DEVELOPMENT OF DNA FINGERPRINTS FOR SELECTED SHELLFISH-BORNE MICROBIAL PATHOGENS USING THE ARBITRARILY PRIMED POLYMERASE CHAIN REACTION (AP-PCR)

Albert L. Smith, III, Michael C.L. Vickery, and Asim K. Bej
Department of Biology
The University of Alabama at Birmingham
Birmingham, Alabama

INTRODUCTION

Bivalve mollusks such as oysters, mussels, and clams are economically important marine food species found in abundance in estuarine and marine waters. These organisms are filter-feeders and are commonly eaten whole in the living state. While feeding on plankton and other microflora, shellfish may concentrate pathogenic bacteria and viruses from polluted waters. Among many of the microbial pathogens that are found in bivalve shellfish *Vibrio cholerae*, *Salmonella typhimurium*, *V. mimicus*, *V. parahaemolyticus*, and *V. vulnificus* are considered to be most significant pathogens due to their frequent outbreaks causing serious illness to humans (often several deaths during each outbreak) in the United States and many countries of Europe and Asia (Table 1). For safe consumption and consequently ensured public health, rapid and effective identification of the source of such contamination in seafood is required.

Routine monitoring of microbial pathogens and indicator microorganisms for possible fecal contamination in shellfish and other seafood is performed by conventional microbiological culture methods which may take weeks to complete the tasks (APHA 1986). In the case of a shellfish-mediated microbial disease outbreak or contamination of a specific batch of shellfish, the source of contamination should be traced. To achieve this criteria, specific detection of the pathogen in combination with the intra-species identification is required to enable us to track the source of contamination. In addition, a rapid and reliable method of detection and intra-specific identification of shellfish-borne bacterial pathogens will prevent further spread of the disease or a possible disease outbreak, thereby ensuring the safety of public health. A rapid and reliable routine monitoring of the microbial pathogens in shellfish and seafood will decrease the disease outbreaks resulting in increasing public confidence in the consumption of seafood.

Conventional PCR is an *in vitro* genetic-based DNA amplification method that exponentially increases the amount of a defined segment of a gene using oligonucleotide primers located at the two ends of the target gene segment to be amplified (Saiki et al. 1988). A modification of the conventional PCR approach called Arbitrarily Primed Polymerase Chain Reaction (AP-PCR) is a rapid technique that generates fingerprints of genomes of target microbial pathogens by using single arbitrarily selected primers for amplification. In AP-PCR, single-stranded DNA oligomers are allowed to anneal to denatured template DNA (Welsh and McClelland 1990). At some frequency, two primers will anneal to each complementary template DNA strand relatively close to one another. In the presence of free nucleotides and DNA polymerase, the oligomers are extended to form a new copy of the target DNA. Then, by increasing the stringency of the reaction, only those products formed in the first few low stringency reactions are amplified. By repeating this process many times, specific fragments of DNA can be amplified, thereby becoming relatively abundant in the mixture of DNA. When the amplified DNA fragments are separated by gel electrophoresis, a readily distinguishable pattern of DNA bands is produced. These bands represent a DNA fingerprint that can be used to identify different microorganisms and even various strains within a specific microbial species.

The AP-PCR fingerprinting method has successfully been applied for rapid and reliable identification of microbial pathogens such as *Staphylococcus aureus* (Welsh and McCleland 1990), *Lactococcus lactis* (Cancilla et al. 1992), *Frankia* (Sellstedt et al. 1992), *Legionella pneumophila* (van Belkum 1993; Gomez-Lus et al. 1993), *Campylobacter* (Giesendorf et al. 1993), *Rhizobium* (Bruijm 1992), *Actinobacillus* (Hennessy et al. 1993), *Clostridium* (McMillin and Muldrow 1992), and *Escherichia coli* (P. Lett and A. Bej unpublished). This procedure takes a maximum of 3 days to complete. However, this approach has not been applied in shellfish borne microbial pathogens such as *V. cholerae*, *V.*

parahaemolyticus, V. vulnificus, and V. mimicus. In this study, we have attempted to optimize the AP-PCR methodology to generate DNA fingerprints for selected strains of shellfish borne microbial pathogens.

