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Convenient and versatile DNA extraction using agarose plugs for ribotyping of problematic bacterial species

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Abstract

We describe a convenient, versatile and safe method for preparing bacterial DNA for ribotyping analysis. In this method, extraction of bacterial DNA from *Salmonella typhi* and *Burkholderia pseudomallei*, and subsequent restriction endonuclease digestion, was performed in agarose blocks/plugs thus minimizing shearing and loss of DNA, problems commonly associated with liquid phase phenol extraction. Digested DNA in the plugs was then electrophoresed directly, transferred to nylon membranes and hybridized with labeled rDNA probes in the usual manner to provide reproducible restriction patterns. This method is particularly useful for bacterial species where standard DNA extraction in the liquid phase using phenol has been problematic (e.g. *B. pseudomallei*) but can be used for any bacterial species. The DNA extracted within the agarose plugs can be stored for long periods and can be used in other, widely-used typing methods such as pulsed-field gel electrophoresis (PFGE) and PCR-based techniques. Embedding live cells directly in agarose plugs also minimizes the risk of exposure to these virulent human pathogens among laboratory workers.

1. Introduction

Ribotyping has become increasingly popular as a powerful and discriminating method in the molecular typing of many bacterial pathogens and allows investigators to address important epidemiological questions. The method essentially involves the extraction of bacterial DNA, digestion with restriction endonucleases, Southern blotting and then hybridization with labeled rRNA (or rDNA) gene probes. The resultant band patterns are then conveniently analysed using a variety of computer programs enabling conclusions to be drawn as to the extent of relationships between individual strains or isolates. Although the standard ribotyping techniques have worked well with a large variety of gram-negative (Altwegg et al., 1989; Lew and Desmarchellier, 1993; Trakulsomboon et al., 1997) and gram-positive bacteria (Thomson-Carter et al., 1989; Bruneau et al., 1994), certain species are known to be problematic, particularly at the DNA extraction stage. This includes *Pseudomonas*, *Burkholderia* and *Klebsiella* spp. which contain large amounts of polysaccharide in their capsules. These compounds tend to co-precipitate with DNA which result in problems in the DNA extraction process, including poor yields and subsequent interference with restriction endonuclease digestion. In an attempt to overcome such problems,

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we evaluated the adaptation of a component of another commonly used method for molecular typing, pulsed-field gel electrophoresis (PFGE), in which agarose plugs or inserts are used to immobilize bacterial cells prior to DNA extraction, digestion with restriction endonucleases and electrophoretic separation. In this report we describe the use of these agarose plugs in ribotyping of *Burkholderia pseudomallei* and *Salmonella typhi* to produce convenient and reproducible DNA band patterns.

2. Materials and methods

2.1. Bacterial strains

Strains of *Salmonella typhi* and *Burkholderia pseudomallei* used in this study were recent clinical isolates obtained from patients in Papua New Guinea and Malaysia with a diagnosis of typhoid fever and melioidosis, respectively. The strains were isolated, identified and maintained using standard bacteriological procedures and methods (Cowan and Steel, 1974). Given the highly pathogenic nature of *S. typhi* and *B. pseudomallei*, extra safety precautions were taken in working with these organisms, including the use of a biohazard (laminar flow) safety cabinet, gloves, face mask and immediate sterilization of contaminated items.

2.2. Preparation of genomic DNA

Bacterial cells were grown in 2 ml of LB broth with shaking at 200 rpm for 24 h at 37°C. Cells were pelleted by centrifugation at 6000 *g* for 10 min at 4°C and resuspended in 0.5 ml of solution 1 (10 mM Tris–HCl pH 7.5, 1 M NaCl). The suspension was then mixed with an equal volume of 1.5% low melting point agarose (Incert agarose, FMC Bioproducts, Rockland, ME, USA), dispensed into plastic slots ($10 \times 6 \times 1$ mm) and allowed to solidify for 10 min at 4°C. For *S. typhi*, agarose blocks/plugs containing the bacterial cells were then transferred into 2 ml of lysis solution (6 mM Tris–HCl, pH 7.5, 100 mM EDTA, pH 8.0, 1 M NaCl, 0.5% Brij-58, 0.2% sodium deoxycholate, 0.5% sodium lauryl sarcosine, 10 mg/ml lysozyme, 10 U/ml RNase) and incubated overnight at 37°C. For *B. pseudomallei*, incubation was performed for 6 h at 37°C. The lysis solution was removed and replaced with 2 ml of solution 2 (0.5 M EDTA pH 9.0, 1% sodium lauryl sarcosine, 0.5 mg/ml proteinase K) and incubated at 50°C for 24 h for *S. typhi* and for 48 h for *B. pseudomallei*. The blocks were then washed four times (each time for 1 h) at room temperature in $1\times$ TE buffer (10 mM Tris–HCl, 10 mM EDTA, pH 7.4) and stored in $1\times$ TE at 4°C. Long term storage of extra, undigested plugs was done by placing them in 10 ml of $1\times$ TE buffer at 4°C.

