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# Genetic diversity and clonal relationships of *Acinetobacter baumannii* strains isolated in a neonatal ward: epidemiological investigations by allozyme, whole-cell protein and antibiotic resistance analysis

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SUMMARY

Sixty-five strains of *Acinetobacter baumannii* which had been isolated from patients and the indoor environment of a neonatal intensive care unit and, for comparative purposes, isolates from three other wards, were examined by means of electrotyping and analysis of whole-cell protein and antibiotic resistance patterns. Fourteen different electrotypes were determined. The predominant type, a multiply resistant acinetobacter clone, persisted in the neonatal ward over several months. The results underline the usefulness of electrophoretic subtyping, in particular by means of allozyme pattern and as a supplement to whole-cell protein pattern analysis, in epidemiological investigations into the routes of transmission of nosocomial *A. baumannii* infections.

## INTRODUCTION

During the last decade *Acinetobacter baumannii* has increasingly become known as an agent of nosocomial infection. Severe disease such as pneumonia, meningitis and septicaemia as well as wound infections have been observed, particularly within intensive care units for adult immunocompromised patients [1, 2]. For this reason, great importance is attached to the recognition and elimination of sources of infection and routes of transmission by analysis of epidemiological data based on the fine differentiation of the agents involved. In the case of *Acinetobacter* spp., this has been more or less successfully accomplished using serotyping [3], biotyping [4], phage-typing [5], determination of resistance to antibiotics [6], examination of cell envelope protein profiles [7] and other methods.

Recently, Ritter and co-workers published a report [8] on clusters of respiratory infections and colonization by *A. baumannii* in an intensive care unit for neonates where the environment of patients was also examined.

This article is a report on the results of subtyping of the acinetobacter strains isolated during the above study. For subtyping, the methods of allozyme patterns analysis (electrotyping) as well as determination of whole-cell protein patterns and

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sensitivity to antibiotics were used. The suitability of these methods for epidemiological analysis of nosocomial infections by *A. baumannii* is discussed.

## MATERIAL AND METHODS

During the period between May 1991 and January 1992, 65 *A. baumannii* isolates were recovered in a number of wards of a large hospital. Most of these were isolated from intubated and artificially respirated neonates at the respective intensive care units. Sixteen of the strains examined had been isolated from the environment of the infected neonates and seven from patients at other wards.

Identification of the acinetobacter strains isolated was based on the taxonomic characteristics described by Bouvet [9], using the API 20 NE system (Biomerieux GmbH, Nürtingen, Germany).

Bacterial extracts for allozyme pattern analysis were obtained by removing the bacteria grown on nutrient agar using Tris buffer, centrifugation, washing and ultrasonic disruption (Branson 250 sonifier cell disruptor) for 5 min with 50% intervals under cooling and stored at −70 °C. Electrophoretic separation and staining of enzymes as well as evaluation of bands were based on a method modified from that described by Selander [10], using agarose and polyacrylamide gel electrophoresis with a limited set of selected enzymes [11].

After preliminary studies involving *A. baumannii*, the electrophoretic variability of the following enzymes was determined: β-naphthylacetate esterases (EST), aconitase (ACO), acid phosphatase (ACP), alcohol dehydrogenase (ADH), fumarase (FUM), glucose-6-phosphate dehydrogenase (G6P), glutamate dehydrogenase (GtDH, NADP-dependent), isocitrate dehydrogenase (ICD), leucine aminopeptidase (LAP), malate dehydrogenase (MDH), malic enzyme (ME) and 6-phosphogluconate dehydrogenase (6PGD).

The electrophoretic types of the individual allozymes, corresponding to the variability of one gene locus each, were determined on the basis of the typing scheme described [11]. They are given as numeric values (e.g. ACO 1) in Table 1. The profiles of all allelic enzyme types (allozyme patterns) were summarized as electrotype (e.g. ET II), being characteristic of the genotype of the bacterial strain in question. Bacterial extracts for whole-cell protein patterns analysis (WCPP) were obtained and electrophoretic analysis performed by means of vertical polyacrylamide gel electrophoresis (SDS–PAGE) as described by Costas [12]. Visual evaluation was done using a standard protein mix (Serva, Heidelberg).

