

Investigation of Hospital-Acquired Infections Due to *Alcaligenes denitrificans* subsp. *xylosoxydans* by DNA Restriction Fragment Length Polymorphism

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We demonstrate that DNA restriction fragment length polymorphism determined by pulsed-field gel electrophoresis is very useful in the investigation of the epidemiology of hospital-acquired infections caused by *Alcaligenes denitrificans* subsp. *xylosoxydans*. This approach showed that hospital-acquired infections caused by this opportunistic pathogen over a 6-month period in 10 patients hospitalized in an intensive care unit and a surgical unit were not a true outbreak. In addition, this molecular typing method established that the respiratory therapy equipment was the source of the contamination of two patients.

Alcaligenes denitrificans subsp. *xylosoxydans* (formerly *Achromobacter xylosoxidans*) is an aerobic, nonfermentative, gram-negative bacillus (6). Although *A. denitrificans* subsp. *xylosoxydans* is often considered as a colonizer, it has also been recognized as a causative agent of various human infections, mostly in immunocompromised hosts or patients with underlying diseases including endocarditis, meningitis, and ventriculitis after neurosurgery, pneumonia, bacteremia, osteomyelitis, arthritis, and peritonitis (7, 14-16, 18). Several outbreaks of hospital-acquired infections due to bacterial contamination of antiseptic solutions (16), intravascular pressure transducers (2), nonbacteriostatic solutions used in diagnostic tracer procedures (8), a diagnostic contrast solution (13), and dialysis fluids (14) have been observed in intensive care units or postsurgical recovery areas. The increasing frequency of isolation of this opportunistic pathogen from clinical specimens might be linked to the extensive use of third-generation cephalosporins and fluoroquinolones, which are not effective against this microorganism (3, 9) and possibly select it.

Until 1992, *A. denitrificans* subsp. *xylosoxydans* was rarely isolated from patients hospitalized at Boucicaut Hospital (Paris). However, during the first six months of 1993 there were 10 cases of infection acquired in this hospital which were caused by this bacterium in a medical intensive care unit and in an otolaryngology ward. This unusually high frequency of isolation of *A. denitrificans* subsp. *xylosoxydans* prompted us to initiate an epidemiological study. We report here an investigation by pulsed-field gel electrophoresis to characterize the isolates.

MATERIALS AND METHODS

Bacterial strains. Twelve clinical and two environmental isolates of *A. denitrificans* subsp. *xylosoxydans* were studied. They were identified with the API 32 GN system (Api, La Balme-lès-Grottes, France). Reference strain CIP 6120 from

the collection of the Pasteur Institute (Paris) was also included in this study. Strains were stored at 4°C on tryptic soy agar medium. Antibiotic susceptibility was determined by the disk diffusion method on Mueller-Hinton medium (bioMérieux, Marcy l'Etoile, France) inoculated with 10⁵ CFU per ml.

Pulsed-field electrophoresis analysis of DNA. Bacterial DNA was analyzed by pulsed-field electrophoresis using the contour-clamped homogenous electric field (CHEF) technique developed by Chu et al. (1). Briefly, bacteria were grown in trypticase soy broth to logarithmic phase, harvested, and then resuspended in TE buffer (10 mM Tris, 1 mM EDTA, pH 7.5) to an A_{600} of 2.0. The bacterial suspension was mixed with an equal volume of low melting point 2% agarose (Bethesda Research Laboratories) to form an agar insert and then incubated for 48 h at 50°C with 0.5 M EDTA (pH 8), 1% (wt/vol) sodium dodecyl sulfate, and 50 mg of proteinase K (Appligene, Illkirch, France) per liter. After treatment with 1 mM phenylmethylsulfonyl fluoride (Sigma Chemicals, St. Louis, Mo.) for 1 h, the inserts were washed extensively twice for 30 min with TE buffer and for 30 min with distilled water. DNA was cleaved overnight with restriction endonuclease *Xba*I or *Dra*I according to the instructions of the manufacturer (New England Biolabs, Beverly, Mass.). DNA fragments were separated in 1% agarose-Tris-borate-EDTA buffer gels by electrophoresis using a CHEF apparatus (CHEF-MAPPER; Bio-Rad, Paris, France) at 14°C at 6 V/cm and alternating pulses at a 120° angle in a 7- to 20-s pulse time gradient for 28 h. The gels were stained with ethidium bromide. DNA from bacteriophage lambda concatemers was used as size markers.