MATERIALS AND METHODS

Bacterial Strains and Growth Media

All of salmonellae and *Vibrio* strains were grown on LB agar [10 g Bactotryptone (Difco), 5 g yeast extract (Difco), 10 g NaCl, and 14 g Bacto Agar (Difco) per liter] at 37°C: *Salmonella typhimurium* ATCC 14028, 29058, 23567, SL1344, and SF598; *V. cholerae* ATCC 25870 and 14035;*V. mimicus* ATCC 33653, 33654, and 33655.

Genomic DNA Purification

Genomic DNA from pure cultures of the target microbial pathogens was purified as described by Ausubel et al. (1987). Briefly, 2 ml of overnight cultures were centrifuged. The cell pellets were resuspended in 567 μl of Tris-EDTA buffer [10 mM Tris·Cl (pH 8.0), 1 mM EDTA], followed by treatment with 30 μl of 10% (w/v) sodium dodecyl sulfate (SDS) and 3 μl of 20 mg per ml (w/v) proteinase K (Sigma) at 37°C for 1 h. Next, the samples were treated with 100 μl of 5 M NaCl and 80 μl of hexadecyltrimethyl ammonium bromide (CTAB)/NaCl solution [10% (w/v) CTAB/0.7 M NaCl] at 65°C for 10 min. Then the sample was purified with $CHCl_3$:isoamyl alcohol (24:1 v/v) and with phenol:$CHCl_3$:isoamyl alcohol (25:24:2 v/v). DNA was precipitated with 0.6 volume of isopropanol and washed with 70% (v/v) ethyl alcohol. The dried DNA was resuspended in TE (pH 8.0) buffer and used for AP-PCR amplification of the target gene.

Selection of Oligonucleotide Primer

A single arbitrarily selected oligonucleotide primer, R-PSE420, based on the nucleotide sequence from pSE420 plasmid (Invitrogen) was used for AP-PCR DNA amplification to generate genomic fingerprints of the target microbial pathogens. This primer was 18 nucleotides long with a molecular weight of 5866.20 Da, melting temperature (Tm) of 50.795°C, and had a 33.3% GC content.

AP-PCR Reaction Parameters

In each PCR amplification reaction, 5 μl of PCR Reaction Buffer C (10x PCR reaction buffer consisted of 500 mM Tris·Cl, pH 8.9, 500 mM KCl, and 25 mM $MgCl_2$), 200 μM of each of the dNTPs (Pharmacia Biotechnology), 2.5 μl of R-PSE420 oligonucleotide primer (1 μM final concentration), 1 μg of the purified target DNA, and 2.5 U of AmpliTaq DNA polymerase (Perkin-Elmer). The total reaction volume was adjusted to a final volume of 50 μl in sterile, distilled water. All AP-PCR amplifications were performed in a DNA thermal cycler (Perkin-Elmer Model 480) using the following cycling parameters: 2 cycles with each cycle consisting of 92°C for 5 min, 37°C for 5 min, and 72°C for 5 min; 10 cycles with each cycle consisting of 92°C for 1 min, 40°C for 1 min, 72°C for 2 min; and 35 cycles with each cycle consisting of 92°C for 1 min, 45°C for 1 min, 72°C for 2 min. Following amplification, final extension of incompletely synthesized DNA fragments was performed at 72°C for 10 min.

Detection of the Amplified DNA

The amplified DNA fingerprints from each microbial pathogens were separated in a polyacrylamide gel [5% (w/v)] using TBE buffer(1x TBE buffer consists of 890 mM Tris·Cl, 890 mM boric acid, 20 mM EDTA pH 8.0). The DNA in the gel was stained with ethidium bromide and visualized on a FotoPrep I transilluminator. The fingerprint profile on the gel was photographed using Polaroid® film for documentation and further analysis.

Analysis of the AP-PCR-Generated DNA Fingerprints

The AP-PCR-generated genomic fingerprint profiles were analyzed by using the Pro-RFLP™ image analysis from DNA ProScan, Inc. and a LogiTech ScanMan model 256 image scanner.

RESULTS AND DISCUSSION

Genomic DNA for AP-PCR Fingerprints

The procedure for purification of genomic DNA took approximately 2 h to complete and the total yield was 50-100 μg per 2 ml overnight culture. The use of purified genomic DNA from each of the microbial pathogens tested produced consistent AP-PCR amplified genomic fingerprint profiles.

