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RAPD-PCR and SDS-PAGE analysis of A*eromonas hydrophila* for defining molecular characterization

Agniswar Sarkar[1]*, Mousumi Saha[2], Avijit Patra[3], Pranab Roy[1]

[1]Department of Biotechnology (Recognized by DBT- Govt. of India), The University of Burdwan, Golapbag More, Burdwan, West Bengal- 713 104, (INDIA)

[2]Department of Biotechnology, Oriental Institute of Science and Technology (Affiliated to Vidyasagar University), Burdwan, West Bengal- 713 102, (INDIA)

[3]Central Inland Fisheries Research Institute (ICAR), Barrackpore, Kolkata- 700 120, (INDIA)

E-mail : ognish@gmail.com

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ABSTRACT

Aeromonas hydrophila isolated from fish *(Labeo rohita)*, pond water, river water, raw meat of chicken and mutton and raw cow milk were characterized through Randomly Amplified Polymorphic DNA (RAPD) analysis and Sodium Dodecyl Sulphate- Polyacrylamide Gel Electrophoresis (SDS PAGE) of cellular proteins. PCR amplification of the DNA from the bacterial isolates using ten random primers (OPA-01 to 10) were used and found some distinct bands on agarose gel in gel documentation system. Absolute polymorphism of RAPD profile was studied with a unique pattern for each isolate, indicating its usefulness as an ideal tool for molecular characterization, which are not revealed by other methods like morphological, serological, biochemical characterization and cellular protein profiling. Cellular protein profile did not produced significant polymorphism as all the isolates revealed uniform pattern indicating its usefulness as a tool for species level identification.

KEYWORDS

RAPD-PCR;
SDS-PAGE;
Aeromonas hydrophila;
Molecular characterization.

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[7]. 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[4, 5]. *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[5, 6, 11]. Plasmid analysis is unhelpful because plasmid carriage is infrequent (20-58%) in *A. hydrophila*[5]. In contrast, rRNA gene restriction patterns provide good discrimination within *A. hydrophila*[6, 11]. In the present paper, the importance *Aeromonas* identification and use of molecular genotyping methods as RAPD analysis has been elaborated and cellular proteins also compared with the RAPD data and given some unique pattern.

MATERIALS AND METHODS

Isolation of bacteria and genomic DNA extraction

Thirty isolates of *Aeromonas hydrophila* from different sources like fish *(Labeo rohita)*, raw cow milk, pond water, river water, raw meat of chicken and mutton in West Bengal, India were used in this study. *Aeromonas hydrophila* were isolated by using a 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.). Selected *Aeromonas hydrophila* colonies were subcultured in Tryptic Soya Broth (Difco) for further characterization.

DNA was isolated by overnight grown bacterial culture in 5ml TSB and centrifuge at 12000rpm for 5min at 4°C. Resuspended pellet in 0.2ml of TE-1 buffer and added 50ul lysozyme (3mg/ml) and keep at 37°C for 15min. then 50ul lysozyme was added with 50ul proteinase K (15mg/ml) and incubated at 56°C for 3-4hrs in a water bath. DNA were extracted by adding equal volume of TE saturated phenol by slow mixing and centrifugation 12000rpm for 5min at 4°C. With the supernatant, equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) was added and again centrifuged. To the supernatant, 200ul chloroform was added and supernatant was collected after centrifugation and 25ul of 5M NaCl. 250ul of chilled absolute alcohol was added and DNA was observed as bunch of threads. Then DNA was collected after wash with 70% alcohol and dried it. Pure DNA was dissolved in TE buffer and stored at 4°C for further use.

Total ten numbers of random primers, designated as OPA-1 to OPA-10 was used for PCR amplification. Out of these 10 primers, viz., OPA-03, OPA-09 and OPA-10 were selected for final screening because they only generated several reproducible amplicons and produced some distinct banding patterns on 1.5% agarose gel slab. In the present paper result of OPA-03 has been elaborated.

PCR amplification and analysis of phylogeny

PCR reactions was standardized by a series experiments and finally carried out by following the protocol of P.C.Thomas[12], 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.

