considered significantly supported. All of these analyses were made with the NTSYS-PC software, version 2.02j and PHY-LIP version 3.6 software. The genetic diversity of all the strains (n=13) were estimated in combined dendrogram of RAPD and ERIC-PCRs.

Results

RAPD-PCR profiles of A. hydrophila

The RAPD profiles of all the strains were produced identical clear bands ranged from 3.6 to 0.6 kDa (Fig. 1). The number of bands varied within the isolates, and it ranged between 2 and 8 bands. Even among the diarrhoeal isolates, no similar profiles have been observed. This revealed the wide genetic diversity of the *A. hydrophila* strains tested. Band with high molecular weight (3.6 kb) was seen in strain *Ah*-D12, *Ah*-D19 and *Ah*-M1, while low molecular weight (0.6 kb) band was produced by the strains *Ah*-M1 and *Ah*-F1. Among the diarrhoeal strains *Ah*-D12 has shown low molecular weight with 0.7 kb. The bands with 1.1 kb (*Ah*-T2, *Ah*-D5, *Ah*-D6, *Ah*-D26 and *Ah*-D29), 1.3 kb (*Ah*-T1, *Ah*-T2, *Ah*-D5, *Ah*-D6, *Ah*-D12, *Ah*-D15,

Ah-D19, *Ah*-D24 and *Ah*-D26) and 1.9 kb (*Ah*-D5, *Ah*-D12, *Ah*-D19 and *Ah*-D26) were common among few strains. Band with the molecular weight of 2.9, 1.7 and 0.6 kDa were common in both the fish and milk isolates.
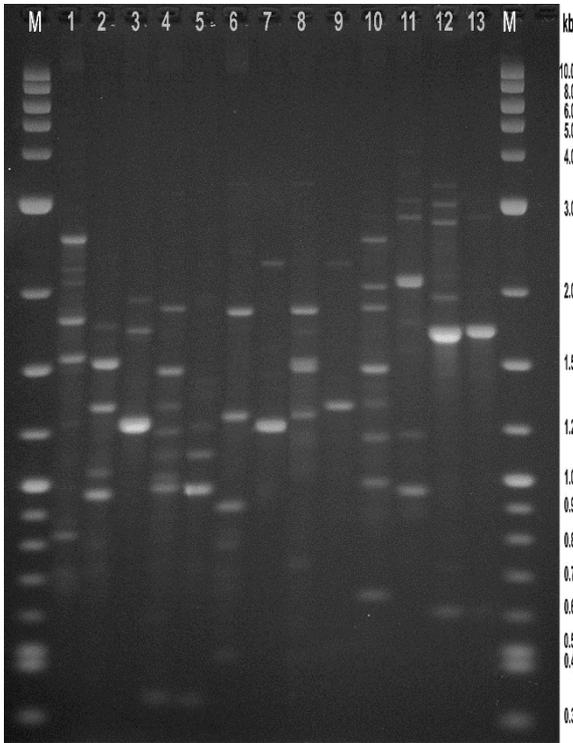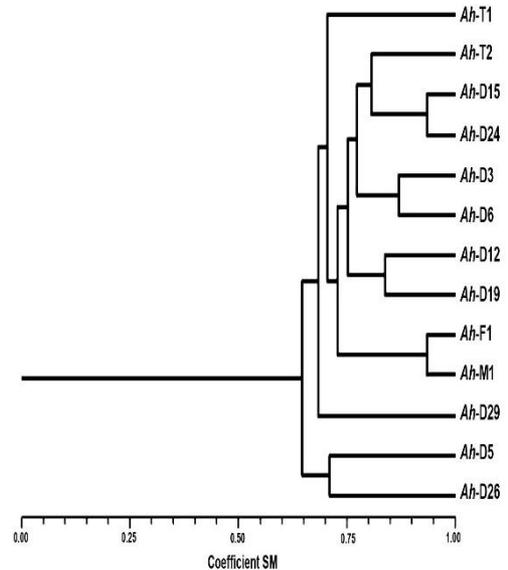

