ANALYSIS OF A MULTIDRUG RESISTANT *ACINETOBACTER SPP.* OUTBREAK IN THE INTENSIVE CARE UNIT OF KING EDWARD VIII HOSPITAL

by

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ABSTRACT

The study arose out of a need to investigate and control a nosocomial outbreak caused by multidrug resistant *Acinetobacter* spp in the fifteen-bed intensive care unit of King Edward VIII Hospital. Following the discovery of the index case, four other patients were found to have a similar strain of *Acinetobacter* spp.

All fifteen patients in the ward were subsequently screened for the organism. Forty-seven isolates were obtained from 12 patients. Eight of the patients were infected with the organism and six of these eight patients subsequently died. Swabs from the ward environment were also screened for the organism, which was found in patients' baths, suction water and urine collection jars. The outbreak was aborted by the use of strict infection control techniques.

Minimum inhibitory concentrations (MICs) of 20 of the 47 isolates were determined for the following antimicrobials: imipenem, ciprofloxacin, gentamicin, amikacin, netilmycin, cefotaxime, ceftazidime and tetracycline. The same 20 isolates were further typed using ribotyping.

Seven different antibiogram patterns were obtained using the MIC data. The majority of isolates (11) fit into a single type, and showed resistance to all drugs tested, except for susceptibility to tetracycline and netilmycin only. Ribotyping revealed 5 different types. There were 9 isolates of ribotype a, 2 of ribotype b, 3 of ribotype c, 5 of ribotype d and 1 of ribotype e.

In conclusion, this study describes a nosocomial outbreak with a multidrug resistant *Acinetobacter* spp. in an intensive care unit. The results showed that there was no correlation between the two typing methods used, ribotyping was more discriminatory than antibiogram types, with the majority of strains belonging to two different ribotypes.

DECLARATION

This study represents original work by the author and has not been submitted in any other form to another University. Where use was made of the work of others, it has been duly acknowledged in the text.

The research described in this dissertation was carried out in the Department of Medical Microbiology, Nelson R Mandela Medical School, University of Natal, Durban, South Africa under the supervision of Professor A.W. Sturm.

F. DEEDAT

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DEDICATION

I hereby dedicate this dissertation to my late father, Mr. Ahmed Deedat, who was the kindest, most wonderful parent any person may wish to have – may God bless him.

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My parents, for their encouragement and support throughout my career, and their continual love and understanding.

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My son, Ahmed Dada, for his presence in my life.

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INTRODUCTION

Acinetobacter spp. may cause infections in all organ systems. The main sites of nosocomial infections by *Acinetobacter* spp. are the lower respiratory tract, peritoneum, urinary tract, surgical wounds, device-related infections, meninges, skin and eye, and these infections may progress to bacteraemia (Bergogne-Berezin and Joly-Guillou, 1991). Numerous outbreaks of infection in intensive care units have been described over the years (Beck-Sague, Jarvis et al., 1990)(Ling, Wise et al., 1996)(Lortholary, Fagon et al., 1995)(Reboli, Houston et al., 1994)(Castle, Tenney et al., 1978)(Stone and Das, 1986)(Hartstein, Rashad et al., 1988).

Several studies have reported that 3 to 5% of nosocomial pneumonias are caused by *Acinetobacter* spp. (CDC, 1987), and this figure increases to 15-24% in the subset of patients who are mechanically ventilated (Fagon, Chastre et al., 1989)(Torres, Aznar et al., 1990), a group in which it can be extremely difficult to distinguish between colonisation and infection. Mortality rates associated with *Acinetobacter* pneumonias are reported to be between 30% and 75%, with the highest rates reported in ventilator-dependent patients (Bergogne-Berezin and Joly-Guillou, 1991) (Fagon, Chastre et al., 1989) (Torres, Aznar et al., 1990). Similar mortality rates (50 – 56%) have been reported for nosocomial pneumonias due to other gram negative bacilli in ventilated patients (Fagon, Chastre et al., 1989).

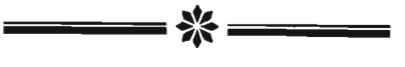
Transmission of *Acinetobacter* to patients in intensive care units is often attributed to environmental sources. In addition, many outbreaks are related to cross contamination by the hands of staff in the unit (Stone and Das, 1986) (French, Casewell et al., 1980) (Patterson, Vecchio et al., 1991). Apart from hands themselves, inadequately used gloves that are not changed between patients may replace hands as an efficient vehicle for *Acinetobacter* transmission (Patterson, Vecchio et al., 1991).

The central hypothesis in epidemiologic typing is that the isolates obtained from an epidemiologic cluster are directly descended from a single common precursor. Typing systems are based on the premise that clonally related isolates share characteristics by which they can be differentiated from unrelated isolates.
Over the decades, many different typing methods have been devised in order to further link and categorise bacteria belonging to the same genus and species. The application of molecular techniques to microbial typing has provided a powerful set of new tools that facilitate epidemiological investigations.

On 8 February 1995, it was realised that there was an outbreak of a multidrug resistant *Acinetobacter spp* in the intensive care unit (ICU) of King Edward VIII Hospital.

The objectives of this study are therefore to:

- describe the course of the outbreak
- describe the investigation of the outbreak
- epidemiologically type the stored isolates from the outbreak, and compare the two different typing systems used.



LITERATURE REVIEW

2.1 Taxonomy of *Acinetobacter* species

The bacteria that belong to the genus today known as *Acinetobacter* have had a long and colourful taxonomic history. They have been classified under at least fifteen different names in this century, including *Diplococcus mucosus* (1908), *Micrococcus calcoaceticus* (1911), *Alcaligenes haemolysis* (1937), *Mima polymorpha* (1939), *Moraxella lwoffi* (1940), *Herellea vaginicola* (1942), B5W (1949), *Neisseria winogradsky* (1952), *Achromobacter lwoffi* (1953), *Achromobacter anitratum* (1954), *Moraxella glucidolytica* (1956), *Acinetobacter anitratum* (1957), *Acinetobacter polymorpha* (1957), *Acinetobacter lwoffi* (1957), *Alcaligenes metalcaligenes* (1963), *Achromobacter haemolyticus* (1963), *Acinetobacter calcoaceticus* (1968) (Allen and Hartman, 1995). It has only been in the last decade that an improved understanding of the microbiology and taxonomy of *Acinetobacter spp.* has emerged.

Transformation and nutritional studies in 1976 have placed the genus *Acinetobacter* within the family Moraxellaceae (Henriksen, 1976). The genus *Acinetobacter* is now defined as including gram negative diplococcoid bacteria, with a DNA G+C content of 39 - 47 mol%, that are nonsporing, strictly aerobic, nonmotile, catalase positive and oxidase negative. With the family name determined, attention subsequently moved to the species delineation of the genus. The 1984 edition of *Bergey's Manual of Systemic Bacteriology* groups *Acinetobacter* under one species, *A. calcoaceticus*, and two subspecies distinguished by their ability to produce acid from glucose - *A. calcoaceticus* var. *anitratus* has this ability, whereas *A. calcoaceticus* var. *lwoffi* does not (Juni, 1984). The 1980 *Approved Lists of Bacterial Names* recognises two species - *A. calcoaceticus* and *A. lwoffi* (Skerman, McGowan et al., 1980).

Rainey in 1994 performed 16S rDNA sequence analysis on the type strains of all validly described *Acinetobacter* species and five unnamed *Acinetobacter* strains. The phylogenetic analyses confirmed that *Acinetobacter* is a coherent genus

within the gamma subclass of Proteobacteria and that the species are phylogenetically well defined. *A. calcoaceticus*, *A. lwoffii*, *A. johnsonii* and *A. haemolyticus* form one cluster of closely related species, the pair *A. junii* and *A. baumannii* forms a second cluster and *A. radioresistens* stands phylogenetically isolated (Rainey, Lang et al., 1994).

Many further efforts to speciate the genus have been made - these include bacteriocin typing, phage typing, outer membrane protein typing, serotyping, phenotyping, ribotyping, and DNA homology studies. These efforts have been largely unsuccessful. Only speciation based on DNA - DNA hybridisation studies have yielded success, and today, 19 genomic species are recognised by the three different laboratories of Bouvet and Grimont (Bouvet and Grimont, 1986) (Bouvet and Jeanjean, 1989), Tjernberg and Ursing (Tjernberg and Ursing, 1989) and Nishimura et al. (Nishimura, Kano et al., 1987). In a first study, Bouvet and Grimont (Bouvet and Grimont, 1986) described a total of 12 genomic species. Tjernberg and Ursing (Tjernberg and Ursing, 1989) found 3 additional genomic species, DNA groups 13, 14 and 15. Concurrently, Bouvet and Jeanjean (Bouvet and Jeanjean, 1989) added 5 more species (species 13 to 17) to the scheme of Bouvet and Grimont (Bouvet and Grimont, 1986). The 19 species have been given numbers by the various authors, but these numbers are not as yet standardised. Seven of the species have also been given formal species names - these are shown in Table 1 below:

Table 1: Formal names and corresponding numbers of *Acinetobacter spp*

Species Name	Genomic Species Number
A. calcoaceticus	1
A. baumannii	2
A. haemolyticus	4
A. junii	5
A. johnsonii	7
A. lwoffii	8
A. radioresistens	12

Genomic species 1, 2, 3 and 13 (groups 3 and 13 are as yet unnamed) have been shown to be extremely closely related genetically (Nishimura, Kano et al., 1987) and are referred to as the *A. calcoaceticus-A. baumannii* complex by some researchers (Gerner-Smidt, Tjernberg et al., 1991). These bacteria mostly acidify glucose, and therefore correspond quite well to the organism formerly named *A. calcoaceticus* subsp. *anitratus*; they are also the most frequently isolated species in clinically significant infections due to *Acinetobacter*. The species from this complex that occurs most frequently in clinical specimens is *A. baumannii*.

Those bacteria that do not acidify glucose and are nonhaemolytic are mainly *A. lwoffii*, *A. johnsonnii*, or *Acinetobacter* genospecies 12. Most haemolytic isolates are identified as *A. haemolyticus* or *Acinetobacter* genospecies 6.

Yamamoto in 1996 sequenced the PCR-amplified fragments of the gyrB genes (DNA gyrase B subunit genes) of 15 *Acinetobacter* strains, including the type and reference strains of genomic species 1 to 12. They found that the gyrB sequence homology among these *Acinetobacter* strains ranged from 69.6 to 99.7%. A phylogenetic analysis, using the gyrB sequences, indicated that genomic species 1, 2, and 3 formed one cluster (87.3 to 90.3% identity), while genomic species 8 and 9 formed another cluster (99.7% identity). These results are consistent with

those of DNA-DNA hybridisation and of biochemical systematics. On the other hand, the topology of the published phylogenetic tree based on the 16S rRNA sequences of the *Acinetobacter* strains was quite different from that of the gyrB-based tree. The numbers of substitutions in the 16S rRNA gene sequences were not high enough to construct a reliable phylogenetic tree. Their gyrB-based analysis indicated that the genus *Acinetobacter* is highly diverse and that a reclassification of the genus would be required (Yamamoto and Harayama, 1996). Thus, the last word on the classification of this organism seems not to be written yet.

The species of *Acinetobacter* other than *A. baumannii* have only rarely been implicated in clinical infection and outbreaks. *Acinetobacter* genospecies 3 and 13 have been implicated in nosocomial outbreaks of infection (Dijkshoorn, van et al., 1993) and *A. johnsonii* has been associated with catheter-related bacteraemia (Seifert, Strate et al., 1993). Only a study in Sweden found *Acinetobacter* genospecies 3 to be the predominant genotype among clinical isolates (Tjernberg and Ursing, 1989).

In a study by Seifert in 1993, a total of 584 *Acinetobacter* strains were isolated from 12 different hospitals over a period of twelve months. *A. baumannii* strains were isolated most frequently (n=420; 72.9%), followed by *Acinetobacter* species 3 (n = 55), *A. johnsonii* (n = 29), and *A. lwoffii* (n = 21). Most isolates were recovered from respiratory tract specimens (42.9%). The rest were from blood cultures (19.9%), wound swabs (15.4%), catheter tips (12.8%), and urinary tract specimens (3.4%). Strains belonging to species other than *A. baumannii* were isolated more frequently (n = 158; 27.1%) than previously reported, mainly from blood cultures, respiratory tract specimens, and central venous catheters (Seifert, Baginski et al., 1993)

2.2 Identification of *Acinetobacter* species

Identification of this group of bacteria to the genus level is not a difficult process; however, identification to the species level is still a problematic and time

consuming process today, and may be performed either phenotypically or, with more accuracy, genotypically.

Acinetobacters are short, plump gram negative rods or cocco-bacilli, forming smooth, sometimes mucoid, pale yellow to greyish-white colonies on solid media. The colonies are similar in size to those of enterobacteria. They are all strict aerobes, oxidase negative, catalase positive, and nonfermentative. Most strains are unable to reduce nitrate to nitrite in the conventional nitrate reduction assay. Most strains can grow in a simple mineral medium containing a single carbon and energy source. A wide variety of organic compounds can be used as carbon sources, although relatively few strains can use glucose. Some strains oxidise glucose and related aldoses due to the production of a nonspecific aldose dehydrogenase and some strains are proteolytic. Besides these properties, they are uniformly inert in most bacteriological tests.

No single metabolic test enables unambiguous differentiation of this genus from other similar bacteria - such unambiguous differentiation relies on the ability of extracted DNA to restore the wild-type phenotype to mutant *Acinetobacter* strain BD413 *trpE27* in a transformation assay (Juni, 1972).

2.2.1 Phenotypic identification of *Acinetobacter* to the species level

In 1986, Bouvet and Grimont described an identification scheme of 28 phenotypic tests to identify *Acinetobacter* isolates to the species level (Bouvet and Grimont, 1986). The tests listed below differentiate between 11 of the initial 12 genomic species described by these authors (Bouvet and Grimont, 1986).

1. Growth at 30, 37, 41 and 44^0C
2. Glucose oxidation
3. Gelatin liquefaction
4. Haemolysis on sheep and human blood agar plates
5. Assimilation of levulinate, citraconate, 4-hydroxybenzoate and L-tartrate

6. Assimilation tests with the following carbon sources:
    - DL-lactate
    - DL-4-aminobutyrate
    - *trans*-aconitate
    - citrate
    - glutarate
    - aspartate
    - azelate
    - *B*-alanine
    - L-histidine
    - D-malate
    - malonate
    - histamine
    - L-phenylalanine
    - phenyl acetate

In 1987, the same authors described a simplified scheme of 16 tests which, except for tests for glucose acidification and detection of haemolysis on sheep blood agar, comprise growth temperature and carbon source utilisation tests (Bouvet and Grimont, 1987). However, this scheme correctly identified only 78% of strains in a study by Gerner-Smidt et al. (Gerner-Smidt, Tjernberg et al., 1991). In 1993, a more detailed and successful scheme using 32 tests was described (Kampfer, Tjernberg et al., 1993) using carbon source utilisation, qualitative enzyme tests and sugar acidification tests, but it seems that no single test or even a few tests can be used for unambiguous phenotypic identification of the different genomic species.

The other problem with proper attempts at phenotypic identification tests is that the information is not of any immediate clinical value, as these tests require specialised media for testing which are not available in a routine laboratory, and prolonged incubation periods (up to 7 days) (Gerner-Smidt, Tjernberg et al., 1991), rendering them useless for management of the individual patient.

Two commercial systems, the API 20NE and Biolog, also do not differentiate *Acinetobacter* strains with any degree of reliability (Bernards, Dijkshoorn et al., 1995) (Bernards, van et al., 1996). Amongst a collection of 130 *Acinetobacter* strains identified by DNA hybridisation to 18 different genomic species which was used to assess the ability of the API 20NE system (bioMerieux, France) to identify *Acinetobacter* genomic species and to determine its accuracy, only 87% of the strains were identified to the appropriate genomic species (Bernards, van et al., 1996). Amongst a collection of 129 *Acinetobacter* strains belonging to genomic species 1-14 which were investigated for their ability to oxidise 95 carbon sources in the Biolog system, the strain groupings obtained by cluster analysis with the Biolog software were compared with the results of DNA-DNA hybridisation studies. The results obtained correlated with the classification of reference strains of the DNA groups by DNA-DNA hybridisation, but six strains of four different DNA groups were not allocated to the clusters of their respective DNA groups. In the case of DNA groups 4, 5, 6, 7, 10, 11 and 14, at least one carbon source oxidation test could be used to differentiate them from the other DNA groups (Bernards, Dijkshoorn et al., 1995). Of the two commercially available systems, the Biolog system seems to be the superior, as it differentiates bacteria on the basis of their oxidation of 95 different carbon sources.

In 1995, Bouvet described an electrophoretic method to differentiate between the common isolates of *Acinetobacter* species. As *Acinetobacter baumannii*, *Acinetobacter* species 3 and DNA group 13 are the most prevalent *Acinetobacter* species in hospitals, and the identification scheme of Bouvet and Grimont is sometimes difficult to differentiate these species from *A. calcoaceticus* (a species of the natural environment that has seldom been found associated with human infection), their study was undertaken, where genetically identified *Acinetobacter* isolates belonging to these species were investigated for electrophoretic separations of L-malate dehydrogenase (MDH), glutamate dehydrogenase (GDH) and catalase (CAT). They found that all *A. calcoaceticus* isolates were easily differentiated from those of the other species investigated by their high MDH values, their low GDH values and CAT values. *Acinetobacter* species 3 was differentiated from *A. baumannii* and DNA group 13 by high CAT values. *A.*

baumannii could not be differentiated from DNA group 13. They concluded that once an *Acinetobacter* is phenotypically identified as one of these four closely related species, electrophoretic analysis of MDH, GDH and CAT might be a useful complement to the identification scheme of Bouvet and Grimont for accurately identifying *A. calcoaceticus* (Bouvet and Jeanjean, 1995). Other authors have not, however, validated the results of this study.

2.2.2 Genotypic tests for identification of *Acinetobacter* species

In order to obtain unambiguous proper identification of *Acinetobacter* species today, and to avoid the problems with phenotypic species identification, genotypic techniques are mandatory. New molecular identification methods are now available, which have shown good correlation when evaluated against DNA-DNA hybridisation.

Ribotyping (Gerner-Smidt, 1992) and PCR ribotyping (ARDRA) (Vaneechoutte, Dijkshoorn et al., 1995) are recently described techniques to identify *Acinetobacter* genomic species. Gerner-Smidt in 1992 performed ribotyping on 70 strains in the *A. calcoaceticus-A. baumannii* complex with known DNA group affiliations with the restriction enzymes EcoR I, Cla I, and Sal I. A nonradioactive digoxigenin-11-dUTP-labeled *Escherichia coli* rRNA-derived probe was used. With any of the three restriction enzymes, banding patterns that were specific for each DNA group were seen. All 70 strains showed banding patterns that could identify them to the correct DNA group by use of any two of the three enzymes. Their results indicate the high discriminatory power of the system (Gerner-Smidt, 1992).

Vaneechoutte in 1995 studied a total of 53 field and reference strains belonging to the 18 genomic species (DNA groups) of *Acinetobacter* by amplified ribosomal DNA restriction analysis (ARDRA). Restriction analysis with the enzymes Alu I, Cfo I, Mbo I, Rsa I, and Msp I of the enzymatically amplified 16S rRNA genes allowed identification of all species except the genomic species 4 (*Acinetobacter haemolyticus*) and 7 (*A. johnsonii*), 5 (*A. junii*) and 17, and 10 and 11, which

clustered pairwise in three respective groups. However, use of a few additional simple phenotypic tests (hemolysis, growth at 37 °C, production of acid from glucose, and gelatin hydrolysis) can be used to differentiate between the species within these clusters. ARDRA proved to be a rapid and reliable method for the identification of most of the *Acinetobacter* genomic species, including the closely related DNA groups 1 (*A. calcoaceticus*), 2 (*A. baumannii*), 3, and 13. The results of this study suggest that ARDRA can be used for the identification of *Acinetobacter* species (Vaneechoutte, Dijkshoorn et al., 1995).

