Appendices

Appendix 1
Luria- Bertani medium

Tryptone 10 g/l
Yeast extract 5 g/l
NaCl 10 g/l

To make LB agar, 15 g/l of agar technical no. 3 was added to the LB broth before autoclaving.

1M Tris (pH7.5)

Tris 12.14 g
Deionized water 80 ml

The pH of the solution was adjusted to 7.5. Top up to 100 ml with distilled water and autoclave.

Solution 1

Tris-Cl (pH7.5) 10mM
NaCl 1M

The solution was made up to the required volume with distilled water and autoclaved.

0.5M EDTA solution (pH8.0)

Disodium EDTA. 2H₂O 18.61 g
Deionized water 60 ml

The pH was adjusted to 8.0 with 1M NaOH. The solution was made up to 100 ml with distilled water and autoclaved.
**EC solution**

1M Tris (pH7.5)  
0.5M EDTA (pH8.0)  
Brij- 58  
Sodium lauryl sarcosine  
Deoxycholate  
NaCl

3 ml  
100 ml  
2.5 g  
2.5 g  
1.0 g  
29 g

The solution was made up to 500 ml using distilled water and autoclaved.

**EC lysis solution**

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</tr>
</thead>
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<tr>
<td>Lysozyme</td>
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<tr>
<td>RNase</td>
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Solution is to be prepared on the day of use.

**ES solution**

<table>
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<tbody>
<tr>
<td>EDTA</td>
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</table>

Distilled water was added to approximately 800 ml and the pH of the solution was adjusted to 9.0 with 10N NaOH. 10 g sodium lauryl sarcosine was added to the solution. The solution was then made to 1 litre with distilled water and autoclaved.

**ESP solution**

<table>
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Solution is to be prepared on the day of use.

**TE Buffer**

(Prepared as a 10X stock solution)

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<td>Tris (base)</td>
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</tr>
<tr>
<td>EDTA (Disodium salt)</td>
<td>10mM</td>
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</tbody>
</table>

pH was adjusted to 8.0 using HCl and the mixture made up to the required volume with distilled water and then autoclaved.
Ethidium bromide (10 mg/ml)

Ethidium bromide 100 mg

Deionized water to 10 ml. The solution was stored in a dark bottle at room temperature.

TBE Buffer

(Prepared in a 10X stock solution)
Tris (base) 121.2 g
Boric acid 61.8 g
EDTA (Disodium salt) 0.745 g

The solution was made up to 1 litre with distilled water and autoclaved. For use, the stock solution was diluted appropriately with distilled water.

TAE Buffer

(Prepared as a 50X stock solution)
Tris (base) 2M
EDTA (Disodium salt) 0.05M

pH was adjusted to 8.0 using glacial acetic acid. For use, the stock solution was diluted appropriately with distilled water.

Depurination solution
HCl 250mM

Denaturation Solution
NaCl 1.5M
NaOH 0.5M

Neutralization solution
NaCl 1.5M
Tris- HCl 0.5M

pH of the solution was adjusted to 7.5.
**20xSSC solution**

- Na$_2$Citrate  0.3M
- NaCl  3.0M

pH of the solution was adjusted to 7.0 using 1N HCl.

**Primary wash buffer**

- Urea (6M)  360 g
- SDS (0.4%)  4 g
- 20xSSC (0.5xSSC)  25 ml

The solution was made up to 1 litre. The buffer can be kept for 3 months in the refrigerator at 2-8°C.

**Secondary wash**

2xSSC

20xSSC was diluted 10X. The buffer can be kept for up to 3 months at 2-8°C.
Appendix 2

SET Buffer
Tris (pH8.0) 25mM
EDTA (pH8.0) 10mM
Sucrose 15%

Lysis solution
NaOH 0.2M
SDS 1%
Freshly prepared for every use.

Solution I
NaCl 0.15M
EDTA (pH8.0) 0.1M

Solution II
SDS 1%
NaCl 0.1M
Tris- HCl (pH8.0) 0.1M
Appendix 3

Silvernitrate solution
Silvernitrate 0.25 g
Formaldehyde (36%) 250 μl
Top up to 250 ml with deionized water.

Colour development solution
Natrium carbonate 6.25 g
Formaldehyde (36%) 250 μl
Natrium thiosulfate 250 μl
Top up to 250 ml with deionized water.

Stop solution
EDTA 3.65 g
Top up to 250 ml with deionized water.

Fixing solution
Ethanol (96%) 150 ml
Glycerol (85%) 23 ml
Top up to 500 ml with deionized water.
Example of an AFLP binary data table. Study carried out on the 25 Papua New Guinea and 5 geographic *S. typhi* isolates using primer combination *EcoRI-AC/MseI-G*. The minor polymorphic character state is shown in gray.
Example of fluorescent AFLP data. Output from GeneScan software displaying typical AFLP electropherograms. Six *S. typhi* Papua New Guinea isolates were analysed using the AFLP primer combination *EcoRI-AC/MseI-G*. 


**Nair, S., Schreiber, E., Thong, K.L., Pang, T. and Altwegg, M. 1999.** Genotypic characterization of *Salmonella Typhi* by amplified fragment length polymorphism fingerprinting provides increased discrimination as compared to pulsed- field gel electrophoresis and ribotyping. *J. Microbiol. Methods.* (accepted)
Presentations at Conferences (Posters)


Awards

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 \textit{Salmonella typhi} and \textit{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. \textit{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. © 1999 Elsevier Science B.V. All rights reserved.

