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Clinical and Epidemiological Investigations of *Acinetobacter* Genomospecies 3 in a Neonatal Intensive Care Unit

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A prospective study of *Acinetobacter* isolates from a neonatal intensive care unit was performed for 24 months. Fifty-six isolates were obtained from 21 patients, and another eight were obtained from environmental specimens. Infection due to *Acinetobacter* organisms was established for 16 patients, 6 with septicemia, 9 with pneumonia, and 1 with a wound infection. Further investigations were performed with 38 representative isolates. Twenty-nine isolates were identified as unnamed DNA-DNA hybridization group (genomospecies) 3, three were identified as genomospecies 2 (*Acinetobacter baumannii*), one was identified as genomospecies 5 (*Acinetobacter junii*), three were identified as genomospecies 14, and two were unclassified. Eight distinguishable protein profiles, coded I through VIII, were found by cell envelope protein electrophoresis. Profile V, a common profile, was observed for 17 isolates that had been recovered from 11 patients and 1 dust specimen. These isolates, all of which belonged to genomospecies 3, had similar antibiograms and biotypes. This study has revealed that genomospecies 3 can be associated with infection and be spread in hospitals.

Acinetobacter are ubiquitous organisms which can be easily isolated from water and soil and have also been recovered from a variety of specimens of biotic origin (20). In general, these bacteria are relatively harmless, but in the last two decades, they have emerged as nosocomial pathogens and numerous reports of epidemic spread of multiresistant *Acinetobacter* strains have appeared (4, 5, 10, 21, 30). Colonization is more common than infection, and careful clinical judgment is required to assess whether isolations represent disease (25). A comprehensive study by Glew et al., published in 1977 (19), has shown that severe and fatal infections occur, especially in patients who require intensive treatment in special care units. Now, patients in these units may be even more at risk from infections because of considerable advances in medical treatment.

The genus *Acinetobacter* has a complex history, and it has long been difficult to find criteria for speciation (38). Since 1986, this genus has been found to consist of at least 17 species which can be identified by DNA-DNA hybridization (6, 8, 35). Seven species have names that are included in the *Index of the Bacterial and Yeast Nomenclatural Changes* (22), *A. calcoaceticus* (genomospecies 1), *A. baumannii* (genomospecies 2), *A. haemolyticus* (genomospecies 4), *A. junii* (genomospecies 5), *A. johnsonii* (genomospecies 7), *A. lwoffii* (genomospecies 8), and *A. radioresistens* (genomospecies 12). A phenotypic scheme for identification of genomospecies 1 to 12 has previously been described (6, 7); however, by using this system, discrepancies with identifications by DNA-DNA hybridization have been found (18). The ecological niches and relation to disease of various species are not yet well-known, although there are indications that genomospecies 2 (*A. baumannii*) and unnamed genomospecies 3 are of clinical significance (6, 7, 35).

A variety of methods for differentiation of clinical strains,

including antibiogram and plasmid typing (29, 37), biotyping (7), phage typing (9), protein electrophoretic typing (1, 12), bacteriocin typing (2), serotyping (36), and ribotyping (17), have been used. Differences between strains can be small (7, 12, 15), and the combined use of typing methods has been advocated (9, 11).

In 1988, *Acinetobacter* were isolated in a neonatal intensive care unit (NICU) from three patients with extraventricular drains in adjoining incubators. Microorganisms were isolated from the extraventricular drain systems of two patients (patients *i* and *ii*) who were considered to have been colonized. In the third patient (patient *iii*), *Acinetobacter* organisms were cultured from cerebrospinal fluid and the drain insertion site. This patient developed a fatal case of ventriculitis. Prior to these isolations, *Acinetobacter* had been recovered from two other patients, in a blood specimen of one and a bronchial aspirate of the other. A review of microbiological data showed that a total of 19 *Acinetobacter* isolates had been recovered from clinical specimens in this ward during 1988, all of which came from these five patients. These observations gave rise to a prospective study of *Acinetobacter* organisms in this NICU.

The aims of this study were threefold. First, the clinical significance of *Acinetobacter* isolates in patients was analyzed. Secondly, epidemiology was investigated by using a combination of typing methods. Thirdly, it was established which genomospecies were involved.

