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Typing of clinical and environmental strains of *Aeromonas* spp. using two PCR based methods and whole cell protein analysis

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ABSTRACT

Two PCR based typing methods i.e. random amplified polymorphic DNA analysis (RAPD) and enterobacterial repetitive intergenic consensus sequence (ERIC)-PCR were evaluated for typing of 42 *Aeromonas* isolates from clinical and environmental sources and whole cell protein (WCP) profiles were analyzed. Both RAPD and ERIC-PCR showed a high level of genetic diversity. Numerical index of the discriminatory (D) values were 0.94 and 0.96 (>0.90) for RAPD and ERIC-PCR, respectively. No correlation in banding pattern and evidence of genetic similarity was found between *Aeromonas* isolates from environmental and clinical sources. Therefore these techniques are highly reproducible and sensitive methods for typing the *Aeromonas* isolate from different sources. WCP profile showed two major variable regions i.e. 20 kDa to 45 kDa region and 70 kDa to 85 kDa region. Though WCP profiling had less discriminatory power, use of this method in combination with other established typing methods such as RAPD and ERIC-PCR may be helpful for reliable typing of *Aeromonas* isolates or to identify new proteins with pathogenic potential.

1. Introduction

Members of the genus *Aeromonas* are facultatively anaerobic, oxidase-positive, gram-negative bacteria whose natural habitat is the aquatic environment. Aeromonads are considered opportunistic pathogens, capable of producing disease in fish or as secondary invaders in fish suffering from other diseases (Camus et al., 1998). Some species are pathogenic for animals and humans (Martin-Carnahan and Joseph, 2005). Clinical stains of *Aeromonas* are responsible for gastroenteritis, wound infections, respiratory tract infections, peritonitis, urinary tract infections, and septicemia in humans (Altwegg, 1999; Austin and Adams, 1996; Janda, 1991). The most common *Aeromonas* species which are associated with clinical infections are *Aeromonas hydrophila*, *Aeromonas caviae* and *Aeromonas veronii* (Janda and Abbott, 1998).

Epidemiological investigations and food safety management programs would require the ability to study genetic relationships between isolates from different sources. Traditionally serotyping of bacterial isolates was performed for epidemiological purposes, but serotyping has limited ability to discriminate between isolates and the availability of antisera would be a major constraint. Typing of microorganisms using DNA methods is very rapid, sensitive and gives information on the genetic relatedness of strains, the source of infection, virulence of strains and the geographical and host distribution of possible variants. Random amplification of polymorphic DNA (RAPD) and enterobacterial repetitive intergenic consensus sequence (ERIC)-PCR are the most commonly accepted methods due to the high level of sensitivity (Davin-Regli et al., 1998; Szczuka and Kaznowski, 2004; Aguilera-Arreola et al., 2005). In addition, PCR-restriction fragment length polymorphism (RFLP) (Abdullah et al., 2003; Soler et al., 2003) of 16S rRNA genes, repetitive extragenic palindromic sequence (REP)-PCR (Soler et al., 2003; Szczuka and Kaznowski, 2004), pulsed-field gel electrophoresis (PFGE) (Abdullah et al., 2003) and whole cell protein fingerprinting followed by sodium dodecyl sulfatepolyacrylamide gel electrophoresis (SDS-PAGE) (Alavandi et al., 2001; Delamare et al., 2002) are also applied for typing. SDS-PAGE of WCP profiling is considered as a rapid, inexpensive and reliable system for identification and taxonomy of *Aeromonas* isolates (Delamare et al., 2002). There are only few comparative studies on the reproducibility and discriminatory power of molecular typing methods in typing *Aeromonas* isolates which are important for deciding on the most appropriate method for any situation. In the present study we evaluated the PCR based typing methods such as RAPD and ERIC-PCR and WCP profiling for typing the clinical and environmental strains of *Aeromonas* species.

2. Materials and methods

2.1. Bacterial cultures and growth

Seven different species of *Aeromonas* i.e. *A. hydrophila*, *A. veronii* biovar *sobria*, *A. veronii* biovar *veronii*, *A. caviae*, *A. trota*, *A. schubertii*, *A. jandaei*, and *A. sobria* were used in this present study. Details of those isolates are listed in Table 1. Environmental strains of *Aeromonas* were

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Table 1
List of isolates used in this study.

Culture name	Source		Stain name
A. hydrophila ($n=16$)	Clinical ($n=5$)	NICED	F20002
		NICED	F12044
		NICED	F15309
		NICED	F22162
		NICED	F17367
	Environmental ($n=11$)	Fish	Ah 12840 (111)
		EUS infected fish	Ah 41
		Tail rot of fresh water fish	Ah 65
		Tail rot of fresh water fish	Ah 71
		Fresh water pond	Ah G49
		Wounded fish (catla)	Ah 38
		UDS fish	Ah UDS
		Infected fish (catla)	Ah 40
		Fish	Ah A
		Hemolysin mutant	Ah AB-3-5-2
		EUS infected fish	Ah (68)
A. veronii biovar *sobria* ($n=5$)	Clinical ($n=5$)	NICED	VTE416
		NICED	F02310
		NICED	F05975
		NICED	F06537
		NICED	F07100
A. veronii biovar *veronii* ($n=5$)	Clinical ($n=5$)	NICED	NT3818
		NICED	VTE338
		NICED	NT3871
		NICED	VTE355
		NICED	VTE599
A. caviae ($n=6$)	Clinical ($n=6$)	NICED	GB1371
		NICED	F05710
		NICED	F04055
		NICED	F12845
		NICED	NT3758
		NICED	NT3762
A. trota ($n=5$)	Clinical ($n=5$)	NICED	F06662
		NICED	F09538
		NICED	F15184
		NICED	F15337
		NICED	NT3817
A. jandaei ($n=1$)	Clinical ($n=1$)	NICED	GB912
A. schubertii ($n=3$)	Clinical ($n=3$)	NICED	F15699
		NICED	G14886
		NICED	VTE599
A. sobria ($n=1$)	Environmental ($n=1$)	Fish	As 77