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, Soutiern 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 Desmarchelier, 1993; Trakulsomboon et al., 1997) and gram-positive bacteria (Thompson-Carter et al., 1989; Bruneau et al., 1994), certain species are known to be problematic, particularly at the DNA extraction stage. This includes \textit{Pseudomonas}, \textit{Burkholderia} and \textit{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,
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 × 6 × 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 × TE buffer (10 mM Tris–HCl, 10 mM EDTA, pH 7.4) and stored in 1 × TE at 4°C. Long term storage of extra, undigested plugs was done by placing them in 10 ml of 1 × 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 × 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 (PstI for *S. typhi*, EcoRI and BamHI 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 × 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 HindIII (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 electroforeosed on a 1.5% TBE gel. The 1.5-kbp fragment was then eluted from the gel using a Glassmax 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. Procedure’s 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 PsI) and B. pseudomallei (digested with EcoRI) 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 (Altweg 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 Desmarchelier, 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 PsI restriction digests of S. typhi hybridized with 16S rDNA gene probe. Lane M = lambda HindIII 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.
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 onto gels for electrophoretic separation, blotting and hybridization with labeled rRNA (or tDNA) 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.

References


Detection of three different types of ‘Tropheryma whippelii’ directly from clinical specimens by sequencing, single-strand conformation polymorphism (SSCP) analysis and type-specific PCR of their 16S–23S ribosomal intergenic spacer region

Hans Peter Hinrikson, Fabrizio Dutly, Satheesh Nair and Martin Altwegg

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The 16S–23S rDNA intergenic spacer region of organisms identical with or closely related to ‘Tropheryma whippelii’, the uncultivated causative agent of Whipple’s disease, was analysed directly from 38 clinical specimens of 28 patients using a specific nested PCR followed by direct sequencing. As compared to the reference sequence in public databases, two novel ‘T. whippelii’ spacer types were recognized. In the absence of DNA–DNA hybridization data it is uncertain whether the three types found represent subtypes of a single species or three different but closely related species. Methods were developed to detect all three variants by single-strand conformation polymorphism analysis and by type-specific PCR assays, thus allowing the screening of large numbers of specimens. Further studies may provide a clue to the possible associations between the type of infecting strain and the various clinical presentations of Whipple’s disease.

Keywords: Whipple’s disease, ‘Tropheryma whippelii’ typing, 16S–23S ribosomal intergenic spacer region, sequence analysis, single-strand conformation polymorphism

INTRODUCTION

Comparative sequence analysis of the 16S rRNA gene (16S rDNA) allowed the hitherto uncultivated presumptive agent of Whipple’s disease to be identified as a actinomycete bacterium constituting the novel but not yet validated taxon ‘Tropheryma whippelii’ (Wilson et al., 1991; Relman et al., 1992). Further, the sequence of the intergenic spacer region between the 16S and the 23S rDNA as well as the 5’ end of the 23S DNA have also been determined (Maiwald et al., 1996).

Several diagnostic ‘T. whippelii’-PCRs that target parts of the 16S rDNA have been established (Relman et al., 1992; Rickman et al., 1995; Altwegg et al., 1996; Dauga et al., 1997). However, such systems may not discriminate between closely related species as illustrated by identical or almost identical 16S rDNA gene sequences of Mycobacterium kansasi and Mycobacterium gastri, Mycobacterium malmoense and Mycobacterium szulgai, or Aeromonas trota and Aeromonas caviae, respectively (Martinez-Murcia et al., 1992; Roth et al., 1998). Since the intergenic spacer is more variable than the flanking structural genes it has been widely used for species identification and particularly for subtyping purposes in many other bacterial groups (Gürtler & Stanisch, 1996). We have designed a nested PCR for the detection of the ‘T. whippelii’ 16S–23S rDNA spacer region directly in clinical specimens. Sequencing of the resulting PCR products from nine independent Swiss patients with Whipple’s disease revealed no spacer polymorphism at all (Hinrikson et
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* T. whippelli*'-specific amplification with primers TW-1 & TW-2 (Altwegg et al., 1996).

* T. whippelli*'-specific nested PCR using primer pairs tws1 & tws2 followed by tws3 & tws4.

Analysis of products derived from *T. whippelli*'-specific nested PCR using primer pairs tws1 & tws2 followed by tws3 & tws4.

Primer combinations: A, twsA1 & twsB1; B, twsA2 & twsB2; C, twsA1 & twsC1; D, twsA2 & twsC1; E, twsA2 & twsC2.

Confirmed by sequencing of products derived from broad-range (universal eubacterial) amplification (Goldenberger et al., 1997). Paraffin-embedded tissue.

Confirmed by sequencing of products derived from *T. whippelli*'-specific semi-nested PCR with primer pairs TW-1 & TW-2 followed by TW-4 & TW-2 (Brändle et al., 1999).

Confirmed by sequencing of products derived from *T. whippelli*'-specific amplification with primers analogous to TW-1 & TW-
‘Tropheryma whippleii’ 16S–23S rDNA spacer region typing

(1999). Nonetheless, specimens from additional patients were investigated using the same approach.