MATERIALS AND METHODS

Patients. A prospective study of *Acinetobacter* isolates was performed in a 24-bed NICU of a 900-bed university hospital from January 1989 until December 1990. The records and clinical conditions of all patients with *Acinetobacter* organisms during this period were studied.

Definition of neonatal nosocomial infections. The Centers for Disease Control and Prevention definitions for nosocomial infections (16) were amended to specify infection or colonization in neonates, including preterms.

Blood stream infection by *Acinetobacter* organisms was diagnosed when one of the following criteria was met: hyper- or hypothermia, spells of apnea or bradycardia, and isolation of this organism from one or more blood cultures. Pneumonia due to *Acinetobacter* organisms was diagnosed when physical findings that are consistent with pneumonia were present; production of respiratory secretions

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was increased; chest roentgenography showed pulmonary infiltrate; apnea, tachypnea, bradycardia, wheezing, rhonchi, or cough was observed; and *Acinetobacter* organisms were isolated as the predominant organism from bronchial aspirate in the absence of other possible pathogens. Patients were said to have omphalitis caused by *Acinetobacter* organisms if erythema and/or serous or purulent umbilical drainage were present and *Acinetobacter* organisms were cultured as the predominant organism in the absence of other pathogens usually found at that site. A diagnosis of ventriculitis due to *Acinetobacter* organisms was made when clinical signs of ventriculitis, such as fever, hypothermia, apnea, and bradycardia, were observed and these organisms were isolated from one or more cerebrospinal fluid specimens. Insertion site infection by *Acinetobacter* organisms was diagnosed if a patient had erythema and/or serous or purulent drainage at that site and this organism was isolated as the predominant organism from the catheter or exit site in the absence of other pathogens usually seen in this type of infection. Urinary tract infections due to *Acinetobacter* organisms were diagnosed when urine samples obtained by bladder puncture showed leukocyturia and this microorganism was cultured from it.

Colonization by *Acinetobacter* organisms was diagnosed when this organism was cultured from a specimen without any sign of infection. All definitions were made in connection with parameters such as temperature, leukocyte and thrombocyte counts, and C-reactive protein.

Isolation of *Acinetobacter* organisms. Specimens were taken from suspected sites of infection, and in addition, routine throat swabs were taken from patients twice a week. During two surveys in 1989 and 1990, 70 and 56 environmental specimens, respectively, were taken from dry and wet sites in this ward, including mattresses, pillows, cleaning equipment, cupboards, sinks, taps, artificial ventilatory equipment, air humidifiers, stock solutions, and air. These specimens also included swabs from the skin and clothes of staff members. Fluid samples were centrifuged, and these sediments as well as other specimens were cultured on blood agar and MacConkey agar and after 18 h of incubation at 35°C inspected for growth. Isolates were identified as belonging to the genus *Acinetobacter* by the following criteria: gram-negative, coccoid, oxidase-negative, nonmotile, and non-fermenting bacteria.

Susceptibility tests. Bacterial susceptibilities to antimicrobial agents were measured by agar disk diffusion according to standard procedures (3). The susceptibilities of isolates to amoxicillin, piperacillin, cefuroxime, imipenem, ceftazidime, gentamicin, tobramycin, co-trimoxazole, and ciprofloxacin were tested.

Typing studies. Thirty-eight isolates (3 from patients *i*, *ii*, and *iii* in 1988; 29 from 21 patients from 1989 to 1990; and 6 environmental isolates) were typed by the following three methods.

(i) **Cell envelope protein electrophoretic typing.** Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of cell envelope protein was performed as described before (12), with minor modifications (13). Briefly, cell envelope fractions were obtained by ultrasonic disruption of cells and subsequent fractional centrifugation. SDS-PAGE was performed with a stacking gel of 3% acrylamide and a running gel of 11% acrylamide. After fast green FCF (Sigma Chemical Co., St. Louis, Mo.) staining, profiles were examined visually.