Figure. 1.   RAPD profiles of *A. hydrophila* isolated from children diarrhoeal sources. M: 2 log DNA ladder (0.1 - 10 kb), 1. *Ah* - T1 (MTCC 646), 2. *Ah* - T2 (ATCC 14715), 3. *Ah* - D3 (Diarrhoea), 4. *Ah* - D5 (Diarrhoea), 5. *Ah* - D6 (Diarrhoea), 6. *Ah* - D12 (Diarrhoea), 7. *Ah* - D15 (Diarrhoea), 8. *Ah* - D19 (Diarrhoea), 9. *Ah* - D24 (Diarrhoea), 10. *Ah* - D26 (Diarrhoea), 11. *Ah* - D29 (Diarrhoea), 12. *Ah* - M1 (Milk isolate), 13. *Ah* - F1 (Fish isolate)

RAPD profiles by coefficient simple matching dendrogram

The molecular data obtained from the RAPD analysis produced consistent dendrogram (Fig. 2). The values of r (*CCCr = 0.76793*) and P (*P = 0.0020*) were obtained using the same polymorphic molecular data. Various groups of *A. hydrophila* were clearly clustered according to the origin of isolation (diarrhoea, milk and fish). All the *A. hydrophila* strains have formed most variable groups, which composed of at least 12 groups. The highest similarity index was observed in two clusters with 0.94 between two diarrhoeal isolates (*Ah*-

D15 and *Ah*-D24) (cluster 1) and fish (*Ah*-F1) and milk (*Ah*-M1) strains (cluster 2). The similarity index value 0.87 was observed with diarrhoeal isolates, *Ah*-D3 and *Ah*-D6 which formed a new cluster (cluster 3).

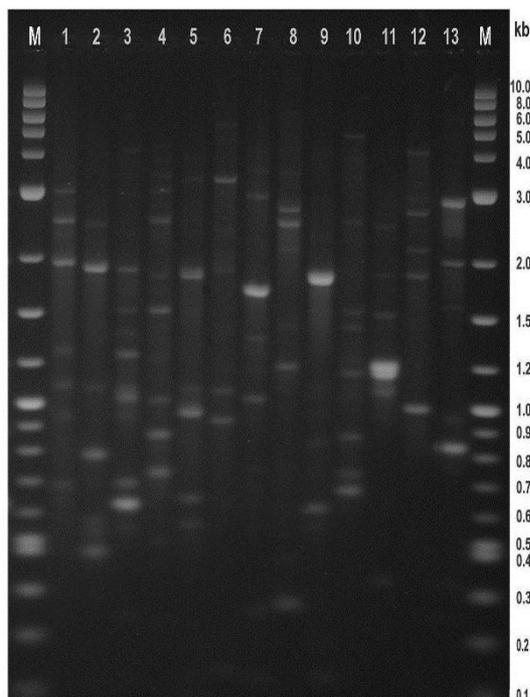**Figure. 2.**   Coefficient Simple Matching dendrogram generated by UPGMA by the RAPD profiles