AP-PCR Reaction Parameters and Selection of Oligonucleotide Primer

The oligonucleotide primer R-PSE420 was arbitrarily selected from a plasmid pSE420 (Invitrogen) based on the size, Tm, molecular weight, %GC content described earlier. The PCR reaction parameters such as concentration of R-PSE420 primer, $MgCl_2$ concentration, and PCR cycling parameters for generating genomic fingerprints from each of the microbial pathogens tested in this study were important for reproducible results. Inconsistent fingerprint profiles were noticed due to a change in the PCR reaction parameters.

Analysis of Fingerprint Profiles

Salmonella typhimurium. Eight DNA bands of molecular weights of 0.41, 0.46, 0.61, 0.78, 1.17, 1.44, 1.6, and 1.81 kbp were identified in all *S. typhimurium* strains tested in this study (Figure 1). In addition to these DNA fragments, a unique 0.539 kbp DNA fragment (Figure 1; lane 2) was found in *S. typhimurium* SL1344 strain. Due to the presence of this unique DNA fragment in combination with the *Salmonella*-specific eight DNA fragments, an intra-species identification of *S. typhimurium* SL1344 can be achieved. Similarly, a 1.28 kbp DNA fragment (Figure 1; lane 4) was noticed in *S. typhimurium* SF598, ATCC 29058 along with the eight *Salmonella*-specific DNA fragments. Lastly, a 2.21 kbp DNA fragment (Figure 1; lanes 2, 3, and 4) was noticed only in *S. typhimurium* SL1344, ATCC 14028, and SF598. These results strongly suggest that by using the appropriate oligonucleotide primers and PCR reactions conditions, total salmonellae along with specific strains within this genus can be identified to trace the source of contaminating pathogen in shellfish and other seafood products.

V. mimicus. All three strains of *V. mimicus* tested in this study showed AP-PCR generated DNA fragments of molecular weight of 0.23 kbp, 0.96 kbp, and a 1.7 kbp that were common (Figure 2). However, unique DNA fragments of molecular weights of 0.62 kbp (Figure 2; lane 2), 0.55 kbp (Figure 2; lane 3), and 1.39 kbp (Figure 2; lane 4) were noticed in *V. mimicus* ATCC 33653, ATCC 33654, and ATCC 33655 strains, respectively. Therefore, from these DNA fingerprint patterns consisting of DNA fragments that are common to all strains along with the unique DNA fragments that are characteristic of a specific strain of this pathogen, detection of a strain of *V. mimicus* is possible. This intra-species identification is useful especially when detection of the source of the contaminating pathogen is required.

V. cholerae. The 2 strains of *V. cholerae* tested in this study showed distinct DNA fingerprint profile when their genomic DNA was amplified using the AP-PCR method. Both strains showed DNA fragments of molecular weight of 2.0 kbp, 1.7 kbp, 1.4 kbp, 1.2 kbp, 1.0 kbp, and 0.76 kbp (Figure 2). However, the DNA fingerprints from *V. cholerae* ATCC 25870 and ATCC 14035 showed unique DNA fragments of molecular weights of 2.6 kbp (Figure 2; lane 7) and 0.96 kbp (Figure 2; lane 8), respectively, which can be used for specific identification of a strain of this pathogen.

CONCLUSIONS

The application of AP-PCR amplification of genomic DNA from selected shellfish borne microbial pathogens such as *S. typhimurium*, *V. mimicus*, and *V. cholerae*, using an arbitrarily selected oligonucleotide primer R-PSE420, generated DNA fingerprints profiles showed the possibility of using this approach for identification of a microbial pathogen that contaminates shellfish and other seafood products. This approach takes a relatively shorter time to complete as compared to the conventional methods of detection. Also, the fingerprint profiles for some of the individual strains of these microbial pathogens showed unique DNA fragments which can be used for intra-species differentiation. This information can be extremely valuable to track the source of the contaminating strain which will help to take appropriate measures at a specific site where the contamination occurred. As a result, these appropriate measures can be focused to a specific location or a specific batch of food samples at that site rather than the entire harvesting area, preventing financial losses to the seafood industry. This study showed that the AP-PCR amplification approach can be used to generate DNA fingerprints for the identification of not only a shellfish borne microbial pathogen, but also a specific strain of a contaminating microbial pathogen. This study establishes a basis for generating DNA fingerprints applying the AP-PCR approach for additional strains of *S. typhimurium*, *V. cholerae*, *V. mimicus*, and other shellfish borne microbial pathogens such as *V. vulnificus* and *V. parahaemolyticus*. Therefore, a DNA fingerprint database can be established as a reference for comparison and identification of microbial pathogens in shellfish and other seafood as part of the routine monitoring process.