In the present study, we describe two new 16S–23S rDNA spacer types of ‘T. whippleii’ or closely related organisms. In addition, two new approaches, single-strand conformation polymorphism (SSCP) analysis and type-specific PCR assays, were developed that are much easier to perform than sequencing and thus allow the screening of large numbers of specimens.

METHODS

specimens. A total of 38 clinical specimens from 28 patients

from all of ‘T. whippleii’ 16S rDNA by semi-nested PCR using primer pairs TW-1 & TW-2 followed by TW-4 & TW-2 (Brändle et al., 1999) were included (Table 1). They included cerebrospinal fluids (n = 3), heart valves (n = 3), a biopsy of an intervertebral disc, synovial fluids (n = 3), intestinal biopsies (n = 17), gastric aspirates (n = 8), and an intraoperative swab from a joint. In 19 specimens representing 13 patients the identity of 16S rDNA amplicons (229 bp) had previously been confirmed by direct sequencing. For an additional eight specimens from eight patients comparable results had been obtained by broad-range (universal eubacterial) amplification and sequencing of a ±530 bp fragment (Goldenberger et al., 1997). No sequence data were available for the remaining 11 samples. Negative controls consisted of intestinal biopsies (n = 23), a bowel aspirate, and gastric aspirates (n = 17) that were negative for ‘T. whippleii’ by semi-nested PCR with the above primer pairs.

Extraction of DNA. Cerebrospinal fluids, synovial fluids, gastric aspirates, the 0.85% NaCl suspension (2 ml) of the intraoperative swab, and the bowel aspirate were centrifuged at 10 min at 14,000 g. These pellets as well as the biopsies were suspended in digestion buffer (50 mM Tris/HCl, pH 8.5, 1 mM EDTA, 0.5% SDS, 200 μg proteinase K ml−1) and incubated at 55 °C for 1 h 30 min with agitation. DNA was extracted with QIAamp DNA-binding columns (QIAGEN) according to the manufacturer’s protocol except for the final step using only 100 μl (instead of 200 μl) of elution buffer AE. Five microliters of the eluate were used for PCR.

PCR assays. Primers used for amplification and/or nested reamplification are listed in Table 2. Amplifications were done in a final volume of 50 μl containing 200 μM each deoxynucleotide, 2.5 U AmpliTag Gold polymerase with the appropriate amount of its optimized buffer (Perkin-Elmer), 25 pmol each primer, 2% (v/v) Tween 20, and 5 μl DNA extract. Reamplification was identical, except that 1–5 μl of amplicon was used as template and Tween 20 was omitted. Each PCR run included molecular-grade H₂O (LAL reagent water; BioWhittaker Europe, Belgium) as negative control. All PCRs were performed on a GeneAmp PCR System 9600 (Perkin-Elmer) and started at 95 °C for 12 min. Amplifications and reamplifications with the same primer pairs tsw1 & tsw2 and tsw3 & tsw4 were done for 40 cycles at 95 °C for 1 min, at 55 °C for 1 min, and at 72 °C for 1 min. Reamplifications with primer combinations (i) tswA1 & tswB1 (system A, targeting spacer type 1), (ii) tswA2 & tswB2 (system B, types 2 and 3), (iii) tswA1 & tswC1 (system C, type 1), (iv) tswA1 & tswC1 (system D, type 2), and (v) tswA2 & tswC2 (system E, type 3) were performed using 20 cycles at 95 °C for 1 min, at 70 °C for 1 min, and at 72 °C for 1 min. All PCRs ended at 72 °C for 10 min. PCR products (10 μl) were separated by electrophoresis on a 2% (w/v) agarose gel, stained with ethidium bromide, and detected under UV light.

Sequence analysis. Direct sequencing of products derived from nested PCR with primer pairs tsw1 & tsw2 followed by tsw3 & tsw4 was performed on an ALFexpress DNA Sequencer (Pharmacia Biotech). Amplicons were purified using the QIAquick PCR Purification Kit (QIAGEN) according to the manufacturer’s instructions. The cycle sequencing reaction was performed in both directions with the 5'-fluorescence-labelled primers tsw3 or tsw4, respectively, and the Thermo Sequenase Kit with 7-deaza-dGTP (Amersham). The cycling conditions were the same as described for the PCR assays except for an initial denaturation at 95 °C for only 5 min. Data analysis included

Table 2. Oligonucleotides used to analyse the 16S–23S rDNA intergenic spacer region polymorphisms of ‘T. whippleii’