PCR reaction was performed with Perkin-Elmer PCR system. Each cycle of initial denaturation at 94°C x 5 min. followed by 35 cycles at 94°C for 1min., 36°C x1min. and 72°C x 2min. and final extension at 72°Cx10 min. PCR amplified products were analyzed on 1.5% agarose gel.

Amplified DNA bands on the 1.5% agarose gel were analyzed by giving scores of zero and one for the absence or presence of bands 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.

Extraction of bacterial protein and SDS-PAGE

Aeromonas hydrophila were inoculated in 5 ml

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Tryptic soya broth for 28 hours at 37°C and cells were collected and centrifuged at 10000 rpm, 4°C, 10 minutes in 1.5mL eppendorf tubes. The supernatant was drained off and 100 µl of B-PER reagent (Bacterial Protein Extraction Reagent) was added to each of the pellets and vortexed. This was incubated at 4°C for 15minutes, mixing gently every five minutes. The cell suspension was then centrifuged at 10000 rpm, 4°C, 10 minutes and the supernatant was collected in eppendorf tubes which contains exocellular proteins and pellets were dissolved with 100 µl of PBS and vortexed, which contain cellular proteins and stored at -20°C for further use.12% SDS-PAGE was carried out in the present study for the total proteiomics analysis.

RESULTS

Aeromonas hydrophila produced yellow colonies in the RS-medium. Gram staining of these colonies gives gram negative reaction, microscopical analysis gives rod shaped, motile, biochemical tests gives oxidase positive, fermentative and antibiotic resistance tests concluded as novobiocin resisitant, primarily indicated that colonies are aeromonads. All isolates were confirmed to the species level *Aeromonas hydrophila* by *Automated Microbial Analyzer* (Biolog, US.).

Analysis of RAPD profile

The RAPD-PCR carried out by following of each cycle of initial denaturation at 94°C x 5 min. followed by 35 cycles at 94°C for 1min., 36°C x1min. and 72°C x 2min. and final extension at 72°Cx10 min. PCR amplified products were analyzed on 1.5% agarose gel, and it produced clear reproducible bands. Amplification of the DNA from each of the 30 isolates with the ten primers named earlier produced a variety of amplicons with distinct bands on 1.5% agarose gel after electrophoresis. The RAPD fingerprints of the isolates generated by these random primers OPA-03 is given in Figure (1-2). In this study, The RAPD fingerprint pattern was more or less unique for each isolates and each banding pattern indicates that there were a number of fragments, which were also homogenous between many of the isolates. These unique fragments may be utilized for the development of species-specific marker

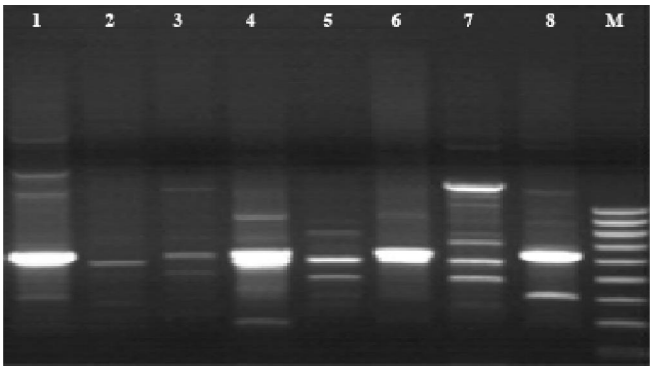

Figure 1 : RAPD profile of *Aeromonas hydrophila* isolates generated by primer OPA-03 using random primer and showing Variable polymorphic and unique DNA bands. Lane 1-11: Ah1- Ah11 isolates; M: Marker (Image analyzed by Gel Doc System, 2000, Biorad).

