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EXPERIMENTAL ARTICLES

Genotyping of *Aeromonas hydrophila* by Box Elements[1]

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Abstract—PCR-based DNA fingerprinting techniques were evaluated to genotype eight diseased, particularly normal and environmental isolates of *Aeromonas hydrophila*. PCR-based fingerprinting method has an advantage of having repetitive sequence also called Box elements that are interspersed throughout the genome in diverse bacterial species. The BOX-PCR fingerprinting technique was evaluated for the discrimination of different isolates of *A. hydrophila*. All the studied isolates have shown major banding patterns ranged from 500–3000 bp. These finding could be advantageous to investigate the strain level specific fingerprints of *A. hydrophila* as potential genotypic markers.

Key words: *Aeromonas hydrophila*, diseases, BOX-PCR, genotyping.

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Aeromonas hydrophila is an opportunistic and zoonatically important bacterial pathogen belongs to family *Aeromonadaceae* [1] and is associated with several diseases of fish, such as hemorrhagic septicemia, fin and tail rot [2]. Conventional identification of *A. hydrophila* is achieved through standard biochemical tests that are time consuming, laborious and also are not always conclusive. Earlier, motile aeromonads have been isolated and characterized from aquatic environment [3]. Several isolates of *A. hydrophila* were recovered from fish and water samples. All the isolates were tested for antibiotic sensitivity and characterized [4]. However, these methods are not accurate for genotyping at strain level identification. Consequently, a need arises for molecular methods for rapid and accurate discrimination of isolates.

The recently developed DNA fingerprinting methods were based on the enterobacterial repetitive intergenic consensus sequence (ERIC), the repetitive extragenic palindromic sequence (REP) and the BOX element have been investigated [5]. Box elements are located outside these genes, an unexpected situation because of the constraints they would impose on protein structure, as pointed for other highly repeated sequences. Box element act at the mRNA level, which is important for Box function a secondary structure rather than primary sequences. Box elements are repetitive sequence elements present in bacterial genomes.

The discovery of a group of highly conserved DNA sequence located in the case studied within intergenic region of the chromosome of the Gram positive *Streptococcus pneumoniae*. Its genome contains approximately 25 of these types of element called Box. The 5' to 3' Box elements are composed of three subunits (BoxA, BoxB and BoxC), which are 59, 45 and 50 nucleotide long, respectively. The Box elements containing one, two or four copies of the BoxB have been observed. Box element sequences have the potential to form stable stem loop structure and minimum one of these is transcribed. Box sequences are regulatory elements shared by several coordinately genes, which include competence specific and virulence related genes [6].

Previously, repetitive-element PCR (rep-PCR) with primers based on repetitive extragenic palindromic (REP) and enterobacterial repetitive intergenic consensus (ERIC) repeated DNA sequences has been reported for genomic finger-printing of *Bartonella* species [7]. RFLP, ERIC and REP have been evaluated for typing isolates of *A. popoffii* from different geographical origins [8]. BOX-PCR fingerprinting technique has been evaluated for the discrimination of strains of some *Aeromonas* species [5]. Single BoxA1

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PCR primers target the repeats and could be used to generate the fingerprint of any bacterial species.

The present study was used to investigate its utility for genotyping of *A. hydrophila* isolates from diseased and normal samples. Box-PCR assay targeted to repetitive intergenic sequences elements of *A. hydrophila* and reported to produce species specific and strain specific genomic fingerprints.

MATERIALS AND METHODS

Bacterial isolates, media and chemicals. All these isolates of *A. hydrophila* used in the genotyping which have been already characterized on the basis of amplification of aerolysin gene [9]. All these isolates of *A. hydrophila* were preserved 15% glycerol in −80°C in the laboratory. *Taq* DNA polymerase, dNTPs, Proteinase K, DNA ladders were obtained (Fermentas) and other media, chemicals were obtained (Sigma, SRL, Himedia). The primers were synthesized from Integrated DNA Technology (IDT, United States).

Isolation of genomic DNA. The isolation of genomic DNA from *A. hydrophila* was done as per the method described by Hiney et al. [10] with some modification. Briefly, a single colony of *A. hydrophila* was inoculated in 2 ml of Nutrient broth (NB) medium and grown at 30°C for over night. Well-grown culture was harvested by centrifuged at 10 000 rpm for 2 min at 4°C. The total 500 µl lysis buffer (100 mM Tris pH 8.0, 10 mM EDTA pH 8.0, 1.25% NaCl and 0.25% Sucrose) was added in the pellet and mix properly. It was incubated at 65°C for 1 hour. The 10 µl proteinase K (10 mg/ml) was added and invert gently again incubated at 37°C for one hour. Equal amount of phenol: chloroform: isoamylalcohol (25 : 24 : 1) was added to cell lysate and mixed by the inversion of the tube properly. The suspension was centrifuged at 10000 rpm for 10 min at 4°C. The aqueous layer from the top was removed carefully to avoid any protein debris and transferred to new tube. This step repeats twice times. Add 1/10 volume sodium acetate (pH 5.2) and two volume of chilled ethanol was added to aqueous phase so as to precipitate the DNA and incubated at −20°C for 30 min. The DNA was pelleted by centrifugation at 10000 rpm for 20 min at 20°C. The pellet was washed with 1 ml of 70% ethanol and air dried and dissolved in 50 µl of TE buffer (pH 8.0) and stored at −20°C for further molecular study.