ERIC-PCR profiles of A. hydrophila

The ERIC-PCR profiles also strongly support the wide genetic diversity of the *A. hydrophila* strains used in this study (Fig. 3). A great heterogeneity was demonstrated among all *A. hydrophila* strains tested. No band was common among all the strains, while few strains shared few common bands with different molecular weights. A band with a high molecular weight of 5.8 kb was shown by *Ah*-D12, while low molecular weight (0.3 kb) was seen in both *Ah*-D19 and *Ah*-D29. A band with a molecular weight of 0.9 kb was observed as a common band among most of the strains except *Ah*-D19, *Ah*-D12 and *Ah*-M1. Thus, the strains have produced non identical profile.
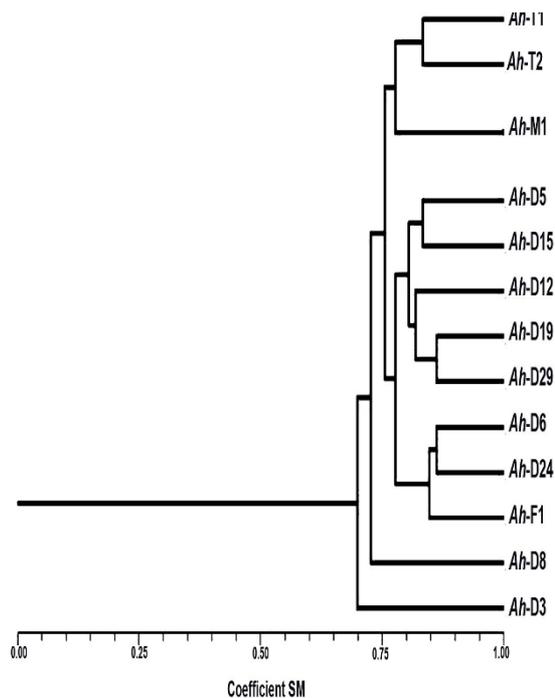
ERIC profiles by coefficient simple matching dendrogram

The ERIC-PCR data were subjected to development of dendrogram and it was found consistent. The values of r (*CCCr = 0.78285*) and P (*P = 0.20*) were obtained when all of the repetitive molecular data were included in the same analysis (Fig. 4). The ERIC data analysis of 36 polymorphic and reproducible DNA fragments (bands) of *A. hydrophila* of several groups were

recognized on the dendrogram and the clusters formed clearly indicates various origins of their isolation (diarrhoea, fish and milk). The strains which were used in this study formed variable groups, and the similarity value were recorded above 0.70. Two clusters with the similarity value of 0.87 were documented and this was the highest similarity value. One is with *Ah*-D19 and *Ah*-D29 (cluster 1) and another is with *Ah*-D6 and *Ah*-D24 (cluster 2). Another group was formed between the strain *Ah*-F1 combined with cluster 2 with a similarity value 0.85 (cluster 3). Two new clusters with a similarity value of 0.83 were seen with the strain *Ah*-D5 and *Ah*-D15 (cluster 4) and the type strain *Ah*-T1 and *Ah*-T2 (cluster 5). The major cluster 11 along with *Ah*-D3 showed a major group, while the similarity value of 0.70 was recorded. Analysis of combined RAPD and ERIC profiles by coefficient simple matching dendrogram.

Figure 4. Coefficient Simple Matching dendrogram generated by UPGMA by the ERIC profiles

Coefficient simple matching dendrogram for combined RAPD and ERIC profiles

The molecular data obtained from the RAPD and ERIC methods were combined together and a combined dendrogram has been developed (Fig. 5) with simple co-efficient. The r and P values were *CCCr = 0.70953* and *P = 0.0020* respectively. In the RAPD analysis 66 markers (or bands) were observed,.of which, 31 were polymorphic markers. In ERIC analysis, 69 markers (or bands) were found of which 36 were polymorphic DNA. All the *A. hydrophila* have formed most variable groups and the genetic diversity of all the strains showed totally 12 clusters. The molecular data indicated highest similarity between *Ah*-D15 and *Ah*-D24 with 0.89 similarity value (cluster 1). Cluster 2 created with cluster 1 and strain *Ah*-D12 (similarity value 0.84). Milk and fish strains formed a new group with 0.83 similarity value (cluster 3). The same similarity value formed between the type strain (*Ah*-T1 and *Ah*-T2) (cluster 4). The diarrhoeal *A. hydrophila* strains of clinical importance are clearly indicating the efficiency of dendrogram and discriminating the origin. The combined RAPD and ERIC dendrogram