Antibiotic resistance was determined in the agar diffusion test according to the German standard DIN 58940, part 3 [13]. Results were confirmed by the broth dilution method according to DIN 58940, part 6 [14]. Resistance to the following antibiotics was determined: amikacin (AMK), ampicillin (AMP), ampicillin/sulbactam (AMP/SUL), azlocillin (AZL), aztreonam (AZT), cefotaxime (CTX), cefoxitin (COX), ceftazidime (CAZ), ciprofloxacin (CIP), doxycycline (DOX), gentamicin (GEN), imipenem (IMP), mezlocillin (MEZ), norfloxacin (NOR), piperacillin (PIP), polymyxin B (POL) and tobramycin (TOB).

## RESULTS

Examination of a total of 65 *A. baumannii* isolates from clinical material and samples taken from the patient's environment revealed 14 different allozyme

Table 1. *Allozyme and whole-cell protein patterns of strains isolated from patients*

| Ward | No. of strains | WCCP | ET | Allozyme pattern | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | EST | ACO | ACP | ADH | FUM | G6P | GtDH | GtDH(P) | ICD | LAP | MDH | ME | 6PGD |
| | | | | Single-locus diversities in various enzymes | | | | | | | | | | | | |
| N11 | 33 | A | I | 42 | 2 | 5 | 2 | 2 | 3 | 1 | 1·5 | 1 | 4 | 4 | 3 | 1 |
| | | A′ | I′ | | | | | | | | | | | | | |
| N11 | 3 | A | II | 42′ | 2 | 5 | 2 | 2 | 3 | 2 | 3 | 1 | 4 | 4 | 3 | 1 |
| N11 | 4 | A | III | 42 | 1 | 5 | 2 | 2 | 3 | 1 | 2 | 1 | 4 | 4 | 3 | −1 |
| N11 | 1 | G | IV | 42 | 1 | 5 | 3 | 1 | 1 | −3 | 3 | −2 | 3 | 3 | 0 | 1 |
| N11 | 1 | D | V | 42′ | 2 | 3 | 0 | −1·5 | 1·5 | 1 | 1·5 | 3 | 0 | 2 | 0 | 1 |
| N11 | 1 | E | VI | 42′ | 0 | 5·2 | 2·2 | 2·8 | 3 | 3 | 2·5 | 1 | 4·5 | 4 | 3 | 1 |
| N11 | 2 | F | VII | 49 | 2 | 3 | 4 | 3 | 4 | 2 | 3 | 3 | 4 | 5 | 2·5 | 2 |
| PW1 | 1 | H | VIII | 42 | 2 | 3 | 4 | 3 | 4 | 2 | 3 | 3 | 4 | 5 | 0 | 2 |
| SW2B | 2 | B | IX | 43 | 1 | 3 | 2·5 | 2 | 3 | 1 | 1·5 | 1 | 3 | 4 | 3 | 1 |
| SW2B | 1 | I | X | 48 | 2 | 6 | 2 | 2 | 3 | 2 | 2 | −1 | 4 | 4 | 3 | 1 |

For abbreviations see under Material and Methods.

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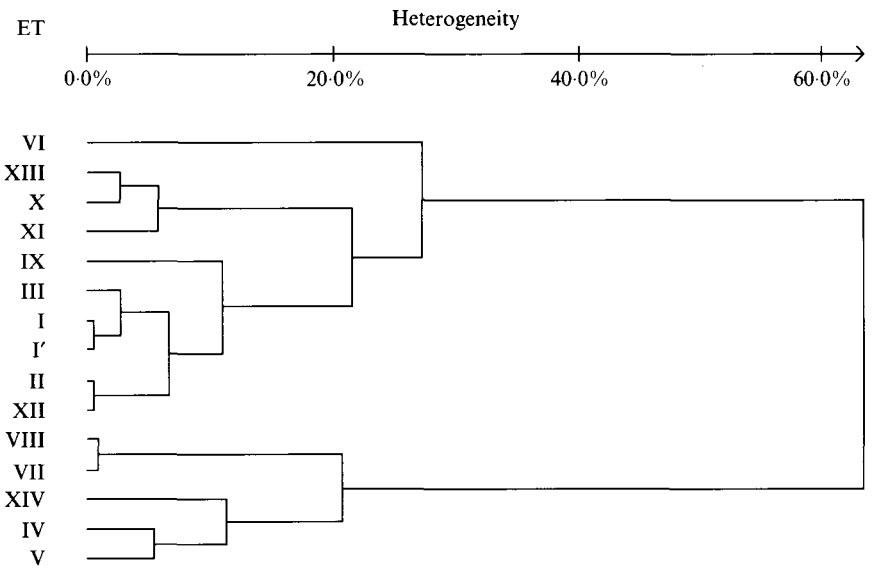


