

## Comparison of Biotyping, Ribotyping, and Pulsed-Field Gel Electrophoresis for Investigation of a Common-Source Outbreak of *Burkholderia pickettii* Bacteremia

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**Over a 3-month period, six immunocompromised patients developed one or more episodes of *Burkholderia pickettii* bacteremia and/or catheter infection. Vials of a commercially available, "sterile" saline for injection which had been used for flushing the patients' indwelling intravenous devices were implicated as the common source of the organisms. No further cases were diagnosed once the use of this saline was discontinued. Twenty-six isolates, including 9 outbreak-related strains from case patients and contaminated saline as well as 17 control strains, were tested comparatively by biotyping, ribotyping with *EcoRI* and *HindIII*, and pulsed-field gel electrophoresis (PFGE) with *SpeI*. Macrorestriction analysis revealed nine PFGE groups and was more discriminating than ribotyping (seven ribotypes) and biotyping (two biovars). Among the outbreak-related isolates, one *B. pickettii* type was found by the three typing methods. Furthermore, PFGE was useful for subdividing ribotypes and for distinguishing isolates involved in the outbreak from all epidemiologically unrelated strains.**

*Burkholderia pickettii* is a nonfermentative gram-negative rod isolated from environmental sources and infrequently from clinical samples (5). It is an occasional agent associated with pseudobacteremias or asymptomatic colonization of patients (8, 13, 19). However, this bacterium has been identified in a few reports as the etiologic agent of true bacteremias (2, 4, 7, 8, 12, 14, 15). Most of the bloodstream infections were traced to the contamination of parenteral fluids or of medical equipment (14).

We report here on an outbreak of *B. pickettii* bacteremias at a 650-bed teaching hospital in Liege, Belgium. From June to August 1994, six immunocompromised patients developed *B. pickettii* bacteremia and/or catheter infection. All had permanent central venous catheters. The patency of these lines was maintained by regular flushing with heparin solution. The organism was isolated from multiple vials containing commercially available, "sterile" saline without preservative (PhysioSterop; Sterop, Brussels, Belgium) used for dilution of heparin. Randomly selected isolates originating from both clinical cultures and contaminated vials, as well as control strains, were characterized by biotyping, ribotyping, and DNA typing by pulsed-field gel electrophoresis (PFGE).

### MATERIALS AND METHODS

**Identification of cases and epidemiological investigation.** Cases were defined as all patients from whom *B. pickettii* was isolated from cultures of blood and/or catheter samples. Six patients harboring this bacterium had permanent indwelling intravenous devices that were regularly flushed with heparin solution. The possible sources of infection considered were (i) the heparin used in the flushing procedure and (ii) the commercially available sterile saline contained in unidose vials (20 ml) and used to make up the heparin solution.

**Microbiological methods.** Unopened vials of heparin and saline were examined in the microbiology laboratory by the following techniques.

(i) **Step 1.** Twelve (5 ml) vials of heparin were incubated into Biphase Hemo-line (bioMérieux, Marcy l'Etoile, France) and were incubated aerobically at 35°C for 7 days. Seventeen samples of 10 ml each from vials of the three saline batches available in the hospital (five samples each for batches F92G07 and F92I09 and seven samples for batch F94E04) were similarly investigated.

(ii) **Step 2.** Large-scale screening cultures were performed for each of the three saline batches. Forty-four vials (880 ml) were cultured by membrane filtration with a 0.45- $\mu$ m-pore-size filter (Millipore); the filters were placed on the surfaces of blood agar plates, which were then incubated aerobically at 35°C for 7 days.

(iii) **Step 3.** Sixteen vials of saline from the positive batch were cultured, one by one, by the procedure described for step 2.

All isolates were identified by using the Vitek GNI card and the API 20NE system (bioMérieux). Disc sensitivity testing was performed by the Kirby-Bauer method and by using the Vitek system.

