However PFGE is expensive and time consuming when compared with other methods.

CHAPTER 8

8. TYPING METHODS

Typing systems are important tools for establishing the sources and mode(s) of transmission for epidemic strains. Early attempts to develop typing systems for *Acinetobacter* spp. have been already reviewed previously (Bouvet, 1991). No single typing method has so far gained acceptance for typing *Acinetobacter* spp., and this area is still the subject of research. The sections below describe the different typing systems that are currently being applied to *Acinetobacter* spp. Some of these are based on the latest taxonomic developments, while others aim simply to discriminate individual strains without determining their precise genomic species. There are inherent advantages and disadvantages in every approach of bacterial typing and this has to be kept in mind when considering the different typing methods.

8.1 Biotyping:

Biochemical profiles comprising binary characteristics (with results being scored as positive or negative) can be used for comparative typing of strains. A biotyping system consisting of five tests (Bouvet et al., 1990; Bouvet and Grimont, 1987; Gerner-Smidt, 1994), devised originally for dividing isolates of *A. baumannii* into 19 biotypes (Table-8.1). This has also been used to type closely related genomic
Table 8.1

_Acinetobacter calcoaceticus-_ Acinetobacter baumannii _- complex (Acb-complex) biotyping reactions_ (Gerner-Smidt, 1994).

<table>
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<th>Assimilation of</th>
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<tbody>
<tr>
<td>Levulinate</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Citraconate</td>
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<tr>
<td>L-Phenylalanine</td>
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<tr>
<td>4-hydroxybenzoate</td>
<td>+</td>
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<tr>
<td>L-tartrate</td>
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species of *A. baumannii* such as genospecies 3 and 13 TU in various hospital outbreaks of infection (Bouvet et al., 1990; Gerner-Smidt and Frederiksen, 1993; Gerner-Smidt and Tjernberg, 1993). The commercial API 20NE system has been used to distinguish 31 different biotypes among 122 different *Acinetobacter* strains (Towner and Chopade, 1987), but this system sometimes has problems with sensitivity and reproducibility (Kropec et al., 1993). Cluster analysis of carbon source growth assays has been used to identify major clusters of isolates that were related to the epidemiological origin of the strains (Dijkshoorn et al., 1990). Other commercial systems with large numbers of substrates may therefore be of use, but such systems have yet to be assessed with epidemiologically defined strains of *Acinetobacter* spp. from different outbreaks.

### 8.2 Antibiotyping:

Numerous studies have used antibiotic susceptibility patterns (antibiograms) to detect emerging resistance patterns and to group similar isolates (Alexander et al., 1988; Allen and Green, 1987; Bergogne-Berezin and Joly-Guillou, 1985; Joly-Guillou and Bergogne-Berezin, 1991; Struelens et al., 1993; Vila et al., 1989; Biendo et al., 1999), often on the basis of MICs, breakpoints, or zone diffusion sizes. Such results are often expressed as resistance, susceptible, or intermediate. A more informative approach uses the actual diameters of inhibition zones in disc diffusion tests for cluster analysis, and such groupings have been shown to correlate well with other typing and epidemiological data (Dijkshoorn et al., 1993). However, it must be emphasized that antibiogram typing results should be interpreted with caution, since
unrelated strains may exhibit the same antibiogram (Joly-Guillou and Bergogne-Berezin, 1991) and changes in susceptibility may occur during episodes of infection.

8.3 Serotyping:

There have been numerous attempts to type *Acinetobacter* strains by serological methods, but only limited success was obtained in early work (Adam, 1979; Das and Ayliffe, 1984; Henriksen, 1973; Kulachandra Singh et al., 1983), and most such schemes have been rendered obsolete by the taxonomic developments in the genus. More recent work involving checkerboard tube agglutinations and reciprocal cross absorption polyclonal rabbit immune sera against heated cells has allowed the delineation of 34 serovars in *A. baumannii* and 26 serovars in genomic species 3 (Traub, 1989, 1990; Traub and Leonhard, 1994). However antigenic differences between *A. baumannii* and genomic spp 3 serovars were not entirely satisfactory, and the relationship with strains belonging to genomic species 13TU is unclear. Only one recent study has successfully used serotyping for typing genomic species 13TU and they were able to differentiate these isolates from that of *A. baumannii* strains (Pantophlet et al., 1999). More work with large numbers of epidemiologically defined strains identified unambiguously by DNA-DNA hybridization are required to test the utility of this method.
8.4 Phage Typing:

Two complementary sets of bacteriophages have been used in a number of different epidemiological studies of *Acinetobacter* strains isolated from France and other European countries (Bouvet et al., 1990; Buisson et al., 1990; Giammanco et al., 1989; Joly-Guillou et al., 1990; Santos Ferreira et al., 1984; Vieu et al., 1979). One comprises 25 phages, allowing the identification of 125 phage types and the other where in 14 phages were used allowing the identification of 25 phage types. Phage type numbers 17 and 124 were the predominant phage types identified in some outbreaks (Santos Ferreira et al., 1984; Vieu et al., 1979). However, this system has been used only at the Phage typing Center of the Institut Pasteur in Paris, and it seems that a substantial proportion of strains from other geographical areas may be untypeable. Moreover, very few centers can afford this expensive facility. Even some doubt has been cast upon the reproducibility of such typing method (Bouvet et al., 1990). This typing may be useful, albeit time consuming, when used in conjunction with other typing methods.

8.5 Bacteriocin Typing:

Only two reports of bacteriocin typing of *Acinetobacter* isolates were published so far. In the first study, 176 strains were typed by means of ten indicator strains that were susceptible to bacteriocins. Overall typeability was 65%, but 56% of strains belonged to only two groups (Andrews, 1986). The second investigation (Stone and Das, 1985) used 19 bacteriocin containing lysates to type 100 strains.
Only 46% of strains were typable, but 16 isolates, including 11 from an outbreak, belonged to the same type. Bacteriocin typing of isolates identified by DNA-DNA hybridization has not been studied, and hence the usefulness of this typing method for typing clinically significant strains remains unexplored.

8.6 Electrophoretic protein analysis:

Whole cell protein as well as cell envelope protein patterns have been used in a number of epidemiological and taxonomic studies of *Acinetobacter* spp. Analysis of cell envelope protein patterns by Sodium Dodecyl Sulphate-Polyacrylamide gel electrophoresis (SDS-PAGE) has shown heterogenicity in unrelated strains, but multiple isolates from patients or outbreaks were indistinguishable (Dijkshoorn et al., 1987a, 1987b). This profiling has been successfully used to trace specific strains during endemic episodes and outbreaks in hospitals (Crombach et al., 1989; Dijkshoorn et al., 1993; Weernink et al., 1995).

Electrophoretic analysis of whole-cell protein fractions has the advantage that the sample preparation is simpler than preparation of cell envelopes. Similarities between *Acinetobacter* isolates from outbreaks and dissimilarities in unrelated control strains have been reported in different studies (Alexander et al., 1984, 1988; Mortensen et al., 1987). However like other phenotypic based methods, apparent differences between isolates from common origin should be interpreted with caution. Protein profiles alone is insufficient to judge the epidemiological data and to elucidate the outbreaks, as it should be used in conjunction of other typing methods (Dijkshoorn et al., 1993)
8.7 **Multilocus Enzyme Electrophoresis typing:**

Multilocus enzyme electrophoresis investigates the relative electrophoretic mobilities of a large number of cellular enzymes (Selander et al., 1986). Investigation of 27 esterases and 2 dehydrogenases in 81 *Acinetobacter* isolates identified between 2 and 17 variants for each enzyme (Picard et al., 1989). A separate study of 13 enzymes in 65 *Acinetobacter* clinical isolates identified 14 different types, of which 1 type was found in 41 multiresistant isolates with common whole-cell protein profiles (Thurm and Ritter, 1993). These results suggest that multilocus enzyme electrophoresis has the potential to be developed into a useful technique for strain identification.

8.8 **Plasmid Profiles:**

Numerous studies have shown plasmid typing as a rapid and simple method for identifying *Acinetobacter* strains (Alexander et al., 1988; Gerner-Smidt, 1989; Gerner-Smidt and Tjernberg, 1993; Getschell-White et al., 1989; Kropec et al., 1993; Seifert et al., 1994). The plasmids found in *Acinetobacter* strains vary considerably in both size and number. In one study, between one and four plasmids varying in size from 2.1 to >100 kb were found in 13 strains belonging to the *Acb*-complex in one particular study (Gerner-Smidt and Tjernberg, 1993), but no plasmids were found in 10 other strains examined. Nevertheless, analysis of plasmid profiles has been useful in delineating several outbreaks of *Acinetobacter* infection (Back-Sague et al. 1990; Hartstein et al., 1988; Kropec et al., 1993; Vila et al., 1989).
Strains with similar plasmid profiles have been differentiated further either by restriction endonuclease digestion of plasmid DNA to generate plasmid fingerprints (Kropec et al., 1993; Vila et al., 1989) or by hybridization of plasmids with a labeled probe from one of the strains (Gerner-Smidt and Tjernberg, 1993). Overall, it can be concluded that plasmid typing may be extremely useful in epidemiological studies, provided the results are interpreted in conjunction with other typing methods and the origin of the strains are known.