(ii) **Antibiogram typing combined with cluster analysis.** Iso-Sensitest agar (CM 471; Oxoid, Basingstoke, Hampshire, United Kingdom) plates were inoculated with a bacterial suspension in saline of standardized density ($10^{-3} \times 0.5$ McFarland) that had been prepared from a 24-h culture on blood agar, yielding confluent or almost confluent growth. The antibiotic disks (Oxoid) used were amoxicillin (25 µg), piperacillin (100 µg), imipenem (10 µg), ticarcillin-clavulanic acid (75 and 10 µg, respectively), cefuroxime (30 µg), cefsulodin (30 µg), cefotaxime (30 µg), ceftazidime (30 µg), aztreonam (30 µg), tobramycin (10 µg), amikacin (30 µg), norfloxacin (10 µg), ciprofloxacin (5 µg), chloramphenicol (30 µg), trimethoprim-sulfamethoxazole (1.25 and 23.75 µg, respectively), and colistin sulfate (10 µg). Plates were incubated for 24 h at 30°C, after which inhibition zones were read. Isolates were comparatively typed on the basis of similarities in inhibition zones of antibiotics. The diameters of inhibition zones were normalized and subjected to cluster analysis. By this method, isolates were grouped on the basis of similarity; this grouping, depicted in a dendrogram, was inspected for correlations with other features, for example, protein profile as well as the site and time of isolation. Similarities between isolates were based on the squared euclidean distance, and grouping was obtained by the unweighted-pair group method by using the arithmetic-average clustering criterion (32) and SPSS software package (26).

(iii) **Biotyping.** Isolates were biotyped by using the API20NE system (bioMérieux, Marcy l'Etoile, France) according to the manufacturer's instructions. In short, standardized bacterial suspensions that corresponded to an optical density of 0.5 McFarland were prepared from 24-h blood agar cultures grown at 30°C with a densitometer. Strips were incubated at 30°C and read after 48 h, except for glucose acidification, which was read after 24 h. All strips were inoculated on the same day by one individual, incubated for the same period, and read by the same individual.

Taxonomic identification by DNA-DNA hybridization. Typed isolates were identified to genomospecies level by a quantitative dot filter method, as described previously (34). Briefly, bacterial preparations were hybridized on a filter with ^{125}I -labeled DNAs from reference strains and the stabilities of duplexes were determined by thermal denaturation. The reference strains and labeling procedures were the same ones that had been used before (35). Criteria for

TABLE 1. *Acinetobacter* isolates from clinical and environmental specimens from this NICU from 1989 to 1990

Specimen type and source	No. of isolates	No. of patients
Clinical		
Blood	8	6
Wound	1	1
Intravascular device	2	1
Bronchial aspirate	44	15
Skin	1	1
Environmental ^a		
Sink	4	
Ventilator (inlet of air)	1	
Dust	1	
Pillow	2 ^b	

^a From 126 specimens collected.

^b No longer available for typing.

inclusion of an isolate in a given genomospecies were based on the difference in thermal denaturation between homologous and heterologous DNA-DNA duplexes or the percentage of DNA removed at a temperature 7°C below the thermal denaturation midpoint of homologous duplexes (34). Each isolate included in a species had a thermal denaturation that was equal to or smaller than 3.0 or a percentage of DNA removed that was equal to or smaller than 17%.

RESULTS

Isolation of acinetobacters. During this prospective study (1989 and 1990), a total of 796 neonates were nursed in this unit. Fifty-six *Acinetobacter* strains were isolated from 21 patients, and another eight were isolated from the environment (Table 1). Most clinical isolates were obtained from bronchial aspirates and blood samples. Environmental isolates were obtained from both wet and dry sources.

Susceptibilities. All isolates were fully susceptible to piperacillin, ceftazidime, imipenem, gentamicin, tobramycin, co-trimoxazole, and ciprofloxacin. Resistance to amoxicillin and cefuroxime was found in 65 and 85% of the isolates tested, respectively.

Patients. Clinical data for the patients in this prospective study (patients A through G from 1989 and patients H through U from 1990) are summarized in Table 2. There were twice as many female neonates as male neonates. Eleven neonates had gestational ages of less than 32 weeks, with 10 having birth weights of less than 1,500 g. All but one patient (patient U) had one or more underlying diseases. Fourteen patients had infant respiratory distress syndrome.