**Epidemiological typing.** (i) **Bacterial strains.** Five strains of *B. pickettii* originating from cultures of blood from different patients as well as four organisms isolated from contaminated saline were tested. Fifteen strains of *B. pickettii* originating from clinical and environmental specimens in five other Belgian hospitals and coming from the Université Catholique de Louvain (UCL) collection and two American Type Culture Collection (ATCC) strains (ATCC 27511 and ATCC 27512) were used as controls (see Table 2). The isolates were stored at -70°C in glycerol until processing.

(ii) **Biotyping.** Biotyping was performed as described by Dimech et al. (3) by oxidation-fermentation basal medium (OFBM) typing with 1% carbohydrate substrates, namely, lactose, maltose, mannitol, and glucose.

(iii) **Ribotyping.** Ribotyping with *EcoRI* and *HindIII* with a nonradioactive probing system (acetylaminofluorene rRNA Kit I from Eurogentec, Seraing, Belgium) was carried out as reported earlier (1).

(iv) **Macrorestriction of genomic DNA and PFGE.** DNA preparation and cleavage were performed as described previously (16), with minor modifications. *SpeI* macrorestriction fragments were separated on a CHEF Mapper system (Bio-Rad Laboratories, Nazareth, Belgium) by using a pulse time of 2 to 40 s for 24 h. A *SmaI* digest of *Staphylococcus aureus* NCTC 8325 was used as a molecular size marker.

(v) **Analysis of DNA relatedness.** The PFGE patterns were compared by using the GelCompar software (Applied Maths, Kortrijk, Belgium), based on the Pearson correlation coefficient, and clustering by the unweighted pair group method with arithmetic means (UPGMA method) (9). The fragments from 40 to 800 kb were used in the comparison. The strains which clustered at a level of  $\geq 80\%$  similarity were considered clonally related and were classified into the same PFGE group. The ribotype patterns were compared by calculating their Dice similarity coefficients and were clustered by the UPGMA method.

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TABLE 1. Clinical and microbiological features of *B. pickettii*-infected patients<sup>a</sup>

Patient no.	Sex/age (yr)	Underlying disease	Unit	Permanent venous catheter	No. of positive blood cultures (mo)	No. of positive catheter cultures (mo)
1	M/44	Tonsil neoplasia	Oncology	Port-à-Cath		1 (June)
2	F/69	Diabetes	Dialysis	Hickman	1 (July)	
3	F/64	Diabetes	Dialysis	Hickman	2 (August)	
4	F/72	Breast carcinoma	Oncology	Port-à-Cath	2 (August)	1 (August)
5	F/72	Breast carcinoma	Oncology	Port-à-Cath	2 (August)	1 (August)
6	M/46	Testicular carcinoma	Hematology	Port-à-Cath	2 (August)	

<sup>a</sup> All patients had pyrexia. M, male; F, female.

## RESULTS

**Epidemiological features.** Six patients with *B. pickettii* bacteremia and/or positive catheter culture were identified from June to August 1994 in three different units (oncology, hematology, and hemodialysis). All were immunocompromised patients. The main clinical and microbiological features are presented in Table 1. Recognition of the outbreak was delayed by the fact that the first three cases appeared to be unrelated, occurring 6 weeks apart and in two different wards. Chronological analysis of the interval between implantation of the catheter and the onset of infection, the type of catheter used for administration of chemotherapeutic agents or for hemodialysis (Hickman or Port-à-Cath), or the antiseptic solutions used for preparation of the skin was not informative. Because catheters were flushed with heparin solution in saline before

each episode, the flushing procedure appeared at the end of August to be related to the acquisition of the organism. The use of the suspected products was then discontinued, and no new case was diagnosed thereafter. All patients rapidly recovered with antibiotic therapy, mainly a combination of ceftazidime and ciprofloxacin, and no relapse was observed after removal of indwelling devices.

**Microbiological studies.** No growth was observed after inoculation of vials of heparin or sterile saline in Biphase Hemo-line. However, filtration of large volumes of saline was effective at detecting contamination in one batch (batch F94E04); the two other batches (batches F92G07 and F92I09) were negative. The contamination was light. Indeed, *B. pickettii* was isolated at a concentration of 1 or 2 CFU/20 ml from 12 of 16 vials of the positive batch which were tested.

