Plasmid DNA Fingerprinting of Acinetobacter Species Other than Acinetobacter baumannii

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Received 14 June 1993/Returned for modification 7 September 1993/Accepted 12 October 1993

During the last years Acinetobacter species have emerged as clinically significant pathogens. Most infections are nosocomially acquired and mainly due to Acinetobacter baumannii. Little is known about the epidemiology and clinical significance of unnamed Acinetobacter species (3) (the second most often encountered member of the genus Acinetobacter) and other Acinetobacter species such as A. johnsonii, A. junii, and A. lwoffi. Seventy-five clinical isolates of Acinetobacter species other than A. baumannii (Acinetobacter species 3, n = 37; A. johnsonii, n = 20; A. junii, n = 8; A. lwoffi, n = 10) recovered from 66 patients over a period of 12 months were analyzed by plasmid DNA fingerprinting. Plasmids were found in 84.4% of Acinetobacter species 3 isolates and in all A. johnsonii, A. junii, and A. lwoffi isolates. Strains harbored up to 15 plasmids each. Almost every isolate gave a unique plasmid pattern. With one exception, identical plasmid profiles were detected only in corresponding isolates recovered from blood cultures and intravascular catheters from a given patient. Plasmid DNA fingerprinting proved to be useful for typing Acinetobacter species other than A. baumannii. There was no evidence of patient-to-patient transmission or hospital outbreaks due to these species. This finding is in contrast to the results obtained in studies of the hospital epidemiology of A. baumannii.

In recent years Acinetobacter baumannii has been increasingly recognized as an important nosocomial pathogen (4, 10, 17). This organism has been implicated as the cause of a wide spectrum of infectious diseases such as pneumonia (10), meningitis (6), bacteremia (4, 25), and device-related infections (4, 27). Outbreaks of infections have been reported from neonatal intensive care units (25), medical and surgical intensive care units (4, 17), and burn units (30) and have been associated with respiratory equipment (10), humidifiers (15), pressure transducers (4), patients' mattresses (30), and contaminated gloves (23).

For epidemiological studies, various typing methods, such as antibiotic resistance typing (1, 19), bacteriocin typing (3), biotyping (8, 9, 19), phage typing (9, 16, 26), serotyping (32, 33), cell envelope protein typing (1, 9, 11, 12), plasmid typing (1, 13, 17, 23), ribotyping (11, 14) and restriction fragment length polymorphisms of chromosomal DNA determined by pulsed-field gel electrophoresis (2), have been developed.

As A. baumannii, formerly classified as A. calcoaceticus subspecies anitratus, has been frequently shown to be the most prevalent species among Acinetobacter strains (8, 16, 20, 33) and was predominantly responsible for nosocomial infection and hospital outbreaks (4, 10, 17, 23), epidemiological studies have focused mainly on A. baumannii. Little is known about the epidemiology and clinical significance of Acinetobacter species other than A. baumannii, previously classified as A. calcoaceticus subspecies lwoffi. The occurrence of bloodstream infections, mainly in three hospitals, due to Acinetobacter species other than A. baumannii as identified on the basis of the latest terminology (7, 8) prompted us to investigate these isolates with regard to their possible epidemiologic relationship. The purpose of this study was to find out whether plasmid typing of unnamed Acinetobacter species 3 (the second most prevalent member of the genus Acinetobacter), A. johnsonii, A. junii, and A. lwoffi would be useful for epidemiological investigations of infections due to these organisms.

(Part of this work has been presented previously [29a].)

MATERIALS AND METHODS

Bacterial strains. During a 12-month survey a total of 584 Acinetobacter isolates from 420 patients were consecutively recovered from different clinical specimens submitted to our institution. Details of these isolates have been presented elsewhere (28). Isolates were identified according to the simplified identification scheme described by Bouvet and Grimont (8) including growth at 37, 41, and 44°C; production of acid from glucose; gelatin hydrolysis; and assimilation of 14 different carbon sources. Identification of isolates at the genus level was confirmed by the transformation assay of Juni (21). The following reference strains served as controls: Acinetobacter species 3 strain CIP 70-29, A. johnsonii ATCC 17909, A. junii ATCC 17908, and A. lwoffi ATCC 15309. Reference strains were obtained through the courtesy of P. J. M. Bouvet and P. A. D. Grimont (Service Enterobactéries, Institut Pasteur, Paris, France). One hundred fifty-eight isolates were identified as species other than A. baumannii. The most common species were unnamed Acinetobacter species 3 (n = 55), A. johnsonii (n = 29), A. lwoffi (n = 21), and A. junii (n = 11). Of these, all isolates from relevant clinical sources, blood cultures, catheter tips, and cerebrospinal fluid, were included in this study. Other isolates were included if they were recovered from the same hospital or ward. Thus, a total of 75 isolates were selected: 37, Acinetobacter species 3; 20, A. johnsonii; 10, A. lwoffi; and 8, A. junii. The origins of the isolates and the distribution of the species are shown in Table 1. All isolates were stored in glycerol broth at −70°C until further use.