Nowak in 1995 described a PCR-based method of restriction fragment length polymorphism (RFLP) analysis of *Acinetobacter* for genospecies identification using the rec A gene as a primer. Primers deduced from known rec A gene sequences of *Acinetobacter calcoaceticus* and *Neisseria gonorrhoeae* allowed the amplification of DNA from all *Acinetobacter* genospecies. The amplified products were examined further by restriction fragment length polymorphism (RFLP) analysis. Restriction analysis with only two enzymes, Mbo I and Hinf I, enabled accurate identification of all known genospecies (Nowak and Kur, 1995).

The same authors also described another PCR-based technique for *Acinetobacter* genospecies identification. They tested strains of 17 reference *Acinetobacter* genospecies by the PCR. They used primers to amplify spacer regions between the 16S and 23S genes in the rRNA genetic loci, then resolved the spacer amplification products by electrophoresis. The resulting patterns could be used to distinguish all of the tested *Acinetobacters* into 15 groups. They also tested clinical strains, which were identified correctly to the genospecies level, and the identifications were confirmed by conventional biochemical tests. On the basis of these results, PCR amplification of the 16S-23S spacer region was shown to be a simple tool for the identification of *Acinetobacter* genospecies. The nucleotide sequences of the primers are sufficiently highly conserved among these organisms as to permit PCR reactions to be carried out with a single set of reaction conditions and amplification parameters (Nowak, Burkiewicz et al., 1995).

Dolzani in 1995 also proposed a simple and rapid method for the identification of the genospecies belonging to the *A. calcoaceticus-A. baumannii* complex based on the combined digestion by the restriction endonucleases Alu I and Nde II of the DNA fragments resulting from the amplification of the 16S-23S rRNA intergenic spacer sequences. They analysed 36 strains previously characterised by DNA-DNA hybridisation and showed that the restriction profiles obtained are highly reproducible and characteristic for each genospecies. They extended the study to 68 clinical strains, which were assigned to the *A. calcoaceticus-A. baumannii* complex by phenotypic tests, and confirmed a panel of limited and well-conserved restriction patterns which allowed the identification of the strains tested (Dolzani, Tonin et al., 1995).

Ehrenstein in 1996 validated the suitability of a rapid identification technique based on tRNA spacer (tDNA) fingerprinting in comparison with that of a commercially available assay involving carbon source utilisation tests (Biolog MicroStation System) for identifying the 19 DNA-DNA hybridisation groups of *Acinetobacter* species. They analysed 128 strains previously identified by DNA-DNA hybridisation by both techniques. Their results showed that tDNA fingerprinting was highly reproducible and classified all strains into 17 groups. The software used with the commercial carbon source utilisation method grouped the 128 strains into 12 clusters, explaining the lower discriminatory power of this system. They concluded that tDNA fingerprinting is a quick and reliable method for the routine differentiation of most *Acinetobacter* species (Ehrenstein, Bernards et al., 1996).

Wiedmann-al-Ahmad in 1994 differentiated *Acinetobacter* type strains and isolates from wastewater treatment plants by PCR fingerprinting. On the first level, PCR fingerprinting with two tRNA-gene specific primers (T5B and T3A) was used for the identification of species (genospecies 1 to 17). On the second level, a single arbitrary primer (DAF 4) was employed for strain differentiation. Upon comparison of *Acinetobacter* type strains with 28 sewage sludge isolates, 2 could be classified as belonging to *A. johnsonii*, 8 isolates could be classified as *A. lwoffii*, 8 could be classified as *A. baumannii*, and 9 isolates were very closely

related to the *Acinetobacter* species *A. junii*; only one isolate could not be classified as one of the *Acinetobacter* type strains. The PCR fingerprinting method was found to be a reproducible and fast method for differentiation and identification of *Acinetobacter* isolates. Because of some resulting discrepancies compared with previously described identification schemes, e.g., DNA-DNA hybridisation methods, the original identification experiments should be repeated and the results should be reassessed (Wiedmann-al-Ahmad, Tichy et al., 1994).

Seifert in 1997 performed an epidemiological study to investigate the colonisation with *Acinetobacter spp.* of the skin and mucous membranes of 40 patients hospitalised in a cardiology ward and 40 healthy controls. Single samples were obtained once from each of nine different body sites, i.e., forehead, ear, nose, throat, axilla, hand, groin, perineum, and toe web. Identification of *Acinetobacter* isolates was achieved by using phenotypic properties and was compared to identification by amplified ribosomal DNA restriction analysis. Selected isolates were further investigated with sodium dodecyl sulfate-polyacrylamide gel electrophoresis, ribotyping, and DNA-DNA hybridisation. Plasmid profile analysis was used for epidemiological typing. Thirty patients (75%) and 17 controls (42.5%) were found to be colonised with *Acinetobacter spp.*, and the colonisation rates of patients increased during their hospital stay. The most frequently isolated species were *Acinetobacter lwoffii* (47%), *A. johnsonii* (21%), *A. radioresistens* (12%), and DNA group 3 (11%). In contrast, *A. baumannii* and DNA group 13TU, the most important nosocomial *Acinetobacter spp.*, were found only rarely on human skin (0.5 and 1%, respectively) and their natural habitat remains to be defined. A good correlation between phenotypic and genotypic methods for identification of *Acinetobacter spp.* was observed (Seifert, Dijkshoorn et al., 1997).

2.3 Nosocomial Infections Caused By *Acinetobacter* species

Since the modification of the taxonomy of this organism, *Acinetobacter baumannii* has been found to be the species most frequently isolated in nosocomial infection. (Kropec, Hubner et al., 1993). The true frequency of nosocomial *Acinetobacter*

infections is difficult to assess, partly because isolation of the organism from clinical specimens is not necessarily proof that it is the cause of the infection, but it may only reflect colonisation of the site of the specimen by the organism (Struelens, Carlier et al., 1993). Other reasons for the variability in morbidity and mortality due to this organism in published reports are the variation in the numbers of susceptible individuals, the presence of predisposing factors in the patients in the wards, and differences in the diagnostic criteria used.

Acinetobacter may cause infections in all organ systems, often together with other pathogens. The main sites of nosocomial infections by *Acinetobacter spp.* are the lower respiratory tract, peritoneum, urinary tract, surgical wounds, device-related infections, meninges, skin and eye, and these infections may progress to septicaemia (Bergogne-Berezin and Joly-Guillou, 1991). Most infections are opportunistic in nature and develop in compromised patients after invasive diagnostic and therapeutic procedures (Beck-Sague, Jarvis et al., 1990). Numerous outbreaks of infection in intensive care units have been described over the years (Beck-Sague, Jarvis et al., 1990) (Ling, Wise et al., 1996) (Lortholary, Fagon et al., 1995) (Reboli, Houston et al., 1994) (Castle, Tenney et al., 1978) (Stone and Das, 1986) (Hartstein, Rashad et al., 1988), and risk factors have been identified.

2.3.1 Identified risk factors for *Acinetobacter* infections

Only a few case-control studies on this issue have been published, and all the studies have been retrospective (see Table 2 below). (Vandenbroucke-Grauls, Kerver et al., 1988) (Sherertz and Sullivan, 1985) (Struelens, Carlier et al., 1993) (Beck-Sague, Jarvis et al., 1990).

Table 2: Identified risk factors for *Acinetobacter* infections

1. ICU stay
2. Previous antibiotic administration
3. Recent major surgery
4. Severe underlying disease (e.g. malignancy, burns, immunosuppression)
5. Presence of invasive devices and foreign bodies
6. Advanced age
7. Prolonged respiratory therapy with mechanical ventilation
8. Duration of hospital stay
9. Hyperalimentation

2.3.2 Transmission of *Acinetobacter* species amongst patients

The bacterium is part of the bacterial flora of the skin and mucous membranes of normal subjects. No nose, throat, vaginal or rectal carriers of outbreak strains have been found among staff members so far, but they have been found from the nose, mouth and rectum of patients in several studies (Allen and Green, 1987). Some authors have suggested that the gastrointestinal tract is an important reservoir for *Acinetobacter*. However, gastrointestinal carriage rates in patients have been lower than skin carriage rates in all studies reporting both (Gerner-Smidt, 1995). Thus, gastrointestinal carriage is more likely to be a sign of massive colonisation of the patients, and outbreaks will not be controlled by eliminating the organism from the gastrointestinal tract (e.g. by selective decontamination) (Gerner-Smidt, 1995).

Transmission of *Acinetobacter* to patients in intensive care units is often attributed to common environmental sources such as mechanical ventilators (Castle, Tenney et al., 1978) (Stone and Das, 1986), ventilator tubing (Castle, Tenney et al., 1978) (Vandenbroucke-Grauls, Kerver et al., 1988) (Cefai, Richards et al., 1990) (Hartstein, Rashad et al., 1988), equipment and solutions used for

respiratory therapy (Castle, Tenney et al., 1978) (Cefai, Richards et al., 1990) (Stone and Das, 1986), arterial pressure transducers (Beck-Sague, Jarvis et al., 1990), resuscitation bags (Castle, Tenney et al., 1978) (Stone and Das, 1986), mattresses (Sherertz and Sullivan, 1985), or air and surfaces in the unit (Allen and Green, 1987) (Getchell - White, Donowitz et al., 1989). The role of air and surface contamination in *Acinetobacter* transmission is less clear than that of the other factors. Prolonged survival of *Acinetobacter* for up to two weeks on dry surfaces has been reported (Getchell - White, Donowitz et al., 1989) (Allen and Green, 1987), and airborne dispersal around colonised patients has been shown (Allen and Green, 1987) (Crombach, Dijkshoorn et al., 1989); these factors suggest that the environment may be an indirect source of the organism, but this has only been documented in two studies; the first was in a burns unit where mattresses were conclusively shown to be the epidemic reservoir (Sherertz and Sullivan, 1985) and the second was in a hospital in the Netherlands which conclusively showed feather pillows to be the reservoir (Weernink, Severin et al., 1995). In addition, many outbreaks are related to cross contamination by the hands of staff in the unit (Stone and Das, 1986) (French, Casewell et al., 1980) (Patterson, Vecchio et al., 1991). Apart from hands themselves, inadequately used gloves that are not changed between patients may replace hands as an efficient vehicle for *Acinetobacter* transmission (Patterson, Vecchio et al., 1991).

2.3.3 Lower respiratory tract infection

Many outbreaks of *Acinetobacter spp.* nosocomial lower respiratory tract infection in intensive care units have now been described in the literature (Beck-Sague, Jarvis et al., 1990) (Ling, Wise et al., 1996) (Lortholary, Fagon et al., 1995) (Reboli, Houston et al., 1994) (Castle, Tenney et al., 1978) (Stone and Das, 1986) (Hartstein, Rashad et al., 1988), and the role played by *Acinetobacter spp.* in ventilator-associated pneumonia appears to be increasing (Bergogne-Berezin and Joly-Guillou, 1991) (Castle, Tenney et al., 1978) (Cefai, Richards et al., 1990) (Hartstein, Rashad et al., 1988) (Stone and Das, 1986) (Vandenbroucke-Grauls, Kerver et al., 1988). Several studies have reported that 3 to 5% of nosocomial pneumonias are caused by *Acinetobacter spp.* (CDC, 1987), and this figure

increases to 15-24% in the subset of patients who are mechanically ventilated (Fagon, Chastre et al., 1989) (Torres, Aznar et al., 1990). Mortality rates associated with *Acinetobacter* pneumonias are reported to be between 30% and 75%, with the highest rates reported in ventilator-dependent patients (Bergogne-Berezin and Joly-Guillou, 1991) (Fagon, Chastre et al., 1989) (Torres, Aznar et al., 1990). A number of risk factors have been identified or suspected to be implicated in these infections in the intensive care unit - these include advanced age, chronic lung disease, immunosuppression, surgery, use of antimicrobial agents, presence of invasive devices such as endotracheal and gastric tubes, and type of respiratory equipment (Bergogne-Berezin and Joly-Guillou, 1991) (Buxton, Anderson et al., 1978) (Castle, Tenney et al., 1978) (Lortholary, Fagon et al., 1995) (Peacock, Sorrell et al., 1988).

2.3.4 Bacteraemia

The most common *Acinetobacter* species causing significant bacteraemia is now identified in most series of patients in whom proper species identification is made, as *A. baumannii.* Seifert in 1993 identified a total of 584 *Acinetobacter* strains from 420 patients from 12 different hospitals over a period of twelve months according to the new taxonomy proposed by Bouvet and Grimont. *A. baumannii* strains were isolated most frequently (n = 426; 72.9%), followed by *Acinetobacter* species 3 (n = 55), *A. johnsonii* (n = 29), and *A. lwoffii* (n = 21). Most isolates were recovered from respiratory tract specimens (n = 251; 42.9%). The others were from blood cultures (n = 116; 19.9%), wound swabs (n = 90; 15.4%), catheter tips (n = 75; 12.8%), and the urinary tract (n = 20; 3.4%) (Seifert, Baginski et al., 1993). It must be remembered, however, that the differentiation between true bacteraemia and contamination of the blood culture with skin inhabitants is sometimes difficult to assess.

Acinetobacter species may be found either as a single pathogen or as part of a polymicrobial bacteraemia. The two groups of patients who are most at risk for *Acinetobacter* bacteraemia seem to be immunocompromised adults and neonates. The commonest source of the bacteraemia is often a respiratory tract

infection; other sources are surgical wound infections, burns, vascular catheters and pressure monitoring transducers.

Seifert in 1994 described the clinical features, possible predisposing factors and treatment outcomes associated with bacteraemia due to *Acinetobacter* species other than *Acinetobacter baumannii*. They reviewed laboratory and medical charts over a period of 18 months, and discovered 61 cases of bacteraemia due to *Acinetobacter* species other than *A. baumannii* occurring in 59 patients. Six of these were considered not significant. Fifty cases represented catheter-related bacteraemia, one case was associated with meningitis following brain surgery, and four cases could not be classified. Clinical courses were usually benign: all but four patients were cured, and death was not related to *Acinetobacter* bacteraemia in any case. Therapy included catheter removal alone (32.8%), appropriate antimicrobials alone (12.7%), or both (49.1%). They concluded that *Acinetobacter* species other than *A. baumannii* are clinically significant organisms with limited pathogenic potential; they are almost exclusively involved in device-related bacteraemia, and that the clinical and epidemiological features of infections due to these organisms are clearly distinct from infections due to *A. baumannii* (Seifert, Strate et al., 1994).

2.4 Virulence factors in *Acinetobacter* species

Acinetobacters are generally considered to be low-grade pathogens (Smego, 1985), especially the species other than *A. baumannii*, but there are some factors that may enhance the virulence of these organisms, although this aspect has not been studied well at all by researchers. However, the presumed virulence factors are enumerated below.

a) Their polysaccharide capsule, formed by L-rhamnose, D-glucose, D-glucuronic acid and D-mannose (Kaplan, Rosenberg et al., 1985). The capsule probably makes the bacteria more hydrophilic.
b) Adhesion to human epithelial cells due to fimbriae and the capsule (Rosenberg, Bayer et al., 1982).

c) The production of exoenzymes, which may damage tissue lipids (Poh and Loh, 1985).
d) The potential toxicity of the lipopolysaccharide of the cell wall (Kaplan, Rosenberg et al., 1985).

2.5 Typing Methods for Bacteria

Over the decades, many different typing methods have been devised in order to further link and categorise bacteria belonging to the same genus and species. The reasons for trying to link them together are manifold, and include the important need to distinguish epidemiological linkages amongst different strains of bacteria. As time passed, the typing methods became more and more sophisticated, and the present boom in technology, especially in the area of molecular methodology, has led to the development of numerous molecular techniques in bacterial strain typing; these techniques are increasing in numbers almost daily. The application of molecular techniques to microbial typing has provided a powerful set of new tools that facilitate epidemiological investigations.

The central hypothesis in epidemiologic typing is that the isolates in a series obtained from an epidemiologic cluster are directly descended from a single common precursor. Typing systems are based on the premise that clonally related isolates share characteristics by which they can be differentiated from unrelated isolates.

There are two major categories of typing methods; these are phenotypic techniques, which detect characteristics expressed by the microbes, and genotypic techniques, which involve direct DNA-based analyses of chromosomal or extrachromosomal genetic elements.

There is no "gold standard" by which to judge a typing method, but the following criteria are useful in evaluating typing systems:

a) Typeability - this refers to the ability to obtain an unambiguous positive result for each isolate analysed. Nontypeable isolates are those that give either a null or an uninterpretable result.
b) Reproducibility - this refers to the ability of a technique to yield the same result when the same strain is tested repeatedly. Reproducibility is influenced by both technical and biologic factors.
c) Discriminatory power - this refers to the ability of the typing technique to differentiate among unrelated strains. Ideally, each unrelated isolate is detected as unique.
d) Ease of interpretation of results.
e) Ease of performance of the test.

To be widely useful, a typing method should be applicable to a broad range of microorganisms as well as inexpensive and technically accessible. It should not require expensive equipment or special expertise. Results should be available rapidly enough to be relevant to patient management or infection control. At this time, no single typing system is optimal by all of these criteria, and no one approach is preferred for all clinical settings or infecting species. In the absence of a gold standard for the evaluation of typing methods, two typing systems can be formally compared only if both have been applied to the same set of isolates.

2.5.1 Phenotypic typing techniques

Biotyping, antibiogram typing, serotyping, bacteriophage typing, bacteriocin typing, immunoblotting, electrophoretic protein typing, multilocus enzyme electrophoretic typing and outer membrane protein typing are the usual methods of phenotypic bacterial strain typing. These techniques are inherently limited by the capacity of microorganisms to alter the expression of the underlying genes (Wachsmuth, 1985). Such changes may occur unpredictably or in response to various environmental stimuli (Mekalanos, 1992). Also, point mutations may result in the abnormal regulation or function of the gene responsible for a particular phenotype. Thus, isolates that are the same strain and are almost genetically indistinguishable can vary in the phenotype detected.

2.5.1.1 Biotyping

Biotyping makes use of the pattern of metabolic activities expressed by an isolate and may include specific biochemical reactions, colonial morphology and environmental tolerances. Such characteristics have classically been used for taxonomy. In general, biotyping has relatively poor discriminatory power, and is not used today in epidemiologic investigations.

2.5.1.2 Antibiogram typing

Antibiogram typing is readily available to investigators as it relies on routinely performed panels of susceptibility tests on isolates. In fact, the identification of a new or unusual pattern of antimicrobial resistance among isolates cultured from multiple patients is often the first indication of an outbreak. However, this typing method is of limited value, as phenotypes vary, and there are multiple genetic mechanisms by which strains may become abruptly resistant to a particular antibiotic (Mickelsen, Plorde et al., 1985), resulting in either of two consequences - different strains may develop similar resistance patterns, and sequential isolates representing the same strain may differ for one or more antibiotics (Mickelsen, Plorde et al., 1985) (Tenover, Arbeit et al., 1994).

2.5.1.3 Serotyping

Serotyping is based on the observation that microorganisms of the same species can differ in the antigenic determinants expressed on their cell surface. Many different surface structures exhibit such antigenic variation - these include, in bacteria, lipopolysaccharides, capsular polysaccharides, membrane proteins, and extracellular organelles. For some organisms, such as pneumococci, salmonellae and shigellae, serotyping remains a primary means of evaluating isolates. As a general means of performing detailed epidemiological analyses, however, serotyping has several critical limitations - it requires high quality commercial reagents, and if these are not available, the preparation of specific typing antibodies is a difficult process generally restricted to reference and research

laboratories. Also, serotyping has poor discriminatory power, because many strains may represent only a few serotypes or may be nontypeable (Tsang, Denner et al., 1992).

2.5.1.4 Bacteriophage typing

Bacteriophage typing is the characterisation of isolates by their patterns of resistance or susceptibility to a standard set of lytic phages - these are viruses that are capable of infecting and lysing bacterial cells. It is a technique that is available only at reference laboratories, and performed only on certain organisms, viz. *Staphylococcus aureus* and *Salmonella* species (Hickman-Brenner, Stubbs et al., 1991) (Blair and Carr, 1960). In addition, it is technically very demanding and has considerable experimental and biologic variability.