TABLE 2. Origins, biotypes, ribotypes, and PFGE groups of *B. pickettii* strains

Isolate <sup>a</sup>	Yr of isolation	Hospital <sup>b</sup>	City <sup>c</sup>	Source	Biotype	Ribosomal DNA pattern with the following enzyme:		Ribotype	PFGE group
						<i>EcoRI</i>	<i>HindIII</i>		
Outbreak									
Pat 1	1994	I	Lg	Bloodstream	Va-1	E2	H2	A	1
Pat 2	1994	I	Lg	Bloodstream	Va-1	E2	H2	A	1
Pat 3	1994	I	Lg	Bloodstream	Va-1	E2	H2	A	1
Pat 4	1994	I	Lg	Bloodstream	Va-1	E2	H2	A	1
Pat 5	1994	I	Lg	Bloodstream	Va-1	E2	H2	A	1
Sal 1	1994	I	Lg	Saline solution	Va-1	E2	H2	A	1
Sal 2	1994	I	Lg	Saline solution	Va-1	E2	H2	A	1
Sal 3	1994	I	Lg	Saline solution	Va-1	E2	H2	A	1
Sal 4	1994	I	Lg	Saline solution	Va-1	E2	H2	A	1
Control									
UCL 3	1981	II	Bxl	Pleural fluid	Va-1	E2	H2	A	2
UCL 4	1972	II	Bxl	Respirator	Va-1	E2	H2	A	2
UCL 6	1974	II	Bxl	Catheter	Va-1	E2	H2	A	2
UCL 17	1992	II	Bxl	Bloodstream	Va-1	E5	H5	B	3
UCL 19	1992	II	Bxl	Bloodstream	Va-1	E5	H5	B	3
UCL 20	1992	III	Bxl	Water	Va-1	E6	H2	C	4
UCL 1	1972	IV	Cha	Bloodstream	Va-1	E1	H1	D	5
UCL 10	1983	II	Bxl	Sputum	Va-1	E3	H3	E	6
UCL 12	1985	II	Bxl	Respirator	Va-1	E3	H3	E	6
UCL 18	1992	V	Gos	Water	Va-1	E3	H3	E	6
UCL 21	1992	II	Bxl	Sputum	Va-1	E3	H3	E	6
UCL 22	1992	II	Bxl	Throat	Va-1	E3	H3	E	6
UCL 24	1994	II	Bxl	Throat	Va-1	E3	H3	E	6
UCL 23	1993	II	Bxl	Sputum	Va-1	E3	H3	E	7
UCL 13	1985	VI	Ott	Bloodstream	Va-1	E4	H4	F	8
ATCC 27511					Va-2	E6	H6	G	9
ATCC 27512					Va-2	E6	H6	G	9

<sup>a</sup> Pat, patient; Sal, saline.

<sup>b</sup> I to VI indicate the six different hospitals in which the strains were isolated.

<sup>c</sup> Lg, Liege; Bxl, Brussels; Cha, Charleroi; Gos, Gosselies; Ott, Ottignies (all cities in Belgium).