Figure 3. ERIC profiles of *A. hydrophila* isolated from children diarrhoeal sources. M: 2 log DNA ladder (0.1 - 10 kb), 1. *Ah* - T1 (MTCC 646), 2. *Ah* - T2 (ATCC 14715), 3. *Ah* - D3 (Diarrhoea), 4. *Ah* - D5 (Diarrhoea), 5. *Ah* - D6 (Diarrhoea), 6. *Ah* - D12 (Diarrhoea), 7. *Ah* - D15 (Diarrhoea), 8. *Ah* - D19 (Diarrhoea), 9. *Ah* - D24 (Diarrhoea), 10. *Ah* - D26 (Diarrhoea), 11. *Ah* - D29 (Diarrhoea), 12. *Ah* - M1 (Milk isolate), 13. *Ah* - F1 (Fish isolate)
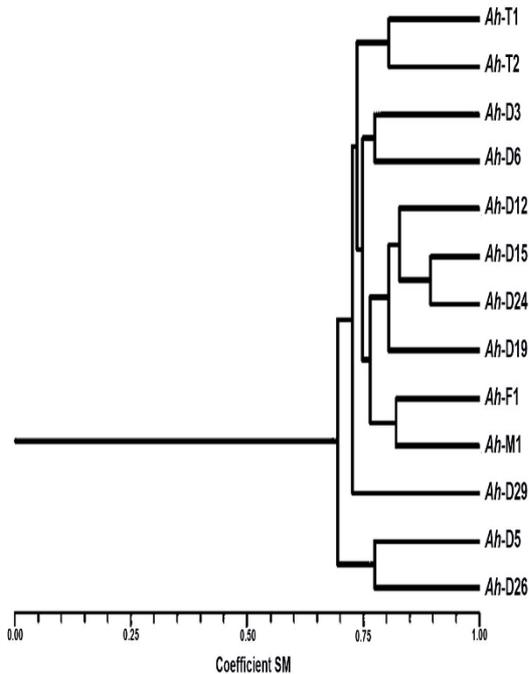
revealed the similarity 0.69 between all the *A. hydrophila*.

Figure 5. Coefficient Simple Matching dendrogram generated by UPGMA by the RAPD+ERIC profiles

Discussion

Molecular typing of A. hydrophila using RAPD and ERIC-PCRs

Taxonomic identification within the genus *Aeromonas* has been subject to considerable debate and differentiation at species level is somewhat perplexed (Blair *et al.*, 1999). The need for a good system to enable identification and classification of *Aeromonas* is justified because of their ecological and clinical importance. Plasmid carriage is infrequent (20 to 58 %) in *A. hydrophila* (Janda, 1991). Although there are some arguments exist against the reproducibility of PCR–based typing methods (van Belkum *et al.,* 1994; Perez *et al.,* 1998). There are reports with excellent correlation and statistically validated between RAPD and ERIC-PCR methods (Davin-Regli *et al.,* 1998; Szczuka and Kaznowski, 2004). In the present study, these methodologies were highly reproducible.

In recent reports, both RAPD and ERIC–PCRs were stated to be powerful tools for differentiating the strains of *A. hydrophila* (Davin-Regli *et al.,* 1998; Aguilera-Arreola et al., 2005; Thayumanavan, 2005; Subashkumar et al., 2006b), while Szczuka and

Kaznowski (2004) reported that both RAPD and ERIC–PCRs have the same discriminatory power of the species of *Aeromonas*. They found that the repetitive extragenic palindromic (REP) PCR was less effective for differentiating *Aeromonas* isolates into species, since the sequence may not be widely distributed in *Aeromonas* sp. genome. In contrast, Alavandi *et al.* (2001) used whole cell proteins and RAPD–PCR to distinguish the clinical strains of *Aeromonas* spp. and concluded that both RAPD and whole cell protein finger printing techniques could not differentiate the phenospecies of the genus *Aeromonas*.