2.5.1.5 Bacteriocin typing

Bacteriocin typing is where an isolate is assessed for susceptibility to a set of bactericidal peptides produced by selected strains. It is useful for certain pathogens, such as *Pseudomonas aeruginosa* (Pitt, 1988), but has limitations similar to those described for bacteriophage typing.

2.5.1.6 Electrophoretic protein typing

Electrophoretic protein typing is performed by isolating proteins or glycoproteins from cells, separating them by sodium dodecyl sulphate-polyacrylamide gel electrophoresis, and staining the proteins in the gel to determine the resulting pattern. In immunoblotting, the electrophoresed bacterial products are transferred ("blotted") onto a nitrocellulose membrane and then exposed to antisera or pooled human sera containing broadly reactive antibodies. The bound antibodies can then be detected using commercially available enzyme-labelled anti-immunoglobulins. The patterns detected using both these techniques are very complex, the comparisons among multiple strains can be difficult, and the

significance of small differences is uncertain (Gaston, Duff et al., 1988). Therefore, these methods are not widely employed for epidemiological typing.

2.5.1.7 Multilocus enzyme electrophoresis (MLEE)

Multilocus enzyme electrophoresis is the analysis of isolates according to the differences in the electrophoretic mobilities of a set of metabolic enzymes (Selander, Caugant et al., 1986), which are electrophoresed in nondenaturing starch gels. For each enzyme analysed, the gel is stained with a specific colorimetric substrate such that the position of the enzyme is detected by the appearance of a visible reaction product. Variations in the electrophoretic mobility of an enzyme are referred to as electromorphs; combinations of electromorphs are designated electrophoretic types, and each distinct electrophoretic type is considered to represent a multilocus genotype. MLEE has been used most effectively to examine the population genetics of bacterial species, where genetic diversity among large collections of isolates can be assessed, and the genetic structure of the population can be represented graphically as a dendrogram (Musser, Kroll et al., 1990). However, MLEE is only moderately discriminatory for the epidemiologic analysis of clinical isolates, and requires techniques and equipment that are available in relatively few laboratories, and therefore has relatively limited application to epidemiologic studies.

2.5.2 Genotypic typing techniques

When DNA-based typing techniques were initially discovered, they were the newest "solution" to all the problems of epidemiological bacteriology; however, they have since been shown to have problems of their own.

2.5.2.1 Plasmid profile analysis

Plasmid profile analysis was among the earliest DNA-based techniques applied to modern epidemiology (Mayer, 1988). The number and sizes of plasmids carried by an isolate are determined by preparing a plasmid extract and subjecting it to

routine agarose gel electrophoresis. However, the problems with such plasmid analyses is that the DNA composition of a plasmid can change rapidly (Lupski, 1987). Furthermore, plasmids may spread rapidly among strains and among different species (John and Twitty, 1986). Also, many clinical isolates lack plasmids, rendering them nontypeable by this technique; other isolates carry only one or two plasmids, which reduces the discriminatory power of the technique.

2.5.2.2 Restriction enzyme analysis of plasmids

Restriction enzyme analysis of plasmids is now the method of choice for plasmid studies. It substantially improves the reproducibility and discriminatory power of plasmid analyses by digesting the plasmids with restriction enzymes and then electrophoretically analysing the number and sizes of the resulting restriction fragments (Mayer, 1988). It is a technically simple technique that requires only modest specialised equipment, and can be performed relatively quickly.

2.5.2.3 Restriction endonuclease analysis of chromosomal DNA

Restriction endonuclease analysis of chromosomal DNA is the digestion of bacterial DNA with restriction endonucleases, and separating the resultant DNA fragments by size using constant-field gel electrophoresis; the pattern of the separation of fragments can then be detected by staining the gel with ethidium bromide and examining it under UV light. Different strains of the same bacterial species will have different patterns, as their genomic DNA composition will vary; and therefore, all isolates are typeable by this technique. However, the major limitation of this technique is the difficulty of comparing the profiles, as they may be extremely complex with hundreds of bands that may be unresolved and overlapping (Bialkowska-Hobrazanska, Jaskot et al., 1990). Furthermore, plasmid DNA can readily contaminate genomic DNA preparations, thereby making isolates that are the same genomically but different only in their plasmid content appear as different strains. In general, therefore, restriction endonuclease analysis of chromosomal DNA has largely been supplanted by other genomic typing methods.

2.5.2.4 Restriction fragment length polymorphisms

Southern blot analysis of restriction fragment length polymorphisms (RFLPs) is the digestion of bacterial genomic DNA using restriction endonucleases, then separating the fragments by agarose gel electrophoresis, then transferring ("blotting") the fragments onto a nitrocellulose or nylon membrane, then detecting patterns by the detection of specific DNA sequences (loci) using a labelled piece of homologous DNA as a probe. The probe binds (hybridises) only to those fragments containing identical complementary nucleotide sequences. Variations in the number and sizes of the fragments detected are referred to as restriction fragment length polymorphisms (RFLPs). Provided the correct probe is used, all strains with homologous loci to the probe are typeable by this technique, and the results are, in general, highly reproducible. Southern blot analyses in which insertion sequences and transposons are used as probes have proven to be reproducible and highly discriminatory (Edlin, Tokars et al., 1992).

2.5.2.5 Ribotyping

Ribotyping refers to a Southern blot analysis in which strains are characterised for the RFLPs associated with the ribosomal operon. Operons are clusters of genes that share related functions. The ribosomal operons comprise nucleotide sequences coding for 16S rRNA, 25S rRNA and one or more tRNAs. Ribosomal sequences are highly conserved, and probes prepared from *E. coli* rRNA hybridise to the chromosomal ribosomal operons of a wide range of bacterial species. All bacteria carry these operons and are therefore typeable. Ribotypes are stable and reproducible. Isolates from an outbreak typically have the same ribotype; however, epidemiologically unrelated isolates also sometimes demonstrate the same pattern, limiting the usefulness of the method (Tenover, Arbeit et al., 1994).

2.5.2.6 Pulsed field gel electrophoresis

Pulsed field gel electrophoresis (PFGE) of chromosomal DNA is the digestion of the bacterial genome with restriction endonucleases with relatively few restriction sites, thereby generating far fewer but much larger fragments of DNA than would be obtained with conventional restriction endonucleases; these fragments are then separated by a modification of the gel electrophoresis technique whereby the orientation of the electric field across the gel is changed periodically ("pulsed") rather than being kept constant as in the conventional gel electrophoresis.

PFGE was developed in 1984 by Drs. Schwartz and Cantor to separate fragments of DNA much larger than the 50kb pieces separated by conventional electrophoresis (Schwartz and Cantor, 1984). DNA fragments up to 12.6mb have been separated by PFGE and fragments much larger most likely can also be separated. PFGE utilises more than one set of electrodes and each set of electrodes is positioned at different angles to the DNA sample. During the electrophoresis run, each set of electrodes alternately switch on and off for a given time period and then the other set is on, thus giving the system the name "pulsed". The theory behind it is that each time the direction of the electrical field changes, the DNA must reorient and realign relative to the electric field. Because larger fragments of DNA take longer to reorient than smaller ones, the larger the fragments of DNA, the longer it will take to migrate down the gel.

DNA that is prepared in solution is spontaneously sheared into random fragments of 100kb or less. Therefore, suitable unsheared DNA is obtained for PFGE by embedding intact organisms in agarose plugs ("inserts") and then enzymatically lysing the cell wall and digesting the cellular proteins. The isolated genomes are then digested in situ with restriction enzymes with few recognition sites.
PFGE provides a chromosomal restriction profile typically composed of 5-20 distinct, well-resolved fragments ranging from 10 to 800kb. All bacterial isolates are theoretically typeable and the results are highly reproducible. The simplicity of the profiles generated renders the analysis and comparison of multiple isolates much easier.

PFGE has two notable limitations. First, because of the need for all buffers and enzymes to be diffused into the agarose insert, the preparation of suitable DNA involves several extended incubations, and takes from two to four days (Maslow, Slutsky et al., 1993). However, this DNA embedded in the agarose is stable for years at 4°C, and can easily be released into solution for use in other protocols. Secondly, PFGE requires relatively expensive, specialised equipment.

2.5.2.7 Polymerase chain reaction (PCR)-based typing

Polymerase chain reaction (PCR)-based typing systems are methods of epidemiological typing that utilise the PCR. The essential feature of PCR is the ability to rapidly and exponentially replicate (amplify) a particular DNA sequence. Briefly, the basic procedure involves several distinct components:

a) The sequence to be amplified (the template) should be a relatively small fragment of DNA (0.5 - 2kb).
b) Two small oligonucleotides (primers), typically 18 - 20bp, corresponding to sequences at opposite ends of the template, are utilised to define the sites of DNA replication.
c) The double-stranded DNA template is first denatured, the primers are bound to each strand of the template, and the complementary strand is then synthesised (polymerised).
d) A rapid, self-contained "chain reaction" is achieved by using thermostable DNA polymerases and programmable thermocyclers. An entire procedure consists of 20-30 cycles, and this generates sufficient product (amplicon) to be visualised and sized directly in an agarose or polyacrylamide gel.

Several variations of PCR have been developed to provide additional information suitable for strain typing:

a) Restriction digestion of PCR products is the most direct modification whereby the PCR product is digested with a restriction endonuclease, and the resulting fragments are analysed for polymorphisms by electrophoresis. The restriction

digests are highly reproducible, but the discriminatory power varies substantially for different species of organisms, loci and restriction enzymes (Goh, Byrne et al., 1992) (Telenti, Marchesi et al., 1993). Strain differentiation can be increased by evaluating digests prepared with several different restriction enzymes (Matar, Swaminathan et al., 1993) (Fujimoto, Marshall et al., 1994). The use of nested PCR (which involves the use of a second set of primers representing sequences located inside the target sequences of the initial primers) is sometimes employed to improve the PCR product and to obtain more distinct restriction fragments (Goh, Byrne et al., 1992); however, this requires more reagents and technical effort than the routine procedure.

b) Rep-PCR is PCR based on repetitive chromosomal sequences, where the primers used are based on short extragenic repetitive sequences. Such sequences are present at multiple sites on the bacterial genome. When two sequences are located near enough to each other, then the DNA fragment between those sites (the "interrepeat fragment") is effectively amplified. The number and sizes of these interrepeat fragments varies from strain to strain. The technique seems to have excellent reproducibility and moderate discriminatory power (Woods, Versalovic et al., 1992).

c) Arbitrary primed PCR is also referred to as the random amplified polymorphic DNA assay. It is based on the observation that short primers (usually 10bp) will hybridise at random chromosomal sites to allow initiation of polymerisation. If two such sites are located within a few kilobases of each other on opposite DNA strands and in the proper orientation, then amplification of the intervening fragment will occur (Welsh and McClelland, 1990). The variation in the number and sizes of the fragments among different strains will be detected by electrophoresis of the amplicon. The approach is theoretically suitable for use with any organism. However, there are problems with the reproducibility and discriminatory power of the technique, for two main reasons. Firstly, reaction conditions are necessarily less stringent than with conventional PCR, resulting in bands being produced that vary widely in intensity, which are difficult to interpret and compare (Saulnier, Bourneix et al., 1993). Secondly, fragments

from a single isolate may vary in different amplification reactions because some of the products represent relatively inefficient reactions. In fact, there may be a need to isolate purified DNA and to quantitate the DNA concentrations in order to obtain reproducible results (van Belkum, Bax et al., 1993).

2.5.2.8 Nucleotide sequence analysis

In today's times of advanced molecular epidemiology, the ability to sequence an entire genome is not outside the realms of possibility. However, for fairly simple strain differentiation, this is not possible, nor is it practicable. By using the PCR to amplify a known DNA segment and automated techniques to sequence the PCR product, it is now feasible to compare multiple isolates by sequencing each one at the same locus. The advantages are that the data is precise, and that extensive databases can be shared with relative ease, thereby facilitating comparative analyses. However, appropriate loci for sequencing must be identified for each bacterial species; and these loci must be present in all strains of the species and be sufficiently variable to allow epidemiologically useful strain differentiation. Furthermore, it is not clear whether sequencing at a single locus will be a reliable and unambiguous tool for epidemiological typing (Bisercic, Feutrier et al., 1991). Finally, automated sequencers are prohibitively expensive for most settings.

2.6 Typing methods described for *Acinetobacter* species

2.6.1 Antibiotic resistance typing

Alexander in 1988 studied 44 isolates of *Acinetobacter* collected during hospital outbreaks using polyacrylamide gel electrophoresis (PAGE), plasmid analysis, antibiograms and biochemical tests to determine their degree of similarity. All methods were able to subdivide the isolates, but results did not always correlate well between methods. Their results suggested that no single biotyping technique is likely to be adequate and that electrophoretic, biochemical and antibiogram

data may complement one another and other epidemiological data in the typing of these organisms (Alexander, Rahman et al., 1988).

Joly-Guillou in 1990 used 2 typing systems to conduct an epidemiological study of *Acinetobacter* and to establish their relationship to antibiotic resistance phenotypes. Biotyping was performed with biochemical tests. Phage typing included two complementary systems: 125 phage-types and 25 subtypes. Resistance phenotype analysis included 11 antibiotics. They found that the three typing systems were complementary but that antibiotic resistance phenotypes and one of the two other typing systems would be required in parallel to provide suitable information for epidemiological purposes (Joly-Guillou, Bergogne-Berezin et al., 1990).

Tankovic in 1994 typed isolates using three methods, viz. antibiotyping, biotyping, and pulsed-field gel electrophoresis in 31 intensive care unit (ICU) patients who were either colonised or infected by imipenem-resistant *Acinetobacter baumannii.* All three methods revealed that two distinct strains were involved in the outbreak and that one of these strains had acquired a higher level of imipenem resistance as well as resistance to all aminoglycosides. They also found that ICU environmental contamination was an important reservoir of this epidemic strain (Tankovic, Legrand et al., 1994).

Ratto in 1995 used ribotyping, biotyping and resistance phenotype to characterise 37 *Acinetobacter baumannii-A. calcoaceticus* complex isolates responsible for nosocomial infections in Buenos Aires. Nineteen isolates were recovered from endemic infections at 2 hospitals and 18 represented an intensive care unit outbreak that occurred in a third hospital. They concluded that combined analysis of biotypes, resistance phenotypes, and ribotypes was an accurate approach for epidemiologic investigation of *A. baumannii.* Furthermore, ribotyping discriminated *Acinetobacter* genospecies 13 isolates which were phenotypically difficult to type (Ratto, Sordelli et al., 1995).

Aubert in 1995 characterised 42 strains of *Acinetobacter baumannii* isolated from 15 patients hospitalised in a French intensive care unit using biotyping, antibiotyping and ribotyping to recognise the transmission of multiresistant strains by transfer of a patient from one hospital to another. They found that the three methods gave a good correlation: the epidemic strains had the same antibiotic resistance pattern, the same biotype, and the same ribotypes obtained with three different endonucleases (Aubert, Grimont et al., 1995).

Vaneechoutte in 1995 studied two successive *Acinetobacter* outbreaks in a neonatal intensive care unit with arbitrarily primed polymerase chain reaction (AP-PCR), cell envelope protein electrophoresis (protein fingerprinting) and antibiotic susceptibility testing. They found that AP-PCR fingerprinting and protein fingerprinting yielded identical clustering of the isolates studied and susceptibility test results were useful for rapid recognition of the outbreaks, but clustering of several isolates was different from the clustering obtained with AP-PCR fingerprinting and protein fingerprinting (Vaneechoutte, Elaichouni et al., 1995).

Hence we can see that antibiotyping is not a tool that may be used on its own for proper epidemiologic typing of *Acinetobacter* species. Its usefulness lies in the initial identification of a possible outbreak situation and in combination with other, more discriminatory, typing methods.

2.6.2 Bacteriocin typing

Andrews in 1986 developed a technique for typing *Acinetobacter spp.* by bacteriocin production. One hundred and seventy-six cultures from patients in outbreaks, in the community and environmental sources were identified, tested for sensitivity to gentamicin and bacteriocin typed; 154 were *A. anitratus* and the remainder *A.lwoffi*. Only one *A.lwoffi* strain produced bacteriocin. Ten of 22 were sensitive to bacteriocins and could be used as indicators. A close association was found between bacteriocin production and gentamicin resistance. Using six indicator strains, 100/104 (96%) gentamicin-resistant strains were typed with 9 distinct patterns of inhibition. Overall typeability was 65% but 100/176 (56%) fell

into only two groups. They concluded that the technique may be of value in studying the epidemiology of *Acinetobacter* species (Andrews, 1986).

2.6.3 Biotyping

Bouvet in 1987 identified a total of 343 *Acinetobacter* strains, most isolated from hospital patients using a 16-test system (acid production from glucose, gelatin hydrolysis and utilisation of 14 carbon sources) associated with tests for growth at 37, 41 and 44 degrees C. Of 299 nosocomial isolates, 253 were identified as *A. baumannii*, 20 as *Acinetobacter* genospecies 3, 8 as *A. haemolyticus*, 8 as *A. lwoffii*, 4 as *A. johnsonii* and 6 as other (at the time) unnamed species. A biotyping system based on the utilisation of levulinate, citraconate, L-phenylalanine, phenylacetate, 4-hydroxybenzoate and L-tartrate allowed recognition of 17 biotypes among 247 *A. baumannii* isolates. (Bouvet and Grimont, 1987).

Bouvet *et al.* in 1990 determined species, biotypes, and phage types for 120 *Acinetobacter* strains from clinical or environmental sources or from culture collections. These characteristics were compared with cell envelope protein profiles obtained by sodium dodecyl sulfate-polyacrylamide gel electrophoresis in previous studies. A considerable heterogeneity of species and types was observed by use of the various methods, in particular among strains from different sources. *Acinetobacter baumannii* was the most commonly found species in isolates from clinical sources, followed by *Acinetobacter* species 3. Nine biotypes were observed among *A. baumannii* strains. Further differentiation within most species and biotypes was achieved by protein profile typing and, to some extent, phage typing. Of 120 strains, 49 (41%) were not typeable by phages. Their results suggest that biotyping was an appropriate method for the screening of strains, whereas protein profile and phage typing could serve as additional methods to establish the identity or nonidentity of strains. They concluded that the combination of the typing methods is useful in epidemiological studies (Bouvet, Jeanjean et al., 1990).

Joly-Guillou in 1990 used 2 typing systems to conduct an epidemiological study of *Acinetobacter* and to establish their relationship to antibiotic resistance phenotypes. Biotyping was performed with biochemical tests. Phage typing included two complementary systems: 125 phage-types and 25 subtypes. Resistance phenotype analysis included 11 antibiotics. They found that the three typing systems were complementary but that antibiotic resistance phenotypes and one of the two other typing systems would be required in parallel to provide suitable information for epidemiological purposes (Joly-Guillou, Bergogne-Berezin et al., 1990).

As seen above (under antibiotyping), Tankovic in 1994 used three methods for typing imipenem-resistant *Acinetobacter baumannii* isolates from 31 patients in two intensive care units (ICUs). These three methods were antibiotyping, biotyping, and pulsed-field gel electrophoresis. They found that two distinct strains were involved in their outbreak and ICU environmental contamination was recognised as an important reservoir of the epidemic strains. They also noted that all three methods were suitable for epidemiologic typing of their isolates (Tankovic, Legrand et al., 1994).

Sire in 1994 conducted an epidemiological survey over a one-week period to assess the spread of *Acinetobacter baumannii* in a medical intensive care unit. Fifty strains were isolated from patients and from a hospital environment. These strains belonged to biotypes 9 or 18. The rRNA gene restriction patterns (using EcoRI and PvuII as restriction endonucleases) and the esterase electrophoretic profiles were determined. They identified four EcoRI ribotypes, four PvuII ribotypes and six esterase profiles. All biotype 9 strains presented the same ribotype after EcoRI digestion, the same ribotype after PvuII digestion and the same zymotype. The same observation was made on most of the biotype 18 strains. They concluded that biotyping was an appropriate method for screening of strains, and ribotyping and esterase electrophoresis could be used as additional methods to delineate outbreaks of nosocomial infections caused by *A. baumannii* (Sire, Gras-Rouzet et al., 1994).