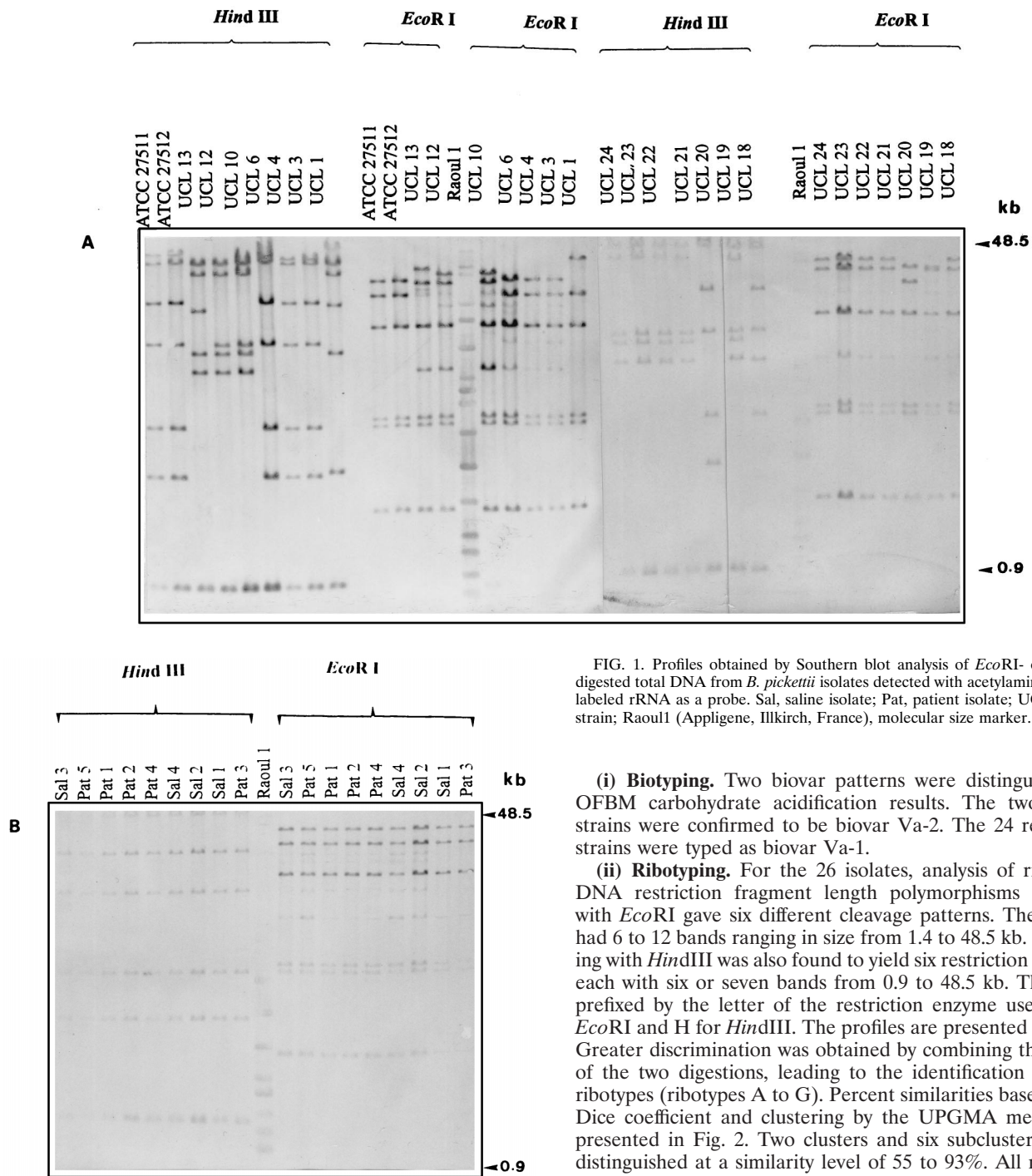


FIG. 1. Profiles obtained by Southern blot analysis of *EcoRI*- or *HindIII*-digested total DNA from *B. pickettii* isolates detected with acetylaminofluorene-labeled rRNA as a probe. Sal, saline isolate; Pat, patient isolate; UCL, control strain; Raoul1 (Appligene, Illkirch, France), molecular size marker.

All isolates of *B. pickettii* recovered from patients and from saline had identical antimicrobial susceptibility patterns: susceptibility to cefuroxime, cefotaxime, piperacillin, imipenem, and ciprofloxacin, but resistance to gentamicin, tobramycin, amikacin, ampicillin, cephalothin, and amoxicillin in combination with clavulanic acid.

**Epidemiological typing.** The distribution of the 26 isolates studied with respect to epidemiological type, patient or environmental sample, specimen type, origin, and time is presented in Table 2.

(i) **Biotyping.** Two biovar patterns were distinguished by OFBM carbohydrate acidification results. The two ATCC strains were confirmed to be biovar Va-2. The 24 remaining strains were typed as biovar Va-1.

