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RAPD marker as an ideal tool for evolutionary genetics for the analysis of *Aeromonas hydrophila*

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ABSTRACT

Aeromonas hydrophila isolates from fish, meats (chicken, Mutton) and water from river were characterized through Randomly Amplified Polimorphic DNA (RAPD) analysis. PCR amplification of DNA produced different identical amplicons and given some unique bands by using RAPD primers (OPA1 to OPA10) on agarose gel electrophoresis and showed genetic heterogeneity within the species. Polymorphism of RAPD profile was evident with a unique pattern and indicating its usefulness as an ideal tool for evolutionary genetics because of morphological and biochemical characterization sometimes gives contradictory results.

INTRODUCTION

Aeromonas hydrophila are Gram-negative, non-spore-forming and rod-shaped to coccoid cell with rounded ends. They are oxidase and catalase positive, reduce nitrate to nitrite, and ferment D-glucose. *A. hydrophila* have their natural habitat in water and grow over a wide temperature range between 0ºC and 45ºC, with a temperature optimum of 22ºC to 32ºC. They have been isolated from water especially in surface water and sewage. They also occur in untreated and treated drinking water, soil and foodstuffs[6]. This species are responsible for severe haemorrhagic syndrome in a variety of fishes and multiple diseases in poikilothermic animals[1]. This species are shown to be potentially pathogenic and associated with several human infections, including gastrointestinal infections and extra-intestinal infections, such as endocarditis, meningitis, septicaemia and urinary tract and wound infections. Abscesses or wound infections associated with exposure to soil or water represent the most prevalent extra-intestinal infections[3, 4]. *Aeromonas* spp. are found to be serologically heterogeneous, with individual serogroups found in more than one species. Most type and reference strains were not serologically representative of a genomospecies. Various genotypic typing methods have been applied for identification to this species[4, 5, 9]. Plasmid analysis is unhelpful because plasmid carriage is infrequent (20-58%) in *A. hydrophila*[4]. In contrast, rRNA gene restriction patterns provide good discrimination within *A. hydrophila*[5, 9]. In the present paper, the importance *Aeromonas* identification and use of molecular genotyping methods as RAPD analysis have been elaborated.

MATERIALS AND METHODS

Bacterial isolates

Thirty isolates of *Aeromonas hydrophila* from Chicken and Mutton, water from rivers and ponds in West Bengal were used in this study. Isolation of Aeromonas hydrophila was done using selective medium, Rimler Shotts agar (Hi Media). The plates were incubated at 37°C for 28hours. All cultures were identified to the species level using *Automated Microbial Analyzer* (Biolog, US.) and as well as different biochemical tests (TABLE 1). Selected *Aeromonas hydrophila* colonies were subcultured in Tryptic Soya Broth (Difco) for further molecular characterization.

RAPD-PCR analysis

DNA extraction

DNA were isolated by lysis of the bacteria in a solution containing 0.5% sodium dodecyl sulfate and 5 mg of lysozyme per ml, followed by extraction with phenol-chloroform[2]. Nucleic acid samples were precipitated with ethanol and dissolved in TE buffer (10 mM Tris chloride, 1 mM EDTA, pH 8.0). The nucleic acid content was quantified by determining the optical density (OD) at 260 nm (OD_{260}) and was adjusted to give a final concentration of 200 g/ml in TE buffer. Template DNA for PCR was prepared by further dilution in distilled H_2O to a concentration of 2µg/ml.

Primers

Total 10 numbers of decamer random primers, designated as OPA-1 to OPA-10 was used for PCR amplification. Three out of these 10 primers, viz., OPA-03, OPA-09 and OPA-10 were selected for final screening as they only generated several reproducible amplicons that could be resolved as distinct bands by agarose gel electrophoresis.

PCR amplification and resolution of RAPD markers

PCR reactions has been carried out by following the protocol of[10], as annealing temperature, concentration of $MgCl_2$, template DNA, Taq DNA polymerase, dNTP's and primers. The PCR reaction components consists of 200 mm dNTP, 20 pico moles of primer, 2 units of Taq DNA polymerase enzyme, assay buffer with working concentration of 1.5 mM $Mgcl_2$, 20-30 ng template DNA in an assay volume of 25mL. These concentrations were determined by a series of preliminary standardizing experiments.