The length of NICU stay before first isolation of *Acinetobacter* organisms ranged from 1 to 76 days, with an average of 21.5 days. Twenty patients were mechanically ventilated at the time of *Acinetobacter* isolation from one or more specimens. The duration of ventilation before first isolation ranged from 1 to 76 days, with an average of 17.5 days.

Antibiotics had been administered or were being given to 17 of these 21 patients before or at the time of the first *Acinetobacter* isolation. Two patients had received a penicillin derivative as a single agent. A penicillin derivative in combination with an aminoglycoside was given to 15 patients. For four of these patients, this regimen was replaced by an expanded-spectrum cephalosporin in combination with an aminoglycoside or a glycopeptide.

Acinetobacter organisms were isolated from the blood samples of 6 patients and the bronchial aspirates of 15 patients. For three patients, *Acinetobacter* organisms were cultured from multiple specimens (patients D, K, and U). In 16 of 21 cases,

TABLE 2. Clinical and microbiological data of patients (n = 21)

Patient	Sex ^a /gestational age (wk)	Birth wt (g)	NICU stay before first isolation/total NICU stay (days)	Duration of ventilation before first isolation/total duration of ventilation (days)	Source(s) of culture ^b (no. of isolates)	Protein profile(s)	Clinical feature(s) ^c	Colonization or infection ^d	Clinical picture affected by <i>Acinetobacter</i> organisms	Clinical outcome
A	F/38	3,310	1/9	1/8	B (2)	I	ASPH, SE, MOF	I	Yes	Died
B	F/30	1,365	8/38	1/3	B (2)	II	IRDS, ODB, PVH, SE	I	Yes	Survived
C	F/25	1,005	13/71	13/24	BA (1)	III	IRDS, ODB, PVH, BPD	C	No	Survived
D	F/28	1,040	13/47	13/47	BA (5), SK (1)	IV	IRDS, ODB, BPD, PN	I	Yes	Died
E	M/33	2,350	35/49	35/39	BA (1)	IV	IRDS, ODB, ASPH, PVH	C	No	Died
F	F/38	2,950	4/7	4/5	BA (1)	II	RI	C	No	Died
G	M/36	2,250	6/14	6/8	BA (1)	V	ASPH, ODB, PN	I	Yes	Survived
H	F/28	1,565	36/46	36/46	BA (4)	VI	IRDS, PFC, PVH, BPD, PN	I	Yes	Survived
I	M/30	1,680	35/112	25/112	BA (14)	V, VI	IRDS, CD, ODB, PN	I	Yes	Died
J	M/31	1,340	23/25	23/25	BA (2)	IV	IRDS, ODB, BPD, PN	I	Yes	Died
K	M/32	1,560	13/19	13/19	B (1), ID (2)	VIII	IRDS, PFC, SE	I	Yes	Died
L	F/27	1,080	7/46	7/20	BA (1)	V	IRDS, ODB, BPD, PN	I	Yes	Survived
M	F/31	1,900	3/4	3/4	B (1)	V	IRDS, PFC, SE	I	Yes	Died
N	F/39	3,050	39/46	39/46	BA (2)	V	RI, CD	C	No	Died
O	F/28	1,110	76/191	76/78	BA (1)	V	IRDS, BPD	C	No	Survived
P	M/34	2,250	19/27	19/19	BA (2)	V	IRDS, PFC, MA, PN	I	Yes	Died
Q	M/28	1,135	36/57	16/24	BA (4)	V	IRDS, ODB, BPD, PN	I	Yes	Survived
R	F/34	1,480	20/46	5/19	B (1), BA (2)	V	RI, CD, ODB, SE	I	Yes	Died
S	F/38	2,805	22/35	22/35	BA (3)	V	RI, CD, PN	I	Yes	Died
T	F/27	590	29/62	1/22	B (1)	IV	IRDS, ODB, VCSS, SE	I	Yes	Died
U	F/35	1,430	12/64	-/-	W (1)	V	WI	I	No	Survived

^a F, female; M, male.

^b B, blood; BA, bronchial aspirate; SK, skin; ID, intravascular device; W, wound.