RESULTS

An outbreak of hospital-acquired infections caused by *A. denitrificans* subsp. *xylosoxydans*. During the first 6 months of 1993, there were 10 cases of mixed bacterial infections involving *A. denitrificans* subsp. *xylosoxydans* at the Boucicaut Hospital in Paris. They occurred an average of 17 ± 5 days (mean ± standard deviation) after patient admission and thus could be considered as hospital-acquired infections. Eight of the cases were pneumonia in patients in a medical intensive care unit (ward A). All patients had been intubated with an

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TABLE 1. Characteristics of the 10 patients infected with *A. denitrificans* subsp. *xylosoxydans*

Patient no., sex, ^a and age (yrs)	Ward	Admission date ^b	Underlying disease	Diagnosis on admission	<i>A. denitrificans</i> subsp. <i>xylosoxydans</i> isolation			Symptoms at the date of <i>A. denitrificans</i> subsp. <i>xylosoxydans</i> isolation
					Site ^c	Strain no.	Pulso-type	
1, F, 84	A	12/23/92	Chronic obstructive pulmonary disease	Acute exacerbation	Sinus aspirates and PTC specimen	93.1	4	Acute respiratory failure
2, M, 42	A	12/29/92	AIDS	Suicide attempt	PTC specimen	93.2	4	Pneumonia
3, M, 69	A	3/15/93	Chronic obstructive pulmonary disease	Acute exacerbation	PTC specimen	93.3	5	Pneumonia
4, F, 50	A	4/1/93	Chronic alcoholism	Suicide attempt	PTC specimen	93.4	4	Acute respiratory distress syndrome
5, F, 43	A	4/20/93	None	Suicide attempt	PTC specimen	93.5	5	Coma
6, F, 64	A	5/5/93	Chronic alcoholism	Alcohol-related seizures	PTC specimen	93.6	5	Coma
7, M, 73	A	6/15/93	Sigmoid adenocarcinoma and metastatic prostate cancer in lung	Pleural effusion	Pleural fluid	93.7	6	Acute respiratory failure
8, F, 64	A	5/7/93	Chronic restrictive pulmonary disease	Postoperative respiratory failure	PTC specimen	93.8	4	Acute respiratory distress syndrome
9, M, 41	B	5/28/93	Pharynx epithelioma	Pharyngectomy	Skin	93.9	7	Cutaneous necrosis
10, M, 73	B	6/7/93	Pharynx epithelioma	Pulmonary embolism and aspiration pneumonia	Bronchial aspiration	93.10	8	Pneumonia

^a F, female; M, male.^b Month/day/year.^c PTC, plugged telescoping catheter.

endotracheal tube (for 16 ± 6 days) before isolation of infecting microorganisms. *A. denitrificans* subsp. *xylosoxydans* was isolated ($>10^3$ CFU/ml) both on 5% horse blood agar and chocolate agar from lower respiratory tract secretions collected with a plugged telescoping catheter as previously described (11) in seven cases (strains 93.1, 93.2, 93.3, 93.4, 93.5, 93.6, and 93.8) and from pleural fluid in one case (strain 93.7). Nosocomial cases of pneumonia are frequently caused by more than one bacterial species (10), and in all cases that we observed except one (case number 3), *A. denitrificans* subsp. *xylosoxydans* was isolated from the specimens in association with various microorganisms (*Pseudomonas aeruginosa*, 5 cases; *Serratia marcescens*, 3 cases; *Xanthomonas maltophilia*, 1 case; *Proteus mirabilis*, 1 case; *Providencia stuartii*, 1 case; and *Staphylococcus aureus*, 1 case). Finally, two strains, designated 93.9 and 93.10, were isolated from two patients (cases 9 and 10) hospitalized in June 1993 at the department of otolaryngology (ward B) (Table 1). These strains were isolated in association with *Escherichia coli* and *Pseudomonas aeruginosa* from skin tissue (patient number 9) and in association with *Serratia marcescens* from a bronchial aspirate (patient number 10). During hospitalization, both patients were treated with a respiratory device that aerosolized tap water in order to humidify oxygen.