Fig. 1. Genetic distance between different electrotypes. The dendrogram was established by clustering of coefficients of distance based on their genetic distance between pairs of strains, calculated as the proportion of mismatches of alleles.

patterns (ET I–XIV; Tables 1 and 2). The majority of isolates from clinical specimens and some of the environmental samples (41 of 65 strains) exhibited electrotype I or a variety with a minor genetic difference, I′. This variant differed from the principal type only in the electrophoretic mobility of a single enzyme (single-locus diversity). In parallel, the WCPP A was found in all 41 isolates, in part, also its variants A′ and A″, with a minor variability in the molecular weight range around 30 kDa and 70 kDa. All these had been isolated in the neonatal intensive care unit (Ward NI1). As can be seen from Table 1, isolates from a few patients and part of the environmental samples from this unit exhibited electrotypes (ET II–XIV). These latter correspond to an allozyme pattern which differs in several gene loci. In some of them, protein pattern A was found to be present. The other ETs, most of them showing major deviations in their enzyme patterns, each correlated with other protein patterns (B–I). This was the case in particular for isolates from two patients of Ward NI1 (ET V, WCPP D and ET VII, WCPP F), comparative isolates from the Children's Ward PW1 (ET VIII, WCPP H) and the Surgical Ward SW2 (ET IX, WCPP B) as well as part of the environmental samples where, during follow-up examinations in January 1992, a new *A. baumannii* clone (ET XIV, WCPP C) was found. The genetic variability of the individual electrotypes and the small genetic distance between ET I and ET I′ can be seen in the dendrogram depicted in Figure 1. As an example, the electrophoretic differences at the LAP locus demonstrating the allelic variability can be seen in Figure 2. The whole-cell protein patterns of some isolates are shown in Figure 3.

The isolates from patients corresponding to ET VII, which exhibited a large genetic diversity in their allozyme patterns, proved to be sensitive to all

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Table 2. *Allozyme and whole-cell protein patterns of strains isolated from environmental samples*

| Sampling date | Sampling site | No. of isolates | WCP | ET | Allozyme pattern | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | EST | ACO | ACP | ADH | FUM | G6P | GtDH | GtDH(P) | ICD | LAP | MDH | ME | 6PGD |
| 16 Nov 91 | Hand (nurse) | 1 | A′ | I | 42 | 2 | 5 | 2 | 2 | 3 | 1 | 1·5 | 1 | 4 | 4 | 3 | 1 |
| 16 Nov 91 | Heater for infusion solution | 1 | A′ | I | 42 | 2 | 5 | 2 | 2 | 3 | 1 | 1·5 | 1 | 4 | 4 | 3 | 1 |
| 16 Nov 91 | Distilled water (incubator) | 2 | A′ | I | 42 | 2 | 5 | 2 | 2 | 3 | 1 | 1·5 | 1 | 4 | 4 | 3 | 1 |
| 16 Nov 91 | Incubators 1 and 2 (inside) | 2 | A | I′ | 42 | 2 | 5 | 2 | 2 | 3 | 1 | 1·5 | 2 | 4 | 4 | 3 | 1 |
| 16 Nov 91 | Washbasin 1 | 1 | A′ | I′ | 42 | 2 | 5 | 2 | 2 | 3 | 2 | 1·5 | 1 | 4 | 4 | 3 | 1 |
| 16 Nov 91 | Washbasin 2 +incubator III | 2 | A | XI | 42 | 1 | 0 | 2 | 2 | 3 | 2 | 2·5 | 2 | 4 | 4 | 3 | 0·8 |
| 18 Oct 91 | Distilled water container | 1 | A | XII | 42′ | 2 | 5 | 4 | 2 | 3 | 2 | 3 | 1 | 3 | 4 | 3 | 1 |
| 19 Jan 92 | Water separator 1 | 2 | A | XIII | 42 | 2 | 0 | 4 | 2 | 3 | 1 | 2·5 | −1 | 4 | 4 | 3 | 1 |
| 19 Jan 92 | Water separator 2 | 2 | C | XIV | 42′ | 1 | 1 | 2 | 3 | 2 | −1 | −1 | −1 | 1 | 1 | 0 | 1·2 |
| 19 Jan 92 | Hand (nurse) | 2 | | | | | | | | | | | | | | | |