^c ASPH, asphyxia; SE, septicemia; MOF, multiple organ failure; IRDS, infant respiratory distress syndrome; ODB, open ductus Botalli; PVH, periventricular hemorrhage; BPD, bronchopulmonary dysplasia; PN, pneumonia; RI, respiratory insufficiency; PFC, persistent fetal circulation; CD, congenital disorder; MA, malignancy; VCSS, vena cava superior syndrome; WI, wound infection.

^d I, infection; C, colonization.

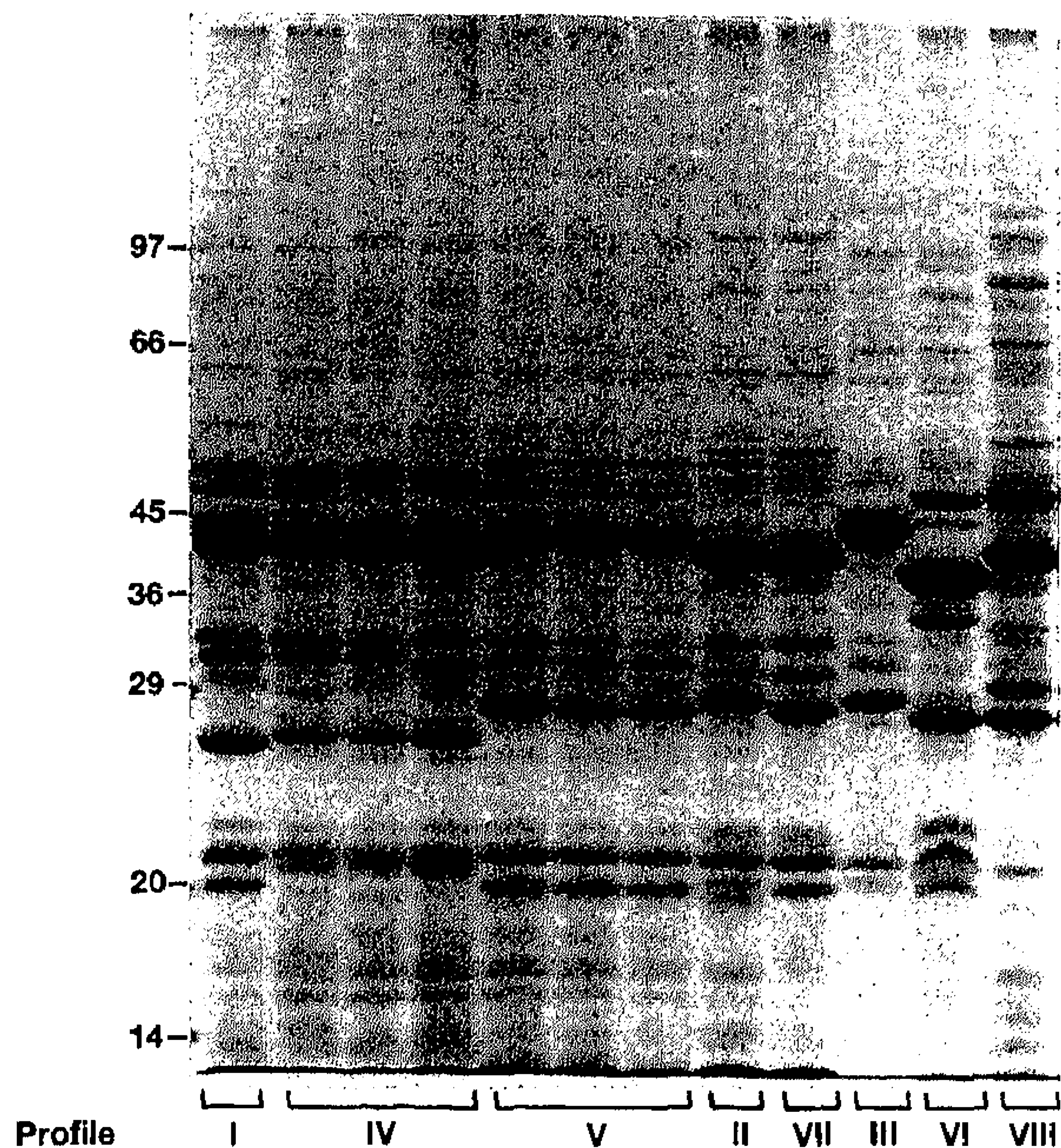
M_r (10³)