Susceptibility testing. MICs of selected antimicrobial agents were determined by a microtiter broth dilution method (MicroScan MIC Plus Type MK Dried Panels; Baxter Healthcare Corp., West Sacramento, Calif.) and by the disk diffusion method on Mueller-Hinton agar.
TABLE 1. Origins of Acinetobacter isolates and distribution of species

<table>
<thead>
<tr>
<th>Organism</th>
<th>No. of isolates from:</th>
<th>Total no. of isolates</th>
<th>Total no. of patients</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Blood culture</td>
<td>Central venous catheter</td>
<td>Sputum</td>
</tr>
<tr>
<td>Acinetobacter species 3</td>
<td>11</td>
<td>13</td>
<td>6</td>
</tr>
<tr>
<td>A. johnsonii</td>
<td>15</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>A. junii</td>
<td>4</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>A. lwoffii</td>
<td>8</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>38</td>
<td>20</td>
<td>7</td>
</tr>
</tbody>
</table>

**Plasmid isolation.** Plasmid DNA was prepared as described by Hartstein et al. (17), with minor modifications. In brief, isolates were grown on brain heart infusion agar plates at 30°C for 24 h. Cells from one quarter of the plate—growth from one-half of the plate when A. johnsonii, A. junii, or A. lwoffii was used—were suspended in 3 ml of 2.5 M NaCl–10 mM EDTA (pH 8.0). After centrifugation, cells were resuspended in 900 µl of 20% sucrose–50 mM Tris–10 mM EDTA (pH 8.0) and 200 µl of lysozyme (10 mg/ml). After incubation at 37°C for 30 min, 1 ml of 2.5 M NaCl–10 mM EDTA (pH 8.0) and a lysis solution containing 0.5 ml of 0.5% mixed alkytrimethylammonium bromide (ATAB) and 0.5 ml 1% Triton X-100 was added. The resulting lysate was incubated in a water bath at 56°C for 15 min and then centrifuged at 33,000 × g for 45 min at 20°C in a Sorvall centrifuge. Five milliliters of 0.5% ATAB was added to the supernatant. Following incubation on ice for 10 min and centrifugation at 2,500 × g for 15 min at 4°C, the pellet was dissolved in 0.25 ml of 2.5 M NaCl–10 mM EDTA (pH 8.0) and 0.5 ml of 10 mM Tris–1 mM EDTA (pH 8.0). After addition of 3 µl of RNase (500 µg/ml; Boehringer GmbH, Mannheim, Germany), the solutions were incubated at 37°C for 30 min. The remainder of the procedure was performed in Eppendorf tubes. Protein was extracted with phenol–chloroform–isoamyl alcohol (25:24:1), and plasmid DNA was precipitated with an equal volume of ice-cold isopropanol. The precipitate was collected by centrifugation, dried under vacuum, and then dissolved in 60 µl of sterile distilled water. Electrophoresis was performed on a horizontal gel containing 0.8% agarose in TAE buffer (0.04 M Tris-acetate, 0.001 M EDTA [pH 8.2]). The samples (20 µl) and 5 µl of running dye were loaded onto the gel and run for 18 h at 30 V. Gels were stained with ethidium bromide and photographed under a UV lamp. Molecular weights were estimated by comparison with molecular size markers. A plasmid type was defined as any plasmid pattern which varied from another pattern with regard to the number and size of plasmid bands. Isolates were considered similar if one of the compared patterns contained one or two additional bands. Isolates were run in duplicate on different gels.

**RESULTS**

**Antibiotic resistance patterns.** Analysis of the antibiotic resistance patterns of Acinetobacter species 3 isolates revealed only three different patterns. Susceptibility pattern A accounted for 35 (95%) of the 37 isolates tested. Isolates were susceptible to amoxicillin-clavulanate, gentamicin, tobramycin, amikacin, trimethoprim-sulfamethoxazole, tetracycline, ciprofloxacin, and imipenem and resistant to ampicillin, mezlocillin, Piperacillin, cefazolin, and cefuroxime; resistance was variable with regard to broad-spectrum cephalosporins. Only two isolates showed greater susceptibility to penicillins (pattern B) or penicillins and older cephalosporins (pattern C), respectively.