<table>
<thead>
<tr>
<th>Primer* /sense†</th>
<th>Sequence (5’ → 3’)</th>
<th>Position‡</th>
<th>‘T. whippleii’ target rDNA</th>
<th>Spacer type(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>tsw1/f</td>
<td>ATCGCAAGGTTGAGGGCAATCT</td>
<td>1213 → 1233</td>
<td>3' end of 16S</td>
<td>1, 2 and 3</td>
</tr>
<tr>
<td>tsw2/r</td>
<td>CGGATTCTGGCCGCCCAC</td>
<td>1940 → 1923</td>
<td>5' end of 23S</td>
<td>1, 2 and 3</td>
</tr>
<tr>
<td>tsw3/f</td>
<td>CCGGTGACTAATCCCTTTGGAAGA</td>
<td>1387 → 1410</td>
<td>3' end of 16S</td>
<td>1, 2 and 3</td>
</tr>
<tr>
<td>tsw4/f</td>
<td>TCCGGAGCTTACGCAATGTGAG</td>
<td>1875 → 1854</td>
<td>5' end of 23S</td>
<td>1, 2 and 3</td>
</tr>
<tr>
<td>tsw1/A1</td>
<td>AAGTGAACCCGCAATGCTCTGAG</td>
<td>1527 → 1551</td>
<td>16S–23S spacer</td>
<td>1</td>
</tr>
<tr>
<td>tsw2/A2</td>
<td>AAGTGAACCCGCAATGCTCTGAG</td>
<td>1527 → 1551</td>
<td>16S–23S spacer</td>
<td>2 and 3</td>
</tr>
<tr>
<td>tsw1/B1</td>
<td>TTCGCGTACCTTGTGGCCAAAAC</td>
<td>1600 → 1577</td>
<td>16S–23S spacer</td>
<td>1</td>
</tr>
<tr>
<td>tsw2/B2</td>
<td>TTCGCGTACCTTGTGGCCAAAAC</td>
<td>1600 → 1577</td>
<td>16S–23S spacer</td>
<td>2 and 3</td>
</tr>
<tr>
<td>tsw1/C1</td>
<td>AAATGTCACACAAGTGCAATAGCA</td>
<td>1667 → 1643</td>
<td>16S–23S spacer</td>
<td>1 and 2</td>
</tr>
<tr>
<td>tsw2/C2</td>
<td>AAATGTCACACAAGTGCAATAGCA</td>
<td>1667 → 1643</td>
<td>16S–23S spacer</td>
<td>3</td>
</tr>
</tbody>
</table>

This study except tsw1, tsw2, tsw3 and tsw4 (Hinrikson et al., 1999).

f, forward; r, reverse.

According to ‘T. whippleii’ sequence, GenBank accession no. X99636 (Maiwald et al., 1996).
**RESULTS**

**Amplification and direct sequencing of the 16S-23S rDNA spacer region**

All DNA extracts were investigated by nested PCR with primer pairs tws1 & tws2 followed by tws3 & tws4 targeting the 'T. whipplei' 16S-23S rDNA spacer and flanking coding regions. Amplicons of the expected size (~490 bp) were detected in all specimens previously shown to harbour 'T. whipplei' 16S rDNA but not in the negative controls (results not shown). All amplicons that were sequenced (n = 30) perfectly matched the revised 'T. whipplei' reference sequence GenBank accession no. AF074933; Hinrikson et al., 1999) in the 3' and 5' terminal regions of the 16S and 23S rRNA genes, respectively. However, sequence variability was repeatedly observed within the 16S-23S rDNA spacer sequence with the following five di-meric types: (i) T or C [position 1551 of original spacer reference sequence (Maiwald et al., 1996)]; (ii) TTTT or CTTT (positions 1578–1581); (iii) T or C (position 1619); (iv) T or C (position 1643); and (v) A or G (position 1652). According to these single-base alterations three spacer types were recognized (Fig. 1).

**SSCP analysis**

All 27 amplicons digested with HaeIII yielded two fragments of the expected sizes (295/294 and 166 bp, respectively) by agarose gel electrophoresis while the remaining 29 bp fragments were not clearly visible.
Type-specific PCR assays

All DNA extracts were amplified with primer pair tws3 & tws4 and directly reamplified in five separate reactions with primer combinations targeting dimorphic spacer sites (Fig. 2). All specimens known to contain ‘T. whippelii’ 16S rDNA were classified according to their type-specific amplicons (74/73 bp and 141/140 bp, respectively), with type-specific reamplification systems A and C being positive for type 1, systems B and D for type 2, and systems B and E for type 3. None of these samples was positive for more than one spacer type (Table 1). The appearance of tws3 & tws4 amplicons and other minor products (> 200 bp; representing amplicons derived from combinations of 1st and 2nd round primers) correlated with the amount of target DNA present in the clinical samples. All 41 controls tested remained negative for spacer products and did not show non-specific bands.

Frequency of the three spacer types in clinical specimens

Spacer type 1 was found in 15 patients, types 2 and 3 in 10 and 3, respectively (Table 1). Spacer types did not correlate with the type of specimen analysed.

DISCUSSION

The various clinical manifestations of Whipple's disease ranging from chronic low grade fever to life-threatening endocarditis (Dobkins, 1987) have often been assumed to represent differences between the infecting strains of ‘T. whippelii’ or to be due to closely related, ‘T. whippelii’-like species. This, however, remained pure speculation as nobody has ever looked at such differences, mainly because of the absence of reliable cultures (Schoeden et al., 1997). In this study, we have analysed the 16S–23S rDNA spacer sequences directly from 38 clinical specimens of 28 patients. These specimens had previously been shown to contain 16S rDNA of ‘T. whippelii’ by a specific assay (Table 1). The identity of the respective amplicons from 19 samples of 13 patients had been confirmed by sequence analysis (Brändle et al., 1999; Ehrbar et al., 1999). For an additional eight specimens from eight patients comparable results had been obtained by broad-range (universal eubacterial) amplification and sequencing of a ± 530 bp fragment (Goldenberger et al., 1997), thus excluding also the presence of significant amounts of DNA from unrelated bacteria.

