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Genetic Analysis of *Aeromonas hydrophila* MTCC 646 by Random Amplified polymorphic DNA

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RAPD profiling of *Aeromonas hydrophila* MTCC 646 strain was performed in order to analyze the possible genetic variability in this bacterium, the causative agent of epizootic ulcerative syndrome and hemorrhagic septicaemia. Out of one hundred sixty random decamer primers of OPA, OPB, OPC, OPE, OPF, OPJ, OPK and OPY screened against genomic DNA of *A. hydrophila*, sixty four amplified good, consistent and reproducible fingerprints. The greatest number of fragments was found with the use of primers OPE 15 (8 bands) and OPK 07 (9 bands). The molecular weight of the amplified products of *A. hydrophila* MTCC 646 against all the tested primers ranged from 0.22 kb to 3.4 kb. The clear, distinct and reproducible DNA fragments of selected primers can be used as standard for identifying new strains of *A. hydrophila*.

Key words: *Aeromonas hydrophila*, RAPD-PCR, Genomic DNA, Primer.

Aeromonas spp. has been known to be pathogenic to reptiles, fish and other cold-blooded animals causing haemorrhagic and ulcerative diseases and septicaemia[1-4]. *A. hydrophila* is gram negative, facultative anaerobic fresh water bacterium which is pathogenic in human beings also[5-8]. Majority of the isolates of *A. hydrophila* and related aeromonads secrete extracellular products, some of which, such as aerolysin, enterotoxins, proteases and acetylcholinesterase, have been characterized and considered as virulence factors in pathogenesis[7, 9-13]. Since *A. hydrophila* was first recognized as a significant opportunistic pathogen for humans, many efforts were made to correctly identify and classify various species belonging to this genus[14-15]. Amplification of specific DNA for PCR provides a highly sensitive and specific tool for identification and strain differentiation, crucial for epidemiological studies. Discriminative methods based on genotypic differences are not affected by the physiological state of the organism and can be easily standardized. Random amplified polymorphic DNA (RAPD) patterns have been successfully employed for discriminating strains of a number of bacterial fish and shellfish pathogens[16-18]. The present study was directed to determine the RAPD fingerprint profiles of *A. hydrophila* MTCC 646, which will useful for identifying the unknown strains of *A. hydrophila*.

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MATERIAL AND METHODS

A. hydrophila reference strain MTCC 646 was procured from Microbial Type Culture Collection Center, Institute of Microbial Technology, Chandigarh, India, grown in Tryptone soya broth (TSB) and incubated at 37°C for 24h in shaking incubator. The pure cultures were maintained on Tryptone soya agar (TSA) slant for extraction of genomic DNA.

DNA isolation and RAPD assays

Generic DNA from *Aeromonas hydrophila* MTCC 646 was obtained from an overnight culture grown on TSB medium as reported by Sambrook *et al.,*[19] with slight modifications. For isolation of genomic DNA, 1.5 ml culture was microcentrifuged at 13000g for 5 min. The supernatant was discarded and another 1.5 ml culture added to the pellet, centrifuged again. The combined pellets were suspended in 367 µL of Tris-EDTA (TE) buffer (10 mM Tris, 1 mM EDTA , pH 8.0), 30 µL of 10% SDS and 5 µL of proteinase K (20 mg mL^{-1}). The suspension was thoroughly mixed and incubated at 37°C for 2 hours[19]. The DNA sample was then extracted with phenol: chloroform: isoamyl alchol (25:24:1). The aqueous phase recovered and precipitated with 1/20 volume of 3M sodium acetate (pH 5.2) with double volume of isopropanol. After precipitation the samples were centrifuged at 13000 g for 10 min at 4°C. Then the pellet was washed with 70% alcohol twice and finally dissolved with 40 µL of Tris-EDTA buffer and stored at 4°C. Absorbance solution was read at 260 and 280 nm in UV-spectrophotometer to determine the concentration and purity. The Genomic DNA sample was also examined on an 0.8% (w/v) agarose gel to check it. The genomic DNA had not become excessively fragmented and stored at-20°C for further use.

