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Typing of Strains from a Single-Source Outbreak of *Pseudomonas pickettii*

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Plasmid profiles, genome restriction fragment polymorphisms, carbohydrate oxidation-fermentation reactions, methylumbelliferyl substrate hydrolysis patterns, antimicrobial susceptibilities, and results obtained with the Biolog GN biochemical substrate kit were used to type 19 common-source, but mixed-biotype, outbreak strains and one epidemiologically distinct strain of *Pseudomonas pickettii*. Biotyping with conventional and methylumbelliferyl substrates failed to distinguish between strains. Plasmid profile testing was found to be inconsistent and not reproducible. The Biolog GN kit allowed greater strain differentiation than restriction fragment polymorphism did (12 biotypes versus 5 biotypes); antimicrobial susceptibility testing yielded 4 biotypes, and oxidation-fermentation tests gave 3 biotypes. Oxidation-fermentation results were consistent with restriction fragment polymorphs in all but 1 of the 20 strains tested. For ease of typing, comprehensive typeability, and reproducibility, oxidation-fermentation tests should be performed initially and followed if necessary by restriction fragment polymorph analysis for the elucidation of *P. pickettii* infection outbreaks.

Pseudomonas pickettii is widely distributed in nature, being a frequent contaminant in water supplies (2, 34). It is increasingly identified as an opportunistic pathogen in nosocomial infections, especially among immunosuppressed patients (21). *P. pickettii* is an infrequent cause of bacteremia (7, 9), meningitis (8), endocarditis (14), pneumonia (36), and osteomyelitis (38). It has also been implicated in common-source nosocomial infection outbreaks due to the addition of contaminated water to parenteral fluids (10, 22, 33, 35) and to medical equipment presumed to be sterile (17, 24, 37). Respiratory and nasopharyngeal colonization in immunosuppressed patients has been described (10, 24, 27). Previous attempts to differentiate between strains in order to elucidate infection clusters have used polyacrylamide gel electrophoresis of whole cell proteins (21) and antibiotic susceptibility profiles (22). This study evaluates plasmid profiles, genomic DNA restriction fragment polymorphism, and biotyping by conventional methods, along with rapid detection of preformed bacterial enzymes by fluorogenic substrate hydrolysis and a commercial enzyme profile system, as typing methods. Strains from an Australia-wide outbreak of bacteremia caused by contamination of a single batch of ampoules of parenteral water commercially produced for injection were used. Nineteen cases of bacteremia due to three biotypes of *P. pickettii* and one case of *Pseudomonas cepacia* infection were reported. Of the 19 patients with *P. pickettii* septicemia, 13 had an underlying neoplasm. The clinical course of this epidemic has been reported elsewhere (35). *P. pickettii* and *P. cepacia* were isolated from numerous ampoules. Nineteen randomly selected isolates originating from both clinical cultures and contaminated ampoules were tested.

MATERIALS AND METHODS

Bacterial strains. Five strains of *P. pickettii* isolated from different patients' blood cultures from a single-source outbreak, as well as 14 organisms isolated from contaminated commercially produced "sterile" water for injection, were tested. An epidemiologically unrelated strain of *P. pickettii* was also included. The isolates were randomly numbered 1 to 20 and stored at −70°C in buffered glycerol broth until processing. Prior to processing, the isolates were subcultured from glycerol broth and inoculated directly into broth for plasmid and genomic typing or onto solid media for antibiogram analysis and biotyping.

Biotyping. The isolates were cultured in tryptone soy broth overnight at 35°C. The optical density was adjusted to a McFarland 0.5 standard with a colorimeter (Hach, Loveland, Colo.), and the isolates were inoculated onto 21 biochemical substrates (6) (Table 1) by a replicator method (5, 11). Lactose, maltose, mannitol, and glucose oxidation-fermentation basal media (OFBM) were also inoculated, and the results were used to determine the biovars (19, 35) of the 20 isolates (Table 2). All tests were read after incubation at 35°C in air for 24 h.

Antibiograms. The susceptibilities of the 20 isolates to 19 antimicrobial agents were determined by the replicator system in accordance with the National Committee for Clinical Laboratory Standards recommended antibiotic susceptibility-testing procedures (29). All tests were performed with Iso-Sensitest agar (Oxoid, Basingstoke, Hampshire, United Kingdom) with 1% *p*-nitrophenolglycerol (Stremfine Chemicals, Newburyport, Mo.). When indicated (Table 3), 5% horse blood (Amadeus International, Altona North, Victoria, Australia) was added to the media.