FIG. 1. Cell envelope protein profiles found among 38 *Acinetobacter* isolates.

infection due to *Acinetobacter* organisms was diagnosed; in the remaining 5 patients, these microorganisms were related to colonization. Of the infected patients, six had septicemia, nine had pneumonia, and one had a wound infection. The clinical course was also considered to be affected by *Acinetobacter* organisms in 15 patients, 10 of whom died. However, this was mainly due to underlying diseases.

Typing studies. (i) **Cell envelope protein electrophoretic typing.** Eight cell envelope protein profiles (I through VIII) were distinguished by SDS-PAGE (Fig. 1). Slight but reproducible differences were observed among isolates that were allocated to profile group IV, while isolates within all other profile groups were indistinguishable (Fig. 1). Of the 38 isolates investigated, 17 had protein profile V and 11 had protein profile IV (Table 3).

(ii) **Antibiogram typing combined with cluster analysis.** The results of cluster analysis of antibiogram typing are presented in the dendrogram of Fig. 2. Ten clusters of multiple and single strains were distinguishable at the cutoff point indicated by the arrow. Cluster 1 at the top consisted of 17 isolates, 16 of which had protein profile V. Except for one, these isolates had been recovered over a period of 7 months (March to September 1990). The isolates from patients *i*, *ii*, and *iii* (cluster 2) also had a similar protein profile and had been obtained over a relative short period (August 1988).

(iii) **Biotyping.** By using the API2ONE system, biotyping resulted in 13 biochemical profiles (Table 3). Profile 0041073 was found for 16 isolates, 15 of which were cell envelope protein profile V. Other API2ONE profiles were observed for one to four isolates. Different API2ONE profiles were recorded for isolates with the same protein profile (profiles IV, V, and VI), while isolates with API2ONE profiles 0041071 and 0041073 were found in different protein profile groups.

Taxonomic identification by DNA-DNA hybridization. Twenty-nine isolates were identified as unnamed genomospecies 3, three isolates were identified as genomospecies 2 (*A.*

TABLE 3. Identification by DNA-DNA hybridization, typing by cell envelope protein electrophoresis, and biotyping of 38 *Acinetobacter* isolates

Genomospecies	Protein profile (no. of isolates)	API2ONE profile ^a (no. of isolates)
2 (<i>A. baumannii</i>)	II (2)	1000051 (2)
	VII (1)	0001051 (1)
3 (unnamed)	I (1)	0041071 (1)
	IV (11)	0041071 (3)
		0001071 (1)
		4041071 (3)
		4041073 (3)
5 (<i>A. junii</i>)	V (17)	0041073 (1)
		0041073 (15)
		0001473 (1)
		1241073 (1)
		0000051 (1)
14 (unnamed)	III (1)	0000051 (1)
	VI (3)	4010053 (2)
Unclassified	VIII (2)	0010053 (1)
		0000071 (2)

^a According to the API2ONE profile index, profiles 1000051, 0001051, 0000051, and 0000071 correspond to *A. junii* (genomospecies 5); profiles 0010053 and 4010053 correspond to *A. haemolyticus* (genomospecies 4), and all other profiles listed correspond to *A. baumannii* (genomospecies 2).

baumannii), and one isolate was identified as genomospecies 5 (*A. junii*) (Table 3). Three hemolytic isolates were identified as genomospecies 14, as described by Tjernberg and Ursing (35), which corresponds to the genomospecies 13 described by Bouvet and Jeanjean (8). Two isolates could not be identified as any known genomospecies. There was no correspondence between phenotypic identifications by API2ONE and those by DNA-DNA hybridization, as only one isolate was allocated to its appropriate genomospecies by API2ONE.