Thermal cycling was performed with Perkin-Elmer thermocycler (GeneAmp PCR System 2400). Each of the 35 PCR cycles standardized for this work consisted of Denaturation at 94oC x 5min followed by 35 cycles of amplification at 94oC x 1min, then 36oC x 1min then 72oC x 2min. Final; extension was given at 72oC x 10 min. The PCR amplification products were resolved by carrying out electrophoresis using a 1.5% agarose gel, stained with ethidium bromide. The marker used was λ DNA cut with HindIII. The DNA bands were visualized and documented using a Gel documentation system.

Phylogenetic analysis

Amplified DNA fragments resolved as bands on the gels were used to generate the data matrix by giving scores of zero and one for the absence or presence of bands, respectively, at each band position. The similarity index between isolates was calculated following the method of Nei and Li (1979. Genetic similarity between isolates): A & B (S_{AB}) was calculated using the formula: - $S_{AB} = 2N_{AB} / (N_A + N_B)$. Where S_{AB} = Genetic similarity between A & B. N_{AB} - Number of amplified bands shared in common between isolate A & B. N_A and N_B - Total number of bands possessed by the isolates A & B, respectively. Further cluster analysis was performed using this matrix in SAS programme to create a dendrogram. Statistical analysis was carried out by one way analysis of variance (ANOVA) in SAS (version 6.12) to test the level of significance.

RESULTS

Aeromonas hydrophila appeared as flat yellow colonies with entire margin in the Rimler shotts medium. In primary characterization tests, they were gram negative, rod shaped, motile, oxidase positive, fermentative novobiocin resisitant, suggesting that colonies are aeromonads. All isolates were confirmed to the species level Aeromonas hydrophila by Automated Microbial Analyzer (Biolog, US.) and differential biochemical tests given in TABLE 1.

FULL PAPER

TABLE 1 : Characteristics of bacteria isolated in the study.

Test	Characte ristics	Test	Characte ristics
Gram stain	-	Arginine dihydrolase	+
Shape	Rods	Lysine decarboxylase	+
Motility	+	H2S production	+
Oxidase	+	Indole	+
Glucose O/F	Fermentative	Ampicillin	Resistant
α galactosidase	+	Chloramphenicol	Sensitive

RAPD profile

The thermal cycle regime optimized for the PCR consisted of Denaturation at 94°C x 5min followed by 35 cycles of amplification at 94°C x 1min, then 36°C x 1min then 72°C x 2min. Final; extension was given at 72°C x 10 min. In our attempt to reduce the background banding, the annealing temperature was increased to 55ºC, and it gave clear reproducible bands. Amplification of the DNA from each of the 30 isolates with the five primers named earlier produced a variety of amplicons, which were consistent and appeared as distinct bands on agarose gel after electrophoresis. The RAPD fingerprints of the isolates generated by these random primers OPA-03 and OPA-09 are given in Figure (1-2).

In this study, The RAPD fingerprint pattern was

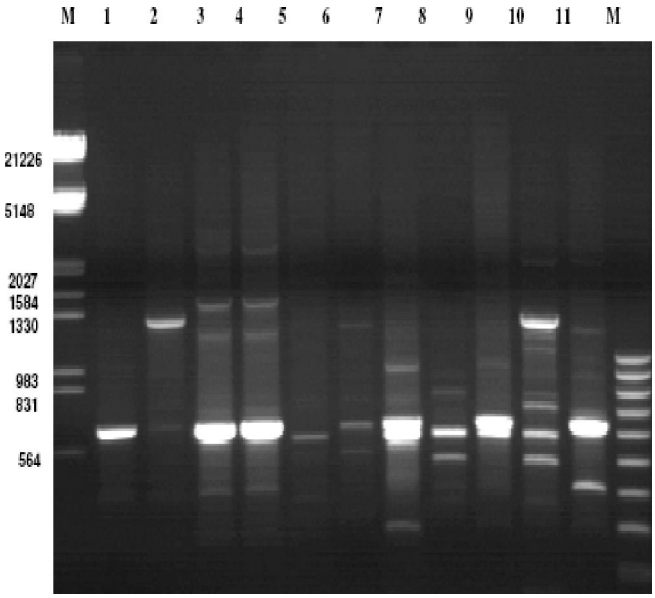

Figure 1 : RAPD profile of 10 *Aeromonas hydrophila* using random primer and showing Variable polymorphic and unique DNA bands (M=Marker)