Ratto in 1995 used ribotyping, biotyping and resistance phenotype to characterise 37 *Acinetobacter baumannii-A. calcoaceticus* complex isolates responsible for nosocomial infections in Buenos Aires. By ribotyping, isolates were classified into five different clones of *A. baumannii* biotype 2, 3 of *A. baumannii* biotype 9, and 3 of *Acinetobacter* genospecies 13. Combination of the three epidemiological markers permitted categorisation of 18 outbreak isolates into four probable strains. They concluded that combined analysis of biotypes, resistance phenotypes, and ribotypes was an accurate approach for epidemiologic investigation of *A. baumannii*, but that ribotyping discriminated *Acinetobacter* genospecies 13 isolates which were phenotypically difficult to type (Ratto, Sordelli et al., 1995).

Aubert in 1995 studied 42 strains of *Acinetobacter baumannii* from 15 patients hospitalised in a French intensive care unit using biotyping, antibiotyping, and ribotyping to recognise the transmission of multiresistant strains by transfer of a patient from one hospital to another. They found that the three methods gave a good correlation: the epidemic strains had the same antibiotic resistance pattern, the same biotype, and the same ribotypes obtained with three different endonucleases (Aubert, Grimont et al., 1995).

Oliviera in 1996 phenotypically identified 255 *Acinetobacter* strains from clinical specimens of inpatients and outpatients. *A. baumannii* was the most frequent species (80.8%). This species underwent biotyping according to the scheme of Traub, and found that 81.2% belonged to biotypes 2, 6 and 9 with a predominance of biotype 2. These clones presented marked multiple resistance patterns and were widespread in different wards. No outbreak was reported during the period studied. The authors concluded that these phenotypical methods proved to be useful in differentiating strains of *A. baumannii* and, if used together, they showed a high discriminatory power (Oliveira, Irino et al., 1996).

2.6.4 Phage typing

Santos-Ferreirra in 1984 isolated 62 strains of *Acinetobacter calcoaceticus* from pathological samples or from the environment in several hospitals in Lisbon, and studied them by means of two complementary phage-typing systems. They found 18 phage-types or sub-types, one group of uncommon types (9.6%) and one group of untypeable strains (20.9%). A new phage-type (No. 104) and a new sub-type (No. 18) were defined among the Portuguese strains (Santos-Ferreirra, Vieu et al., 1984).

Giammanco in 1989 compared biotyping, phage typing, and the analysis of the bacterial envelope protein profiles using 64 multiresistant *Acinetobacter* strains isolated from clinical specimens. The antibiotic susceptibility of the strains was also considered. After geno-species identification, biotyping allowed the recognition of a relatively large and long-lasting presence of two *A. baumannii* biotypes at an Intensive Therapy Unit. Phage-typing and the analysis of the susceptibility to antibiotics allowed for the differentiation of strains belonging to different geno-species and biotypes, and in some cases also to the same biotypes. On the contrary, the analysis by polyacrylamide gel electrophoresis of the cell-envelope proteins failed to show any diversity not only within, but also between some of the biotypes of *A. baumannii* (Giammanco, Vieu et al., 1989).

Bouvet *et al.* in 1990 determined species, biotypes, and phage types for 120 *Acinetobacter* strains from clinical or environmental sources or from culture collections. These characteristics were compared with cell envelope protein profiles obtained by sodium dodecyl sulfate-polyacrylamide gel electrophoresis in previous studies. A considerable heterogeneity of species and types was observed by use of the various methods, in particular among strains from different sources. *Acinetobacter baumannii* was the most commonly found species in isolates from clinical sources, followed by *Acinetobacter* species 3. Nine biotypes were observed among *A. baumannii* strains. Further differentiation within most species and biotypes was achieved by protein profile typing and, to some extent, phage typing. Of 120 strains, 49 (41%) were not typeable by phages. Their results

suggest that biotyping was an appropriate method for the screening of strains, whereas protein profile and phage typing could serve as additional methods to establish the identity or nonidentity of strains. They concluded that the combination of the typing methods is useful in epidemiological studies (Bouvet, Jeanjean et al., 1990).

2.6.5 Serotyping

Traub in 1989 serologically examined 152 clinical isolates of *Acinetobacter baumannii* from 152 patients. The isolates were identified by carbon source utilisation tests. Polyclonal rabbit immune sera against *A. baumannii* strains were used in checkerboard tube agglutination tests, and 20 serovars were identified. They found that serovar 19 cross-reacted with genospecies 3. They also delineated several outbreaks of nosocomial cross-infection caused by serovars 4 and 10. (Traub, 1989).

The same author in 1990 identified a total of 156 clinical isolates of *Acinetobacter* genospecies 3, the second most commonly encountered member of the genus *Acinetobacter*, with carbon source utilisation. Checkerboard tube agglutination tests and reciprocal cross-absorption studies with polyclonal rabbit immune sera against heated cells of serovar candidate strains of genospecies 3 permitted identification of 13 serovars. They found crossreactions between serovars 10 and 8, serovars 2 and 12, serovars 13 and 2, and serovars 4 and 12. Genospecies 3 serovars 3, 7, 8, and 9 cross-reacted with *Acinetobacter baumannii* serovars 19, 21, 6, and 15, respectively (Traub, 1990).

Traub in 1996 comparatively examined triplets of isolates representing 20 putative clusters of nosocomial cross-infection due to *Acinetobacter baumannii* and genospecies 3 using serotyping and analysis of restriction fragments (Sma I and Apa I) of genomic DNA with the aid of pulsed-field gel electrophoresis. Carbon source assimilation tests disclosed phenotypic variation among 6 to 20 triplets of isolates. Two misleading results of serotyping were encountered. A strain of *A. baumannii* serovar 15 had infected 8 patients in a surgical intensive care unit,

while a second, genotypically totally different strain with the identical serovar had caused infection in one additional patient. With this exception, they concluded that the correlation between serotyping and analysis of macrorestriction profiles was excellent (Traub, Leonhard et al., 1996).

Oliveira in 1996 examined 255 *Acinetobacter* strains from clinical specimens of inpatients and outpatients. They were identified phenotypically according to the taxonomy of Bouvet and Grimont, and found *A. baumannii* to be the most frequent species (80.8%). This species underwent biotyping and serotyping according to the scheme of Bouvet and Grimont, and that of Traub, respectively. They found that 81.2% of samples belonged to biotypes 2, 6 and 9 with a predominance of biotype 2 and 86.6% of the strains could be serotyped. Serotype 29 was the most frequently isolated, and was related to biotype 2 (86.6%), whereas serotype 13 was related to biotype 6 (84.8%). These clones were widespread in different wards. No outbreak was reported during the period studied. They concluded that these phenotypical methods were useful in differentiating strains of *A. baumannii* and, if used together, they showed a high discriminatory power (Oliveira, Irino et al., 1996).

2.6.6 Cell envelope protein typing

Dijkshoorn in 1987 analysed the cell envelope protein patterns of 78 strains of *Acinetobacter calcoaceticus*, mainly isolated in hospitals, by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). They found the patterns to be stable and reproducible. The protein profiles made possible differentiation between two groups of strains. The patterns of the first group could be classified on the basis of concordance. The second group consisted of strains with unique patterns, which could not be classified. They concluded that the comparison of SDS-PAGE patterns appeared to be a suitable method for the relative classification of *A. calcoaceticus* strains of nosocomial origin (Dijkshoorn, Michel et al., 1987).

Alexander in 1988 studied 44 isolates of *Acinetobacter calcoaceticus var anitratus* collected during hospital outbreaks using polyacrylamide gel electrophoresis (PAGE), plasmid analysis, antibiograms and biochemical tests to determine their degree of similarity. They found that all methods were able to subdivide the isolates, but results did not always correlate well between methods. Reproducibility data indicated that careful attention to technique is required when organisms are examined by PAGE sequentially. They concluded that no single biotyping technique is likely to be adequate and that electrophoretic, biochemical and antibiogram data may complement one another and other epidemiological data in the typing of these organisms (Alexander, Rahman et al., 1988).

Bouvet in 1990 determined species, biotypes, and phage types for 120 *Acinetobacter* strains. These characteristics were compared with cell envelope protein profiles obtained by sodium dodecyl sulfate-polyacrylamide gel electrophoresis in previous studies. A considerable heterogeneity of species and types was observed by use of the various methods. *Acinetobacter baumannii* was the most commonly found species in isolates from clinical sources, followed by *Acinetobacter* species 3. Nine biotypes were observed among *A. baumannii* strains. Further differentiation within most species and biotypes was achieved by protein profile typing and, to some extent, phage typing. Of the 120 strains, 49 (41%) were not typeable by phages. They determined that biotyping was an appropriate method for the screening of strains, whereas protein profile and phage typing could serve as additional methods to establish the identity or nonidentity of strains. They suggest that a combination of typing methods is useful in epidemiological studies (Bouvet, Jeanjean et al., 1990).

Dijkshoorn in 1993 typed 58 *Acinetobacter baumannii* isolates from 49 patients by cell envelope protein electrophoresis and by a quantitative carbon source growth assay. Most isolates were from respiratory tract specimens from intensive care patients, whose charts were reviewed to differentiate between colonisation and infection. Twelve protein profiles were distinguished in the isolates. Forty-two isolates were of the same protein profile (profile I); other profiles were observed in a few or single isolates. Cluster analysis of carbon source growth divided profile I

isolates into two groups - one of isolates from 1984 and one from 1985. They were associated with infections in eight patients. Four other infections were caused by acinetobacters with other protein profiles (three of *A baumannii*, one of the unnamed DNA group 3). They concluded that apart from sporadic strains, two strains of the same protein profile, but distinguishable by carbon source growth, were successively endemic, and that cluster analysis was a valuable tool in the interpretation of typing and epidemiological data (Dijkshoorn, van et al., 1993).

Thurm in 1993 examined 65 strains of *Acinetobacter baumannii* which had been isolated from patients and the indoor environment of a neonatal intensive care unit 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 clone, persisted in the neonatal ward over several months. The results underline the usefulness of electrophoretic typing in epidemiological investigations into the routes of transmission of nosocomial *A. baumannii* infections (Thurm and Ritter, 1993).

Sire in 1994 carried out an epidemiological survey over a one-week period to assess the spread of *Acinetobacter baumannii* in a medical intensive care unit. Fifty strains were isolated from patients colonised or infected by the organism and from the hospital environment. These strains belonged to biotypes 9 or 18. The rRNA gene restriction patterns (using EcoRI and PvuII as restriction endonucleases) and the esterase electrophoretic profiles were determined on 31 strains. Four EcoR I ribotypes, 4 Pvu II ribotypes and 6 esterase profiles were identified. All biotype 9 strains isolated presented the same ribotype after EcoR I digestion, the same ribotype after Pvu II digestion and the same zymotype. The same observation was made on most of the biotype 18 strains. They concluded that biotyping is an appropriate method for screening of strains, and ribotyping and esterase electrophoresis could be used as additional methods to delineate outbreaks of nosocomial infections caused by *A. baumannii* (Sire, Gras-Rouzet et al., 1994).

Vaneechoutte in 1995 studied two successive *Acinetobacter* outbreaks in a neonatal intensive care unit with arbitrarily primed polymerase chain reaction (AP-PCR), cell envelope protein electrophoresis (protein fingerprinting) and antibiotic susceptibility testing. AP-PCR fingerprinting and protein fingerprinting yielded identical clustering of the isolates studied. Susceptibility test results were useful for rapid recognition of the outbreaks, but clustering of several isolates was different from the clustering obtained with AP-PCR fingerprinting and protein fingerprinting. Typing results indicated that the two outbreaks were each caused by a single strain, and that both strains differed from the strains prevailing in the hospital. The strain of one outbreak was identified as *A. junii*, a species commonly not involved in outbreaks (Vaneechoutte, Elaichouni et al., 1995).

Horrevorts in 1995 conducted a prospective study of *Acinetobacter* isolates from a neonatal intensive care unit for 24 months. Fifty-six isolates were obtained from 21 patients, and another 8 were obtained from environmental specimens. Infection due to *Acinetobacter* was established for 16 patients, 6 with septicaemia, 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 hybridisation group 3, three were identified as *Acinetobacter baumannii*, one was identified as *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 (Horrevorts, Bergman et al., 1995).

2.6.7 Plasmid typing

Alexander in 1988 typed 44 isolates of *Acinetobacter calcoaceticus var anitratus* collected during hospital outbreaks using polyacrylamide gel electrophoresis (PAGE), plasmid analysis, antibiograms and biochemical tests to determine their

degree of similarity. All methods were able to subdivide the isolates, but results did not always correlate well between methods (Alexander, Rahman, et al., 1988).

Hartstein in 1990 typed 34 *Acinetobacter calcoaceticus subspecies anitratus* isolates by plasmid DNA analysis, two biotyping systems and antimicrobial susceptibility to 24 drugs. These isolates were obtained from mechanically ventilated patients in five intensive care units (ICUs) of one hospital over a 16-month period. They found that plasmid DNA fingerprints were distinct in 18 isolates (they differed from each other and all others), similar in two and identical or similar in ten. The latter group of isolates were recovered from patients in four ICUs. Reproducibility of biotyping was poor. Neither biotyping nor antimicrobial susceptibility was successful in identifying sameness among the group isolates nor differences among other isolates. They concluded that plasmid DNA fingerprinting should be used to assess the possibility of multiple patient transmissions of the same *A. anitratus* strain in the absence of an obvious outbreak (Hartstein, Morthland et al., 1990).

Patterson in 1991 conducted an epidemiologic investigation to identify reservoirs and modes of transmission during an outbreak of *A. anitratus* in their intensive care unit. Latex gloves were being used for universal precautions without routine changing of gloves between patients. Environmental sources culture-positive for *A. antitratus* included a small volume medication nebuliser and gloves in use for patient care. Plasmid typing showed that plasmid profiles of isolates from two symptomatic patients, two colonized patients, the nebulizer, and the gloves were identical. Other *A. anitratus* ICU isolates had distinct plasmid profiles. The need for changing gloves between patients was reinforced. They concluded that gloves used incorrectly for universal precautions may potentially transmit *A. anitratus* (Patterson, Vecchio et al., 1991).

Seifert in 1994 studied the epidemiological, microbiological, and clinical features of infections due to *Acinetobacter baumannii* in a complex endemic situation over an 18-month period and determined the clinical usefulness of plasmid DNA analysis of *A. baumannii* in epidemiological investigations. Antibiotic resistance

patterns, biotyping, and plasmid profile analysis were used to characterise clinical and environmental isolates. Pulsed-field gel electrophoresis (PFGE) of chromosomal DNA was performed to verify results obtained with the other typing methods. This was done in four different intensive care units of an 800-bed tertiary care centre. They found that 240 patients were colonised or infected with *A baumannii* during the study period. Five different epidemic strains were identified: one each was *A. baumannii* biotype 2 and 6, and three were biotype 9. *A baumannii* biotype 9 accounted for the vast majority of isolates (88%), which were clustered into three epidemic strains demonstrating distinct plasmid profiles. Two of these were considered genetically related as shown by PFGE. They concluded that *Acinetobacter* strains representing multiple biotypes and plasmid types were present in this endemic setting and that plasmid DNA analysis proved to be useful in epidemiological typing of *A. baumannii* strains and may serve as a complementary typing system to traditional epidemiological methods (Seifert, Boullion et al., 1994).

Seifert, again in 1994, studied a set of 103 epidemiologically well defined *Acinetobacter baumannii* isolates obtained from nine hospital outbreaks and 21 unrelated strains by pulsed-field gel electrophoresis (PFGE) of total genomic DNA digested with ApaI. Among outbreak strains, eight different patterns and five possible variants were identified by PFGE. Results were compared with those from plasmid profile analysis, antimicrobial susceptibility, and biotyping. Plasmid analysis revealed six different and two related patterns; one outbreak strain lacked plasmids. A total of 16 of the 21 unrelated strains harboured plasmids and exhibited unique patterns. Epidemiologically unrelated strains were placed into only two biotypes and had similar antimicrobial susceptibility patterns but were clearly distinguished by PFGE.They concluded that plasmid profile analysis may provide a cost-effective first step in epidemiological typing of *A. baumannii* isolates obtained from well-defined hospital outbreaks, but that PFGE yielded reproducible and easily readable results and showed excellent discriminatory power (Seifert, Schulze et al., 1994).

Seifert, also in 1994, studied the epidemiology and clinical significance of unnamed *Acinetobacter* species 3, *A. johnsonii*, *A. junii,* and *A. lwoffii.* Seventy-five clinical isolates of *Acinetobacter* species other than *A. baumannii* from 66 patients over a period of 12 months were analysed by plasmid DNA fingerprinting. Plasmids were found in 84.4% of *Acinetobacter* species 3 isolates and in all *A. johnsonii, A. junii*, and *A. lwoffii* isolates. Strains harboured 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, in contrast to the results obtained in studies of the hospital epidemiology of *A. baumannii* (Seifert, Schulze et al., 1994).

Garcia in 1996 used plasmid profiles to analyse 39 *Acinetobacter baumannii* isolates from 36 patients at 3 hospitals, which were previously classified by biotyping and rDNA fingerprinting. Ribotyping was useful to establish the lineage of isolates and to confirm genospecies identification. Thirty-seven isolates (94.9%) contained plasmids. The variable number of plasmids with different molecular weights in each isolate enabled the identification of 13 profiles without the need for endonuclease digestion. Fifteen isolates of similar ribotype and antibiotype contained identical plasmids over a two-month outbreak at one hospital. Plasmid typing discriminated these isolates from sporadic *A. baumannii* isolates of close ribotype obtained from different hospitals. A few isolates of different lineage, however, showed similar plasmid profile. Their results suggest that plasmid typing is a practical method a to assist infection control of nosocomial *A. baumannii.* A combination of plasmid typing and ribotyping is suggested to confirm genospecies classification and to identify strains against reference band profiles (Garcia, Nociari et al., 1996).

2.6.8 Ribotyping

Gerner-Smidt in 1992 used ribotyping to identify 70 strains in the *A. calcoaceticus-A. baumannii* complex with known DNA group affiliations by use of restriction enzymes EcoRI, ClaI, and SalI. A nonradioactive digoxigenin-11-dUTP-labeled *Escherichia coli* rRNA-derived probe was used. With any of the three restriction enzymes, banding patterns that were specific for each DNA group were seen. All 70 strains showed banding patterns that could identify them to the correct DNA group by use of any two of the three enzymes. In addition, banding patterns that could separate strains within any one DNA group were present. Their results also indicated the high discriminatory power of the system when used for epidemiological typing (Gerner-Smidt, 1992).

Dijkshoorn in 1993 used 4 methods, namely, biotyping, cell envelope protein electrophoresis, ribotyping, and comparison of antibiograms, for strain identification of *Acinetobacter* isolates from five outbreaks in hospitals. They found good agreement among the methods for the identification of an index strain, but biotyping and the comparison of antibiograms were the least discriminatory (Dijkshoorn, Aucken et al., 1993).

Vila in 1994 compared arbitrarily primed polymerase chain reaction (AP-PCR) and ribotyping in an investigation of an outbreak of *Acinetobacter baumannii* infections. Twenty-five clinical isolates shown previously by other criteria to belong to two different groups, and nine randomly selected *A. baumannii* clinical isolates from other hospitals were investigated. They observed nine different EcoR I rRNA gene restriction pattern fingerprints, which distinguished clearly between the two *A. baumannii* groups defined in the outbreak. Two of the nine strains selected randomly had the same ribotype as those strains involved in the outbreak, whereas the remaining seven strains each had a different ribotype. When the strains were tested by AP-PCR with M13 forward primer, 10 different profiles were obtained. They concluded that ribotyping and AP-PCR exhibited a similar discriminatory power, although AP-PCR had the additional advantages of speed and simplicity (Vila, Marcos et al., 1994).