We considered the analysis of the 16S–23S rDNA spacer region a promising approach to possibly detect strain differences as it has been shown for a variety of organisms that the spacer region is more variable than the flanking regions encoding 16S and 23S rRNA, respectively (Gürtler & Stanisch, 1996). In a first series of nine patients no variability had been found (Hinrikson et al., 1999) and we, therefore, decided to analyse as many different samples as possible to verify these findings using the same approach, including sequence analysis of PCR products. This resulted in the detection of five dimorphic sites constituting three different spacer types (Fig. 1). In each of the eight patients with more than one positive specimen the same type was found in all independently processed samples (Table 1). The most frequent type detected, i.e. spacer type 1, perfectly matched the original ‘T. whippelii’ spacer sequence (294 bp) previously deposited in GenBank (Mairwald et al., 1996). As compared to this reference sequence, spacer types 2 and 3 differed solely at two and five nucleotide positions, respectively (Fig. 1). Nonetheless, these slight DNA alterations could be confirmed by SSCP analysis of HaeIII-generated spacer fragments spanning all variable nucleotide positions found (not shown). As anticipated from the sequencing data, a total of three distinct SSCP profiles were obtained, each corresponding to one of the spacer types. Because both sequencing and, to some extent, SSCP analysis are tedious and time-consuming, we decided to establish type-specific PCR assays based on the polymorphisms found (Fig. 2). For each single specimen, the results of the five different assays were as expected (Table 1), thus confirming the reliability of each of the three methods used. While type-specific PCR is straightforward for detecting the presently known three types, SSCP and sequence analysis have the potential of recognizing additional types not related to the currently known five dimorphic sites.

In all specimens analysed only one single spacer type was detectable. Assuming that each patient is infected with only one single strain as suggested by the eight patients with multiple specimens, this may be explained by the presence of one single rRNA operon, i.e. spacer region, per strain as described for other actinobacteria (Gürtler & Stanisch, 1996). If so, any fixed single-base mutation in the rRNA spacer leading to an inappropriately folded molecule would have to be compensated by further sequence variation. Interestingly, the spacer types now found seem to have evolved mainly by pairwise DNA alterations (position 1551 ↔ position 1578, and position 1643 ↔ position 1652, Fig. 1) as indicated by the fact that five dimorphic sites (with 32 possible combinations) but only three different spacer types have been found.
The variations found raise the question whether they represent three different, closely related species or three subtypes of the single species 'T. whippelli'. Partial 16S sequences determined for most specimens included in this study did not reveal any differences as compared to the published sequence. However, as has been shown for other organisms, even complete identity of 16S rDNA sequences does not prove species identity (Fox et al., 1992) but suggests a very close relationship (Stackebrandt & Goebel, 1994). To solve the problem DNA-DNA hybridization studies are definitely needed but not feasible due to the fact that 'T. whippelli' has not yet been cultured on artificial media. As long as hybridization data are not available, we suggest that the three types found be regarded as subtypes of the single species 'T. whippelli' for practical reasons and because of the small number of variable nucleotides which is similar or even less than the variations found within other species (Gurtler & Stanisch, 1996).

We conclude that at least three different 'T. whippelli' 16S-23S rDNA spacer types can be detected with different frequencies directly from human clinical specimens. Further studies including samples from other geographic areas as well as from environmental sources (Maiwald et al., 1998) may show whether additional spacer types can be found. Such investigations are much easier to perform when using either type-specific PCR assays and/or SSCP analysis as compared to sequencing. Although an obvious association of spacer types and types of specimens analysed was not found, it remains to be shown whether the 'T. whippelli' spacer types can be correlated with clinical presentations of Whipple's disease even with the possible carrier state for Whipple bacilli as postulated previously (Ehrbar et al., 1999).

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REFERENCES


Genotypic characterization of *Salmonella typhi* by amplified fragment length polymorphism fingerprinting provides increased discrimination as compared to pulsed-field gel electrophoresis and ribotyping

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Abstract

Amplified fragment length polymorphism (AFLP) is a recently developed, PCR-based high resolution fingerprinting method that is able to generate complex banding patterns which can be used to delineate intraspecific genetic relationships among bacteria. In the present study, AFLP was evaluated for its usefulness in the molecular typing of *Salmonella typhi* in comparison to ribotyping and pulsed-field gel electrophoresis (PFGE). Six *S. typhi* isolates from diverse geographic areas (Malaysia, Indonesia, India, Chile, Papua New Guinea and Switzerland) gave unique, heterogeneous profiles when typed by AFLP, a result which was consistent with ribotyping and PFGE analysis. In a further study of selected *S. typhi* isolates from Papua New Guinea which caused fatal and non-fatal disease previously shown to be clonally related by PFGE, AFLP discriminated between these isolates but did not indicate a linkage between genotype and virulence. We conclude that AFLP (discriminatory index = 0.88) has a higher discriminatory power for strain differentiation among *S. typhi* than ribotyping (DI = 0.63) and PFGE (DI = 0.74). © 2000 Elsevier Science B.V. All rights reserved.