All clinical isolates were resistant (zone diameter, <6 mm) to cephalothin, cefuroxime, cefoxitin, cefotaxime, aztreonam, gentamicin, netilmicin, amikacin, tobramycin, pefloxacin, and ciprofloxacin, as has been previously described for this bacterial species (3, 9). *A. denitrificans* subsp. *xylosoxydans* strains resistant to ticarcillin, piperacillin, and azlocillin by production of an OXA or CARB type penicillinase have been reported (12); however, these penicillins could not be used to distinguish between the ten isolates, which were all highly susceptible to these carboxy- and acylpenicillins. Nevertheless, the patterns of susceptibility to two antibiotics, amoxicillin (AMX) and

sulfonamide (S), allowed the strains to be classified into four groups: (i) AMX- and S-susceptible isolates (93.3, 93.5, and 93.6), (ii) AMX-susceptible and S-resistant isolates (93.1, 93.2, 93.4, and 93.8), (iii) AMX-resistant and S-susceptible isolates (93.7 and 93.10), and (iv) an AMX- and S-resistant isolate (93.9).

Identification of the source of contamination. An epidemiological investigation was conducted in the medical intensive care unit after the third case of *Alcaligenes* infection had occurred (in April), to determine the origin of the contamination. The bacterium was recovered in all of the first three cases from lower respiratory tract secretions collected from mechanically ventilated patients. The respiratory therapy equipment was therefore suspected to be the source of *Alcaligenes* organisms. Cultivating water from the humidifier on a selective agar medium containing 100 mg of aztreonam (a β -lactam to which *Alcaligenes* spp. are resistant) per liter allowed isolation of *A. denitrificans* subsp. *xylosoxydans* (strain 93.3a) from this site. Soaps and antiseptic solutions used for patient nursing were also systematically analyzed; all cultures of undiluted samples on nonselective nutrient agar were sterile after incubation for 3 days at 30°C. Following these results, the disinfection procedure was revised and thereafter, antibacterial and humidifier filters were used. Nevertheless, *A. denitrificans* subsp. *xylosoxydans* infection developed in four other patients in May and June, but we failed to isolate the bacterium from the patients' environment. Infections that developed in the otolaryngology unit were related, in one case, to the contamination of nebulizer water, from which we isolated strain 93.10a. The two environmental strains, 93.3a and 93.10a, displayed the same antibiotic susceptibility pattern as clinical isolates 93.3 and 93.10, respectively.

Pulsed-field gel electrophoresis analysis of *A. denitrificans* subsp. *xylosoxydans* DNA. DNA restriction fragment length polymorphism (RFLP) was investigated by pulsed-field gel

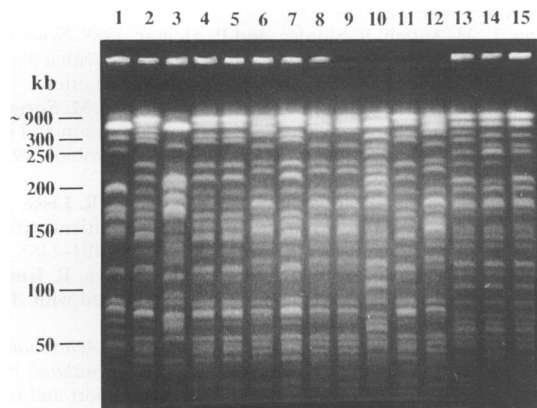


FIG. 1. Restriction patterns of *Xba*I-restricted DNA from *A. denitrificans* subsp. *xylosoxydans*. Lanes: 1, reference strain CIP 6120 (pulsotype 1); 2, clinical strain 93.L (pulsotype 2) from Laennec Hospital; 3, clinical strain 93.HD (pulsotype 3) from Hôtel-Dieu Hospital; 4 to 11, clinical strains 93.1 (pulsotype 4), 93.2 (pulsotype 4), 93.3 (pulsotype 5), 93.4 (pulsotype 4), 93.5 (pulsotype 5), 93.6 (pulsotype 5), 93.7 (pulsotype 6), and 93.8 (pulsotype 4), respectively, from Boucicaut Hospital (ward A); 12, strain 93.3a (pulsotype 5) from nebulizer water of patient number 3; 13 and 14, clinical strains 93.9 (pulsotype 7) and 93.10 (pulsotype 8), respectively, from Boucicaut Hospital (ward B); 15, strain 93.9a (pulsotype 7) from nebulizer water of patient number 9.