(ii) **Ribotyping.** For the 26 isolates, analysis of ribosomal DNA restriction fragment length polymorphisms (RFLPs) with *EcoRI* gave six different cleavage patterns. The profiles had 6 to 12 bands ranging in size from 1.4 to 48.5 kb. Ribotyping with *HindIII* was also found to yield six restriction patterns, each with six or seven bands from 0.9 to 48.5 kb. They were prefixed by the letter of the restriction enzyme used: E for *EcoRI* and H for *HindIII*. The profiles are presented in Fig. 1. Greater discrimination was obtained by combining the results of the two digestions, leading to the identification of seven ribotypes (ribotypes A to G). Percent similarities based on the Dice coefficient and clustering by the UPGMA method are presented in Fig. 2. Two clusters and six subclusters can be distinguished at a similarity level of 55 to 93%. All nine outbreak-related strains, isolated from case patients as well as contaminated saline, fell into one subcluster, but they could not be differentiated from three control strains. The 14 remaining control strains, including the two ATCC strains, were distributed into five other subclusters, each with one to seven isolates.

(iii) **Macrorestriction analysis.** Digestion with *SpeI* enzyme was chosen because it revealed fewer fragments than digestion with *DraI* or *XbaI* (data not shown). *SpeI* produced PFGE patterns of 15 to 20 DNA fragments (Fig. 3). A total of 26 PFGE patterns were found and were classified into nine PFGE groups at a cutoff level of 80% correlation on the dendrogram. All outbreak-related strains were clustered together into clonal

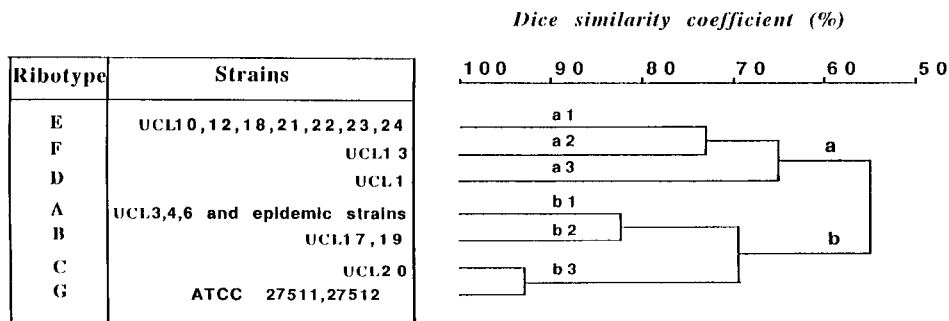


FIG. 2. UPGMA cluster dendrogram of the isolates of *B. pickettii* based on combined ribotype patterns of *Eco*RI and *Hind*III digests.

group 1. This group showed some heterogeneity with two subgroups of six and three strains, respectively. Each subgroup included isolates from both case patients and contaminated saline. The 17 control strains were distributed into eight other clonal groups, each with one to six strains. Six of the control strains were subdivided into three clonal groups (clonal groups 2, 3, and 4) which were related to outbreak-related strains at a similarity level of 70 to 77%. The remaining 11 control strains were classified into clonal groups 5 to 9, which were less related (similarity,  $\leq 40\%$ ) to the other strains.

(iv) **Correlation of PFGE and ribotyping.** There was a good correlation between PFGE and ribotyping (Fig. 4). However, PFGE was superior with regard to discrimination: ribotypes A and E were each subdivided into two PFGE groups. Macrorestriction analysis could distinguish, at a similarity level of 77%, the outbreak-related strains (clonal group 1) from three control strains (clonal group 2), whereas all these strains belonged to ribotype A.

**DISCUSSION**

Isolation of unusual organisms from the bloodstream of patients with prolonged intravenous catheterization may sug-

gest an exogenous source such as contaminated solutions (8). In this report, we describe six patients who developed *B. pickettii* bacteremia and/or catheter infection in association with colonization of a permanent indwelling intravenous device. Epidemiological investigation incriminated contamination of saline solution containing heparin intended for flushing the patients' indwelling lines. All patients promptly recovered with antimicrobial therapy, and no further cases occurred when it was advised that use of these products be stopped.