Biolog GN MicroPlate. A commercially available, standardized microtiter plate with 95 biochemical tests was used to type all 20 *P. pickettii* isolates. The Biolog GN microtiter plates (Biolog, Hayward, Calif.) were processed in accordance with the manufacturer's instructions (26). The isolates were subcultured on horse blood agar and incubated overnight at 35°C in air. A McFarland 3.0 suspension was made in 20 ml of sterile saline, and 150 μl of the suspension was aseptically pipetted into each of the 96 microtiter plate wells. After 18 h of incubation at 37°C in air, the wells were visually compared with the negative control well (A1) and those

TABLE 1. Reaction[a] of *P. pickettii* isolates with conventional biochemical substrates

Biochemical substrate or test	No. of positive isolates (n = 20)
Acidified substrates	
Adonitol	0
Arabinose	0
Glucose	0
Inositol	0
Lactose	0
Mannitol	0
Sorbitol	0
Sucrose	0
Arginine dihydrolase	20
Citrate (Simmons)	20
DNase	0
Esculin	0
Gelatinase	0
H_2S production	0
Indole	0
Lysine decarboxylase	20
Ornithine decarboxylase	20
Oxidase	20
5% Sodium chloride	0
Tryptophan deaminase	0
Urease	20

[a] Growth on 5% sodium chloride and H_2S production were measured.

resembling A1 were scored as negative. A definite homogeneous purple color was recorded as positive. Substrate reaction profiles were assigned a species probability value by using the computer package Micrologg 2, version 3.00 (Biolog).

4MUB-labelled substrates. Twenty-seven 4-methylumbelliferyl (7-hydroxy-4-methylcoumarin) (4MUB)-labelled substrates were used to type the 20 strains of *P. pickettii* by a modification of previously published methods (12, 13, 15). The 4MUB-conjugated substrates were obtained from Sigma Chemical Co., St. Louis, Mo., with the exception of laurate and 2-acetam-2-deoxy-β-D-glucopyranoside (ICN Biochemicals, Cleveland, Ohio). The substrates were reconstituted as suspensions (Table 4), filter sterilized, and stored at temperatures between −10 and −20°C. Prior to use, the substrates were thawed and 200-μl aliquots were dispensed into borosilicate glass tubes. Known positive and negative controls were included with each test.

Test and control organisms were taken from the horse blood agar plate with a wooden applicator stick and inoculated into the substrate to give a dense suspension equivalent to a McFarland 4.0 standard. The tubes were covered with foil to prevent evaporation and incubated for 20 min at 35°C in air. When necessary, 50 μl of saturated sodium bicarbonate was added to the tube prior to reading. Each test and

TABLE 2. *P. pickettii* biotyping by oxidation-fermentation testing

Biovar	Outbreak isolate no.	Acid production on OFBM[a] with:			
		Glucose	Maltose	Mannitol	Lactose
1	1–9, 11–14, 16–19	+	+	−	+
2	10, 15	+	−	−	−
3	20	+	+	+	+

[a] 1% Carbohydrate was added to the OFBM. +, acid produced; −, no acid produced.

TABLE 3. Antibiogram results for *P. pickettii* isolates

Antimicrobial agent	No. of susceptible isolates (n = 20)
Ampicillin[a]	1 (isolate 20)
Cefotaxime	All
Ceftazidime	1 (isolate 9)
Cephalothin	All
Ciprofloxacin	All
Colistin	None
Erythromycin[a]	None
Fusidic acid	None
Gentamicin	1 (isolate 2)
Imipenem	All
Methicillin[a]	None
Nitrofurantoin	None
Novobiocin[a]	None
Oxacillin[a]	All
Penicillin G[a]	1 (isolate 20)
Piperacillin	All
Rifampin[a]	None
Trimethoprim	All
Vancomycin[a]	None

[a] 5% Horse blood was added.

each control were read under long-wave UV light at 365 nm (Spectroline; Spectrotonics Corp., Westbury, N.Y.). Hydrolysis of the 4MUB-conjugated substrates by bacterial enzymes releasing the 4MUB produced a strong blue fluorescent emission at 365 nm. When bacterial enzymes failed to hydrolyze the substrate, the 4MUB remained conjugated and no fluorescent product was obtained.