PCR amplification of Box elements. BOX-PCR was performed according to the protocol of Coenye et al. [11]. The Box-PCR was performed in 50 µl reaction mixture with consisted of 10 ng of genomic DNA, 2.0 U of *Taq* DNA polymerase, 5 µl of 10× PCR amplification buffer (100 mM Tris HCl, 500 mM KCl pH 8.3), 300 mM dNTP, 10 pmoles single BoxA primer (5'TACGGCAAGGCGACGCTGACG3') and 1.5 mM $MgCl_2$ was used. Amplification included initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation 94°C for 30 s, annealing temperature of primers at 48°C for 1 min and extension at 72°C for 2 min. A final extension at 72°C for 10 min was used. Twenty µl of the PCR amplified was loaded on 2.5% agarose gel with ethidium bromide at 8 V/cm and the reaction product were visualized under Gel doc/UV transilluminator. The 100 bp DNA ladder was used as size estimation.

Construction and analysis of phylogeny. It is intensive to relate the relative concentration of bands between fingerprints, discontinuous noise and overall density of fingerprints. Box-PCR patterns, a band-matching algorithm (Match-matching tolerance of 1.0%) were used to calculate pairwise similarity matrix with the similarity coefficient. Cluster analysis of similarity matrices were performed by the unweighted pair group method using arithmetic average (UPGMA). The major DNA bands were considered for construction of phylogenetic tree with the help of Tools for population genetic analysis (TFPGA).

RESULTS AND DISCUSSION

In Box-PCR there are two common bands (500 bp and 1000 bp), which were present in all the isolates of *A. hydrophila*. Several other bands were also amplified and used for generating the phylogenetic relationship on the basis of results. The difference in the intensity of the same size in different isolates resulted from point mutation; the template leads to reduced stability or poor annealing of the primer to these templates. In this study, we have used different annealing temperature and $MgCl_2$ concentrations. The best result was obtained while using the 48°C annealing temperature and 1.5 mM concentration of $MgCl_2$ (Fig. 1). In our study, the isolates AHPN3-5, AH19, AH21 and AH16 amplified better and given more prominent bands in comparison to other isolates. In our previously reported isolate AH14 *A. hydrophila* has been potentially pathogenic to fish based on molecular level amplification of aerolysin and hemolysin genes which was present in same isolates but in distinct location [12, 13] and it showed similar banding pattern in Box PCR with AH13. In the present study critical examination of the Box-PCR results were analyzed and showed that 20 isolates could be placed in 6 groups. Eight of similar biochemical tests yields a similar banding pattern. Box-PCR, using a primer is specific to the highly conserved repeated sequence. It has also been reported to be useful for the discrimination of *A. hydrophila* isolates. Novel Box repeat PCR assay has been described for high resolution typing of 28 strains of *Streptococcus pneumoniae* [14].

The 120 strains isolated from stool specimens collected from humans suffering from gastroenteritis and from environmental samples have been analyzed by PCR RAPD, REP-PCR and ERIC-PCR. Species of *A. hydrophila, A. bestiarum, A. salmonicida, A. caviae, A. media*, and *A. veronii* revealed clonal structure. No genetic similarity between clinical and environmental

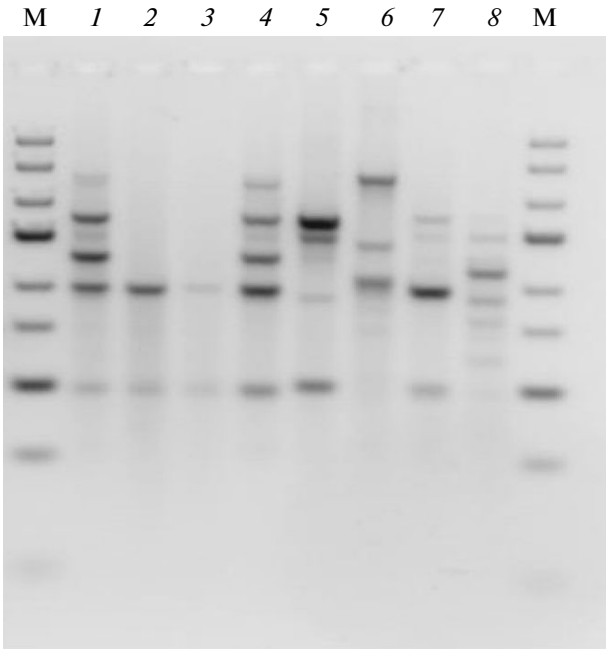

Fig. 1. PCR amplification of BoxA element of *A. hydrophila*. Lane M: DNA ladder 100 bp (Fermentas), lane *1*: isolate AHPN3-5, lane *2*: isolate AH13, lane *3*: isolate AH14, lane *4*: isolate AH16, lane *5*: isolate AH19, lane 6: isolate AH21, lane *7*: isolate AH22, lane *8*: isolate AH24.

strains of *Aeromonas* sp. isolated from distinct geographical areas as well as from the same geographical area was obtained [15].