Epidemiology. Isolates that were highly similar in typing characteristics and had been obtained over the same period were considered to be related. This was certainly the case for the nine isolates (from seven patients [N, O, P, Q, R, S, and U]) of antibiogram cluster 1 (Fig. 2). These isolates were cell envelope protein profile V and had been obtained over a short period, thus indicating the spread of a single strain. Furthermore, all isolates with protein profile V, except for two, had similar API2ONE profiles (Table 3). Cross-colonization or -infection among patients *i*, *ii*, and *iii* and between patients H and I was also suggested.

Except for the environmental isolate with protein profile VII, environmental isolates shared typing characteristics with isolates from patients. One isolate from dust belonged to protein profile V. Strains with the same protein profile were found in 11 patients (Table 2). Nine of these eleven patients were infected; two of them had septicemia.

DISCUSSION

In the last 2 decades, the genus *Acinetobacter* has been increasingly associated with hospital infection and colonization. Most outbreaks of nosocomial *Acinetobacter* infections have occurred in adult intensive care units, with the respiratory tract as the predominant site of infection, but other sites have also been described (14, 19, 23, 31). In reported outbreaks in NICUs, *Acinetobacter* organisms have been associated with septicemia and pulmonary infection (24, 27, 28, 33). Intravascular nutritional fluids, intestinal flora, air humidifiers, and resuscitation bags have been found to be reservoirs, and the spread of *Acinetobacter* organisms by hands or air humidifiers has been suggested.