Keywords: AFLP fingerprinting; *Salmonella typhi*; Typhoid fever

1. Introduction

Typhoid fever remains as an important global health problem, especially in the developing countries (Pang, 1998), and effective epidemiological surveillance is crucial in monitoring the presence and spread of the disease. It is estimated that more than 16 million cases of typhoid fever occur annually worldwide with more than 600,000 deaths, accompanied by a belief among public health experts that these estimates are possibly 5–10 times too low (Ivanoff, 1998; Pang, 1998). In recent years, many DNA-based, molecular approaches have been utilized in the molecular typing of *Salmonella typhi,*
including ribotyping (Alteweg et al., 1998), pulsed-field gel electrophoresis (PFGE) (Thong et al., 1995, 1996a,b), IS200 fingerprinting (Navarro et al., 1996) and RAPD-PCR (Shogkuan and Lin, 1998). These newer techniques have been most useful in delineating epidemiological relationships between various isolates of S. typhi, including investigations of outbreaks (Thong et al., 1994; Gruner et al., 1997), geographical distribution of clones (Thong et al., 1995), antibiotic resistance (Hermans et al., 1996), environmental sources (Thong et al., 1996a,b) and associations with fatal illness (Thong et al., 1996a,b).

In addition, a more recently developed technique for DNA fingerprinting, AFLP (Vos et al., 1995), which is based on the selective PCR amplification of restriction fragments from a total digest of genomic DNA, has been reported to be even more superior in discriminative power and able to differentiate strains of bacteria which were highly related or identical by other typing methods (Janssen et al., 1996; Aarts et al., 1998; Boumedine and Rodolakis, 1998). Although AFLP has been used in the genotypic analysis of several species of pathogenic bacteria (Janssen et al., 1996; Aarts et al., 1998; Boumedine and Rodolakis, 1998; Gibson et al., 1998), including Salmonella spp. (Aarts et al., 1998), it has not been used in the molecular typing of S. typhi. In the present report we compare the use of AFLP in the genotypic profiling of S. typhi with ribotyping and PFGE. Two main questions were addressed: (i) is AFLP able to discriminate strains of S. typhi from various geographical regions? (ii) Is AFLP able to discriminate S. typhi strains which were clonally related by PFGE and ribotyping?

### 2. Materials and methods

#### 2.1. Bacterial strains

Strains of S. typhi used in this study were clinical isolates obtained from patients in Malaysia, Indonesia, India, Chile, Papua New Guinea and Switzerland with a culture confirmed diagnosis of typhoid fever (Table 1). The strains were isolated, identified and maintained using standard bacteriological procedures and methods (Cowan and Steel, 1993).

<table>
<thead>
<tr>
<th>Strain</th>
<th>Origin</th>
<th>PFGE patterns</th>
<th>Ribotyping patterns</th>
<th>AFLP patterns</th>
</tr>
</thead>
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<tr>
<td>PNG fatal X1</td>
<td>P1</td>
<td>A1</td>
<td>56</td>
<td></td>
</tr>
<tr>
<td>PNG fatal X1</td>
<td>P1</td>
<td>A3</td>
<td>57</td>
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<tr>
<td>PNG fatal X1</td>
<td>P1</td>
<td>A2</td>
<td>58</td>
<td></td>
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<tr>
<td>PNG fatal X1</td>
<td>P2</td>
<td>A5</td>
<td>59</td>
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<td>P1</td>
<td>A3</td>
<td>61</td>
<td></td>
</tr>
<tr>
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<td>A4</td>
<td>62</td>
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<td>P1</td>
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<td>P3</td>
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<td>A4</td>
<td>87</td>
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</tr>
</tbody>
</table>

PNG, Papua New Guinea.

#### 2.2. Ribotyping and PFGE analysis

Procedures for ribotyping and PFGE analysis of the various S. typhi isolates were as described previously (Nair et al., 1994, 1999).

#### 2.3. Data analysis

Restriction enzyme analysis (REA) patterns generated by PFGE and ribotyping were subjected to comparative analysis, either visually or using RFLP (restriction fragment length polymorphism) pattern analysis software (GelCompar; Applied Maths, Kortrijk, Belgium), in order to assess diversity. The objective measure of similarity/dissimilarity between
strains was the $F$ value (coefficient of similarity) which calculates the number of shared bands between two strains relative to the total number of bands (El-Adhami et al., 1991). The formula used to calculate this coefficient was $F = n_x + n_y / 2n_0$, where $n_x$ is the number of bands in strain $x$, $n_y$ is the number of bands in strain $y$, and $n_0$ is the number of bands shared between the two strains. By this measurement, an $F$ value of 1.0 indicates identity of two strains and a value of 0 indicates total dissimilarity.

2.4. AFLP fingerprinting

AFLP fingerprinting was performed essentially as described by Vos et al. (1995). Genomic S. typhi DNA was digested with EcoRI and MseI and ligated with EcoRI and MseI AFLP adaptor pairs (PE Applied Biosystems, Foster City, CA, USA) using T4 DNA ligase. These serve as unique primer binding sites for subsequent PCR amplification. Using the AFLP™ Microbial Fingerprinting Kit (PE Applied Biosystems), subsets of genomic DNA fragments with three distinct fluorescent endlabels are generated in subsequent PCR amplifications according to protocols supplied by the manufacturer. Resulting fragments are electrophoresed along with an internal size standard (GeneScan® OX-500 size standard, PE Applied Biosystems) on a PE Applied Biosystems ABI Prism® 310 instrument using parameters supplied by the manufacturer in the AFLP protocol. Fragment patterns were then analysed using ABI Prism GeneScan® Software (PE Applied Biosystems) enabling the data to be standardized and normalized as binary data for further analysis.