2.3. Digestion with restriction endonucleases and electrophoresis

A single DNA block/plug was equilibrated in 20 μl of $1\times$ restriction endonuclease buffer for 1–2 min on ice as recommended by the manufacturer. The microfuge tube containing buffer and DNA plug was then placed in a 75°C water bath for 10 min and then transferred to a 37°C water bath to ensure that the melted plug cools before digestion and also to prevent the plug from solidifying. Digestion of the DNA was carried out overnight at 37°C with 20 U of the appropriate restriction endonuclease (*Pst*I for *S. typhi*, *Eco*RI and *Bam*HI for *B. pseudomallei*, Promega, Madison, WI, USA). After digestion, the tube was placed in a 65°C waterbath for 10 min. The plugs containing digested DNA were then loaded into wells of a 0.8% agarose gel in $0.5\times$ TBE buffer (45 mM Tris–HCl, 45 mM boric acid, 10 mM EDTA, pH 8.7). Electrophoresis was performed for 20 h at 60 V for *S. typhi* and for 6 h at 60 V for *B. pseudomallei*. Lambda DNA digested with *Hin*dIII (New England Biolabs, Beverly, MA, USA) was used as a molecular weight marker. After electrophoresis, the gel was stained with 1 μg/ml of ethidium bromide for 15 min and photographed under UV illumination using a Polaroid MP4 camera with an orange filter.

2.4. Southern blotting, hybridization and detection

Southern blotting of DNA fragments to Hybond-N nylon membranes (Amersham International, Amersham, UK) was performed according to standard methods (Maniatis et al., 1982). The DNA was subsequently fixed to the membrane by a 5-min

exposure to UV illumination. The PCR-amplified 16S rRNA (rDNA) probe was generated as described by Weisburg et al. (1991) and subsequently electrophoresed on a 1.5% TBE gel. The 1.5-kbp fragment was then eluted from the gel using a Glassmix DNA isolation matrix system (Gibco, Gaithersburg, MD, USA) and DNA at a concentration of 20–30 ng/ml was labelled with horseradish peroxidase (ECL Direct Nucleic Acid Labelling and Detection System, RPN 3000/3001/3005, Amersham International) according to the manufacturer's instructions. Procedures for hybridization of the labeled probe to the digested DNA on the nylon membrane were as described previously (Poh et al., 1992). After hybridization, membranes were exposed to autoradiographic film (Hyperfilm-ECL, Amersham International) which was then developed according to standard procedures.

3. Results

The DNA fragments of *S. typhi* (digested with *Pst*I) and *B. pseudomallei* (digested with *Eco*RI) were subsequently transferred to nylon membranes and probed with a 16S rDNA gene probe to produce rDNA restriction patterns for the various isolates tested. Polymorphic restriction patterns were observed for both *S. typhi* (Fig. 1) and for *B. pseudomallei* (Fig. 2). In the analysis of *S. typhi* strains, for example, it was noted that strains from Papua New Guinea possess several distinct, but related patterns (Fig. 1, lanes 1–3, 4–6) which were quite different from a Malaysian isolate (Fig. 1, lane 7). Similarly, for *B. pseudomallei,* strains isolated from different parts of Malaysia show distinct but related patterns (Fig. 2), with an environmental isolate producing a rather different profile (Fig. 2, lane 3). Using different restriction endonucleases, we have also used the same plugs successfully for PFGE analysis (data not shown).

4. Discussion

The preparation of DNA from gram-negative bacteria for ribotyping has been a relatively straightforward process as bacterial cells are usually easily lysed by detergents and DNA extracted by conventional methods, e.g. in *S. typhi* (Altwegg et al., 1989). However, other genera such as *Pseudomonas*, *Burkholderia* and *Klebsiella* have been known to be problematic. *B. pseudomallei*, the causative agent of melioidosis, has been a particular problem in our laboratory, although several reports have been published on the ribotyping of this organism (Lew and Desmarchellier, 1993; Trakulsomboon et al., 1997). The major problems encountered have been the low yield of DNA and its unsuitability to subsequent digestion with restriction endonucleases (RE). It is probable that these problems are related to the presence of a pseudocapsule in *B. pseudomallei* (Chambon and Fournier, 1956) which has been reported to consist of amorphous, slime-like substances containing polysaccharides, including galactose, glucose, mannose and rhamnose (Leelarasamee and Bovornkitti, 1989; Denisov, 1985; Kapliev et al., 1990). The existence of a capsular exopolysaccharide in *B. pseudomallei* has also been reported (Masoud et al., 1997) which may co-extract with the DNA, thus complicating the extraction process. Also, fresh clinical isolates of *B. pseudomallei* possess non-