ACKNOWLEDGMENTS

This research was funded in part by the National Oceanic and Atmospheric Administration (NOAA), U.S. Department of Commerce under Grant# NA56RG0129, the Mississippi-Alabama Sea Grant Consortium (MASGC), and The University of Alabama at Birmingham.

REFERENCES

APHA. 1986. Laboratory procedures for the examination of sea water and shellfish. 7th Edition. Amer. Pub. Health Assoc. Washington, D.C.

Ausubel, F. M., R. Brent, R. E. Kingston, D. Moore, J. G. Smith, J. G Sideman, and K. Struhl (ed.). 1987. Current protocols in molecular biology. New York: John Wiley.

Bej, A.K., J.P. Southworth, R. Law, M. H. Mahbubani, and D.D. Jones. 1996. Detection of Salmonella in chicken meat using PCR. Food Testing & Analysis. 1: 3.

Brauns, L.A., M. C. Hudson, and J. D. Oliver. 1991. Use of the polymerase chain reaction in detection of culturable and nonculturable *Vibrio vulnificus* cells. Appl Environ Microbiol. 57: 2651-2655.

Bruijn, de, F.J. 1992. Use of repetitive (repetitive extragenic palindromic and enterobacterial repetitive intergenetic consensus) sequences and the polymerase chain reaction to fingerprint the genome of *Rhizobium meliloti* isolates and other soil bacteria. Appl Environ. Microbiol. 58:2180-2187.

Cancilla, M.R., I.B. Powill, A.J. Hillier, and B.E.Davidson. 1992. Rapid genomic fingerprinting of *Lactococcus lactis* strains by arbitrarily primed polymerase chain reaction with ^{32}P and fluorescent labels. Appl. Environ. Microbiol. 58:1772-1775.

Giesendorf, B.A., J.A. van Belkum, A. Koeken, H. Stegeman, M.H.C. Henkens, J. vander Plas, H. Goossens, H.G.M. Niesters, and W.G.V. Quint. 1993. Development of species-specific DNA probes for *Campylobacter jejuni, Campylobacter coli*, and *Campylobacter lari* by polymerase chain reaction fingerprinting. J. Clinical Microbiol. 31:1541-1546.

Gomez-Lus, P., B.S. Fields, R.F. Benson, W.T. Martin, S.P. O'Conner, and C.M. Black. 1993. Comparison of arbitrarily primed polymerase chain reactions, ribotyping, and monoclonal antibody analysis for subtyping *Legionella pneumophila* serogroup 1. J. Clinical Microbiol. 31:1940-1942.

Hennessy K.J., J.J. Iandolo, and B.W. Fenwick. 1993. Serotype identification of *Actinobacillus pleuropneumoniae* by arbitrarily primed polymerase chain reaction. J. Clinical Microbiol. 31:1155-1159.

Hill, W.E., S. P. Keasler, M. W. Trucksess, P. Feng, C. A. Kaysner, and K. A. Lampell. 1991. Polymerase chain reaction identification of *Vibrio vulnificus* in artifically contaminated oysters. Appl. Environ. Microbiol. 57(3):707.

Jones, D.D., R. Law, and A.K. Bej. 1993. Detection of *Salmonella* spp. in contaminated oysters using polymerase chain reaction (PCR) and gene probes. Journal of Food Science. 58(6): 1191-1197.

Lett, P. W., J. P. Southworth, D. D. Jones, and A.K. Bej. 1995. Detection of Pathogenic *Escherichia coli* in ground beef using Multiplex PCR. Food Testing & Analysis. 1:3.

McMillin, D.E. and L.L. Muldrow. 1992. Typing of toxic strains of *Clostridium difficile* using DNA fingerprints generated with arbitrary polymerase chain reaction primers. FEMS Microbiol. Ltrs. 92:5-10.

Saiki, R.K., D. H. Gelfand, S. Stoffel, S. J. Scharf, R. Higuchi, G. T. Horn, K. B. Mullis, and H. A. Erlich. 1988. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science. 239:487-494.

Sellstedt, A., B. Wullings, J. Nystrom, and P. Gustafsson. 1992. Identification of Casuarina-Frankia strains by the use of polymerase chain reaction (PCR) with arbitrary primers. FEMS Microbiol. Ltrs. 93:1-6.