Plasmid profile analysis. Plasmid DNA was prepared by a modification of a previously published method (25). Briefly, isolates were grown in 5 ml of tryptone soy broth overnight at 35°C with vigorous shaking. A 1.5-ml aliquot was pelleted by centrifugation and resuspended in 100 μl of lysis buffer (50 mM glucose, 10 mM EDTA, and 25 mM Tris-HCl [pH 8.0]), and then it was incubated for 5 min at room temperature. Twenty microliters of denaturing buffer (0.2 N NaOH, 1% sodium dodecyl sulfate) was added, and the solution was mixed by inversion and left at 0°C for 5 min. One hundred fifty microliters of 3 M potassium acetate was added, and incubation continued for a further 5 min. The preparation was centrifuged at 10,000 × g for 5 min, and the supernatant was harvested. RNase was added to a final concentration of 20 μg/ml, and the preparation was incubated for 20 min at 37°C. Following phenol-chloroform extraction (23), the DNA was ethanol precipitated, reconstituted in 50 μl of distilled water, and held at 4°C until used. Three microliters of running dye was added to 30 μl of preparation, and the mixture was electrophoresed in a 0.75% agarose gel in TBE buffer (89 mM Tris-HCl [pH 7.6], 89 mM boric acid, 2 mM EDTA) at 2.5 V/cm for 18 h. The gel was stained with 0.5 μg of ethidium bromide per ml and photographed under UV light.

Total genomic DNA analysis. Total DNA for restriction endonuclease fragment polymorphism analysis by pulsed-field gel electrophoresis (PFGE) was prepared by a modification of a previously described procedure (28). Briefly, isolates were grown in 10 ml of tryptose soy broth overnight at 35°C with vigorous shaking. A 200-μl aliquot of the overnight culture was dispensed into 10 ml of fresh broth, and incubation was continued for 4 h. Cells were pelleted by centrifugation and resuspended in 10 ml of PIV buffer (1 M NaCl–10 mM Tris-HCl [pH 7.6]) and maintained at 37°C. The bacterial suspension was mixed with an equal volume of

TABLE 4. Preparation of 4MUB substrates

Substrate	Concn of substrate in solvent	Buffer[a]
Glycosides 2-Acetam-2-deoxy-β-D-glucopyranoside L-L-Arabinofuranoside L-L-Arabinoside β-D-Cellobiopyranoside β-D-Fucoside L-D-Galactoside β-D-Galactoside L-D-Glucoside β-D-Glucoside β-D-Glucuronide β-D-Lactoside L-D-Mannopyranoside β-D-Mannopyranoside *N*-Acetyl-β-D-galactosaminide β-D *N,N',N"* Triacetychitotriose	5 mg in 200 μl of DMSO[b]	0.05 M acetate (pH 5.2)
N-Acetyl-L-D-glucosaminide	0.02 M[c] in DMF[d]	Phosphate[e] (pH 7.4)
Laurate	0.02 M[c] in DMSO	0.05 M citrate[f] (pH 4.5)
Butyrate *p*-Trimethylammonium-cinnamate chloride	0.02 M[c] in DMSO	0.05 M acetate[f] (pH 5.2)
Oleate[g]	5 mg in 200 μl of DMSO	0.05 M acetate[f] (pH 5.2)
Palmitate Propionate Acetate	0.02 M[c] in DMSO	0.05 M Tris[f] (pH 7.0)
Phosphate (acid)	5 mg in 10 ml of buffer	0.05 M acetate (pH 5.2)
Phosphate (alkaline)	5 mg in 10 ml of buffer	0.05 M Tris (pH 9.0)
Sulfate	5 mg in 10 ml of buffer	0.05 M Tris (pH 7.0)

[a] Added to give a final volume of 10 ml.
[b] DMSO, dimethyl sulfoxide (Sigma Chemical Company).
[c] 0.02 M in 1 ml of solvent; 200 μl made up to 10 ml in buffer.
[d] DMF, dimethyl formamide (BDH Chemicals, Poole, United Kingdom).
[e] Dulbecco A (Oxoid).
[f] Contains 20% absolute ethanol.
[g] Deliquescent form.

2.4% (wt/vol) low-melting-point agarose (Bio-Rad Laboratories, Richmond, Calif.) in PIV buffer, dispensed into 100-μl insert molds (Karba Pharmacia Ltd., Uppsala, Sweden), and allowed to solidify at 4°C. The agarose pellets produced were placed in a lysis solution (1 M NaCl, 6 mM Tris-HCl [pH 7.6], 100 mM EDTA, 0.2% sodium deoxycholate, and 0.5% Sarkosyl to which 20 μl of freshly prepared lysozyme [50 mg/ml] was added) and incubated overnight at 37°C. Proteinase K was added to a final concentration of 1 mg/ml, and incubation was continued for a minimum of 4 h at 50°C. The agarose pellets were washed three times for 30 min in TE (10 mM Tris-HCl [pH 7.6], 1 mM EDTA). One-quarter of each pellet was used for restriction endonuclease analysis. After equilibration in the appropriate restriction buffer, 30 U of restriction endonuclease *Dra*I (Bresatec Pty. Ltd., Adelaide, Australia) was added. The plugs were incubated overnight at 37°C and loaded on a 1% agarose gel (DNA grade agarose; INC Biomedicals, Seven Hills, Australia). DNA fragments were resolved by PFGE (CHEFF-DR II; Bio-Rad) at 14°C for 22 h at 170 V with a linear-ramped pulsed time from 10 to 90 s. Following electrophoresis, the gel was stained in 0.5 μg of ethidium bromide per ml, destained for a minimum of 3 h in 0.5× TBE, visualized, and photographed under UV light.