With only a few exceptions, isolates identified as A. johnsonii, A. junii, or A. lwoffii were susceptible to ampicillin, amoxicillin-clavulanate, broad-spectrum cephalosporins, the aminoglycosides, trimethoprim-sulfamethoxazole, tetracycline, ciprofloxacin, and imipenem. Resistance was variable with respect to mezlocillin, piperacillin, and older cephalosporins.

**Plasmid profiles.** Twenty-seven different plasmid profiles were observed among the 37 Acinetobacter species 3 isolates obtained from 33 patients. Plasmids were found in 32 (86.5%) of 37 isolates, and no plasmids were found in 5 isolates (13.5%). If apparently identical isolates were eliminated, plasmids were detected in 27 (84.4%) of 32 strains. There was a median number of four plasmids in each strain, with a range of 0 to 10 plasmids. The sizes of the plasmids were in the range of 2 to >30 kb. Representative plasmid profiles are shown in Fig. 1.

Plasmid profiles were identical, with one exception, only in isolates recovered from the blood culture and intravascular catheter or cerebrospinal fluid from a given patient (Fig. 1, lanes 3 to 8, patients B to D). Plasmid profiles were also identical in two isolates obtained from two different patients in the same hospital. Profiles were similar for two isolates recovered from a blood culture and a central venous catheter in a patient with device-related infection; the latter isolate showed an additional plasmid with a low molecular weight (Fig. 1, lanes 1 and 2, patient A).

Plasmids were found in all A. johnsonii isolates tested; each strain harbored a median number of 10 plasmids (range, 3 to 15). Sixteen different profiles were detected in 20 isolates obtained from 16 patients; identical profiles were found only in those isolates that were recovered from the blood culture and intravascular catheter of the same patients (Fig. 2, lanes 1 to 8, patients A to D).

Similar results were obtained with A. junii (data not shown). All isolates harbored plasmids, with an average of six plasmids in each strain (range, 2 to 15). Eight different plasmid profiles were detected among the eight isolates tested.

Among the 10 A. lwoffii isolates tested, nine different plasmid profiles were found (data not shown). All strains harbored plasmids, with a median number of nine plasmids in each strain (range, 4 to 16). Only two isolates from a blood culture and a peripheral venous catheter of a patient with catheter-related sepsis were shown to have identical plasmid profiles.

**Reproducibility.** For comparison and reproducibility testing, one strain was retested on each gel. In addition, four Acinetobacter species 3 strains and two A. johnsonii strains were maintained at room temperature for up to eight weeks and subcultured weekly. Longitudinal reproducibility of
plasmid profiles was studied by running the isolates side by side on the same gel with corresponding isolates that were kept frozen. Plasmid profiles were identical; no loss of plasmid bands was found.

**DISCUSSION**

Because *Acinetobacter* species are ubiquitous in the environment and are commensals on human skin and mucous membranes (24, 31), they are common contaminants and there is difficulty in determining whether the isolation of *Acinetobacter* species indicates infection or merely contamination. The diagnosis of infection with *Acinetobacter* species often depends on the isolation of the same strain from the lesion, blood, intravascular device, urine, cerebrospinal fluid, or peritoneal dialysate. Epidemiologic typing is therefore applied in the diagnosis of a single patient. Transmission of a strain between patients or staff is another level at which typing can elucidate the epidemiology. Examples of this type of transmission exist for *A. baumannii* and *Acinetobacter*...
species 3 (11, 18), but it is currently not known if patient-to-patient transmission or hospital outbreaks occur with other Acinetobacter species.

Various methods for epidemiological typing of Acinetobacter strains have been developed and have recently been reviewed (1, 5). A biotyping system has been introduced by Bouvet and Grimont (8) and has proved useful in the delineation of hospital outbreaks. This system, however, has been developed only for A. baumannii and Acinetobacter species 3; but, whereas 19 different biotypes of A. baumannii were identified, only 3 biotypes were observed among Acinetobacter species 3 strains (9). The bacteriophage typing system was based on the use of two complementary sets of bacteriophages. This typing system has been used only in a single laboratory in the world. Typeability among A. baumannii strains was about 75% (9, 26), whereas, when strains other than A. baumannii were considered, 68% of strains were untypeable (9). Antibiotyping typing was used in several studies as an epidemiological marker (1, 16, 19) and may be valuable when there are marked similarities or dissimilarities in resistance patterns. This method has not been used for Acinetobacter strains other than A. baumannii, but our results indicate that antibiotic resistance typing of Acinetobacter species 3, A. johnsonii, A. junii, and A. Iwoffii has only poor discriminative power and cannot be used as a reliable marker in epidemiological investigations. Antibiograms of these isolates revealed that organisms with identical susceptibilities belonged to as many as 25 different plasmid types. In two recent studies a serotyping system has been designed for A. baumannii and Acinetobacter species 3 with polyclonal rabbit immune sera; 20 and 13 serovars, respectively, have been identified (32, 33). This system seems promising for the study of outbreaks of nosocomial infection but is rather labor intensive.