*B. pickettii* was cultured from multiple unopened single-dose vials of the commercially available "sterile" saline that was used. Only one of three batches available in our hospital was positive. The level of contamination was very low (equal to or less than 1 CFU/10 ml). Validation procedures for the positive batch, performed by the manufacturer according to the European Pharmacopoeia, did not detect the contamination. This underlines the pitfalls of the European standard quality control tests used to ensure the sterility of products for parenteral use. Investigation also failed to reveal a specific source for the product contamination.

A few features associated with this outbreak suggest the low intrinsic pathogenicity of *B. pickettii*. Although vials of the

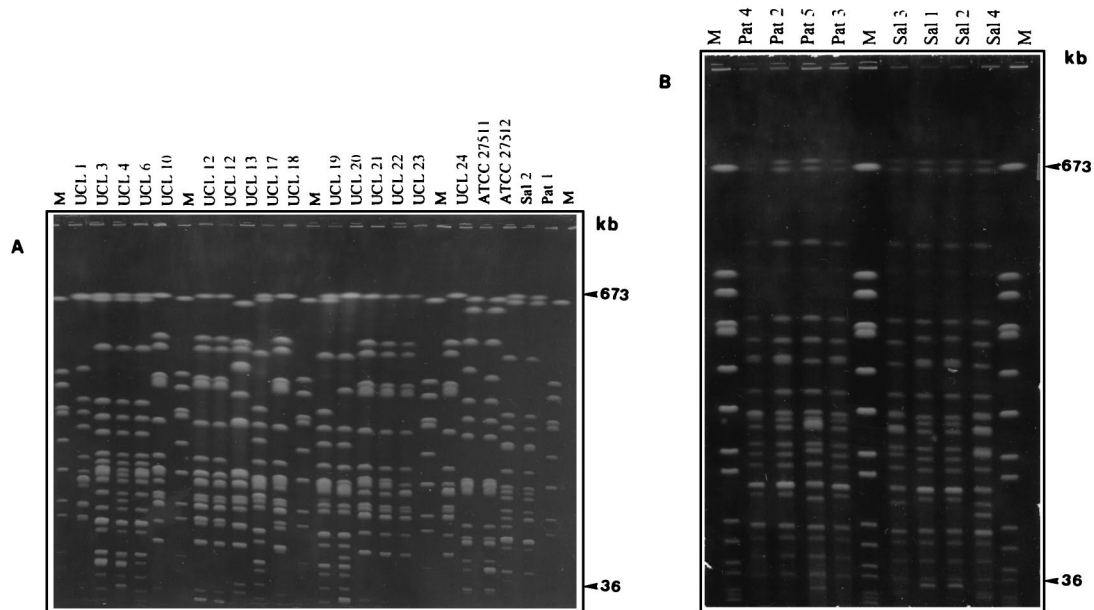


FIG. 3. PFGE separation of *Spe*I macrorestriction fragments of *B. pickettii* genomic DNA from all outbreak-related and control isolates. Sal, saline isolate; Pat, patient isolate; UCL, control strain. Lanes M, molecular size markers.