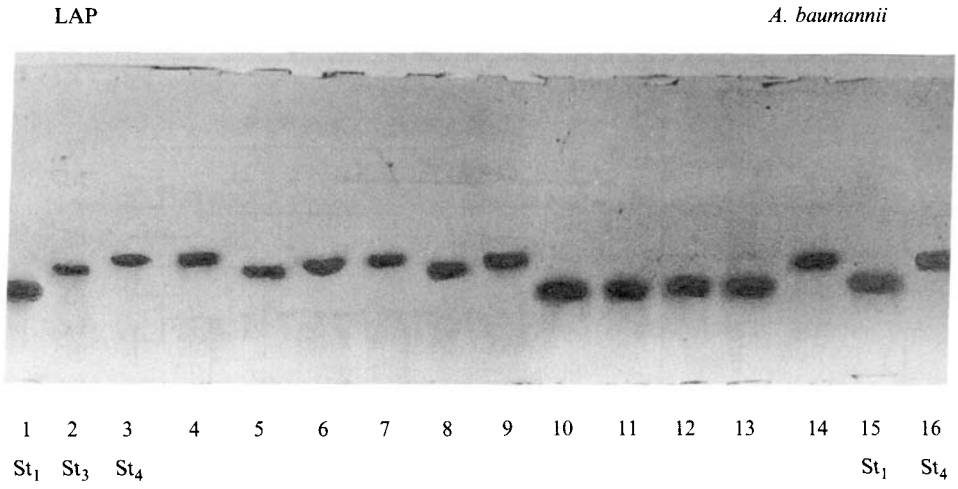


Fig. 2. Leucine aminopeptidase patterns. Nos. 1–3, 15, 16 standards; nos. 4, 6, 7, 9, strains from patients (ET I station NI1); no. 5, strain from patients (ET IX station SW2B); no. 8, strain isolated from distilled water (ET XII); no. 14, strain from the environment (ET I, incubator, isolated 16 Nov. 1991); nos. 10–13, strains from the environment (ET XIV, water separator, isolated 19 Jan. 1992).

antibiotics tested. Most of the isolates, however, were multiply resistant to AMK, AMP, AZL, AZT, CTX, MEZ and NOR; some were resistant to additional chemotherapeutic agents, e.g. CIP. Only the isolate which was of the highly different electrotype VI exhibited additional resistance to the combination AMP/SUL. However, correlations could not always be confirmed between the resistance patterns on the one hand and the enzyme and protein patterns, on the other. In contrast, the results of allozyme pattern analysis were largely supported by the protein patterns and they were closely related to the epidemiological results, e.g. those of the examinations in the environment of patients (Table 2).

## DISCUSSION

From the results of the allozyme pattern analysis available, it may be concluded that an *A. baumannii* clone (characterized as ET I (I′) and WCPP A (A′)) had spread within the neonatal intensive care unit of the hospital involved and persisted there over months, colonizing and infecting neonates. In addition incubators, other medical equipment, water used to raise humidity, and hands of staff were contaminated and constituted potential vehicles of cross-infection.

Minor deviations (single locus diversities) in the enzyme pattern (ET I′) have been rated as spontaneous mutations which had taken place in single isolates in the course of the epidemic. Similarly minor heterogeneity was observed by Dijkshoorn [15] in the biotype of strains isolated over an extended period in a hospital study. These small changes obviously lack a specific influence on the epidemiological status, at least in the case of acinetobacter.