The binary data were then analysed further using the phylogenetic analysis program PAUP*4 (Sinauer Associates, Cambridge, MA, USA) and computed using parsimony or distance analysis (UPGMA or neighbour joining) algorithms.

2.5. Statistical analysis

The discrimination index (DI), the probability that two unrelated strains sampled from the population would be placed into different typing groups, was calculated by Simpson’s index of diversity (Hunter and Gaston, 1988).

3. Results

Molecular characterization of 30 human isolates of Salmonella typhi was performed by using pulsed-field gel electrophoresis (PFGE) after digestion of chromosomal DNA with restriction endonuclease XbaI. Patterns consisted of 15–20 fragments ranging in size from 23 to 540 kb. Of the 30 strains tested, 10 Papua New Guinea (PNG) strains were obtained from patients with fatal typhoid fever; 15 PNG strains were obtained from patients with non-fatal disease and the other five strains were of diverse geographic origin (Table 1). The 25 PNG strains showed limited genetic diversity as evidenced by only two major PFGE patterns and a few minor clonal variants (with one to three band differences from the major PFGE patterns) detected following digestion with XbaI ($F=0.81–1.0$). The 10 PNG strains isolated from fatal cases were closely related ($F=0.95–1.0$) with PFGE X1 pattern being dominant (eight out of the 10 strains), while patterns X1a and X1b were the other two clonal variants. This data corresponds to work previously done (Thong et al., 1996a,b) and suggests that there could be an association among strains of S. typhi between genotype patterns and the capability to cause fatal illness. In contrast, the 15 non-fatal PNG strains had various PFGE patterns (X1, X1a, X1d, X2, X2a, X2b) and they were grouped in two clusters at 84% similarity (Fig. 1). Geographical strains were highly diverse with $F$ values ranging from 0.65 to 0.88.

Genomic DNAs of the 30 strains were then digested with restriction enzyme PstI. Electrophoresis of the digested DNA on agarose gels produced too many fragments and the resolution of these fragments was generally poor. By probing the DNA fragments with a 1.6-kb PCR generated fragment of the 16S rDNA gene (Nair et al., 1999), the number of bands in the fingerprints were greatly reduced thus making it easier to compare restriction patterns (ribotypes) between strains. PstI generated six ribotypes which contained five to six bands ranging from 6 to 21.5 kb. Eighty percent of the PNG fatal strains had ribotype pattern P1 and the other 20% had pattern P2. $F$ values between 0.6 and 1.0 showed diversity among the PNG fatal isolates. The PNG non-fatal strains were diverse as well with three ribotype patterns (P1 (eight strains), P3 (six strains)
and P6 (one strain)) and F values between 0.2 and 1.0. These three ribotypes fell into different dendrogram clusters (Fig. 2). The geographic strains also showed considerable diversity with F values between 0.2 and 1.0 (Fig. 2). However, these geographic strains also had ribotype patterns similar to that of Papua New Guinea strains and in one instance the strain from Chile had an identical pattern to an Indonesian strain. The rather high genetic diversity between strains of various ribotype patterns could be due to the unstable nature of the ribosomal genes in S. typhi (Liu and Sanderson, 1996). Limited number of ribotype patterns were also observed, as ribotyping is a technique to detect polymorphisms at the ribosomal gene level.

Like PFGE, AFLP analysis provides a way of examining DNA segments distributed over the entire genome of an organism. It offers this advantage over other methods that examine restriction site changes in single genes (e.g., ribotyping). The purpose of this study was to identify diversity among all the strains studied and the possible trait for lethality in the PNG strains. Genomic DNA for the 30 strains of S. typhi were analysed with 18 AFLP primer combinations.
Fig. 2. Dendrogram showing the cluster analysis of 30 *S. typhi* strains generated by GelCompar program using the UPGMA method, based on the *F* values. (Ribotyping Analysis). Strain numbers correspond to that of Table 1. G depicts geographic origin (st26, India; st27, Malaysia; st28, Zurich; st29, Chile; and st30, Indonesia), F and NF represent Papua New Guinea fatal and non-fatal strains, respectively.

(Table 2). A total of 780 fragments were scored. This represents about 2.5% of the *S. typhi* genome, assuming an average fragment length of 150 bp. Fifty-eight fragments (7.4%) were polymorphic. A dendrogram was generated using the PAUP*4 program to ascertain the genetic relationship among the strains studied. Based on the cluster analysis (Fig. 3) it was shown that the geographic strains are genetically diverse. Genetic diversity was evident as well for the PNG strains from fatal and non-fatal cases as they fell into different clusters in the dendrogram (Fig. 3).

Discriminatory index analysis showed that AFLP with a DI of 0.88 was more discriminatory than PFGE (DI = 0.74) and ribotyping (DI = 0.63).