The RAPD reaction was conducted in a total volume of 25 µL containing 3 U Taq DNA polymerase, 2.5mM dNTPs mix, 25mM $MgCl_{2}$, 10X PCR buffer, bacterial DNA solution (50ng) and oligonucleotide primer (20 Pmol), at 95°C for 5 min, followed by 40 cycle of denaturation at 95°C for 45 sec, annealing at 36°C for 45 sec and extension at 72°C for 1 min 30 sec. The final extension was carried out at 72°C for 7 min in a thermocycler. Six microlitre of the reaction product was analyzed in a 1.2% agarose gel along with 1.0 kb DNA ladder and visualized by ethidium bromide staining. The images of DNA fingerprinting of the 1.2% run gel was stored in a floppy disc using Gel documentation system for further analysis. Oligonucleotide primers were purchased from Operon Technologies Inc. Eight series of decamer primers (OPA, OPB, OPC, OPE, OPF, OPJ, OPK and OPY) were taken for amplification; primers amplifying more than 2 bands were chosen for screening. Selected oligonucleotide primers and their sequences used in this experiment are shown in Table 1.

Table 1. Nucleotide sequence (5'-3') of various primers used in the study.

S. No.	Primer code	Nucleotide sequence (5'-3')
1.	OPC-06	GAACGGACTC
2.	OPC-07	GTCCCGACGA
3.	OPC-09	CTCACCGTCC
4.	OPC-10	TGTCTGGGTG
5.	OPC-11	AAAGCTGCGG
6.	OPC-12	TGTCATCCCC
7.	OPC-13	AAGCCTCGTC
8.	OPC-15	GACGGATCAG
9.	OPC-16	CACACTCCAG
10.	OPE-02	GGTGCGGGAA
11.	OPE-07	AGATGCAGCC
12.	OPE-08	TCACCACGGT
13.	OPE-09	CTTCACCCGA
14.	OPE-14	TGCGGCTGAG
15.	OPE-15	ACGCACAACC
16.	OPE-16	GGTGACTGTG
17.	OPE-19	ACGGCGTATG
18.	OPE-20	AACGGTGACC
19.	OPF-20	GGTCTAGAGG
20.	OPK-01	CATTCGAGCC
21.	OPK-02	GTCTCCGCAA
22.	OPK-07	AGCGAGCAAG
23.	OPK-08	GAACACTGGG
24.	OPK-09	CCCTACCGAC
25.	OPK-11	AATGCCCCAG
26.	OPK-12	TGGCCCTCAC
27.	OPK-13	GGTTGTACCC
28.	OPK-15	CTCCTGCCAA
29.	OPK-16	GAGCGTCGAA
30.	OPK-19	CACAGGCGGA
31.	OPK-20	CTGTCGCGAG
32.	OPA-01	CAGGCCCTTC
33.	OPA-02	TGCCGAGCTG
34.	OPA-03	AGTCAGCCAC
35.	OPA-04	AATCGGGCTG
36.	OPA-07	GAAACGGGTG

37.	OPA-09	GGGTAACGCC
38.	OPA-10	GTGATCGCAG
39.	OPA-11	CAATCGCCGT
40.	OPA-12	TCGGCGATAG
41.	OPA-13	CAGCACCCAC
42.	OPA-14	TCTGTGCTGG
43.	OPA-15	TTCCGAACCC
44.	OPA-16	AGCCAGCGAA
45.	OPA-18	AGGTGACCGT
46.	OPA-19	CAAACGTCGG
47.	OPA-20	GTTGCGATCC
48.	OPB-01	GTTTCGCTCC
49.	OPB-04	GGACTGGAGT
50.	OPB-05	TGCGCCCTTC
51.	OPB-06	TGCTCTGCCC
52.	OPB-10	CTGCTGGGAC
53.	OPB-11	GTAGACCCGT
54.	OPB-12	CCTTGACGCA
55.	OPB-13	TTCCCCCGCT
56.	OPB-14	TCCGCTCTGG
57.	OPB-15	GGAGGGTGTT
58.	OPB-16	TTTGCCCGGA
59.	OPY-03	ACAGCCTGCT
60.	OPY-04	GGCTGCAATG
61.	OPY-05	GGCTGCGACA
62.	OPY-13	GGGTCTCGGT
63.	OPJ-18	TGGTCGCAGA
64.	OPJ-20	AAGCGGCCTC