Sire in 1994 carried out an epidemiological survey over a one-week period to assess the spread of *Acinetobacter baumannii* in a medical intensive care unit. Fifty strains were isolated from patients colonised or infected by the organism and from the hospital environment. These strains belonged to biotypes 9 or 18. The rRNA gene restriction patterns (using EcoR I and Pvu II as restriction endonucleases) and the esterase electrophoretic profiles were determined on 31 strains. Four EcoR I ribotypes, 4 Pvu II ribotypes and 6 esterase profiles were identified. They concluded that although biotyping is an appropriate method for screening of strains, ribotyping and esterase electrophoresis could be used as additional methods to delineate outbreaks of nosocomial infections caused by *A. baumannii* (Sire, Gras-Rouzet et al., 1994).

Aubert in 1995 isolated 42 strains of *Acinetobacter baumannii* from 15 patients hospitalised in a French intensive care unit. An epidemiological study based on the typing of these isolates was carried out using biotyping, antibiotyping, and ribotyping to recognise the transmission of multiresistant strains by transfer of a patient from one hospital to another. Fifteen strains from the outbreak (1 strain for each patient), five strains isolated before the outbreak, and five strains isolated in another hospital were included. The three methods gave a good correlation: the epidemic strains had the same antibiotic resistance pattern, the same biotype, and the same ribotypes obtained with three different endonucleases (Aubert, Grimont et al., 1995).

Crowe in 1995 described an outbreak in a Nottingham intensive therapy unit where 11 patients were infected with multi-resistant *Acinetobacter* strains and 26 patients were colonised. Multi-resistant strains were isolated most frequently from the respiratory tract, and eight patients had probable or suspected pneumonia caused by a multi-resistant *Acinetobacter spp.* Multi-resistant *Acinetobacter spp.* were isolated from various environmental sites in the unit, and patient and environmental isolates were found to be related closely by biotyping, antibiograms, pulsed-field gel electrophoresis of chromosomal fingerprints and ribotyping. The outbreak was controlled ultimately by transfer of infected or

colonised patients to an isolation cubicle, cohort nursing, emphasis on the importance of hand washing before and after patient contact and when handling case notes, and the use of disposable aprons and gowns during patient contact (Crowe, Towner et al., 1995).

Seifert in 1995 typed 73 isolates of the *Acinetobacter calcoaceticus-Acinetobacter baumannii* complex, including 26 isolates from 10 hospital outbreaks, by ribotyping with EcoR I and Cla I and by pulsed-field gel electrophoresis (PFGE) of genomic DNA after digestion with Apa I. Ribotyping with EcoRI distinguished 31 ribopatterns. Digestion with Cla I generated another eight ribotypes. PFGE, in contrast, identified 49 distinct patterns with seven variants. Both methods detected all outbreak-related isolates. By ribotyping, nine epidemiologically unrelated strains could not be differentiated from outbreak strains, in contrast to only one isolate not identified by PFGE. Thus, PFGE was more discriminating than ribotyping. However, ribotyping is known to generate banding patterns specific to each DNA group in the *A. calcoaceticus-A. baumannii* complex that may be used for taxonomic identification of the strains. PFGE was shown to lack this property. They concluded, therefore, that both methods are useful for strain differentiation in epidemiological studies of *Acinetobacter* isolates (Seifert and Gerner-Smidt, 1995).

Lyytikainen in 1995 studied 97 *Acinetobacter spp.* isolates from clinical samples as well as isolates from the environment and the hands of staff by antibiogram, plasmid profile and ribotyping, as they observed an increased number of tobramycin- and imipenem-resistant *Acinetobacter spp.* causing colonisation, wound infections, and bacteraemias in a burns and plastic surgery unit. They identified two dominant multi-resistant *A. baumannii* clones. There was a close correlation between the results obtained by plasmid profiling and ribotyping (Lyytikainen, Koljalg et al., 1995).

Ratto in 1995 used ribotype, biotype and resistance phenotype to characterise 37 *Acinetobacter baumannii-A. calcoaceticus* complex isolates responsible for nosocomial infections in Buenos Aires. Nineteen isolates were recovered from

endemic infections at 2 hospitals and 18 represented an intensive care unit outbreak that occurred in a third hospital. By ribotyping, isolates were classified into five different clones of *A. baumannii* biotype 2, 3 of *A. baumannii* biotype 9, and 3 of *Acinetobacter* genospecies 13. Combination of the three epidemiological markers permitted categorisation of 18 outbreak isolates into four probable strains, one of which was responsible for the outbreak. They concluded that combined analysis of biotypes, resistance phenotypes, and ribotypes was an accurate approach for epidemiologic investigation of *A. baumannii.* Furthermore, ribotyping discriminated *Acinetobacter* genospecies 13 isolates which were phenotypically difficult to type (Ratto, Sordelli et al., 1995).

Ling in 1996 compared 202 isolates of *Acinetobacter anitratus* from 126 patients in 36 wards of a university teaching hospital by ribotyping and restriction enzyme digest analysis (REA) of total DNA. Forty-six groups were defined by both techniques. Only two groups were endemic and circulating in the whole hospital while others were less common. Burns and intensive therapy units had the highest number of isolates and these were mainly of the two endemic groups while renal dialysis and neonatal units had isolates belonging to the less common groups. Of the 32 patients with multiple isolates, 17 were infected or colonised at different sites by two and up to four groups of *A. anitratus.* They concluded that both ribotyping and REA of total DNA are discriminatory methods for typing *A. anitratus*, however, the latter is a simpler and more rapid method and it can be used in a routine clinical laboratory (Ling, Wise et al., 1996).

Garcia-Arata in 1997 conducted an epidemiological survey of the *Acinetobacter* species isolates occurring in the intensive care unit of a Spanish teaching hospital during 1993 and 1994. Different laboratory methods were used to find out whether there was a genetic linkage. Using API 20NE biotyping, eight different types were found. Five different plasmid profile types were observed, although plasmids were only demonstrated in 40% of the isolates. Ribotyping with EcoR I, Sal I and Cla I enzymes revealed 10, 9, and 8 different patterns, respectively. In total, 15 different ribotypes were identified using these three enzymes. Twenty-one isolates belonged to exactly the same ribotype, and 13 were associated with

two highly related ribotypes. The ribotyping method produced 100% typeability and ribotypes were easy to compare; it also had taxonomic value. They concluded that ribotyping allowed the determination of the genetic linkage between *Acinetobacter* isolates recovered from their ICU patients (Garcia-Arata, Gerner-Smidt et al., 1997).

2.6.9 Restriction fragment length polymorphism of chromosomal DNA determined by pulsed-field gel electrophoresis (PFGE)

Allardet-Servent in 1989 used pulsed-field gel electrophoresis to investigate an outbreak of *Acinetobacter calcoaceticus* in a urologic department and bronchial colonisation of artificially ventilated patients by *Pseudomonas aeruginosa* in an intensive care unit. They found that the method allowed a clear distinction between epidemic and self-contaminating strains in these different epidemiological situations (Allardet-Servent, Bouziges et al., 1989).

Tankovic in 1994 typed isolates of imipenem-resistant *Acinetobacter baumannii* using three methods - antibiotyping, biotyping, and pulsed-field gel electrophoresis. During a 13-month period, 31 patients in two intensive care units were either colonised or infected by this strain. Typing by PFGE revealed that two distinct strains were involved in the first 9 cases of the outbreak and that one of these strains accounted for 21 of 22 cases in the second part of the outbreak. Environmental contamination of the units was recognised as an important reservoir of this epidemic strain (Tankovic, Legrand et al., 1994).

Seifert in 1994 obtained a set of 103 epidemiologically well-defined *Acinetobacter baumannii* isolates from nine hospital outbreaks and 21 unrelated strains, and characterised them by PFGE of total genomic DNA digested with Apa I. Among outbreak strains, eight different patterns and five possible variants were identified. The results were compared with those from traditional typing methods such as plasmid profile analysis, antimicrobial susceptibility, and biotyping. Plasmid analysis revealed six different and two related patterns; one outbreak strain lacked plasmids. Epidemiologically unrelated strains were placed into only two

biotypes and had similar antimicrobial susceptibility patterns but were clearly distinguished by PFGE. They concluded that PFGE of *A. baumannii* chromosomal DNA yields reproducible and easily readable results and shows excellent discriminatory power (Seifert, Schulze et al., 1994).

Marcos in 1994 recovered 114 isolates of *Acinetobacter baumannii* from urine samples of 57 patients in a Spinal Cord Unit during a period of 28 months. An unusual increase in the number of *A. baumannii* isolates was observed between February 1991 and January 1992. Six different typing methods (biotyping, antimicrobial susceptibility, whole cell and cell-envelope protein analysis, plasmid analysis and chromosomal DNA analysis by PFGE were used to study the isolates to establish any potential relationships among them. Chromosomal DNA analysis by digestion with Apa I and separation of the fragments by PFGE was concluded by the authors to be the most powerful tool to determine the relatedness of isolates. Their results suggested that the isolates from 1991 and 1992 may have originated from strains present in 1990 that subsequently acquired resistance to amikacin and tobramycin during the epidemic (Marcos, Abdalla et al., 1994).

Marcos in 1995 performed a comparative study of biotyping, antimicrobial susceptibility, whole-cell protein analysis, plasmid analysis, PFGE of chromosomal DNA and polymerase chain reaction with arbitrary primers of *Acinetobacter baumannii* isolates from three large hospitals to determine the best markers for epidemiological purposes. Ninety-two isolates were included. They found that biotyping, whole-cell protein and plasmid analysis were the least discriminatory methods, whereas antimicrobial susceptibility and polymerase chain reaction with arbitrary primers showed moderate discriminatory power. Typing based on PFGE of chromosomal DNA appeared to be the best discriminatory method. Furthermore, the addition of polymerase chain reaction with arbitrary primers or antimicrobial susceptibility to PFGE of chromosomal DNA did not further increase the discriminatory power (Marcos, Jimenez et al., 1995).

Seifert in 1995 typed 73 isolates of the *Acinetobacter calcoaceticus-Acinetobacter baumannii complex*, including 26 isolates from 10 hospital outbreaks, by ribotyping with EcoR I and Cla I and by PFGE of genomic DNA after digestion with Apa I. Ribotyping with EcoR1 distinguished 31 ribopatterns. Digestion with Cla I generated another eight ribotypes. PFGE, in contrast, identified 49 distinct patterns with seven variants. Both methods detected all outbreak-related isolates. By ribotyping, nine epidemiologically unrelated strains could not be differentiated from outbreak strains, in contrast to only one isolate not identified by PFGE. Thus, they concluded that PFGE was more discriminating than ribotyping (Seifert and Gerner-Smidt, 1995).

Traub in 1996 comparatively examined triplets of isolates representing 20 clusters of presumed nosocomial cross-infection due to *Acinetobacter baumannii* and genospecies 3 using serotyping and analysis of restriction fragments (Sma I and Apa I) of genomic DNA with the aid of PFGE. Two misleading results of serotyping were encountered. A strain of *A. baumannii* serovar 15 had infected 8 patients in a surgical intensive care unit, while a second, genotypically totally different strain of identical serovar had caused infection in one additional patient. With this exception, they found that the correlation between serotyping and analysis of macrorestriction profiles was excellent (Traub, Leonhard et al., 1996).

Sader in 1996 evaluated the spread of *Acinetobacter baumannii* strains among three hospitals in Sao Paulo, Brazil. A total of 46 isolates were typed by chromosomal DNA analysis with use of PFGE. Isolates with an identical PFGE pattern (pattern B) that were susceptible only to carbapenems, polymyxin B, and ampicillin/sulbactam were recovered in all three hospitals. In addition, isolates with PFGE pattern A that were susceptible only to polymyxin B and ampicillin/sulbactam were recovered in two hospitals. The results of their study strongly suggested the interhospital transmission of multiresistant epidemic strains of *A. baumannii* in Sao Paulo (Sader, Mendes et al., 1996).

2.6.10 Polymerase chain reaction (PCR)-based typing techniques.

In a study by Struelens in 1993 in a university hospital, four ventilated patients developed colonisation, followed by pneumonia in two patients, with *A. baumannii,* resistant to multiple antimicrobials, over a three-week period. Cultures of samples from respiratory equipment and ICU surfaces as well as from hands of personnel failed to yield *A. baumannii*, except for one sample of respiratory tubing. Antibiogram, biotype, chromosomal DNA macrorestriction profiles and PCR-mediated fingerprints of 31 *A. baumannii* isolates indicated that this outbreak was caused by two strains, one of which later spread to another hospital where it caused a second outbreak. Both strains were clearly discriminated from control strains from cases of sporadic infection. Transmission was controlled by implementing contact isolation precautions and routine sterilisation of ventilator tubing. The authors suggest that wider use of sensitive genotypic methods like DNA macrorestriction analysis and PCR-mediated fingerprinting for typing nosocomial pathogens should improve the detection of micro-epidemics, which are then amenable to early control (Struelens, Carlier et al., 1993).

Graser in 1993 applied a PCR technique to the fingerprinting of different strains of *Acinetobacter baumannii* from a cluster of patients infected or colonised with the organism. The DNA was subjected to PCR amplification by using the core sequence of the M13 phase as a single primer. The amplified products were separated by agarose gel electrophoresis and were detected by staining with ethidium bromide. In one intensive care unit, 45 of 49 outbreak isolates obtained from 12 patients showed the same PCR patterns, indicating the epidemiological relatedness of these strains. Four strains isolated from the same patient belonged to another genetic group, as revealed by a distinct amplification pattern. Another single subtype of *A. baumannii* was identified as the causative agent in patients during a second outbreak at a different intensive care unit in the same hospital. Seventeen isolates recovered from 10 immunocompromised patients had the same amplification patterns, which were distinct from all other PCR profiles. Five strains were obtained from two other hospitals; three isolates from one hospital had identical PCR patterns that, however, could be clearly distinguished from the

patterns of all other strains. The remaining two isolates displayed individual patterns. They concluded that PCR fingerprinting may provide a useful and particularly rapid identification technique for epidemiological investigations of nosocomial infections (Graser, Klare et al., 1993).

Reboli recovered 84 isolates of *A. baumannii* from 50 hospitalised patients during the 5-month period from April through August 1990. Biotyping, comparison of antibiograms, plasmid analysis, and DNA polymorphisms of 20 isolates from 20 different patients, determined by the use of repetitive element PCR with primers aimed at repetitive extragenic palindromic sequences and enterobacterial repetitive intergenic consensus sequences, were used to investigate this apparent outbreak. Biotyping, antibiograms, plasmid analysis, and enterobacterial repetitive intergenic consensus PCR were not useful epidemiologically. Repetitive element PCR-mediated DNA fingerprinting using repetitive extragenic palindromic primers was found to discriminate between epidemic and sporadic strains of *A. baumannii* and demonstrated four discrete clusters which were unique epidemiologically (Reboli, Houston et al., 1994).

Vila in 1994 compared arbitrarily primed polymerase chain reaction (AP-PCR) and ribotyping in an investigation of an outbreak of *Acinetobacter baumannii* infections. Twenty-five clinical isolates shown previously by other criteria to belong to two different groups, and nine randomly selected *A. baumannii* clinical isolates from other hospitals were investigated. Nine different EcoR I rRNA gene restriction pattern fingerprints were observed. When the strains were tested by AP-PCR with 0.25, 0.5 or 1 microM of M13 forward primer, 10 different profiles were obtained. However, 11 profiles were observed if two different primer concentrations (0.25 and 1 microM) were used. It was concluded that ribotyping and AP-PCR exhibited a similar discriminatory power, although AP-PCR had the additional advantages of speed and simplicity (Vila, Marcos et al., 1994).

Grundmann in 1995 evaluated a rapid method for genotyping *Acinetobacter baumannii* based on PCR-fingerprinting with fluorescent primers. Automated laser fluorescence analysis (ALFA) enabled on-line generation of high resolution DNA-

fingerprints during polyacrylamide gel electrophoresis of randomly amplified polymorphic DNA (RAPD) products. The results were in concordance with macro-restriction fragment patterns produced by pulsed-field gel electrophoresis (PFGE) of Apa I digests of chromosomal DNA. RAPD-ALFA was able to identify homologous strains suggestive of horizontal transmission in < 8 hours after colonies were obtained on solid media, whereas PFGE analysis took approximately 90 hours. They concluded that the speed and digitised data format makes RAPD-ALFA attractive for epidemiological screening of isolates (Grundmann, Schneider et al., 1995).

Vaneechoutte in 1995 studied 2 successive *Acinetobacter* outbreaks in a neonatal intensive care unit with arbitrarily primed polymerase chain reaction (AP-PCR), cell envelope protein electrophoresis (protein fingerprinting) and antibiotic susceptibility testing. AP-PCR fingerprinting and protein fingerprinting yielded identical clustering of the isolates studied. Susceptibility test results were useful for rapid recognition of the outbreaks, but clustering of several isolates was different from the clustering obtained with AP-PCR fingerprinting and protein fingerprinting. Typing results indicated that the two outbreaks, which occurred at a three-month interval, were each caused by a single strain, and that both strains differed from the strains prevailing in the hospital (Vaneechoutte, Elaichouni et al., 1995).

Repetitive extragenic palindromic (REP) elements have been identified in numerous bacteria and these genomic sequences provide useful targets for DNA amplification. A method for amplifying inter-REP DNA sequences, REP-multiple arbitrary amplicon profiling (REP-MAAP) was applied by Sheehan in 1995 to 29 strains of *Acinetobacter baumannii* from clinical samples. Amplified polymorphic DNA patterns were demonstrated for all isolates and those displaying identical REP-MAAP patterns were considered identical at the genetic level. In the spring of 1993, 10 intensive care unit patients had endotracheal colonisation with *A. baumannii* (five with REP-MAAP I and five with REP-MAAP II patterns). These findings suggested nosocomial transmission of organisms, which was terminated by standard infection control measures. No further *A. baumannii* were detected

until the winter of 1993 when isolates of different REP-MAAP groups emerged, suggesting that factors other than nosocomial transmission were implicated (Sheehan, Lynch et al., 1995).

Sheehan in 1996 isolated 13 *Acinetobacter baumannii* strains from intensive care patients. These were initially typed using the API-20 NE biotyping system and antibiogram analysis. Results obtained using these methods failed to convincingly characterise the organisms. They then utilised a modified PCR where purified chromosomal DNA was subjected to amplification using the M13 universal sequencing primer. Polymorphic DNA bands produced was visualised after agarose gel electrophoresis and ethidium bromide staining. Results demonstrated that 6 of the 13 clinical isolates represented one group and a second group of 2 isolates displayed identical fingerprint patterns. The remaining four organisms were all unique. They concluded that this genotype based method is rapid, simple and reproducible (Sheehan, Boissel et al., 1996).

Snelling in 1996 reported the development and optimisation of a rapid repetitive extragenic palindromic sequence-based PCR (REP-PCR) typing protocol for members of the *Acinetobacter calcoaceticus-A. baumannii* complex that uses boiled colonies and consensus primers aimed at repetitive extragenic palindromic sequences. A cluster of *Acinetobacter baumannii* isolates from five patients in the adult intensive therapy unit of their tertiary-care teaching hospital led to the development of the method. Four of the 5 patient isolates gave the same REP-PCR typing pattern as isolates of *A. baumannii* obtained from the temperature probe of a Bennett humidifier; the fifth isolate had a unique profile. Disinfection of the probe with 70% ethanol, as recommended by the manufacturer, proved ineffective, as *A. baumannii* with the same REP-PCR pattern was isolated from it 10 days after cleaning, necessitating a change in their decontamination procedure. Results obtained with REP-PCR were subsequently confirmed by ribotyping. To evaluate the discriminatory power of REP-PCR for typing members of the *A. calcoaceticus-A. baumannii* complex, compared with that of ribotyping, they applied both methods to a collection of 85 strains that included representatives of six DNA groups within the complex. Overall, REP-PCR typing

proved to be slightly more discriminatory than ribotyping. Their results indicate that REP-PCR typing using boiled colonies is a simple, rapid, and effective means of typing members of the *A. calcoaceticus-A. baumannii* complex (Snelling, Gerner-Smidt et al., 1996).