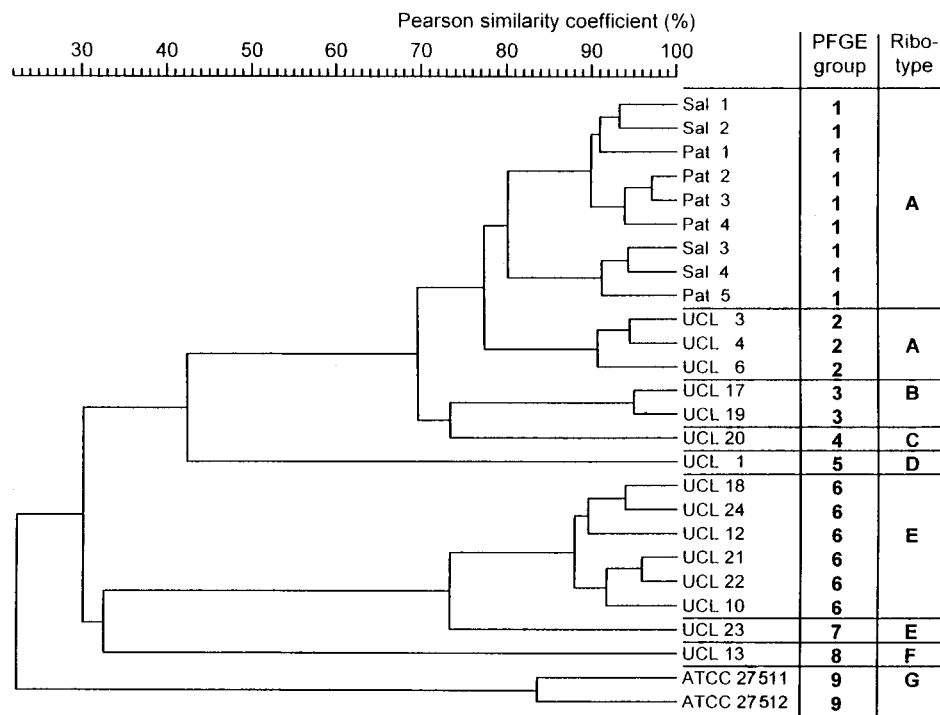


FIG. 4. Dendrogram of outbreak-related and control strains of *B. pickettii* by the UPGMA method of the Pearson correlation coefficients of *SpeI* macrorestriction patterns.

contaminated saline had been used throughout the hospital, proven infections were recorded only in immunocompromised patients, as in the study by Lacey and Want (8). The mild clinical signs of the patients and their rapid response to antibiotic therapy also support this assertion.

The challenge to the laboratory is to perform an analysis that can accurately differentiate between epidemic and sporadic isolates of the same species. Biotyping by the OFBM method did not provide useful information in our study. Indeed, all 26 *B. pickettii* isolates studied (9 outbreak-associated and 17 epidemiologically unrelated isolates), with the exception of two ATCC strains, belonged to one biovar (biovar Va-1). Molecular epidemiology appeared to be more appropriate. For the 26 isolates, analysis of ribosomal DNA RFLPs with *EcoRI* and *HindIII* gave seven ribotypes (ribotypes A to G). All nine epidemic strains (five isolated from case patients and four isolated from contaminated saline) were distributed into ribotype A, but they could not be differentiated from three control strains. Genomic DNA analysis by PFGE with *SpeI* revealed nine groups at a cutoff level of 80% correlation. All outbreak-related strains were clustered together into one group, while 17 control strains were placed into eight other clusters.

As expected, ribotyping yielded a lower number of patterns and a lesser degree of pattern divergence than PFGE. This is in agreement with comparative studies for typing *Listeria monocytogenes* (11) and *Salmonella enterica* subsp. *enterica* serovar *dublin* (10). Several studies suggest that PFGE is the most discriminating of the available genotypic methods because it allows for the detection of minor genomic rearrangements (6, 17, 18). In fact, a good correlation between the two molecular typing methods was observed, but PFGE could discriminate isolates from two of seven given ribotypes into different subsets. So, it differentiated the isolates involved in the

outbreak from the three control strains which were indistinguishable by ribotyping and appeared to be a more effective epidemiological tool.

However, both methods suffered to various degrees from a lack of discriminatory power to subtype among some control isolates that were presumably unrelated epidemiologically. Two clusters of related PFGE patterns found among control strains (clusters 2 and 6) were apparently not associated with recognized outbreaks of infection. Thus, for example, PFGE group 6 characterized six isolates originating from the respiratory tracts of patients and their environments, including a respirator, in two Belgian hospitals over periods ranging from 1983 to 1994 (Table 2). A difficulty in the interpretation of molecular typing of *B. pickettii* is perhaps the present finding that in groups of patients without any known contact, isolates with related PFGE patterns were detected. The possibility of limited genetic diversity within this species is suggested by these data but needs to be clarified by further study.

Epidemiological and microbiological data, including biotyping, ribotyping, and PFGE typing, conclusively traced this outbreak of *B. pickettii* bacteremias. The common-source exposure was a commercially available contaminated "sterile" saline product for injection.

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