RESULTS AND DISCUSSION

Eight operon primer series as OPA, OPB, OPC, OPE, OPF, OPJ, OPK and OPY including 160 individual primers were originally assessed for RAPD PCR and 64 primers were used on their ability to produce consistent and distinguishable fragment patterns. The results of RAPD analyses are shown in Fig 1 and 2. OPA–20 (6 bands), OPC-11(6), OPC-13(7), OPJ-20(6), OPE-2(6), OPE-7(6), OPE-14(7), OPE-15(8), OPE-19(6), OPE-20(6), OPK-07(9), OPK-12(6), OPK-16(6) and OPK-20(7). In OPK series of primers, OPK-12 and OPK-15 amplified bands of *A. hydrophila* was quite similar, but bands amplified by primers OPK-1, OPK-2, OPK-8 and OPK-20 were quite different and showed greatest difference (Fig. 1). Delamare *et al*.,[20] studied RAPD analysis of the DNA of *A. hydrophila* CECT 839 and *A. trota* ATCC 49657 by using 40 decamer primers of kits OPA and OPB of operon technologies and he found that 14 primers as OPA-02, OPA-04, OPA-09, OPA-11, OPA-19, OPB-04, OPB-06, OPB-07, OPB-10, OPB-12, OPB-13, OPB-15, OPB-16 and OPB-17 gave at least three to four intense amplification products and chosen for the amplification of the other *A. hydrophila* and *A. trota* strains respectively. In OPA series, bands amplified using OPA-16 and OPA-19 were quite similar but bands amplified using OPA-3 and OPA-4 were different from each other (Fig. 2). The molecular range of the amplified product of *A. hydrophila* reference strain (MTCC 646) against OPA, OPB, OPC, OPY, OPJ, OPE, OPK and OPF ranged as 0.24 kb to 3.4 kb, 0.3 to 2.3, 0.27 to 0.29, 0.23 to 2.4, 0.8 to 1.95, 0.22 to 2.4 and 0.4 to 1.75 kb respectively. Over all the amplified bands in all the primers varied between 0.22 kb to 3.4 kb.

There was close similarity in the amplified fragments of *A. hydrophila* reference strain, when analyzing with several random primers. 1.1 kb fragment was common when amplified using primers as OPC-6, OPC-9, OPC-11, OPC-12, OPC-13, OPK-2, OPK-15, OPE-9, OPA-2, OPA-3, OPA-9, OPA-10, OPB-4, OPB-5, OPB-11, OPB-15, OPB-16, OPB-5, OPY-13 and OPJ-20; 1.2 kb amplified band was common in OPE-7, OPE-14, OPE-15, OPE-16, OPE-20, OPC-16, OPK-11, OPK-19, OPA-12, OPA-13, OPB-1 and OPB-6; 1.3 kb was common in OPC-7, OPC-10, OPC-11, OPC-15, OPE-2, OPE-8, OPE-20, OPK-2, OPK-8, OPK-20, OPA-8, OPA-11, OPA-14, OPA-16, OPB-4, OPB-5, OPB-6, OPB-16, OPY-13 and OPJ-20; 1.4 kb was common in OPC-9, OPC-15, OPE-2, OPF-20 and OPK-11; 1.6 kb was common in OPC-10, OPK-2, OPK-15, OPK-19, OPY-13 and OPA-15; 1.7 kb was common in OPC-6, OPC-15, OPK-20, OPA-2, OPA-4, OPA-7, OPA-13, OPA-14, OPB-5, OPB-13 and OPB-15; 1.8 kb was common in OPF-20, OPK-13, OPE-14, OPA-18, OPA-19 and OPJ-20; 1.9 kb was common in OPE-19, OPE-20, OPC-7, OPB-6, OPB-10, OPB-12, OPB-15, OPY-5, OPA-4, OPA-11, OPA-12, OPA-13, OPA-16 and OPA-20; 2.1 kb amplified band was common in OPA-13, OPA-14 and OPY-4 respectively.