Belkum, van, A., M. Struelens, and W. Quint. 1993. Typing of *Legionella pneumophila* strains by polymerase chain reaction-mediated DNA fingerprinting. J. Clinical Microbiol. 31:2198-2200.

Vickery, M.C.L, A. L. Smith, III, A. DePaola, S. Morgan, D.D. Jones, D. Cook, and A. K. Bej. 1996. Identification of virulent strains of *Vibrio vulnificus* in artificially contaminated shellfish from DNA fingerprints generated by arbitrarily-primed polymerase chain reaction. In Proceedings of the 26th Mississippi Water Resources Conference, April 2-3, 1996. Edited by B. Jean Daniel. Water Resources Research Institute: Mississippi State University.

Welsh, J. and M. McClelland. 1990. Fingerprinting genomes using PCR with arbitrary primers. Nuc. Acid. Res. 18:7213.

FIGURE LEGEND

Figure 1

A computer image of an original polyacrylamide gel showing AP-PCR Generated DNA Fingerprints of *Salmonella typhimurium*. Lane 1,123 bp Ladder as DNA size standard; Lane 2, *S. typhimurium* SL1344 showing DNA fingerprint profiles consisting of DNA fragments common to *S. typhimurium* tested of molecular weights of 0.41 kbp, 0.46 kbp, 0.61 kbp, 0.78 kbp, 1.17 kbp, 1.44 kbp, 1.6 kbp and 1.81 kbp, along with a unique DNA fragment of molecular weight of 0.539 kbp; Lane 3, *S. typhimurium* 14028 showing DNA fingerprint profiles consisting of DNA fragments common to *S. typhimurium* tested of molecular weights of 0.41 kbp, 0.46 kbp, 0.61 kbp, 0.78 kbp, 1.17 kbp, 1.44 kbp, 1.6 kbp and 1.81 kbp, along with a unique DNA fragment of molecular weight of 2.21 kbp; Lane 4, *S. typhimurium* SF598 showing DNA fingerprint profiles consisting of DNA fragments common to *S. typhimurium* tested of molecular weights of 0.41 kbp, 0.46 kbp, 0.61 kbp, 0.78 kbp, 1.17 kbp, 1.44 kbp, 1.6 kbp and 1.81 kbp, along with unique DNA fragments of molecular weights of 1.28 kbp and 2.21 kbp; Lane 5, *S. typhimurium* 29058 showing DNA fingerprint profiles consisting of DNA fragments common to *S. typhimurium* tested of molecular weights of 0.41 kbp, 0.46 kbp, 0.61 kbp, 0.78 kbp, 1.17 kbp, 1.44 kbp, 1.6 kbp and 1.81 kbp, along with a unique DNA fragment of molecular weight of 1.28 kbp; Lane 6, *S. typhimurium* 23567 showing DNA fingerprint profiles consisting of DNA fragments common to *S. typhimurium* tested of molecular weights of 0.41 kbp, 0.46 kbp, 0.61 kbp, 0.78 kbp, 1.17 kbp, 1.44 kbp, 1.6 kbp and 1.81 kbp, along with a unique DNA fragment of molecular weight of 0.539 kbp; Lane 7, blank; Lane 8, blank, Lane 9, 123 bp DNA ladder as size standard.

Figure 2

A computer image of an original polyacrylamide gel showing AP-PCR generated DNA fingerprints of *Vibrio mimicus* and *V. cholerae*. Lane 1, 123 bp ladder as DNA size standard; Lane 2, *V. mimicus* ATCC 33653 showing DNA fingerprint profiles consisting of DNA fragments common to *V. mimicus* tested of molecular weights of 0.23 kbp, 0.96 kbp, and 1.7 kbp, along with a unique DNA fragment of molecular weight of 0.62 kbp; Lane 3, *V. mimicus* ATCC 33654 showing DNA fingerprint profiles consisting of DNA fragments common to all V. *mimicus* tested of molecular weights of 0.23 kbp, 0.96 kbp, and 1.7 kbp, along with a unique DNA fragment of molecular weight of 0.55 kbp; Lane 4, *V. mimicus* ATCC 33655 showing DNA fingerprint profiles consisting of DNA fragments common to *V. mimicus* tested of molecular weights of 0.23 kbp, 0.96 kbp, and 1.7 kbp, along with a unique DNA fragment of molecular weight of 1.39 kbp; Lane 5, blank; Lane 6, 123 bp ladder as size standard; Lane 7, *V. cholerae* ATCC 25870 showing DNA fingerprint profiles consisting of DNA fragments common to *V. cholerae* tested of molecular weights of 2.0 kbp, 1.7 kbp, 1.4 kbp, 1.2 kbp, 1.0 kbp, and 0.76 kbp along with a unique DNA fragment of molecular weight of 2.6 kbp; Lane 8, *V. cholerae* ATCC 14035 showing DNA fingerprint profiles consisting of DNA fragments common to *V. cholerae* tested of molecular weights of 2.0 kbp, 1.7 kbp, 1.4 kbp, 1.2 kbp, 1.0 kbp, and 0.76 kbp along with a unique DNA fragment of molecular weight of 0.96 kbp; Lane 9, blank.

