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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.

Acinetobacters 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 multidrug-resistant *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 Ogle 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. lwaffii* (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, acinetobacters 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, acinetobacters 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.

**Bloodstream infection by Acinetobacter organisms.** This patient developed a fatal case of ventriculitis. Prior to these isolations, acinetobacters 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.
was increased; chest roentgenography showed pulmonary infiltrate; apnea, tachycardia, 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 onchitis 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, hypothyphemia, apnea, and bradycardia, were observed and these organisms were isolated from one or more cerebrospinal fluid specimen. 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 tromboocyte 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, coccoïde, oxidase-negative, nonmotile, and nonfermenting 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, cefazidime, gentamicin, tobramycin, cotrimoxazole, and ciprofloxacin were tested.

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

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

(ii) **Biofilm typing combined with cluster analysis.** Biofilm typing was performed with a one-inclusion method (CM 471; Oxoid, Basingstoke, Hampshire, United Kingdom) plates were inoculated by adding a bacterial suspension in saline of standardized density (10^5.x 0.5 McFarland) that had been prepared from a 24-h culture on blood agar, yielding confluent to almost confluent growth. After incubation of antibiotic disks (Oxoid) with amoxicillin (25 μg), piperacillin (100 μg), imipenem (10 μg), ticarcillin-clavulanic acid (75 and 10 μg, respectively), ceftazidime (30 μg), cefuroxime (30 μg), cefotaxime (30 μg), cefamandole (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 sulfatol (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 measured, and profiles were examined visually.

(iii) **Biotyping.** Isolates were biotyped by using the API20E 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 loop. Strips were incubated at 30°C and read after 48 h, except for glucose acidification, which was read after 24 h. All strips were incubated on the same day by one individual, incubated for the same period, and read by the same individual.

**Taxonomic identification by DNA-DNA hybridization.** Typing 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 123-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 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 0.3 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, cotrimoxazole, 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 received antibiotics before birth, 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-two patients were mechanically ventilated at the time of *Acinetobacter* isolation from one or more species. 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 species (patients D, K, and U). In 16 of 21 cases,
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<th>Duration of ventilation before first isolation/total duration of ventilation (days)</th>
<th>Source(s) of culture(^b) (no. of isolates)</th>
<th>Protein profile(s)</th>
<th>Clinical feature(s)c</th>
<th>Colonization or infection(^d)</th>
<th>Clinical picture affected by <em>Acinetobacter</em> organisms</th>
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\(^{a}\) 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 hemorrhagiae; 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.
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 API20NE 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 API20NE profiles were observed for one to four isolates. Different API20NE profiles were recorded for isolates with the same protein profile (profiles IV, V, and VI), while isolates with API20NE 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. 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 API20NE and those by DNA-DNA hybridization, as only one isolate was allocated to its appropriate genomospecies by API20NE.

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 API20NE 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). Intravenous 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.
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-colo­

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.

References


119.

176.