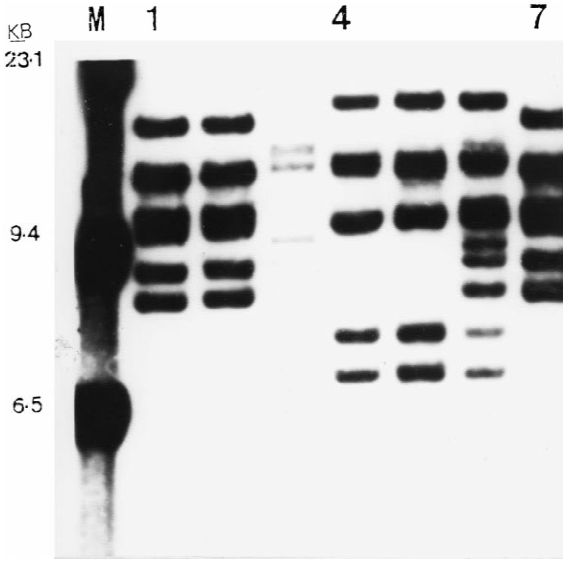

Fig. 1. Autoradiogram of *Pst*I restriction digests of *S. typhi* hybridized with 16S rDNA gene probe. Lane M = lambda *Hin*dIII marker, lanes 1–6 = *S. typhi* isolates from Papua New Guinea, lane 7: *S. typhi* isolate from Malaysia. Figures in kB (kilobases) refer to position of marker bands.

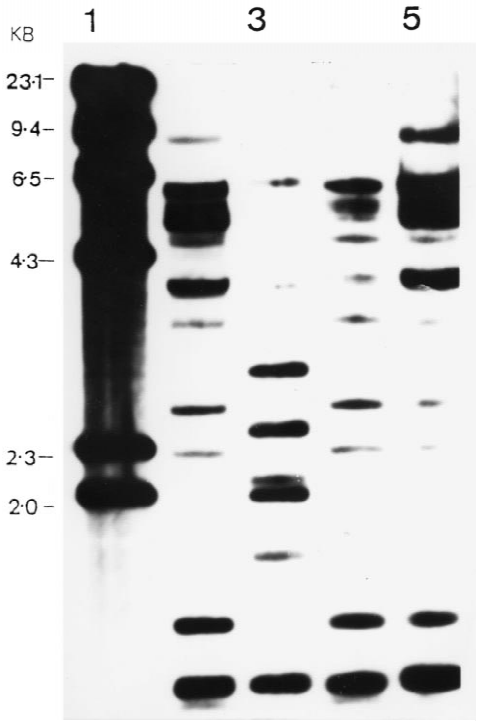

Fig. 2. Autoradiogram of *Eco*RI restriction digests of *B. pseudomallei* hybridized with 16S rDNA gene probe. Lane 1, lambda *Hin*dIII marker, lanes 2–5, *B. pseudomallei* isolates from Malaysia (lanes 2,4,5 are human isolates and lane 3 is an environmental isolate). Figures in kB (kilobases) refer to position of marker bands.

specific acid phosphatase activity (Kondo et al., 1991) which may also interfere with DNA extraction. Efforts to overcome this problem have included the use of toxic and hazardous chemicals such as cetyltrimethylammonium bromide (CTAB) and guanidium thiocyanate. Repeated cycles of phenol–chloroform extraction are often required to generate DNA of sufficient purity, which frequently means low yields of highly sheared material unsuitable for ribotyping. Additional dialysis steps are sometimes required to remove traces of low-molecular-weight chemicals which might inhibit subsequent digestion with REs. The low yields also mean that larger numbers of cells are usually required as the starting material. Some of these technical problems have been alluded to by Lew and Desmarchellier (1993) where *B. pseudomallei* DNA of inadequate purity had to be further treated with proteases, re-extracted and re-precipitated.

Our adaptation of the use of agarose plugs in the DNA extraction process has overcome many of these problems. By using agarose plugs, a 1–2 ml liquid culture of bacterial cells provides sufficient genomic DNA for ribotyping, as opposed to the larger amounts required using the conventional procedures. The extraction process is faster and more convenient as all steps are performed with the cells embedded in agarose, including proteolytic digestion, RNase treatment, dialysis, and RE digestion. Agarose plugs are then loaded directly on to gels for electrophoretic separation, blotting and hybridization with labeled rRNA (or rDNA) probes. The cleanliness and purity of the DNA is indicated by the ease of RE digestion and the intactness ensured as there is no mechanical shearing as is seen during extraction in the liquid phase. DNA prepared in this form can also be used for PFGE analysis, is very stable and can be stored in the agarose plugs for 6 to 18 months in TE buffer at 4°C. An additional advantage is improved safety as a result of immediately embedding live bacterial cells in agarose and thus reducing exposure risk to laboratory workers. Melioidosis (Ashdown, 1992) and typhoid fever (Thong et al., 1996) are not trivial diseases and infection can have severe, even fatal consequences. A potential drawback of the method is the longer time needed for genomic DNA preparation, but this needs to be performed only once. The longer preparation periods are necessary to allow complete diffusion of digested debris, chemicals and other impurities from the agarose blocks. The amounts of restriction endonucleases required for digestion may also be slightly larger compared to the conventional technique.

In conclusion, a simple adaptation of using agarose plugs has provided a convenient, versatile and safe method of preparing bacterial DNA for ribotyping analysis. The method is generally applicable to all bacterial species and should also be applicable to DNA from other cellular sources.

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