NICED, National Institute of Cholera and Enteric Diseases, Kolkata, India; EUS, Epizootic ulcerative syndrome; UDS, ulcerative disease syndromes.

isolated from the southwest coast of India and the clinical isolates were kindly provided by the National Institute of Cholera and Enteric Diseases (NICED), Kolkata. They were isolated from stool samples collected from patients with acute diarrhea admitted in the Infectious Diseases Hospital in Kolkata. Isolated oxidase-positive yellow colonies from selective Rimler–Shotts (RS) medium (HiMedia, Mumbai) were subjected to a battery of biochemical tests (Abbott et al., 2003). Further all the isolates of *A. hydrophila* were confirmed by PCR amplification of *lip* gene (Cascon et al., 1996). Bacterial cultures were maintained in our laboratory stock deep freezer (Sanyo Corporation, Japan) at −80 °C in Luria Bertani (LB) broth (HiMedia) with 30% of glycerol (v/v). The cultures were retrieved in brain heart infusion (BHI) broth (HiMedia) by incubating at 30 °C for overnight with constant shaking and used for the study.

2.2. Genomic DNA isolation

Aeromonas isolates were grown in 3 ml LB broth at 37 °C overnight with shaking. DNA was extracted by cetyl trimethyl ammonium bromide (CTAB)-proteinase K method (Ausubel et al., 1995). Briefly, about 1.5 ml of the culture was taken in a microcentrifuge tube and centrifuged at 10,000 rpm for 10 min. The pellet was resuspended in 567 µl of TE buffer (10 mM Tris–HCl, pH 8.0; 1 mM EDTA) with 30 µl of 10% (w/v) SDS and 3 µl Proteinase K (20 mg/ml) (Bangalore Genei, Bangalore, India). The cells were allowed to lyse for 1 h at 37 °C. Then, 100 µl of 5 M NaCl and 80 µl of CTAB–NaCl was added and incubated for 10 min at 65 °C. DNA thus released was purified by extraction with chloroform-isoamyl alcohol (24:1) followed by extraction with phenol-chloroform-isoamyl alcohol (25:24:1). The purified DNA was then precipitated with 0.6 volumes of isopropanol and washed once with 70% ethanol. The DNA pellet was dried under vacuum and finally resuspended with 1× Tris–EDTA (TE) buffer (pH 8.0). Concentration and purity of the DNA were measured using a NanoDrop® spectrophotometer (ND-1000, V3.3.0, USA).

2.3. RAPD

Four oligonucleotide primers (CRA22, CRA23, CRA25 and CRA26; Neilan, 1995) were tested for typing of *Aeromonas* isolates by RAPD. In RAPD analysis, the target sequence(s) to be amplified is unknown. A single short primer with an arbitrary sequence is used for performing PCR. Amplification takes place when multiple copies of a single arbitrary primer anneal in a particular orientation (such that they point towards each other) and within a reasonable distance of one another. Details of the RAPD primers used in this study are described in Table 2. Among them CRA22 produced a good typable banding pattern to discriminate the strains. Hence, this primer was selected for further analysis. Polymerase chain reactions (PCR) were carried out in a 50 µl containing 5 µl of 10× buffer (100 mM Tris–HCl pH 8.3, 20 mM $MgCl_2$, 500 mM KCl, 0.1% gelatin) (Bangalore Genei, India), each of the four deoxynucleotide triphosphates at a concentration of 100 µm, 50 pmol of primer and 2 U of *Taq* polymerase (Bangalore Genei, India). Programmable thermocycler (MJ Research, USA) was used for nucleic acid amplification with a program consisting of initial delay at 95 °C for 5 min followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 45 °C for 30 s, primer extension at 72 °C for 1 min and final delay at 72 °C for 5 min. The electrophoresis of PCR amplified products was performed on 1.8% of agarose gel stained with ethidium bromide and photographed using a gel documentation system (Herolab, Weisloch, Germany).

2.4. ERIC-PCR

ERIC-PCR was performed in a 50 µl reaction containing 5 µl of 10× PCR buffer (Bangalore Genei, India), 100 µm of each of the four deoxynucleotide triphosphates, 50 pmol of each primer (Millemann et al., 1996) (Table 2) and 2 U of *Taq* polymerase (Bangalore Genei, India). The thermocycling conditions included denaturation at 94 °C for 30 s, annealing at 52 °C for 1 min and primer extension at 65 °C for 8 min for 30 cycles with an initial denaturation at 95 °C for 7 min and final delay at 65 °C for 16 min. Amplified DNA bands were resolved on 1.8% of agarose gel followed by staining with ethidium bromide and

Table 2
Details of the primers used in this study.

Primer name	Primer sequence (5′–3′)	Source/reference
CRA22	CCGCAGCCAA	Neilan (1995)
CRA23	GCGATCCCCA	Neilan (1995)
CRA25	AACGCGCAAC	Neilan (1995)
CRA26	GTGGATGCGA	Neilan (1995)
ERIC-PCR (sense)	ATGTAAGCTCCTGGGGATTCAC	Millemann et al. (1996)
ERIC-PCR (anti-sense)	AAGTAAGTGACTGGGGTGAGCG	Millemann et al. (1996)
lip (sense)	AACCTGGTTCCGCTCAAGCCGTTG	Cascon et al. (1996)
lip (anti-sense)	TTGCTCGCCTCGGCCCAGCAGCT	Cascon et al. (1996)