electrophoresis in each of the four groups of isolates as individualized by antibiotyping. To evaluate the discriminative capacity of this method for *A. denitrificans* subsp. *xylosoxydans*, we included in the study three epidemiologically unrelated strains: reference strain CIP 6120 and two clinical isolates (strains 93.L and 93 H.D) from two other hospitals in Paris (Laennec Hospital and Hôtel-Dieu Hospital). Since the G+C content of *A. denitrificans* subsp. *xylosoxydans* is 66 to 70% (6), the restriction endonucleases *Xba*I and *Dra*I, which recognize AT-rich restriction sites (TCTAGA and TTTAAA, respectively), were used to cleave genomic DNA.

The restriction patterns obtained after digestion of the genomic DNA by *Xba*I are depicted in Fig. 1. Twenty to thirty DNA fragments ranging in size from 50 to ~900 kb were produced by digestion with *Xba*I. This enzyme allowed the three epidemiologically unrelated strains to be distinguished, as each displayed a different electrophoretic pattern. Five other different pulsotypes were observed among the 10 clinical isolates from Boucicaut Hospital. The two strains isolated from the environment of patients 3 and 9 displayed pulsotypes identical to those of the corresponding clinical isolates. A greater number of bands were generated by the restriction endonuclease *Dra*I (not shown), and consequently, DNA cleavage by this enzyme increased the sensitivity of the typing method. The 15 isolates were separated in eight groups according to their electrophoretic patterns again, and the distribution of the strains in each of the groups was in agreement with that determined by *Xba*I digestion.

DISCUSSION

In this work, DNA RFLP determined by pulsed-field gel electrophoresis was used to analyze strains of *A. denitrificans* subsp. *xylosoxydans* isolated over a 6-month period from 10 patients hospitalized in an intensive care unit and in a surgical unit. This molecular typing method has previously been shown to be very useful for the epidemiological investigation of

hospital-acquired infections caused by *Staphylococcus aureus* and *Acinetobacter baumannii* (4, 5). Our results show that DNA polymorphism and RFLPs can be used as epidemiological tools for the species *A. denitrificans* subsp. *xylosoxydans* (Fig. 1).

In general, hospital-acquired infections are either sporadic or epidemic. By determining the RFLPs of clinical isolates, we have demonstrated that clusters of *A. denitrificans* subsp. *xylosoxydans* infections occurring in two hospital wards were not due to the spread of a single bacterial strain and consequently were not a true outbreak. Three different strains were identified among eight clinical isolates from ward A, one being recovered from four patients, another one from three patients, and one from one patient. The two patients from ward B were infected by strains different from those infecting patients from ward A. In addition, the results of antibiotyping and pulsotyping correlated well: isolates belonging to pulsotype 4 were all AMX susceptible and S resistant, those of pulsotype 5 were all AMX and S susceptible, and that of pulsotype 7 was AMX and S resistant. However, pulsotyping was more discriminative than antibiotyping since it permitted differentiation of two strains, both resistant to AMX and susceptible to S.

The natural habitat of *A. denitrificans* subsp. *xylosoxydans* is an aqueous environment, and several human infections reported in the literature were water borne (14, 17). In our study, the source of contamination of two patients in different wards was formally identified as the respiratory therapy equipment. Water from a nebulizer and a humidifier was heavily contaminated with *A. denitrificans* subsp. *xylosoxydans*, and we demonstrated that the two environmental isolates displayed RFLP patterns identical to those of isolates recovered from the two patients. Because the eight remaining patients developed pneumonia following respiratory assistance, contamination of equipment, although not proven, appears likely.

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