Vila in 1996 compared different PCR-based DNA fingerprinting techniques for typing 26 clinical isolates belonging to the *Acinetobacter calcoaceticus-A. baumannii* complex. Seven isolates belonged to a previously defined outbreak while 19 isolates were unrelated epidemiologically. The PCR-based DNA fingerprinting techniques used were: (i) repetitive extragenic palindromic (REP) PCR; (ii) enterobacterial repetitive intergenic consensus (ERIC) PCR; (iii) randomly amplified polymorphic DNA with M13 forward primer; (iv) restriction analysis of the amplified 16S rRNA gene (ARDRA-16S); and (v) restriction analysis of an amplified region containing the 16S-23S rRNA spacer region and part of the 23S rRNA gene (ARDRA 23S + spacer). The discrimination index for these techniques was: 0.99 for REP; 0.94 for ERIC; 0.87 for M13; 0.60 for ARDRA-16S digested with Hpa II and <0.50 for ARDRA 23S + spacer. They concluded that REP-PCR possessed high discriminatory power and reproducibility in comparison with the other PCR-based DNA fingerprinting techniques, and is a simple and rapid typing method for use in epidemiological studies of isolates belonging to the *A. calcoaceticus-A. baumannii* complex (Vila, Marcos et al., 1996).

Webster in 1996 compared the relationships between isolates suggested by a novel DNA typing method (RAPD-ALFA) that combines randomly amplified polymorphic DNA with automated on-line laser fluorescence analysis of DNA fragments with those suggested by four other computer-assisted typing strategies (biotyping, antibiogram typing, pulsed-field gel analysis of chromosomal fingerprints and arbitrarily-primed DNA amplification with three different primers) for 25 isolates of *Acinetobacter baumannii.* The results obtained by cluster analysis with two different software packages confirmed that the relationships suggested by RAPD-ALFA were essentially similar to those suggested by the other more laborious computer-assisted typing methods. The technique of RAPD-

ALFA appears to offer the possibility of routine on-line molecular identification and typing of isolates from particular hospital wards or units (e.g. intensive care units), and could, therefore, play a key role in the early recognition and prevention of outbreaks of infection (Webster, Towner et al., 1996).

The different typing methods for *Acinetobacter spp.* are summarised in Table 3 below.

Table 3: Summary of results of different typing methods for *Acinetobacter spp.* cited in the text

No	1st Author	Year	Isolates	Methods used	Results
1	Santos-Ferreirra	1984	62 *A. calcoaceticus*	Phage typing	20% untypeable
2	Andrews	1986	154 *A. anitratus* 22 *A. lwoffii*	Bacteriocin typing	Poor typeability
3	Bouvet	1987	343 *Acinetobacter spp.*	Developed biotyping scheme	Allowed recognition of 17 biotypes among 247 *A. baumannii* isolates
4	Dijkshoorn	1987	78 *A. calcoaceticus*	Cell envelope protein profile	Stable and reproducible
5	Alexander	1988	44 *A. anitratus*	PAGE Plasmid profiles Antibiogram Biochemical tests	Single technique has poor correlation.
6	Giamman-co	1989	64 *Acinetobacter spp.*	Antibiogram Biotyping Cell envelope protein profile Phage typing	Biotyping good. Phage typing and antibiogram better. Cell envelope protein profile - no diversity.

No	1st Author	Year	Isolates	Methods used	Results
7	Traub	1989	152 *A. baumannii*	Serotyping	Delineated several outbreaks
8	Allardet-Servent	1989	*A. calcoaceticus*	PFGE	Clear distinction between strains
9	Joly-Guillou	1990		Antibiogram Biotyping Phage typing	All 3 complementary. Antibiogram + 1 other necessary for epidemiology.
10	Bouvet	1990	120 *A. baumannii* *Acinetobacter* genospecies 3	Biotyping Phage typing Cell envelope protein profile	Biotyping suitable for screening of strains. Protein profile and phage typing better for epidemiology. 41% not typeable by phage typing.
11	Traub	1990	156 *Acinetobacter* genospecies 3	Serotyping	Found cross reactions with *A. baumannii*.
12	Hartstein	1990	34 *A. anitratus*	Antibiogram Biotyping Plasmid profiles	Antibiogram and biotyping not useful to identify similarity. Biotyping showed poor reproducibility. Plasmid profiles best method.
13	Patterson	1991	*A. anitratus*	Plasmid profiles	Gave distinct profiles
14	Gerner-Smidt	1992	70 *A. calcoaceticus-A.baumannii* complex	Ribotyping	High discriminatory power

No	1st Author	Year	Isolates	Methods used	Results
15	Struelens	1993	31 *A. baumannii*	Antibiogram Biotyping Chromosomal DNA restriction profile PCR-mediated fingerprints	Antibiogram and biotyping not useful to identify similarity. Chromosomal DNA restriction profiles and PCR-mediated fingerprints detected 2 outbreak strains.
16	Graser	1993	74 *A. baumannii*	PCR(M13 primer)	Useful and rapid technique
17	Dijkshoorn	1993	5 hospital outbreaks	Antibiogram Biotyping Cell envelope protein profile Ribotyping	Good agreement but antibiogram and biotyping least discriminatory.
18	Dijkshoorn	1993	58 *A. baumannii*	Cell envelope protein profile Quantitative carbon source growth assay	2 methods worked well in combination.
19	Thurm	1993	65 *A. baumannii*	Antibiogram Whole cell protein typing	Whole cell protein typing useful to delineate routes of transmission.
20	Tankovic	1994	31 patients *A. baumannii*	Antibiogram Biotyping PFGE	PFGE best

No	1st Author	Year	Isolates	Methods used	Results
21	Sire	1994	50 *A. baumannii*	Biotyping Ribotyping Esterase electrophoretic profiles	Biotyping suitable for screening of strains. Ribotyping and esterase electrophoretic profiles better to delineate outbreaks.
22	Reboli	1994	84 *A. baumannii*	Antibiogram Biotyping Plasmid profiles REP-PCR	REP-PCR best to discriminate between epidemic and sporadic strains. Other methods not useful.
23	Seifert	1994	75 *Acinetobacter spp.* other than *A. baumannii*	Plasmid profiles	Useful for typing these strains.
24	Vila	1994	34 *A. baumannii*	AP-PCR Ribotyping	Similar discriminatory power but AP-PCR has the advantages of speed and simplicity.
25	Markos	1994	114 *A. baumannii*	Cell envelope protein profile Whole cell protein typing	PFGE most powerful tool to determine relatedness of isolates.
26	Seifert	1994	240 *A. baumannii*	Antibiogram Biotyping Plasmid profiles PFGE	Plasmid profiles and PFGE were the best methods.

No	1st Author	Year	Isolates	Methods used	Results
27	Seifert	1994	124 *A. baumannii*	Antibiogram Biotyping Plasmid profiles PFGE	Plasmid profiles are a cost-effective first step in epidemiological typing. PFGE gives reproducible, easily readable results with excellent discriminatory power.
28	Ratto	1995	37 *A. calcoaceticus-A.baumannii* complex	Antibiogram Biotyping Ribotyping	Combined analysis accurate. Ribotyping best discrimination.
29	Aubert	1995	42 *A. baumannii*	Antibiogram Biotyping Ribotyping	Good correlation with all 3 methods.
30	Vannee-choutte	1995	2 outbreaks in a neonatal ICU	Antibiogram Cell envelope protein profile AP-PCR	Antibiogram not suitable on its own. Cell envelope protein profile and AP-PCR showed identical clustering.
31	Crowe	1995	37patients + environment isolates. *Acinetobacter spp.*	Antibiogram Biotyping Ribotyping PFGE	Ribotyping and PFGE best discrimination.
32	Seifert	1995	73 *A. calcoaceticus-A.baumannii* complex	Ribotyping PFGE	PFGE more discriminating but both methods did detect all outbreak isolates.

No	1st Author	Year	Isolates	Methods used	Results
33	Lyytikainen	1995	97 *Acinetobacter spp.*	Antibiogram Plasmid profiles Ribotyping	Close correlation between plasmid profiles and ribotyping.
34	Horrevorts	1995	38 *Acinetobacter spp.*	Antibiogram Biotyping Cell envelope protein profile	Similar results with all 3 methods.
35	Grundma-nn	1995		RAPD ALFA PCR PFGE	Results concordant but RAPD ALFA much quicker.
36	Sheehan	1995	29 *A.baumannii*	REP MAAP PCR	Suitable technique
37	Oliviera	1996	206 *A.baumannii*	Antibiogram Biotyping	High discriminatory power when used together.
38	Sader	1996	46 *A.baumannii*	PFGE	Detected inter-hospital transmission.
39	Sheehan	1996	13 *A.baumannii*	Antibiogram Biotyping PCR (M13 primer)	Characterisation with antibiogram and biotyping not convincing. PCR was good, rapid, simple and reproducible.
40	Snelling	1996	91 *A.baumannii*	REP-PCR Ribotyping	REP-PCR more discriminatory, also simple, rapid and effective.

No	1st Author	Year	Isolates	Methods used	Results
41	Vila	1996	26 *A. calcoaceticus-A.baumannii* complex	REP-PCR ERIC-PCR PCR (M13 primer) ARDRA-16S ARDRA-26S + spacer	REP-PCR had highest discriminatory power and reproducibility, also simple and rapid.
42	Webster	1996	25 *A.baumannii*	Antibiogram Biotyping RAPD ALFA PCR PFGE AP-PCR	RAPD ALFA PCR, PFGE and AP-PCR had similar results, but RAPD ALFA PCR could play a key role in early recognition and prevention of outbreaks.
43	Garcia	1996	39 *A.baumannii*	Biotyping Ribotyping Plasmid profiles	Plasmid profiles found to be a practical method to assist infection control. The authors suggest a combination of plasmid profiles and ribotyping to confirm.
44	Ling	1996	202 *A. anitratus*	Ribotyping REA of total DNA	Both were discriminatory, but REA of total DNA simpler and quicker.

No	1st Author	Year	Isolates	Methods used	Results
45	Traub	1996	Triplets from 20 clusters of nosocomial infection due to *A.baumannii* and *Acinetobacter* genospecies 3	Serotyping PFGE	Showed excellent correlation, except for 2 misleading results with serotyping.
46	Garcia-Arata	1997	*Acinetobacter spp.*	Biotyping Ribotyping Plasmid profiles	Ribotyping was most discriminatory.

MATERIALS AND METHODS

3.1 Outbreak Description and Resolution

The intensive care unit (ICU) of King Edward VIII Hospital is a fifteen-bed unit comprising mainly surgical and post trauma patients. It is a busy ward with a fairly rapid turnover of patients. On 22 January 1995, the first multidrug resistant *Acinetobacter spp.* strain was isolated from the patient on bed number one. This was therefore the index patient of the outbreak. On 8 February 1995, it was realised that four of the other patients in the ICU harboured a similar strain of the multiresistant *Acinetobacter spp.* All fifteen patients in the ward were subsequently screened for the organism. The screen comprised endotracheal aspirates, catheter urine samples, stool or rectal swabs, and any other specimens that may have proved useful (such as intraoperative specimens where appropriate). These specimens were plated onto MacConkey agar plates; Gram negative bacilli which were non lactose fermenting and resembled the typical colony morphology of *Acinetobacter spp.* were identified using the API 20E identification system, and routine laboratory susceptibility testing was performed on the isolates. Multiresistant strains of *Acinetobacter spp.* were detected in twelve of the fifteen patients in the ward. Swabs from the ward environment were also screened for the organism - these were taken from patients' lockers, urine collection jars, taps, wash basins, nasogastric tube suction water, endotracheal tube suction water, ventilator circuits, floors, walls, patients' beds and patients' baths. The organism was cultured from the patients' baths, the suction water and the urine collection jars.

Of the sixteen patients present in the ICU during the period of the outbreak, twelve patients were found to be colonised with a multiresistant *Acinetobacter* strain. Of these twelve colonised patients, eight were regarded, based on clinical evaluation, to have an infection with the organism that warranted antimicrobial therapy, and six of these eight patients subsequently died. The antimicrobial therapy used was intravenous tetracycline and amikacin. There was also one other death in the ward at the time of the outbreak, but this death was definitely

thought not to be due to the *Acinetobacter*. Measures to control the outbreak were implemented from the day following the realisation of the outbreak, i.e. 9 February 1995. These control measures were as follows:

a) Patients who were colonised with *Acinetobacter spp.* were cohorted together, and separated from those patients who were not colonised. These two groups of patients were separated by as much distance as the ward would allow, and were nursed by different teams of nurses as far as was possible.

b) Ward rounds were performed in reverse order as far as possible, i.e. rounds began with patients who were not colonised with the organism, and progressed towards the group of colonised patients, so as to avoid transmission of the organism to the noncolonised patients.

c) Hand disinfection was more strictly enforced. Staff members, which included doctors, nurses, general assistants, physiotherapists, ward clerks and cleaners, and all visitors to the ward were well warned of the risks of improper hand disinfection; and hand disinfectant (chlorhexidine and alcohol) was easily and freely available at all patients' bedsides and at any other strategic points in the ICU. Special infection control staff (the hospital infection control nurses, the ICU matron and the microbiologists) acted as "policemen" in order to ensure that all involved people obeyed strict hand disinfection both before and after touching patients.

d) Proper collection procedures for urine were instituted - it was observed that the nurses collected urine from all patients into one collection jar, then discarded the contents. These collection jars were the same ones that *Acinetobacter spp.* was cultured from as part of the ward surveillance. This improper collection technique was corrected; i.e. the urine was collected from one patient into one collection jar, and discarded immediately. Proper disinfection of these collection jars was

then instituted i.e. they were washed in hot water with soap or with Biocide D.

e) The baths used to wash patients, from whom *Acinetobacter spp.* was cultured during the ward surveillance screen, were washed and disinfected properly, i.e. using hot water and soap or Biocide D. These were then retested randomly for *Acinetobacter spp.*, which was not detected after the new cleaning procedure.

f) Patients' bedside lockers were also thoroughly cleaned out and disinfected, as they may be a source of *Acinetobacter spp.* The disinfectants used were Biocide D and hot soapy water.

g) The water used for endotracheal tube suctioning was taken from the same container for all patients being suctioned. This was the water that had cultured *Acinetobacter spp.* in the ward surveillance screen cultures. Asking the central sterilising department of the hospital to aliquot the water into smaller volumes that could be used for a single suctioning procedure only subsequently altered this practice. Thus, suctioning of endotracheal tubes was performed only with sterile water used for a single patient only.

h) The environment surrounding patients was thoroughly cleaned or disinfected as far as was possible while still operating the ward as an intensive care ward i.e. the floors, walls and equipment (including ventilators, beds, trolleys, cabinets, sinks, cupboards, monitors, etc.) were systematically cleaned or disinfected in order to create an *Acinetobacter* - free environment. Unfortunately, the hospital was unable to completely close down the ICU, as there was no place else to transfer the patients to.

i) Although the hospital could not close down the ICU completely, the decision was taken not to admit new patients into the unit until the

outbreak was properly controlled. It was also decided that patients who were colonised with the *Acinetobacter* while in the ICU would only be discharged from the ICU if they could be discharged to their homes, or if they could be transferred to a ward where they would not be able to transmit the *Acinetobacter* to any other patient in the hospital i.e. if they could go out to a ward with no other patients in it (in King Edward VIII Hospital, there are very few wards with such single bedded "side wards"; as a result it meant that most of these patients could only be discharged to their own homes).

j) When discharging colonised patients from the ICU to other wards in the hospital, they could only be discharged after removal of all invasive devices from their persons, i.e. they were only discharged after removal of intravenous lines, arterial lines, temperature probes, urinary catheters, nasogastric tubes, endotracheal tubes, epidural catheters, etc. This was done in order to ensure that everything possible was done not to transmit the outbreak organism to another ward in the hospital i.e. to try and contain the outbreak to the ICU only.

k) The application of aseptic techniques for the performance of ANY procedure in the unit took on a new meaning during this outbreak. The use of hand disinfectant and strict hand washing has been discussed earlier. However, proper disinfecting and the use of gloves for any other procedure being performed in the ICU was emphasised to all personnel, no matter how "small" or "insignificant" the procedure; this included the ICU doctors, nurses, physiotherapists, ward clerks, visiting doctors, pharmacists and patients' visitors.

l) The movement of staff between patients was restricted and controlled as far as was possible. Doctors were assigned to either *Acinetobacter*-colonised or noncolonised groups of patients and were strictly advised not to move between the two groups. Similarly, the nurses were also

assigned to a particular patient and were specifically advised not to go over to another patient's bed except in an emergency situation.

m) All staff was reminded daily and repeatedly about all the control measures mentioned in the above twelve points in an effort to obtain full co-operation in their execution. "Policing" was also considered to be a required activity; this meant that people were constantly being observed and assessed as to whether they were complying with the "law", and was performed by the infection control nurses, the ICU matron and the medical microbiologists.

The above thirteen measures seemed to be effective, as no further multidrug resistant *Acinetobacter* strains were isolated from the 20 February onwards.

The course of the outbreak may be illustrated in the 5 figures following.