RESULTS

Biotyping. Three biovar patterns of *P. pickettii* were distinguished by OFBM carbohydrate acidification results (Table 2). Isolates 10 and 15 were shown to be biovar 2, and isolate 20, the epidemiologically unrelated isolate, was biovar 3. The remaining isolates were typed as biovar 1. The 20 isolates had identical results when tested with conventional biochemical substrates (Table 1).

Antibiograms. The results of the antimicrobial susceptibility testing showed some variation (Table 3). Discrepant results were isolate 9, susceptible to ceftazidime; isolate 2, susceptible to gentamicin; and isolate 20, susceptible to both ampicillin and penicillin. Results for all other isolates concurred.

Biolog GN MicroPlate. The 20 *P. pickettii* strains had identical results in 90 of the 95 biochemical substrates. The data for the five substrates which yielded differing results are summarized in Table 5. Twelve distinct biotypes were demonstrated by this method. All 20 isolates were identified as *P. pickettii* by the Microlog 2 program, with similarity indices ranging from 0.751 to 0.998, indicating high-level confidence in the identification.

TABLE 5. Results for *P. pickettii* isolates tested with the Biolog GN MicroPlate

Outbreak isolate no.	Result[a] with Biolog GN MicroPlate substrate				
	Cellobiose	L-Ketovaleric acid	D-Mannose	L-Ornithine	L-Phenylalanine
16–20	+	+	+	+	+
4, 5, 7	+	−	−	−	−
1, 2	+	+	+	−	+
3, 6	+	−	+	−	+
8	−	+	+	−	+
9	−	+	−	−	+
10	−	−	+	−	−
11	−	−	+	+	+
12	−	−	−	−	−
13	−	−	−	−	+
14	+	−	−	−	+
15	−	−	+	−	+

[a] +, positive reaction; −, negative reaction.

4MUB-labelled substrates. All 20 isolates of *P. pickettii* had uniform results when tested with 4MUB-labelled substrates; therefore, no biotypes could be distinguished by this method. The 20 isolates were positive when tested with the substrates acetate, butyrate, β-D-glucoside, laurate, acid and alkaline phosphate, and propionate. All other substrates gave negative results (Table 4).

Plasmid profile analysis. Results for plasmid detection were inconsistent. Plasmid presence could be demonstrated by the method described. The isolates had up to two bands detected at times; however, the profile patterns were not consistently reproducible, and analysis of the results was not considered useful.

Total genomic DNA analysis. PFGE resolution of DNA restricted with *Dra*I (Fig. 1) showed five restriction profiles. Of the 19 outbreak strains, 16 had identical profiles. Four strains, including the epidemiologically unrelated isolate, were shown to have individual profiles. Repeat analysis by *Dra*I restriction showed consistent patterns and identical clustering. PFGE restriction analysis with *Ssp*I (data not shown) produced identical groupings.

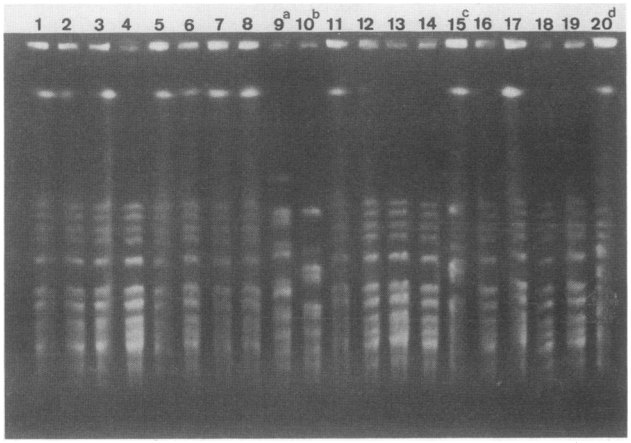

FIG. 1. Agarose gel subjected to PFGE of total DNA restricted with *Dra*I. Lanes 1 through 19 contained DNAs from *P. pickettii* isolates associated with the outbreak, and lane 20 contained DNA from an epidemiologically unrelated strain. With the exception of the isolates in lanes 9^a, 10^b, 15^c, and 20^d, the isolates have identical restriction patterns.