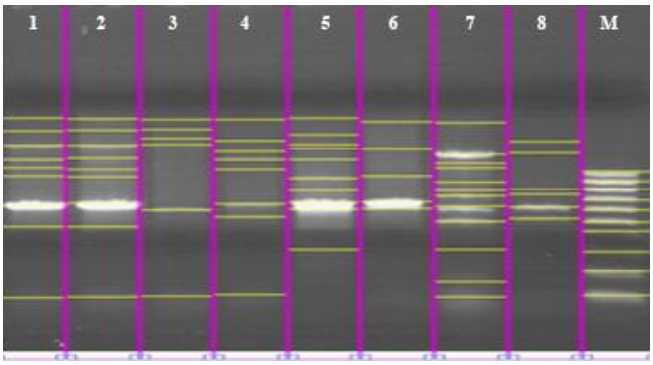

Figure 2 : Scanning of RAPD gel using Gel documentation system and visualization of DNA bands. (Total 70 bands were identified without marker lane).

Scanning of RAPD gel gives total of 70 bands where some scorable as distinct bands in the gel. Whereas many of the amplicons were produced by all the isolates, some of amplicons showed variation between isolates. Polymorphism of the RAPD pattern was quite apparent among the different isolates. The RAPD fingerprint pattern was unique for each of the isolates. Comparison of the amplicons at each loci indicated there were variety of polymorphism. However, perusal of the banding pattern indicates that there were a number of fragments, which were homogenous among many of the isolates. The analysis indicated that the overall polymorphism of these fragments may be utilized for species-specific marker development and other aeromonads can also be examined with these primers.

Analysis of cellular protein

Cellular proteins were extracted from the isolates and analyzed through 12% SDS-PAGE tech-

nique (Figure 3). No significant polymorphism was found (Other than in lane 4). Banding pattern of all the isolates were more or less uniform. Molecular weight marker was used to identify the molecular weight and density of each band of each lane in the gel of all the isolates in the study.

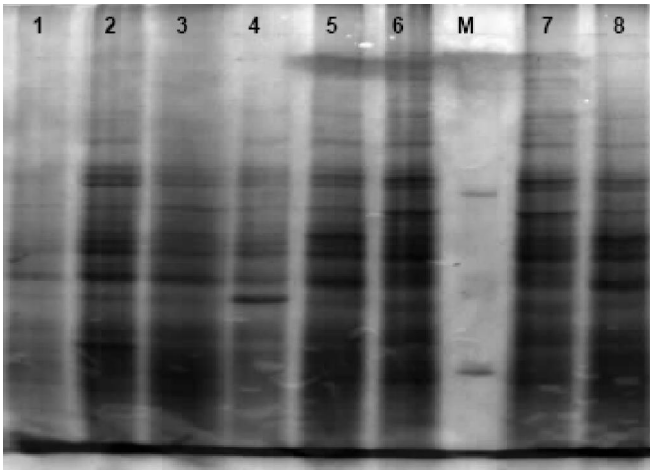

Figure 3 : Cellular protein profile of *A. hydrohila* resolved through SDS PAGE (12% resolving gel and 5% stacking gel) and stained with coomassie brilliant blue R250. Lane 1-06: Ah1- Ah 6 isolates; M: Marker.

DISCUSSION

A. hydrophila are phenotypically, serologically and genetically quite diverse, many conventional methods of identifying these microorganisms like cultural-biochemical properties 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[12]. All the isolates were typable using selected primers. But while performing screening for 10 primers, only primer 09 amplified with scorable bands and others had very poor reproducibility

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. Miyata et al.[10] observed that the DNA required for RAPD is less than one hundredth of the amount required for other methods. This study also performed to generate reproducible RAPD profiles with as little amount of DNA in the PCR reaction in the molecular characterization of *A. hydrophila* strains from different sources of West Bengal, India. Results of this study established the observation by other workers that motile aeromonads are genetically diverse[9]. RAPD-PCR fingerprints have been used for typing and diagnosis of bacteria. 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.

CONCLUSION

RAPD marker could be used as molecular markers of *A. hydrophila* pointed towards the amplicons generated by OPA-03. The species specificity can be confirmed only by checking these primes with isolates of other aeromonads. Large number of RAPD genotypes can be an ideal method for species identification. The results show that RAPD fingerprinting is on of the best molecular tool for identification but protein profiling has some limits to differentiate *Aeromonas* species, and the present study indicates that SDS-PAGE technique is not suitable for the characterization within a species.

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