The technique described here can be used for initial identification of species or strains of *A. hydrophila*. After an initial isolation and characterization by plating of presumably contaminated samples from fish, reliable results

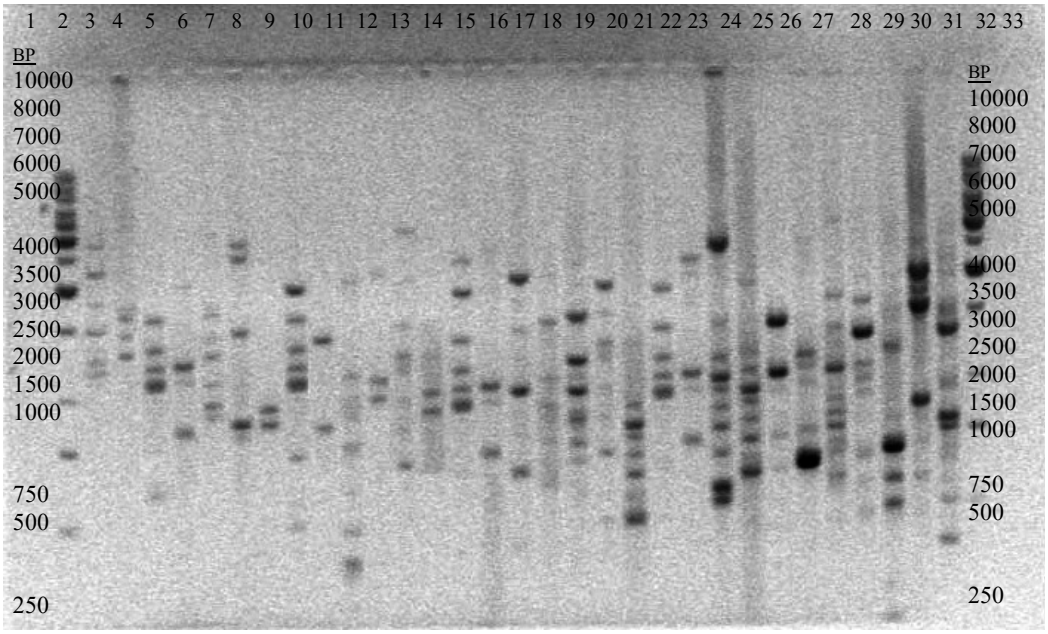

Fig 1. RAPD profile generated by PCR using *A. hydrophila* reference strain (MTCC 646) with OPC, OPE, OPF and OPK primers. (L-R) Lane 1-1kb ladder, Lane 2-10- OPC 6, OPC 7, OPC 9, OPC 10, OPC 11, OPC 12, OPC 13, OPC 15, OPC 16, Lane 11-19- OPE 2, OPE 7, OPE 8, OPE 9, OPE 14, OPE 15, OPE 16, OPE 19, OPE 20, Lane 20-OPF 20, Lane 21-32-OPK 1, OPK 2, OPK 7, OPK 8, OPK 9, OPK 11, OPK 12, OPK 13, OPB 15, OPK 16, OPK 19, OPK 20, Lane 33-1 kb ladder.