In contrast, other bacterial clones were characterized by the clearly deviating ETs II–XIV. Four of these (XI–XIV) were present in the patients' environment.

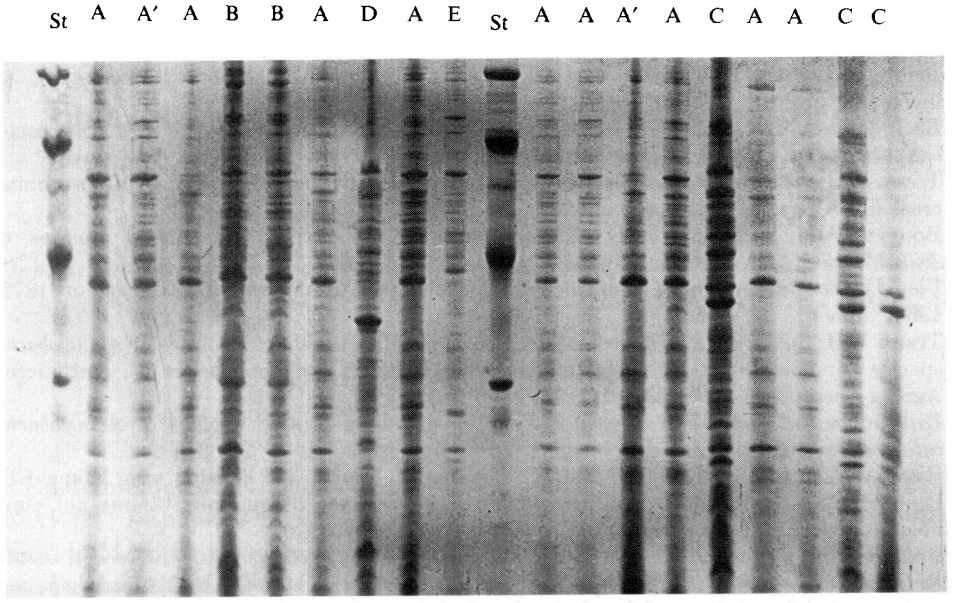


Fig. 3. Whole-cell protein patterns of isolated strains. Pattern A, patients ward NI1 (nos. 2, 4, 7, 9, 12, 13, 17, 18) and incubator (no. 15); pattern A′, washbasin 1 (no. 3) and hand nurse (no. 14); pattern B, patient's ward SW2B (nos. 5, 6); pattern C, water separator 2 (no. 16) and hand nurse, 19.01.1992 (nos. 19, 20); pattern D and E, patients single cases ward NI1 (nos. 8, 10); St, standard protein mix (nos. 1, 11).

without being involved in processes of colonization or infections. The genetic diversity (cf. dendrogram; Fig. 1) of the *A. baumannii* strains isolated from other wards (PW1 and SW1) demonstrates that spread of the 'epidemic' clone to the wards had not taken place. Also, electrotyping has permitted a clear-cut follow-up of a new acinetobacter clone after its emergence (16 January 1992, ET XIV; WCPP C) and its spread from the water separator of the respirator via the hands of nurses. Based on these results, the discriminatory value of the allozyme patterns can obviously be rated higher within the hierarchy of epidemiological typing methods than that of whole-cell protein patterns, some of which include more than one bacterial clone. While the partially plasmid-encoded resistance patterns of the *A. baumannii* strains reported here proved to be useful for epidemiological statements, the proteins patterns turned out to be a meaningful supplement to the enzyme patterns. We can only corroborate corresponding conclusions drawn by Bouvet and Dijkshoorn [15, 16] with regard to the expediency of using several methods for epidemiological purposes, to achieve comprehensive typing.

The results of the present study whose interpretation is based on Selander's principal statements [10, 17] on the evidence provided by multilocus enzyme electrophoresis, are in conformity with the authors experience on other occasions with regard to *Acinetobacter* (unpublished results). They underline the suitability of allozyme pattern analysis for a number of purposes, among them the epidemiology of infections in the practice of hospital hygiene.

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