8.9 Analysis by Pulsed-Field Gel Electrophoresis:

Analysis by pulse-field gel electrophoresis (PFGE) of restriction fragment length polymorphisms generated from intact chromosomal DNA has been used to compare finger prints obtained from *Acinetobacter* strains following restriction with *Apa* I (Graser et al., 1993; Seifert et al., 1994; Tankovic et al., 1994), *Sma* I (Allardet-Servent et al., 1989), *Apa* I, *Sma* I (Gouby et al., 1992), and *Nhe* I and *Sma* I (Struelens et al., 1993). These studies have indicated considerable DNA polymorphism in the clinically important genomic species 2 (*A. baumannii*), even within biotypes, and good correlation between strains from within defined outbreaks or multiple isolates from single patients. Equipment for pulsed-field gel electrophoresis is costly, while the preparation of intact chromosomal DNA in subsequent digestion and electrophoresis require several days. Nevertheless, pulsed-field gel electrophoresis seems to provide highly discriminatory results and extremely useful epidemiological information.
8.10 Ribotyping:

This method has been described in detail for *Acinetobacter* spp. (Gerner-Smidt, 1992). Briefly, purified chromosomal DNA is digested with restriction enzymes, electrophoresed, blotted, and then hybridized with a labeled cDNA probe derived from *E.coli* rRNA. Patterns generated by restriction with *EcoRI*, *ClaI*, or *SalI* have been used to investigate 70 strains that had been identified as either *A. calcoaceticus*, *A. baumannii*, or genomic species 2 or 13TU by DNA-DNA hybridization (Gerner-Smidt, 1992). Excellent reproducibility was observed, and combined use of the 3 enzymes generated 52 different types among the 70 unrelated strains studied. Ribotype patterns within outbreaks have been shown to be stable and to correlate with the results obtained by other typing methods (Dijkshoorn et al., 1993). Ribotyping has several advantages, including patterns which are easy to interpret because of limited number of hybridized fragments, and ribotypes are stable and reproducible after continued subculture (Vila et al., 1994; Gerner-Smidt, 1992). However, although the diversity of ribotypes with in the *A. calcoaceticus - A. baumannii* complex is considerable, common patterns in apparently unrelated strains have also been observed (Dijkshoorn et al., 1993; Gerner-Smidt, 1992). Again, this is a very laborious technique, but it can provide valuable epidemiological information, particularly when used in combination with other typing methods.
8.11 *Polymerase Chain Reaction-based methods:*

Fingerprinting on the basis of PCR amplification of DNA sequences by specific and random primers is used increasingly now. The core region of bacteriophage M13 has been used as a single primer to determine the relatedness of *A. baumannii* strains (Graser et al., 1993). Enterobacterial repetitive intergenic consensus PCR (ERIC1 & 2) primers (Versalovic et al., 1991) were also successfully used in one study (Struelens et al., 1993). ERIC primers were not useful epidemiologically in one study whereas the repetitive elements such as REP-1 and REP-2 combined generated PCR fingerprints that discriminated between the epidemic and sporadic strains of *A. baumannii* and demonstrated four discrete clusters that were unique epidemiologically (Reboli et al., 1994). Arbitrarily primed PCR (AP-PCR) and ribotyping were compared in one investigation to detect outbreaks (Vila et al., 1994). It was concurred that ribotyping and AP-PCR exhibited similar discriminatory power, however AP-PCR had an additional advantage of speed and simplicity. A multicentric study recently evaluated the reproducibility of PCR-based fingerprinting of *Acinetobacter* spp. using standard protocols and reagents (Grundmann et al., 1997) in 7 laboratories of six European countries. Four primers with 4 PCR protocols were used. The epidemiological conclusions reached by the participating laboratories were substantially correct, with 96.4% of the total isolate grouping allocations agreeing with the consensus view. Such kind of results can be obtained, only if one uses quality controlled reagents, standardized protocols for extraction of DNA and standardized amplification conditions.