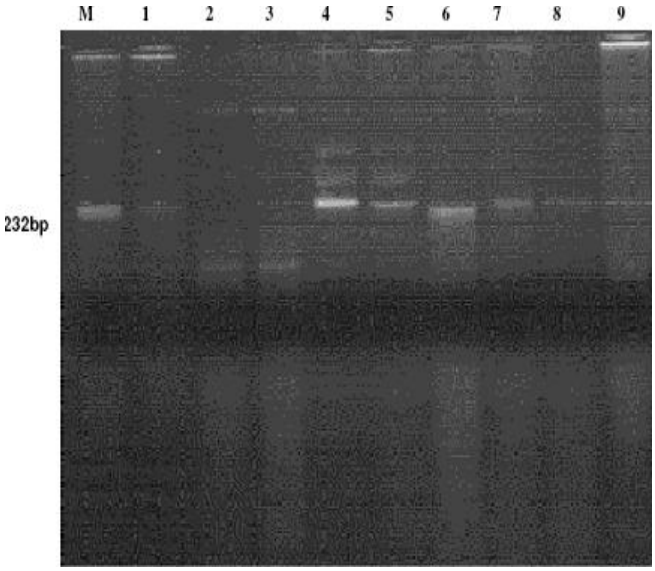

Figure 2 : Detection of *Aeromonas hydrophila* using PCR amplification of a 232 bp DNA band visualized on 1.5% agarose gel.

unique for each of the isolates. Comparison of the amplicons at each loci indicated that all but four were polymorphic. However, each banding pattern indicates that there were a number of fragments, which were homogenous among many of the isolates. These fragments may be utilized for species-specific marker development, only if amplification of other aeromonads are also examined with these primers OPA-01 and OPA –10.

Similarity Index, genetic distance and phylogenetic relationships

The average similarity index between Aeromonas hydrophila isolates was calculated as Nei's original measures of genetic identity and genetic distance, considering all the amplicons resulting from all the primers. The coefficient of genetic identities ranged from 0.413 to 0.848, most of them were of high magnitude. Estimates of genetic distances were of lower magnitude. However, no correlation in genetic distance/similarity could be made between isolates from moribund fishes and those from brackishwater farms.

DISCUSSION

Epidemiological tracking of *A. hydrophila* requires evaluation of genetic diversity and phylogenetic relationship among the isolates. Since *A.hydrophila* are

phenotypically, serologically and genetically quite diverse, many conventional methods of identifying these microorganisms like cultural-biochemical properties, protein profile analysis, and serotyping, give contradictory results compared to the molecular tools. Because of the complexity of methodologies, time taking and difficult interpretation of these results, genomic analysis methods have been commonly employed to characterize the microbial pathogens. All the isolates were typable using selected primers. But while performing screening for 20 primers, only two primers amplified with scorable bands and others had very poor reproducibility. The selected primers were able to generate distinct RAPD profiles for 10 isolates under this study. Moreover, all the primers could generate isolate- specific bands. The presence of isolate-specific unique bands will definitely aid in epidemiological studies. The technique being simple, specific and cost effective is being widely used as an alternate to other fingerprinting methods. Requirement of very small amount of DNA, without any prier information on genomic DNA sequence and use of universal primers make RAPD-PCR a popular DNA fingerprinting method in genomic analysis. It does not require any prior information on the sequence of the DNA being characterized. The RAPD technique requires the least quantity of DNA among the various techniques. Miyata et al.[8] observed that the DNA required for RAPD is less than one hundredth of the amount required for other methods. We were able to generate reproducible RAPD profiles with as little as 20ng of DNA per PCR reaction in the Molecular characterization of *A. hydrophila* strains from Japan by Miyata et al.[8] also revealed considerable heterogeneity within the species. Results of this study corroborate the observation by other workers that motile aeromonads are genetically diverse[7]. RAPD-PCR fingerprints have been used for typing and differentiation of bacteria and increasingly. Very few of the variants, which have selective advantage, are only allowed to get established in the population and result in heterogeneity. Whereas, major portion of the DNA in the cell is the non-coding region that can accumulate genetic variation as they are not subjected to natural selection and therefore, accumulate variations, which can be detected by nucleic acid based techniques. So, this molecular typing method could be used as a new strategy for epidemiological investigations. This information can be used to improve quality control and bio-security protocols, to check *Aeromonas* disease outbreaks and this concept can be applied to other bacterial pathogens.

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