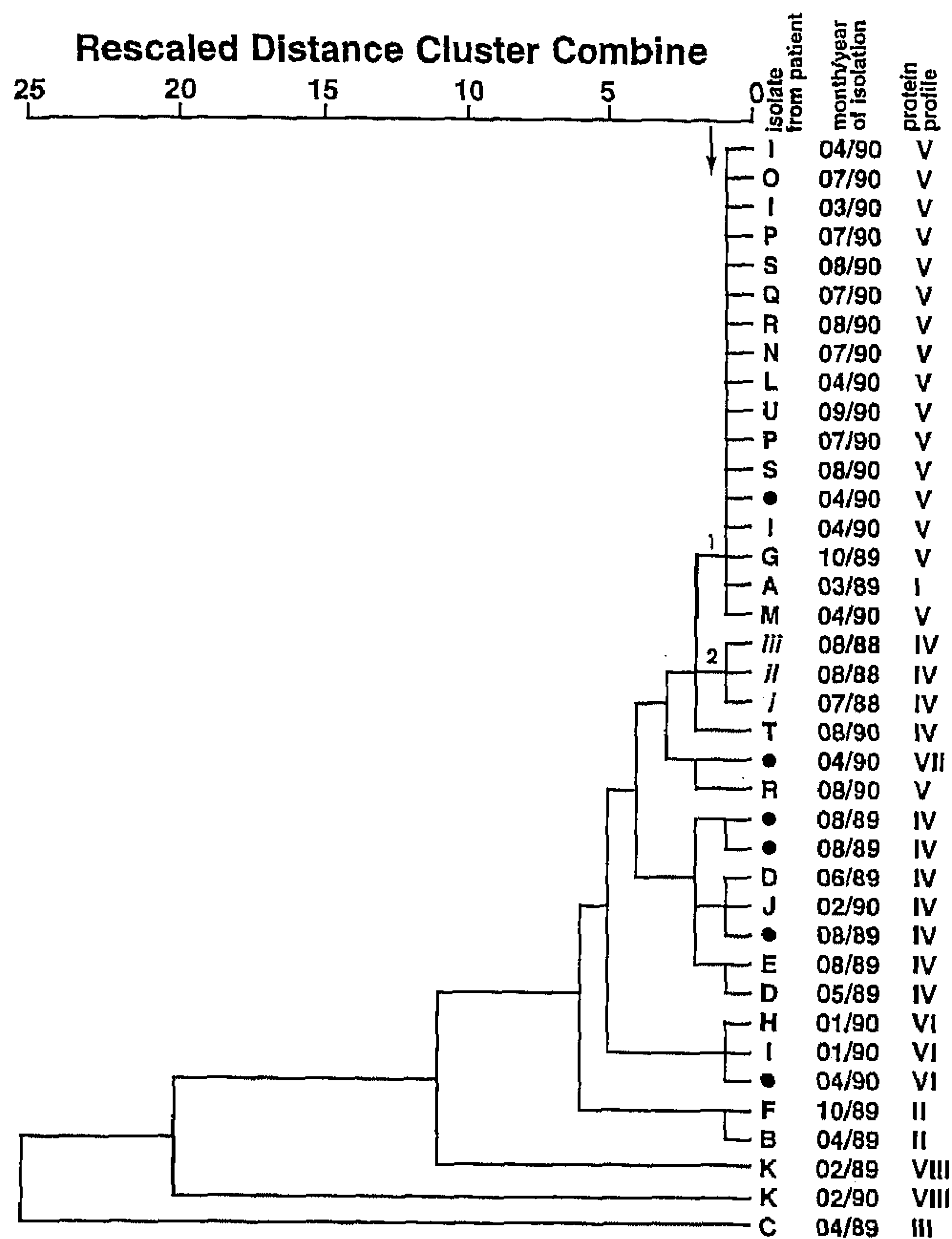


FIG. 2. Grouping of 38 *Acinetobacter* isolates according to antibiotic susceptibility patterns associated with information on source, isolation period, and cell envelope protein profile of organisms. A through U, patients in prospective study (1989 to 1990); i, ii, and iii, patients from 1988; ●, environmental specimens; ↓, cutoff point; 1, cluster 1; 2, cluster 2.

Our report has been concerned with *Acinetobacter* organisms in an NICU, with most clinical isolates coming from bronchial aspirates and blood samples. To discriminate between colonization and infection, the Centers for Disease Control and Prevention criteria for nosocomial infections in patients under the age of 12 months were adapted to specifically define infections in very young neonates, including preterms. Using these criteria, we found that 17 patients were infected with *Acinetobacter* organisms and that in 16 of these cases, the clinical picture was affected by this organism. This illustrates that in these vulnerable patients, *Acinetobacter* strains can be a serious threat.

A combination of typing methods must be used to discriminate between the *Acinetobacter* strains that are involved in outbreaks (9, 11). We used three typing methods. Cross-colonization or -infection was suggested when strains that were indistinguishable by cell envelope protein profile and highly similar in biotype and antibiogram were recovered over a short period. In this study, a number of these cases were confirmed and one strain seemed to have spread epidemically. Several environmental isolates with traits that were common to clinical isolates were obtained during these surveys. These isolates were found at both wet and dry sites. Because of the isolation of *Acinetobacter* organisms from dust, sink basins, and pillows, procedures for cleaning and disinfection of the environment have been reinforced. In addition, the use of alcohol dispensers in hand washing has been introduced. Supporting pillows filled with foam grains have been more frequently changed, and

washing temperatures have been increased from 60 to 90°C. In 1991, the number of *Acinetobacter* isolations in this ward dropped to 5, compared with 17 and 47 during 1989 and 1990, respectively. The reservoir and mode of spread have not been elucidated.

Identification by DNA-DNA hybridization showed that most of the isolates in our study belonged to the unnamed genomospecies 3, which is closely related to genomospecies 2 (*A. baumannii*). Genomospecies 2 is usually implicated in nosocomial outbreaks and infections (6, 7, 9). To the best of our knowledge, the involvement of genomospecies 3 in outbreaks has been reported only once (11). Because of the lack of sufficient phenotypic discriminating criteria (18), unambiguous identification of genomospecies must be performed by DNA-DNA hybridization. It is possible that isolates of genomospecies 3 have been incorrectly identified as genomospecies 2 (*A. baumannii*), as was the case with API2ONE in this study. This may have resulted in a general underestimation of the clinical significance of genomospecies 3.

Many studies of nosocomial *Acinetobacter* outbreaks have focused on the epidemiology of the strains involved. We have combined clinical aspects with epidemiology and taxonomy to obtain a comprehensive picture of *Acinetobacter* organisms in an NICU. The strains involved gave rise to a high number of infections and spread through the ward; most of the isolates belonged to the unnamed genomospecies 3. To elucidate the clinical impact of this entity, rapid and reliable methods for identification need to be developed.

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