Table 1. Proposed shellfish-borne microbial pathogens that will be investigated for AP-PCR based detection.

Pathogen	Disease Caused	Conventional PCR	Reference	AP-PCR
Salmonella spp.	salmonellosis, typhoid fever, gastroenteritis, diarrhea	Yes	Jones et al., 1993	No
Vibrio cholerae	cholera, diarrhea	Yes	Bej and Jones, 1993	No
V. mimicus	diarrhea	No	Not applicable	No
V. parahaemolyticus	gastroenteritis	Yes	A. Bej (in preparation)	A. Bej (In progress)
V. vulnificus	septicemic shock, wound infection	Yes	Brauns et al., 1991; Hill et al., 1991	Vickery et al., 1996

Figure 1

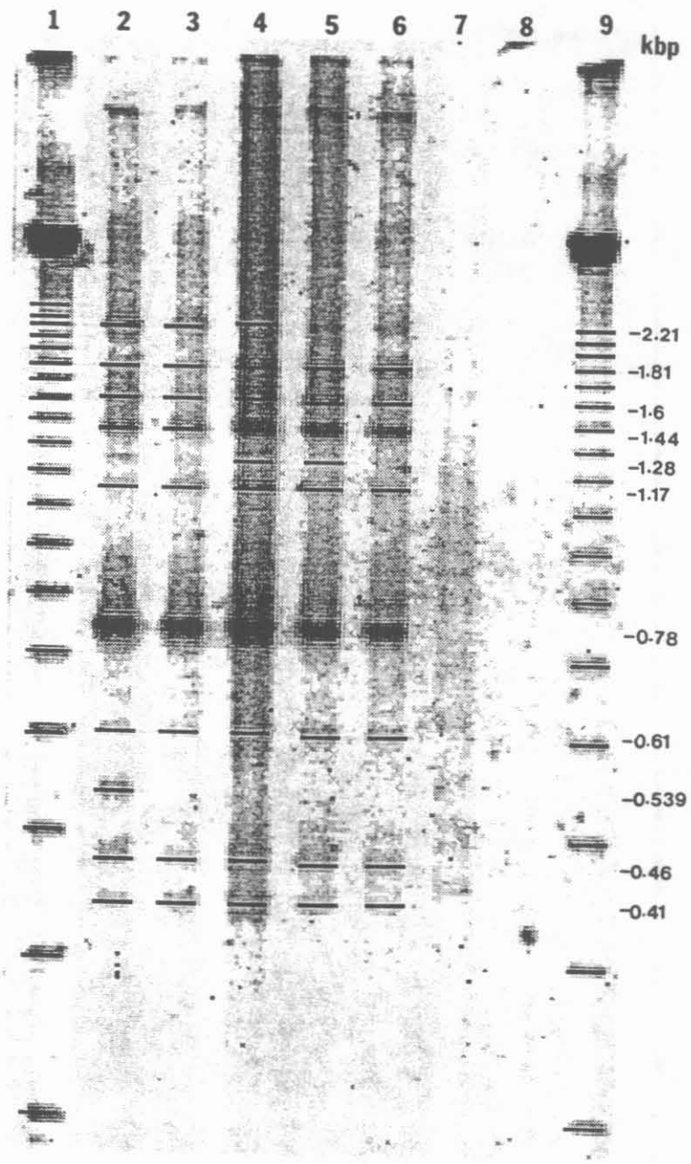

Figure 2