DISCUSSION

Various procedures, including ribotyping (1, 20), polyacrylamide gel electrophoresis (21), and cellular fatty acid analysis (32), have been used previously to type pseudomonads. Phenotypic analyses, including biotyping and antibiograms, are often inconsistent because of changes in chromosome-mediated metabolic controls and plasmid population (11). Several previous reports of outbreaks of *P. pickettii* infection have relied on these methods to identify strain variation (17, 22, 24, 35, 37). The aim of this study was to compare conventional typing methods with both novel and rapid methods. Typing schemata were assessed by reproducibility, ease of technique, typeability, and discrimination power.

Apart from oxidation-fermentation typing, the traditional method of biotyping *P. pickettii*, all the phenotypic analyses were unsuccessful. There were no detectable differences with the conventional biochemical testing method or the rapid method using 4MUB-labelled substrates. 4MUB-labelled substrates have been successful in profiling bacteria including *Pseudomonas maltophilia* (30), members of the family *Enterobacteriaceae* (18), mycobacteria (15), streptomycetes (12), members of the order *Mycoplasmatales* (4), and yeasts (3). The method is simple and rapid, resulting in same-day results, and therefore it would be useful as a rapid diagnostic tool; but in our study the method provided no useful information for biotyping *P. pickettii* isolates. Susceptibility to two antimicrobial agents distinguished isolate 20 (biovar 3) from the other isolates. Isolates 10 and 15 (biovar 2), however, had antimicrobial-susceptibility profiles identical to those of 14 of the 16 biovar 1 isolates. These results confirm a previous report (21), which showed that antibiogram profiles differentiated *P. pickettii* strains poorly. The other rapid method for detecting preformed bacterial enzymes, the Biolog GN MicroPlate system, identified all 20 isolates as *P. pickettii*, but this method's use as a predictor of strain relatedness is questionable, as it yielded 12 profiles compared with the 3 profiles detected by the established OFBM typing method and the 4 profiles detected by PFGE. The Biolog GN method failed to distinguish isolate 20, the epidemiologically distinct strain (biotype 3), from isolates 16 to 19 (biotype 1). Some variation in result interpretation may be due to subjective manual reading. Further studies on the reproducibility of this method as a typing tool may be indicated.

Although plasmid typing is straightforward to perform, the results are often inconsistent and unreliable (11, 16, 31). Plasmid detection is dependent upon the method employed, and special procedures to detect large pseudomonal plasmids may be required. One study testing six isolates of *P. pickettii* failed to detect plasmids (22). Conjugal transfer of plasmids, molecular rearrangement and deletion, or the loss of plasmids during processing can produce conflicting results (11). The transfer of plasmids to unrelated species also reduces the value of plasmid profiles in indicating genetic relatedness.

Linear electrophoresis of genomic DNA digested with frequent-cutting restriction endonucleases has been shown to differentiate strains, but analytical difficulties due to the generation of large numbers of fragments can arise (16). There is evidence that for some closely related strains, differences may be limited to larger genomic fragments and consequently, unless digestion is complete, these differences may be missed and strains may be erroneously presumed identical. Preparation of chromosomal DNA for aqueous

analysis subjects the DNA to unavoidable shearing forces, which may result in smearing during linear resolution.

PFGE has been shown to be a sensitive and reproducible means of differentiating DNA fragments from closely related bacterial strains (16, 28). The use of two infrequent-cutting enzymes enhances the technique. By this method, 16 of the 20 isolates tested had identical genomic DNA restriction profiles and were biotype 1 by OFBM. Four strains had different profiles. Isolate 9 was biotype 1, isolates 10 and 15 were biotype 2, and isolate 20 was biotype 3. Although OFBM typing has been the established standard for biotyping *P. pickettii*, no molecular confirmation of this technique has been published to our knowledge. The results of our limited study indicate that there is a degree of correlation between these phenotypic and genotypic methods, with only 1 of the 20 isolates discrepant.

The rapid methods described in this study failed to differentiate biovars of *P. pickettii*, indicating that they have the same limitations as other phenotyping techniques. These methods may be more successful when used to type other species. Plasmid isolation may be useful in some circumstances, but it can be unreliable. Initial testing by OFBM typing with 1% carbohydrate substrates is a simple and relatively quick method of typing strains of *P. pickettii*. If this method is inconclusive, or if further elucidation is necessary, PFGE resolution of restriction endonuclease DNA fragment polymorphism is appropriate.

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