In the present study, we construct the phylogenetic tree on the basis of highly intense bands of *A. hydrophila* isolates. Total six loci were considered in all 8 isolates and were showed good relationship between diseased and other isolates. The two major clades were obtained in phylogenetic tree (Fig. 2). Isolates AH13, AH14 and AH24 were present in same clades because all these isolates are from diseased fish. The three isolates AHPN3-5, AH16 AH19 were isolated from water and normal fish samples present in other clades. Therefore, our result indicates that the diseased isolates may be pathogenic and others are less pathogenic or environmental isolates. Box based method is important for rapid and accurate discrimination of isolates. On this basis phylogenetic relationship was carried in a short period of time at a reasonable cost for large number of isolates.

Fifty isolates of *Clostridium difficile* have been analyzed by three PCR-based typing methods in order to determine genomic diversity within this strain that may form the basis of a subtyping method. Three methods were used for repetitive extragenic palindromic elements (REP), conserved repetitive DNA elements (BOX), and enterobacterial repetitive PCR intergenic consensus sequences (ERIC) and these methods had satisfactory levels of typeability and reproducibility as determined by blind-coded repeats. BOX-PCR generated between two and five major amplicons with four distinct BOX profiles [16].

The evaluation of the effectiveness of three different molecular techniques, REP-PCR, ERIC-PCR and RAPD-PCR for rapid typing of *Photobacterium damselae* ssp. piscicida strains isolated from different species of marine fish and geographic areas. The DNA banding patterns generated by both molecular methods (RAPD and ERIC-PCR) remarkably separate the strains into two main groups that strongly correlate with their geographic origin [17]. BOXA1R primer has been found the most optimal choice for the establishment of a taxonomical framework of 80 *Bifidobacterium* type and reference strains. Subsequently, the BOX-PCR tested for the identification of 48 unknown

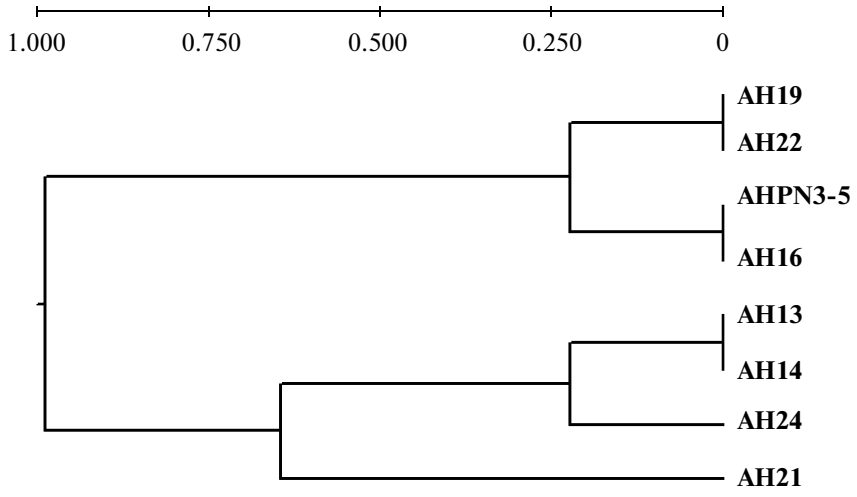

Fig. 2. Dendrogram constructed based on the DNA bands of the Box elements present in different isolates of *A. hydrophila* using the UPGMA method.

bifidobacterial isolates originating from human faecal samples and probiotic products [18].

In conclusion, we consider this method is of invaluable potential for further investigation of *A. hydrophila* epidemiology and its pathogenic implementations from fishes to humans. Modern epidemiologists are well equipped to have a variety of tools which provide good molecular differentiation and can be tailored to fit the needs of both the laboratory and clinical studies. Several of these molecular methods could enable us for the creation of large reference libraries of typed organisms to which new outbreak strains can be compared across laboratories in order to monitor change in bacterial populations. All these techniques would be useful for species and strains differentiation for a wide variety of bacteria and it should be applicable to studies of epidemiology, diagnosis, virulence and molecular taxonomy. It is rapid, easy to perform and reproducible method that is appropriate for genotyping of *A. hydrophila* at the strain level.

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