4. Discussion

AFLP allows the differentiation of related bacterial species through comparison of complex banding patterns produced by PCR-amplified restriction fragments. In this study, *S. typhi* genomic DNA was digested using two restriction enzymes (MspI and EcoRI), and adaptor DNA sequences were ligated to the fragments prior to PCR. Although AFLP has
Table 2

AFLP primer combinations used in this study and number of fragments

<table>
<thead>
<tr>
<th>Primer combination</th>
<th>EcoRI</th>
<th>MseI</th>
<th># Fragments</th>
<th>Polymorphisms</th>
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been used in the genotyping of various species of bacteria (Janssen et al., 1996; Aarts et al., 1998; Boumedine and Rodolakis, 1998; Gibson et al., 1998; Savelkoul et al., 1999), including Salmonella serotypes of poultry origin (Aarts et al., 1998), there have been no reported studies with S. typhi, a strictly human pathogen. In the study of Aarts et al. (1998), the 62 different serotypes were characterized by unique profiles by AFLP fingerprinting and different strains previously identified as identical could be distinguished. It was thus of great interest to evaluate the usefulness of AFLP in the genotyping of individual isolates of S. typhi. The overall results in the present study have shown that AFLP (DI=0.88) was able to clearly discriminate and differentiate geographically diverse strains of S. typhi as well as closely related strains which were identical or very similar in most cases by PFGE (DI=0.74) and ribotyping (DI=0.63). However, it was noted that AFLP patterns A2, A4 and A15 had strains which could be differentiated by PFGE and ribotyping, respectively. Discrepancies like these arise which indicates that the strains in questions have no mutations/polymorphisms within the MseI and EcoRI restriction sites or no mutations in the sequence adjacent to the restriction sites which are complementary to the selective primer extensions, as compared to the polymorphisms within the XbaI sites (detected by PFGE) or rearrangements of the rrm genes (detected by ribotyping).

Beyond their direct application in differentiating and discriminating between strains for epidemiological purposes, molecular techniques have also contributed a great deal to phylogenetic and evolutionary studies. In addition, these approaches have also improved our understanding of the biology of pathogens and also their pathogenesis and virulence. For example, the proposal that significant genetic diversity exists among S. typhi strains globally (Pang, 1998), which may be related to the fact that their genome is highly plastic in nature (Liu and Sander son, 1996), was based largely on studies using approaches such as PFGE. In addition, we have previously shown a correlation between the molecular profile of S. typhi, as defined by PFGE, and its ability to cause fatal disease among typhoid fever cases in Papua New Guinea (Thong et al., 1996a,b).

We concluded from this previous study that a possible association exists between genotype, as assessed by PFGE, and the capability to cause fatal illness. The advent of AFLP, a potentially more discriminative technique, has allowed us to analyze these strains in more detail in the present study. AFLP analysis was able to further subtype these S. typhi strains, and this finding is in agreement with other studies which have also demonstrated the superior discriminatory power of AFLP towards the differentiation of closely related bacterial strains belonging to the same species or even subspecies/biovar (Janssen et al., 1996; Aarts et al., 1998; Boumedine and Rodolakis, 1998; Gibson et al., 1998). The AFLP data in this study suggested no correlation between genotype and virulence unlike a recent study with C. psittaci, where AFLP permitted differentiation among highly related strains in relation to host origin and clinical syndromes (Boumedine and Rodolakis, 1998). Our results show that strains with identical AFLP profiles were present among those which came from fatal and non-fatal cases and that no particular profile was uniquely associated with disease outcome. Based on these findings, we assume that no particular S. typhi genotype assessed by either AFLP or PFGE analysis
Fig. 3. Dendrogram representing the genetic relationships among 30 *S. typhi* strains using PAUP*4.0. (AFLP Analysis). Strain numbers correspond to that of Table 1. G depicts geographic origin (s26, India; s27, Malaysia; s28, Zurich; s29, Chile; and s30, Indonesia), F and NF represent Papua New Guinea fatal and non-fatal strains, respectively.

Seems to be particularly more virulent, as the ability to cause fatal disease is not associated with the banding patterns of either techniques. Therefore, other factors (including differences between hosts), which are as yet unknown, may be involved in the causation of fatal disease.
A number of high-resolution molecular typing systems for bacterial pathogens have been developed in recent years. As pointed out recently (Struelens et al., 1998), many of these methods (e.g., ribotyping, PFGE, RAPD-PCR) are largely comparative, allowing differentiation between closely related strains from those markedly different in genetic background (e.g., outbreak investigations). However, for the purposes of epidemiological surveillance, monitoring clonal spread and prevalence in populations over extended periods, as would be important in diseases like typhoid fever, requires what is referred to as ‘library’ typing systems (Struelens et al., 1998). Such methods must be easily standardized, have a high throughput, be quantitative and also adopt a uniform nomenclature (Struelens et al., 1998). The issue of standardization is particularly important as it would enable results from different laboratories to be compared. Of the many library typing systems, it has been proposed that genotypic profiles generated by AFLP are particularly suitable, especially if they are more reproducibly and objectively analysed by using electrophoresis with automated laser detection (Struelens et al., 1998). In addition, AFLP has other advantages, including the small amounts of DNA needed, good reproducibility, no requirement for prior knowledge of DNA sequence and the possibility of direct cumulative analysis with appropriate software. The minor drawbacks of this technique previously experienced were its cost and technical complexity, but in light of recent modifications this technique has become relatively rapid and technically simpler (Gibson et al., 1998). AFLP may become an important future method in the epidemiological surveillance of pathogenic bacteria.

5. Uncited reference

Pang et al., 1998

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