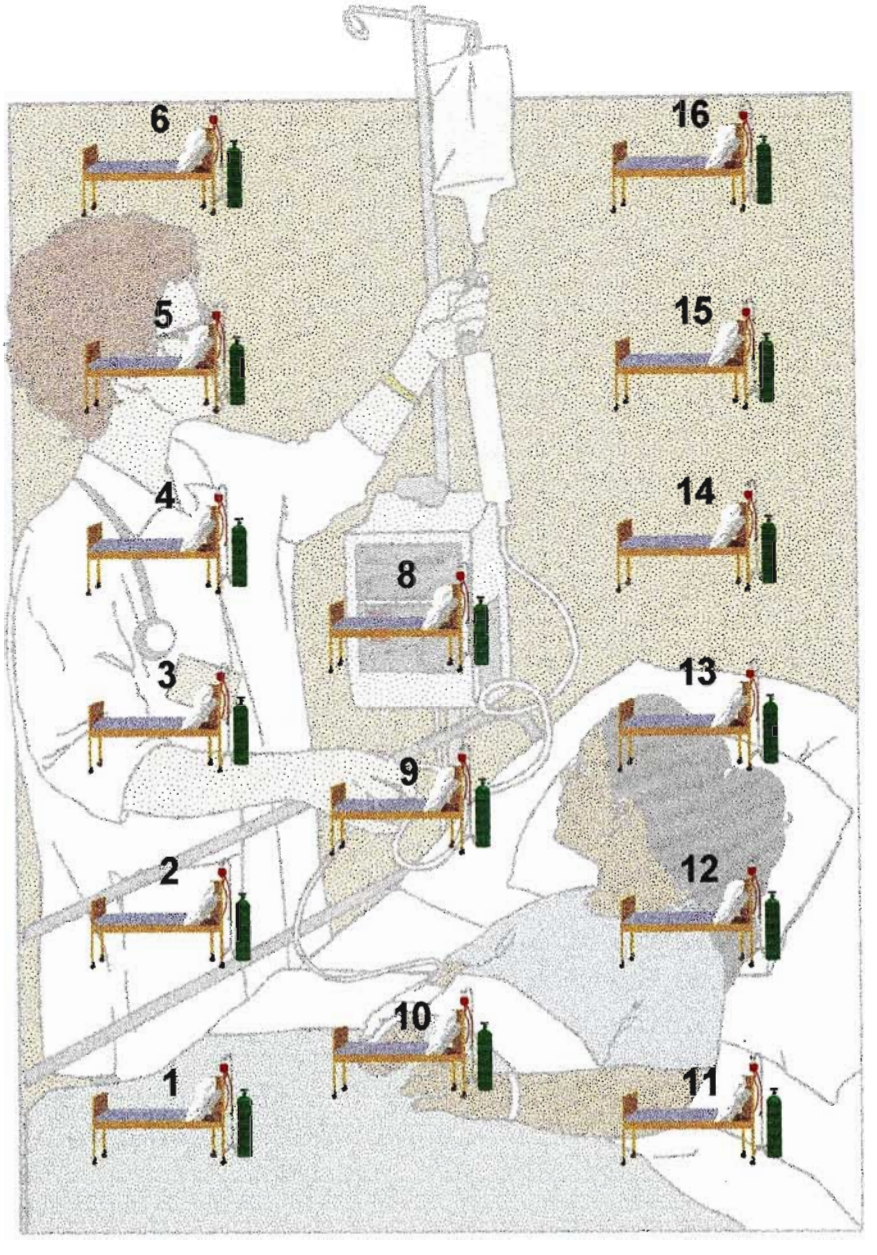


Figure 1: Schematic representation of the bed layout in the ICU at King Edward VIII Hospital (Bed 7 is not in use in the ICU)

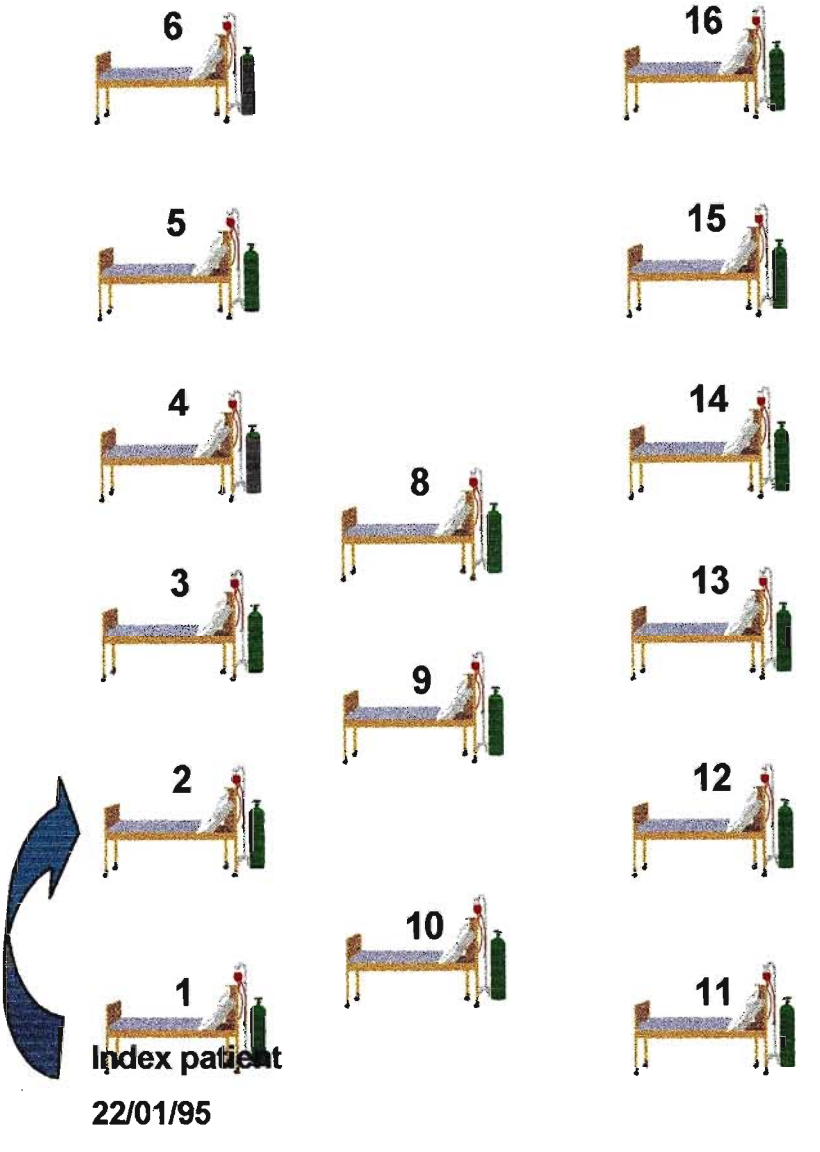


Figure 2: **Schematic representation showing the index patient and the direction of the first known transmission of multiresistant isolates**

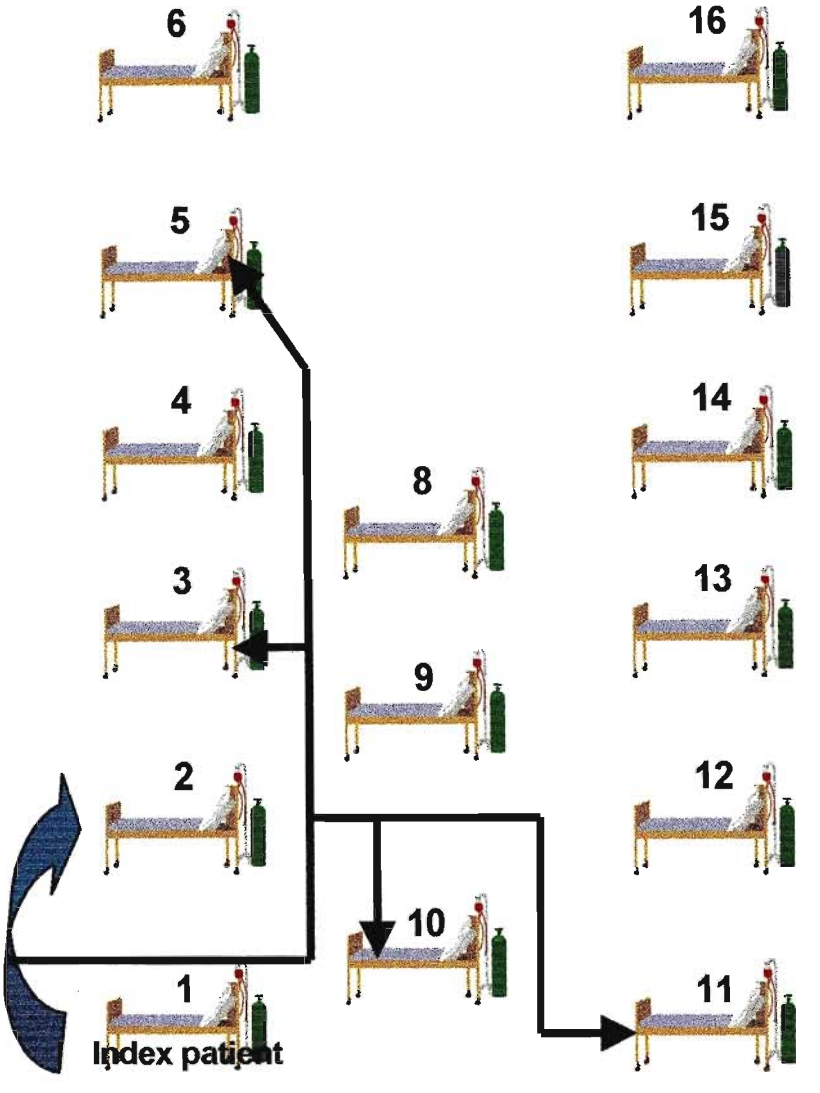


Figure 3: Schematic representation showing the simultaneously colonised patients at the time the outbreak was recognised

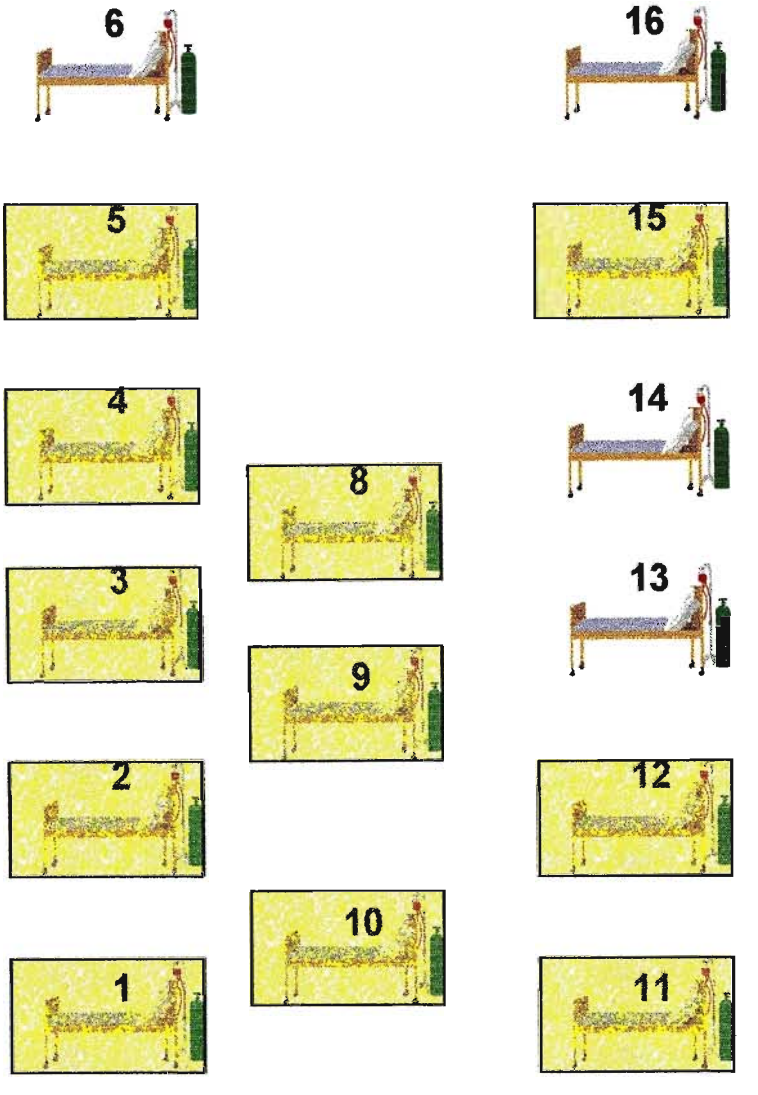


Figure 4: Schematic representation showing the 12 patients (shaded yellow) who were found to be colonised with multiresistant isolates on screening

(Two patients who were in bed 4 sequentially were colonised)

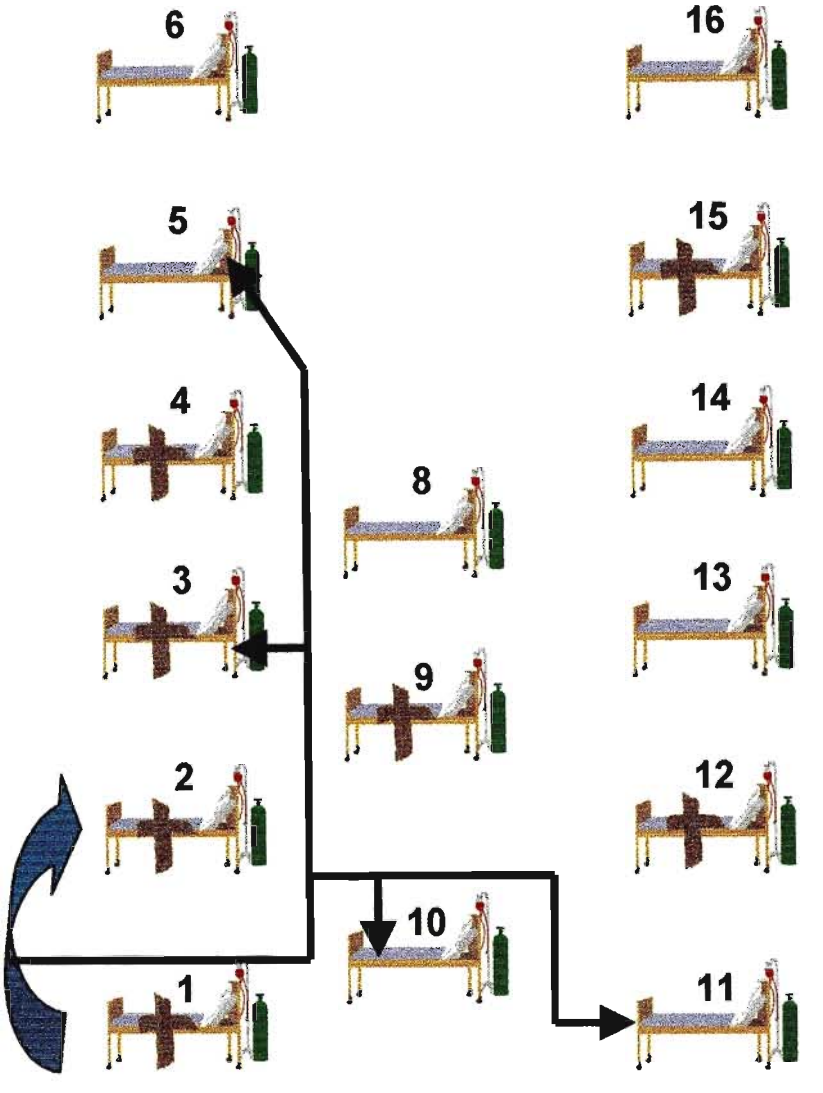


Figure 5: Schematic representation showing all the patients who died (✝) during the outbreak

(Two patients who were in bed 4 sequentially, died)

3.2 Determination of Minimum Inhibitory Concentrations of isolates

The plate method of the agar dilution procedure was performed.

The antimicrobials tested were:

- imipenem
- ciprofloxacin
- gentamicin
- amikacin
- netilmycin
- ceftazidime
- cefotaxime
- tetracycline

The control organisms used were:

- *Staphylococcus aureus* ATCC 2921
- *Eschericia coli* ATCC 25922

Twenty of the 47 outbreak strains of *Acinetobacter* were tested.

For detailed methodology refer to Appendices 1 and 2.

3.3 Ribotyping

A summary of the method used is presented below. For a more detailed method, see appendix 3.

Twenty isolates were obtained in patients from the intensive care unit of King Edward VIII Hospital in Durban. They were cultured on blood agar plates and incubated for 24 hours at 37^0C. The DNA was purified using 3 extraction steps: once with phenol, followed by sodium perchlorate extraction; twice with chloroform, precipitated with isopropanol; washed 5 times with 70% ethanol, and

air dried before being resuspended in TE buffer (10 mM TrisCl, 1mM EDTA (pH 8,0)). During the DNA extraction stages, vortexing was performed for 1 minute after addition of phenol, sodium perchlorate and chloroform. This markedly reduced the viscosity and allowed easier DNA quantification.

About 3ug of purified DNA was digested separately according to the manufacturer's (Boehringer Mannheim) instructions with the enzyme Pvu II. Restriction fragments were separated on a 1% agarose gel overnight at 1V/cm in Tris-borate-EDTA (TBE) buffer and then transferred onto nylon membranes by vacuum blotting.

16S and 23S rRNA from *Eschericia coli* served as a template for synthesis of first-strand cDNA with a first-strand cDNA kit (Boehringer Mannheim). cDNA and 1 kb-molecular weight DNA fragments were random prime labelled with digoxigenin-II-dUTP with a digoxigenin DNA labelling and detection kit (Boehringer Mannheim). The digoxigenin cDNA and 1 kb-molecular weight probes were denatured by boiling for 5 minutes and then rapidly cooled on ice for 5 minutes. Hybridisation and detection were performed according to the manufacturer's (Boehringer Mannheim) instructions with the following changes: after hybridisation, the second wash step was extended to 20 minutes and repeated, and removal of unbound antibody conjugate during detection was achieved by washing twice for 20 minutes each time.

The isolates were typed visually according to the ribotype pattern produced by the enzyme Pvu II.

RESULTS

4.1 Results of patient screening

The number of isolates of *Acinetobacter spp.* cultured from each site in the 12 patients is shown in Table 4. In total, there were 47 isolates, 23 were from endotracheal tube aspirates (ETT), 9 from urine specimens, 5 from arterial (A) line tips, 4 from central venous (CVP) line tips, 3 from blood cultures (B/C), 1 from an intraabdominal pus swab (P/S), 1 from a stool specimen and 1 from an intercostal (IC) drain swab.

Table 4: Number of isolates of *Acinetobacter* spp. cultured from each site in the 12 patients (Total number of isolates = 47)

Pt.	Bed No.	ETT	Urine	A line	CVP tip	B/C	P/S abdo.	Stool	IC drain
1	1	1	3	1	1	1			
2	2	2	2	1	1				
3	2	1							
4	3					1			
5	3	5	1				1		
6	4	1							
7	5	3		1	1				
8	9					1			
9	10	3	2	1				1	
10	11	5	1						1
11	12			1	1				
12	15	2							
Totals		**23**	**9**	**5**	**4**	**3**	**1**	**1**	**1**

Key

Pt. = patient
ETT = endotracheal tube aspirate
A line = arterial line
CVP = central venous pressure catheter
B/C = blood culture
P/S abdo. = abdominal pus swab
IC = intercostal

4.2 Results of minimum inhibitory concentrations (MICs)

Minimum inhibitory concentrations (MICs) were performed on all 20 isolates stored. Table 5 shows the results of MIC testing, and the interpretation of the values obtained. All isolates showed resistance to ciprofloxacin (MIC range 4-64 mg/l), gentamicin (MIC range 64-256 mg/l) and cefotaxime (MIC range 64->256 mg/l). Thirteen isolates showed resistance to imipenem (MIC range 16 -32 mg/l), while 7 were susceptible to this drug. Sixteen isolates showed resistance to amikacin (MIC range 64 -256 mg/l), while 4 were susceptible to this drug. All isolates were susceptible to netilmycin. Nineteen isolates showed resistance to ceftazidime (MIC range 32 -64 mg/l), while 1 was susceptible to this drug. Five isolates showed resistance to tetracycline (MIC >256 mg/l), while 15 were susceptible to this drug.

Table 5: The minimum inhibitory concentrations (MICs), and their interpretations, of the 20 *Acinetobacter spp*.

Spec	Imi		Cip		Gent		Ami		Net		Caz		Ctx		Tet	
708	1	S	4	R	64	R	64	R	2	S	64	R	>256	R	4	S
709	32	R	64	R	64	R	256	R	4	S	64	R	64	R	4	S
712	1	S	4	R	64	R	64	R	2	S	64	R	>256	R	4	S
714	32	R	64	R	64	R	256	R	4	S	64	R	64	R	4	S
770	16	R	64	R	128	R	128	R	4	S	64	R	64	R	4	S
784	1	S	8	R	64	R	128	R	1	S	64	R	>256	R	4	S
785	16	R	64	R	64	R	128	R	8	S	64	R	64	R	4	S
786	4	S	16	R	64	R	16	S	8	S	32	R	64	R	>256	R
798	32	R	64	R	64	R	128	R	4	S	64	R	64	R	4	S
800	32	R	64	R	64	R	128	R	4	S	32	R	>256	R	4	S
802	2	S	64	R	64	R	1	S	4	S	32	R	>256	R	4	S
803	16	R	64	R	128	R	128	R	4	S	64	R	>256	R	4	S
804	16	R	64	R	64	R	16	S	4	S	32	R	>256	R	>256	R
805	16	R	64	R	64	R	1	S	2	S	8	S	64	R	>256	R
926	4	S	4	R	64	R	64	R	8	S	32	R	>256	R	>256	R
931	32	R	4	R	64	R	64	R	2	S	64	R	>256	R	4	S
932	32	R	64	R	64	R	256	R	4	S	32	R	>256	R	4	S
935	32	R	64	R	64	R	64	R	2	S	64	R	>256	R	4	S
936	32	R	64	R	256	R	256	R	4	S	64	R	>256	R	4	S
944	4	S	4	R	64	R	64	R	8	S	32	R	64	R	>256	R

Key

Spec = specimen number	
Imi = imipenem	Tet = tetracycline
Caz = ceftazidime	S = susceptible
Ctx = cefotaxime	R = resistant
Cip = ciprofloxacin	Gent = gentamicin
Ami = amikacin	Net = netilmycin

Seven different antibiogram patterns were obtained using the MIC data. These are shown in table 6 below. The majority of isolates (11) fit into the last type, and showed resistance to all drugs tested, except for susceptibility to tetracycline and netilmycin only.