In the present investigation, we have observed a perfect correlation between RAPD and ERIC–PCR profiles. A greater heterogeneity among within the strains of *A. hydrophila* has been demonstrated by RAPD and ERIC–PCRs. Even though, all the isolates were of clinical origin, and it was observed that none of the *A. hydrophila* isolates produced identical profiles. This clearly reflects the genetic diversity of the strains tested. This was well reputable with RAPD and ERIC-PCRs. This is strongly supported by the findings of Davin-Regli *et al.* (1998) who also observed a similar type of correlation between RAPD and ERIC–PCR profiles for the isolates of *A. hydrophila* isolated from hospital patients and tap water in France. In their study, Davin-Regli *et al.* (1998) found 6 different patterns among 10 clinical isolates of *A. hydrophila* tested. Moyer *et al.* (1992) and Davin-Regli *et al.* (1998) also found no fingerprints were common among environmental and clinical isolates of *A. hydrophila*. Thayumanavan (2005) reported a wide spread variation in clones of *A. hydrophila* isolated from freshly caught and marketed seafood. Work on *A. hydrophila* isolated from river water (Sharma *et al.*, 2005) and raw milk (Subashkumar *et al.*, 2006b) has also supported the findings of the present investigation.

Dendrogram of RAPD and ERIC profiles

The UPGMA cluster analysis on the basis of RAPD and ERIC profile of *Aeromonas* depicted all the strains having the several clusters and it suggests that their profiles are species specific. Visible RAPD profiles revealed the substantial wide genetic diversity among the strains tested. Strains *Ah*–T2, *Ah*–D3, *Ah*–D6, *Ah*–D12, *Ah*–D15, *Ah*–D19 and *Ah*–D24 formed a minor cluster (cluster 7) while *Ah*–D5 and *Ah*–D26 formed a separate minor cluster against the cluster 10. This indicates the genetic relationship among the strains within the clusters. It is clear that one can

easily understand the percentage of genetic similarities between the strains.

Similarly, none of the ERIC profiles have been found to be identical, but greater heterogeneity (0.87) was observed for the strain *Ah*-D3 from the other strains of *A. hydrophila*. It formed a separate cluster showing a wide genetic diversity from other strains. A total of 12 clusters were observed with a maximum number of 8 strains grouped into major cluster-8. However, all of them have been isolated from diarrhoeal sources and one from fish source. But the type strains formed a separate cluster-5 and this formed a new cluster-9 along with the milk isolate (*Ah*-M1). Here it is clear that all diarrhoeal isolates have been arranged in a big cluster (cluster 9). The dendrogram clearly exhibited that clinical and environmental isolates of *A. hydrophila* are not genetically similar. Moyer *et al.* (1992), Davin-Regli *et al.* (1998) and Szczuka and Kaznowski (2004) also reported that none of the *Aeromonas* species were genetically similar and were further confirmed by dendrogram. The combined RAPD and ERIC profiles also supported the co-existence of genetically varied *A. hydrophila* among the samples. Here also none of the strains have been observed identical profile, similar to the findings of Davin-Regli *et al.* (1998) and Bauab *et al.* (2003). Ramalivhana *et al.* (2010) found 12 unique RAPD finger prints revealed a tendency of the clinical and environmental isolates to cluster according their origin of isolation. The rationale for performing molecular typing was to understand whether any particular clone of *Aeromonas* species was more often associated with diarrhoea. Both RAPD and ERIC profiles clearly revealed that all strains tested in this study are having high heterogeneity.

Dendrogram of RAPD and ERIC profiles

The most consistent dendrogram was obtained when the RAPD and ERIC data were combined. Interestingly, the dendrogram reflects the major cluster of diarrhoeal strains and showed the significant variation among them. Whereas the type strains and environmental strains did not follow any such kind of pattern. This strongly supports the previous reports that both the clinical and environmental strains of *A. hydrophila* varied and this variation (s) are due to the genetic structure of the clones.

Both RAPD and ERIC-PCRs are highly reproducible, reliable and accurate. These techniques could be used in the epidemiological exploration of bacterial diseases. The results reveal a certain degree of similarity and variable pattern was observed between isolates of clinical and environmental sources, which indicated genetic relatedness. This investigation strongly supports the wide genetic diversity of *A. hydrophila* strains of diverse origin. On the basis of the previous reports and the present investigation, the coexistence of genetic diversified strains of *A. hydrophila* among the diarrhoeal samples collected from different areas was well established. On public health point of view-the importance of monitoring and evaluating infection-control measures for improved hygiene and to avoid cross-contamination are warranted.

Conflict of interest

All authors declare to have no conflict of interest.

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