The typing procedures mentioned so far involved the use of conventional systems based mainly on phenotype characteristics of the bacteria. Some of these techniques, such as phage typing and serotyping, involve the use of specific reagents that are not readily available and often difficult to develop. The application of modern molecular biology has permitted new approaches for epidemiologic typing of Acinetobacter species. These methods are being applied to an ever-increasing number of organisms. Conflicting results were obtained by using the electrophoretic patterns of cell envelope proteins. Whereas several studies showed that cell envelope protein patterns were a valuable tool in typing Acinetobacter strains and allowed differentiation among strains associated with hospital outbreaks (9, 11, 12), Giammanco et al. (16) failed to show any diversity not only within but also between some of the biotypes of A. baumannii. Strains identified as species other than A. baumannii mostly showed unique profiles, but epidemiologically well-defined strains have only rarely been studied. Recently, however, two hospital outbreaks due to Acinetobacter species 3 have been described and characterized by cell envelope protein profiles. All isolates gave identical patterns (11, 18).

Plasmid profile typing and plasmid fingerprinting were shown to be useful methods of epidemiological typing of various bacterial species (22). Few studies only have been performed with Acinetobacter species. Analysis of plasmid profiles of A. baumannii strains proved to be helpful in delineating nosocomial outbreaks (17, 23). Gerner-Smidt (13) studied the plasmid content of 93 clinical Acinetobacter strains; unfortunately, strain identification was based on older taxonomic schemes of the genus. The investigator reported that 81% of the strains harbored plasmids.

We studied the value of plasmid profile typing of 75 Acinetobacter isolates identified as species other than A. baumannii. A total of 84.4% of Acinetobacter species 3 isolates and all A. johnsonii, A. junii, and A. Iwoffii isolates, contained plasmids. With one exception, identical profiles were found only in corresponding blood culture and intravascular catheter or cerebrospinal fluid isolates from a given patient, thus providing evidence that Acinetobacter species other than A. baumannii may be involved in device-related infections. This was recently demonstrated by us for A. johnsonii (29). Restriction endonuclease digestion of plasmid DNA may increase the discriminatory power of plasmid analysis (17), in particular for those strains that contain only a few plasmids with similar sizes. Digestion of plasmids was not considered necessary in our study because the high number of plasmid bands in the strains investigated facilitates interpretation and comparison of plasmid profiles and contributes to the discriminative power of this typing method. The demonstration of such diversity among plasmid patterns supports the assumption that isolates with different plasmid profiles represent different strains and that plasmid differences do not reflect only acquisition or loss of R-plasmids in a highly selective environment. Reproducibility of plasmid profiles was excellent, even after prolonged storage of strains at room temperature.

Although our primary intention was to assess the diversity and reproducibility of plasmid types in Acinetobacter strains other than A. baumannii, the data we have accumulated suggest some specific epidemiologic conclusions. Patient-to-patient transmission, acquisition of strains from a common source, or outbreaks of infections seem to be rather unusual with these species. We have no conclusive proof for this, because colonization in patients was not monitored prospectively so that a possible hospital outbreak might have gone undetected. However, we evaluated all isolates that were consecutively recovered at our institute during the study period. With one exception, identical or similar profiles in isolates obtained from two different patients were not detected, though all isolates were obtained from the same geographical area and the majority of isolates were obtained from only three different hospitals. It is therefore unlikely that we might have missed an outbreak of infection or colonization. No clustering of type by ward or time was observed. All but two patients were treated in normal wards and not in intensive care units. Though all infections were nosocomially acquired, these cases may represent infections by strains resident in the patients even before hospitalization. These findings are in contrast to the results obtained in epidemiological studies of A. baumannii, as patient-to-patient transmission or common-source outbreaks in intensive care units have often been reported for A. baumannii (2, 4, 17, 23). Similar data were reported by Traub (33), who did not find hospital outbreaks or nosocomial cross-infections due to Acinetobacter species 3. However, nosocomial spread of Acinetobacter species 3 may occur, as was recently demonstrated for the first time in a medical and a neonatal intensive care unit (11, 18).

No single typing system has generally been accepted, and most investigators advocate the use of at least two different methods in epidemiological studies of Acinetobacter species (1, 5, 9, 11, 19). With strains belonging to species other than A. baumannii, species identification and plasmid typing seem to be a good approach in epidemiological investigations, whereas antimicrobial susceptibility testing would not be able to distinguish among most organisms.
REFERENCES


