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

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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

Key words: Aeromonas hydrophila, diarrhoea, heterogeneity, RAPD-PCR, ERIC-PCR

RAPD ve ERIC – Akut diyareli çocuklardan izole edilen virulan Aeromonas hydrophila’nın PZR ile tiplendirilmesi


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 ATCC 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 manufacturer’s instructions. The extracted DNA was diluted into 100 ng/µl 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 °C for 10 sec, annealing at 37 °C for 30 sec and extension at 72 °C 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 °C for 3 min and then subjected to 35 cycles of denaturation at 94 °C for 30 sec, annealing at 45 °C for 1 min and extension at 72 °C for 5 min and final extension at 72 °C 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.


**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
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 phenspecies 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 fingerprints 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.

**References**