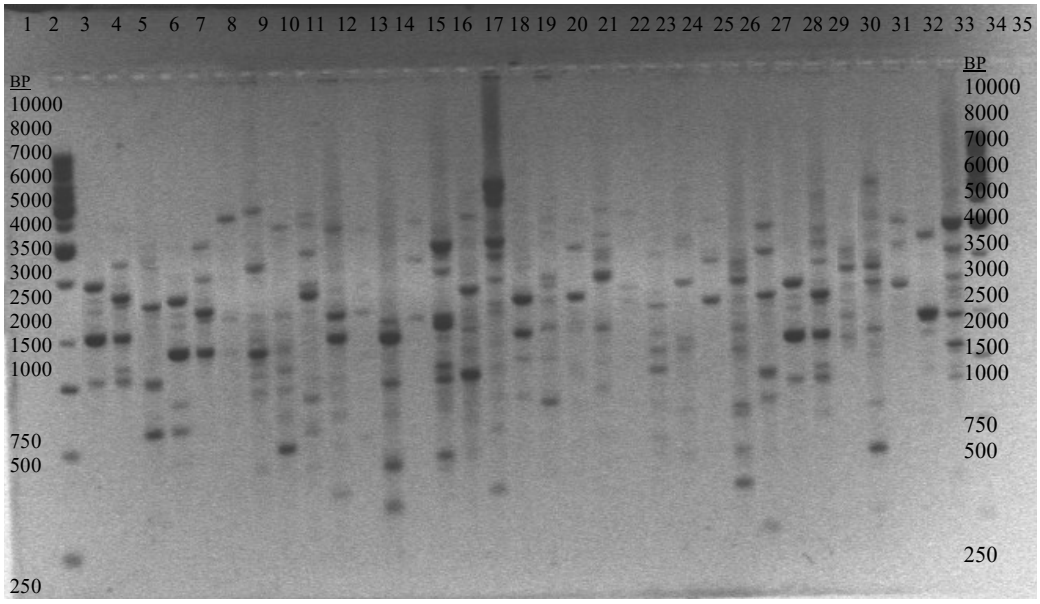

Fig 2. RAPD profile generated by PCR using *A. hydrophila* reference strain (MTCC 646) with OPA, OPB, OPY and OPJ primers. (L-R) Lane 1-1kb ladder, Lane 2-17- OPA 1, OPA 2, OPA 3, OPA 4, OPA 7, OPA 9, OPA 10, OPA 11, OPA 12, OPA 13, OPA 14, OPA 15, OPA 16, OPA 18, OPA 19, OPA 20, Lane 18-28- OPB 1, OPB 4, OPB 5, OPB 6, OPB 10, OPB 11, OPB 12, OPB 13, OPB 14, OPB 15, OPB 16, Lane 29-32- OPY 3, OPY 4, OPY 5, OPY 13, Lane 33-34- OPJ 18, OPJ 20, Lane 35-1kb ladder.

confirming the presence or absence of the pathogenic organism can be confirmed by RAPD PCR. O'hIci *et al*.,[21] described that the RAPD technique produced 100 loci for strain comparison, which was twice in number of scrorable loci produced by PFGE. Based on this results, modifications of both the RAPD and PFGE conditions or other genotype methods producing a lower number of camparable loci may be more suitable for the identification of strain clusters of epizootiological significance among the atypical *A.salmonicida* strains. The result obtained with the RAPD analysis showed that this molecular method was sensitive enough to reveal inter and intraspecific genetic differences among *A. hydrophila* strains. More over, the RAPD PCR is easier to adopt and more sensitive than other molecular techniques[22]. Genotyping system including PFGE and RAPD analysis may prove to be useful tools for epidemiological studies on *A. hydrophila* and *A. sorbia* isolated from different sources. Based on RAPD PCR, the genomic variations between the bacterial species plus the genomic polymorphism between the bacterial isolates can be identified by the differences in the sizes and numbers of DNA fragments. Lee *et al*.,[23] studied RAPD profiles of eight Korean isolates of *A. hydrophila* isolates from rainbow trouts and the type strain produced various DNA fragments using 5 random decamer primers and no species-specific fragments are produced. As expected, the arbitrary primers used in this study for *A. hydrophila* MTCC 646; can be used to study and characterize the different strains of *A. hydrophila*.

CONCLUSION

In the present study, the genotyping of the reference strain of *A. hydrophila* (MTCC 646) by RAPD revealed a number of operon series primers can be used for strain differentiation and epidemiological studies. The RAPD PCR can be used as a rapid, inexpensive and reliable system to help in identification and taxonomy of the *Aeromonas* isolates. The common typing bands as revealed by various primers will further be useful for preliminary identification purposes for unknown strains of *A. hydrophila*.

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