Table 6: The 7 different antibiogram types found in the 20 isolates using the MICs

Antibiotype	No. of isolates and specimen no.	Susceptibility pattern
1	3 - 708, 712, 784	S to imi, net & tet
2	1 - 786	S to imi, net & ami
3	1 - 802	S to imi, net, ami & tet
4	1 - 804	S to ami & net only
5	1 - 805	S to ami, net & caz
6	2 – 926, 944	S to imi & net only
7	11 – rest of isolates	S to tet & net only

Key

imi = imipenem
ami = amikacin
net = netilmycin
caz = ceftazidime
tet = tetracycline
S = susceptible

4.3 Results of Ribotyping

The 20 isolates were typed using ribotyping. Table 7 shows the distribution of ribotype patterns and the specimen type of each pattern. There were 9 isolates of ribotype a, 2 of ribotype b, 3 of ribotype c, 5 of ribotype d and 1 of ribotype e. Figure 6 shows the photograph of the blot following ribotyping.

Table 7: Ribotype distribution of the 20 *Acinetobacter* spp typed

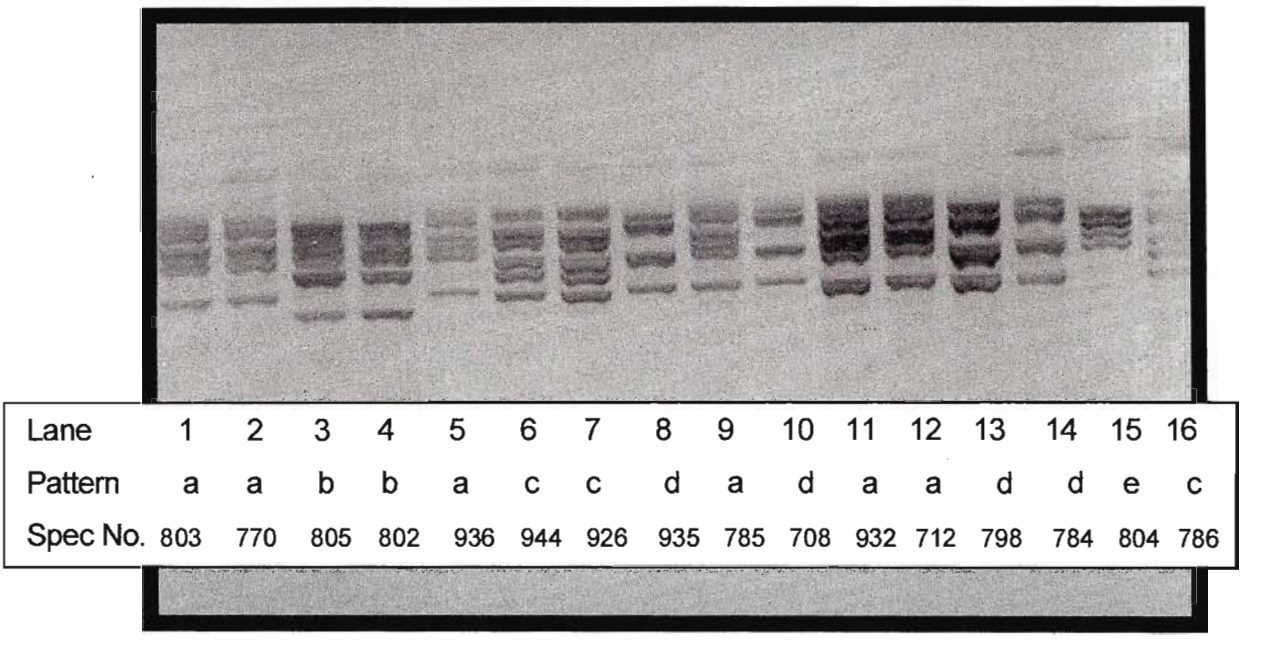
Specimen No.	Specimen type	Bed No.	Ribotype pattern
708	ETT	3	d
709	Urine	Unknown	a
712	CVP	5	a
714	B/C	9	a
770	ETT	12	a
784	ETT	4	d
785	ETT	2	a
786	Water dish	1	c
798	ETT	3	d
800	Urine	3	a
802	Enviro	-	b
803	Enviro	-	a
804	Enviro	-	e
805	Enviro	-	b
926	Urine	10	c
931	ETT	3	d
932	A line	5	a
935	ETT	10	d
936	ETT	5	a
944	Stool	10	c

Key

ETT = endotracheal tube aspirate
CVP = central venous pressure catheter
B/C = blood culture
A line = arterial line
Enviro = Environmental swab

Table 8: Summary of ribotype patterns obtained

Ribotype Pattern	No. of isolates
a	9
b	2
c	3
d	5
e	1



Lane	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Pattern	a	a	b	b	a	c	c	d	a	d	a	a	d	d	e	c
Spec No.	803	770	805	802	936	944	926	935	785	708	932	712	798	784	804	786

Figure 6: Scanned image of a photograph of the DNA blot showing a selection of the ribotype patterns obtained

DISCUSSION

An outbreak of infection is defined as a cluster of infections by the same organism originating from a common source, and is therefore caused by a single strain of that organism. Pseudo-outbreaks are clusters of infection caused by the same species, but originating from diverse sources. The number of strains therefore in a pseudo-outbreak may vary from one to many, depending on the number of sources of the causative organism.

Nosocomial outbreaks with *Acinetobacter spp* may be due to a single strain originating from a common environmental source, or may be single or multiple strain outbreaks caused by a breakdown in infection control procedures. The strains causing outbreaks may be endemic in wards for many months and recognised only by an increasing rate of patient colonisation.

Acinetobacter spp. have unique characteristics among nosocomial gram-negative bacteria that promote their persistence in the hospital environment. These organisms spread easily in the environment surrounding infected or colonised patients and can persist for many days, a factor that may explain their propensity for causing outbreaks of extended duration.

The variety of potential sources of contamination or infection with *Acinetobacter spp.* in the hospital environment makes control of outbreaks caused by these organisms one of the most difficult challenges in infection control. Outbreaks may result from intrinsic contamination of medical equipment (e.g. respiratory equipment, intravenous catheters, needles) used in patients for monitoring or therapy, or from contamination of the environment, either by the airborne route or by contact with patients (e.g. mattresses, pillows, air humidifiers). The emphasis of initial control measures should be on strict isolation of infected or colonised patients to limit dissemination of outbreak strains in the environment. In some instances, extensive measures, including closing of the unit for complete disinfection, is necessary. When exposure to a particular item of equipment is implicated, it is necessary to determine whether extrinsic contamination has

occurred during use in infected or colonised patients or whether intrinsic contamination (e.g. via ineffective sterilisation or contamination by staff carriers during handling) is the cause.

Outbreaks with no obvious common source have been controlled by either closing and disinfection of an affected unit, or by other measures directed at breaking transmission by contact or by the air-borne route, such as cohort nursing, strict attention to proper hand washing and disinfection after contact with patients, and improved disinfection of equipment and other utensils used on patients. Isolation and cohorting of patients and staff is often insufficient to control outbreaks. If substantial contamination is found in the vicinity of infected or colonised patients, housekeeping practices should be reviewed and reinforced.

Multiresistant *Acinetobacter spp.* are also selected out in the hospital environment in response to increasing antibiotic pressure. Control of antibiotic use is therefore also a very important part of preventive measures against the emergence of epidemic *Acinetobacter* infection.

In summary, *Acinetobacter spp* are increasingly important nosocomial pathogens that are capable of rapid adaptation to and persistence in the hospital environment. It is disturbing to know that these organisms will pose continuing problems in the future because of their ever-increasing antibiotic resistance. A combination of control measures is required to contain these organisms. Continual awareness of the need to maintain good housekeeping and control of the environment, including equipment decontamination, strict attention to hand-washing and isolation procedures, and control of antibiotic usage, appears to be the combination of measures most likely to control the previously unabated spread of *Acinetobacter spp* in hospitals.

Of the 47 *Acinetobacter spp.* strains isolated from the patients in the ICU at the time of the outbreak described in this thesis, and the numerous environmental specimens taken, only 20 strains were stored and later recovered. This was an unfortunate circumstance, as less than half of the isolates were subsequently

available for strain typing. This could have easily biased the results. It would have been ideal to be able to recover all isolates at the time of the outbreak. In one sense, this was not as big a bias as it would seem, as the strains were not related to any particular time period in the course of the outbreak; therefore although few strains were obtained, they were fairly evenly spread out over the course of the outbreak, and they were fairly representative of the various specimens and sites of recovery of isolates.

The situation we found in our ICU during this period certainly looks like an outbreak, but the number of strains tested in detail were actually too few to confirm that; but one conclusion that we can reach is that there certainly is transmission of *Acinetobacter spp.* in the ward. The question that requires clarification, but is not answered in this study is whether this saw the introduction of a new organism to the ICU or was it an increase in transmission of an already endemic strain? To answer this question, we would require isolates from patients prior to the onset of this particular outbreak, and strains of organisms isolated after the end of the outbreak. It is possible to obtain the latter strains, but the former strains were unfortunately not stored. Thus it is not possible to determine whether these were newly acquired strains or strains of endemic origin.

Antibiogram typing has been shown in many studies not to be useful for proper strain differentiation. It often does not correlate with results of molecular typing methods, and is not sufficiently discriminatory to delineate outbreak-related isolates from endemic strains. This was shown in this particular study also. In fact, use of the minimum inhibitory concentrations, as was performed in this study, was as non-discriminatory as routine laboratory disc diffusion testing, especially in strains that are as multidrug resistant as these *Acinetobacter* isolates were, as the more resistant the organism, the less discriminative antibiogram typing is. Also, the *Acinetobacter* has the capacity to become resistant and possibly to revert back to in vitro susceptibility, making antibiogram typing an even more unreliable tool for typing.

In this study, there were 7 different antibiogram patterns obtained, with the majority (11 of 20) of strains belonging to one antibiogram type, and the rest of the patterns including not more than 3 isolates each. This is totally different from the ribotype distribution of the same strains. This was probably due to the fact that the strains were as resistant as they were, allowing for little discrimination between types. However, this method is unfortunately the only initial tool available to us to detect the possibility of an outbreak at a relatively early stage in its course. For an organism that is not as multidrug resistant as these *Acinetobacter spp.* were, examination of the antibiogram pattern would be a slightly more reliable screening technique than for this particular multidrug resistant organism. For *Acinetobacter spp,* a molecular typing technique is essential to delineate outbreaks.

Review of the literature on typing techniques for *Acinetobacter spp.* seems to suggest that, for a particular outbreak of a relatively short duration, the best technique to use is pulsed field gel electrophoresis. Ribotyping is also a highly discriminatory technique, but is not as easy to perform as pulsed field gel electrophoresis. In the beginning, we did attempt to use pulsed field gel electrophoresis, but due to lack of experience in the use of the equipment and equipment failure, we were forced to abandon that method. As ribotyping was already in use in our laboratory for typing of other organisms, application of the technique for this *Acinetobacter* outbreak seemed reasonable. In order to truly test the discriminatory power of ribotyping as performed in this particular outbreak, it would be a good idea in the future to test the same isolates using another molecular typing technique, and to compare the patterns obtained.

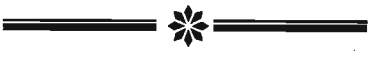
The outbreak described in this study was successfully aborted due to the implementation of strict infection control policies, which should be followed routinely in clinical practice. This is obviously not being done in our intensive care unit, hence the resultant outbreak. The enforcement of infection control guidelines is an unpleasant task, and results in the enforcer becoming unpopular with nursing and medical staff. It should be the responsibility of all members of staff to actively follow these guidelines without requiring reminders from an infection control enforcer, but human nature is such that people forget, and find these

routines time consuming and cumbersome to perform, especially when staff shortages are prevalent, and staff rush from one emergency situation to another. Therefore, continuous education of staff is an essential requirement to prevent such outbreaks from occurring. Strict enforcement of infection control rules with policing is generally only achievable in a once-off situation, but is, sadly, not sustainable on a continuous basis.

An interesting phenomenon occurred during the period of the outbreak. One of the patients in the ICU at the time of the outbreak was a close relative of one of the senior nurses in the unit. This patient was in the ward during the entire outbreak period, and beyond, but no *Acinetobacter* was isolated from this patient during his entire stay in the unit. This was probably due to the fact that, since he was a relative of a senior nurse, he was afforded special care. He was placed in bed 16, where the chance of cross infection was less, he was nursed exclusively by a dedicated team of nurses, and proper hand disinfection was practised. No one else was allowed to touch him or any of his equipment. Ward rounds were started at bed 16, after all members of the team washed their hands thoroughly first. He was also constantly monitored and "policed" by his nursing relative. This incident shows that with the proper care and nursing attitude, it is possible to prevent nosocomial infection and colonisation in any patient in the ICU.

In conclusion, we described a probable nosocomial pseudo-outbreak with a multidrug resistant *Acinetobacter spp.* in the intensive care unit of King Edward VIII Hospital. Twenty outbreak strains were typed using antibiogram typing and ribotyping. There was no correlation of isolates between the two typing techniques, and ribotyping was found to be the superior technique, with the majority of strains belonging to two different ribotypes, resulting in the conclusion that this was a pseudo-outbreak as opposed to a true outbreak. The source of the outbreak was not determined, and the outbreak was aborted by the use of strict infection control techniques without resorting to closure of the unit.

A recommendation for future work would be to confirm the strain differentiation found with ribotyping with another molecular typing technique, either pulsed field gel electrophoresis or a PCR-based method.



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Appendix 1: Method for performance of Minimum Inhibitory Concentrations (MIC's)

Day 1

1. 9mls of distilled water is placed in bottle 1
2. 5mls of distilled water is placed in bottles 2-10
3. 1ml of antibiotic solution is added to bottle 1
4. 5mls from bottle 1 is transferred to bottle 2
5. Double diluting is continued until the last bottle. The last 5mls from bottle 10 is discarded
6. The plates are poured using 5mls diluent + 20mls agar into a sterile petri dish, and dried
7. Two plates are incubated overnight in order to perform a sterility test

Day 2

1. The organisms are picked off in 5mls Mueller-Hinton broth and incubated for 2-5 hours to bring the organism to the lag phase of growth
2. The turbidity is adjusted to achieve an inoculum of 10^5 orgs/ml (i.e. use a 0.5 MacFarlands standard) so that the final inoculum on the plates is 10^4 orgs/ml (taking into account that the inoculating pin in the inoculum replicator dispenses 0.15ul onto the plates).
3. The surface of the plates is dried before inoculation
4. Using a replicator, the plates are inoculated with the organisms, including the 2 control strains
5. A growth control plate (i.e. without antibiotic) is inoculated first, followed by the antibiotic-containing plates, from the lowest to the highest concentration of antibiotic. A second control plate is inoculated last to ensure that no contamination has occurred
6. The inoculated plates stand at room temperature until their surfaces are dry i.e. the inoculum spots are absorbed into the agar
7. The plates are then incubated at 35^{o} C overnight

Day 3

The plates are read such that the MIC is the lowest concentration of antibiotic that completely inhibits growth of the organism, disregarding a single colony or a faint haze caused by the inoculum. It is first ensured that the control plates and control strains have worked out.

Appendix 2: Timetable for performing MIC's

Day 1

Equipment required:

- 350 McCartney bottles
- 80 Bijou bottles
- 5ml pipette tips
- 1ml pipette tips
- distilled water
- inoculation pins
- inoculation wells
- 350 Petrie plates

Day 2

- Autoclave equipment
- Prepare antibiotic stock solutions
- Label bottles
- Dispense distilled water into bottles
- Double dilute antibiotics
- Label plates

Day 3

- Prepare DST media
- Prepare nutrient broth
- Pour plates

Day 4

- Plate out organisms (and controls)
- Dry plates

Day 5

- Check purity of organisms
- Re-plate out organisms if necessary

Day 6

Prepare a 0.5 Mc Farland standard of the organisms (and controls)

Inoculate plates

Day 7

Read MIC's

Appendix 3: Ribotyping Method

1. Isolation of strains

Twenty isolates were obtained from patients in the intensive care unit of King Edward VIII hospital in Durban. They were cultured on blood agar plates and incubated for 2 hours at 37°C. The bacterial cells were then scraped off the agar surface and transferred to a sterile cryotube.

2. DNA Isolation

Chromosomal DNA was isolated by the method of Pillay et al (1996) with some modifications. Cells were suspended in 1ml 1XSSC, in an eppendorf tube, and centrifuged in a microcentrifuge at 12000rpm for 1min. The supernatant was discarded and replaced with 500μl STE buffer, the tube inverted a few times and then recentrifuged as above. This was done rapidly as STE buffer can lyse cells.

The supernatant was aspirated and the pellet resuspended in 500μl STE, 30μl SDS and 10μl RNAse and was incubated at 37°C for 30min. Thereafter 6μl proteinase K (20 mg/ml) was added to the tubes and incubated for a further 30min. The tubes were removed from the waterbath and allowed to cool to room temperature. 550μl phenol was added to the tubes, vortexed for 1min and centrifuged at 12000 pm for 15min.

The aqueous phase was transferred to a new eppendorf tube and sodium percholate (5M) was added to the reaction tube to obtain a final concentration of 1M. The contents were mixed by vortexing for 1min, and DNA was extracted twice with 600ul chloroform: isoamyl alcohol and centrifuged for 15min at 12000rpm

The aqueous phase was transferred to a new eppendorf tube and chromosomal DNA was precipitated with 750μl of anhydrous isopropanol at 4°C for 16hours to

increase the yield of DNA. The DNA was pelleted by centrifugation at 12 000rpm for 15min and washed 5 times in ice-cold 70% ethanol. Pellets were dried for 15min and resuspended in 80-100µl TE buffer at pH8.

3. Quantitation of DNA

DNA was electrophoresed on a 0.8% agarose gel and quantified visually.

4. Restriction analysis

The purified DNA was digested separately according to the manufacturer's (Boehringer Mannheim) instructions with the enzyme Pvu 11. The components were mixed well, centrifuged for 5 seconds before incubating in a waterbath for 16hrs at 37°C. Restriction fragments were separated on a 1% agarose gel for 20hrs at 25V in TBE buffer and then transferred onto a nylon membrane by vacuum blotting.

Restriction Analysis

Strains	DNA (µl)	Buffer (µl)	Enzyme (µl)	dH_2O (µl)	Total volume (µl)
1 714	12	2,0	1,6	4,4	20
2. 931	10	2,0	1,6	6,4	20
3. 800	9	2,0	1,6	7,4	20
4. 803	10	2,0	1,6	6,4	20
5. 770	10	2,0	1,6	6,4	20
6. 805	5,5	2,0	1,6	10,9	20
7. 802	5,0	2,0	1,6	11,4	20
8. 936	8	2,0	1,6	8,4	20
9. 944	6,5	2,0	1,6	9,9	20
10. 926	7,5	2,0	1,6	8,9	20
11. 935	13	2,0	1,6	3,4	20
12. 785	7	2,0	1,6	9,4	20
13. 708	7	2,0	1,6	9,4	20
14. 932	11	2,0	1,6	5,4	20
15. 712	6,5	2,0	1,6	9,9	20
16. 798	9,5	2,0	1,6	6,9	20
17. 784	10	2,0	1,6	6,4	20
18. 804	7,8	2,0	1,6	8,6	20
19. 786	11,5	2,0	1,6	4,9	20
20. 709	13,5	2,0	1,6	2,9	20

Master Mix = 44ul Buffer + 35,2ul Enzyme

= 79,2ul/22

= 3,6ul/tube

5. Transfer of DNA by vacuum blotting

A mask was cut out of parafilm so that it was 1-2mm shorter than the gel on all four sides. Hybond N+ nylon membrane and two pieces of Whatmann 3mm filter paper were cut to the same size as the gel. The nylon membrane and filter paper were soaked in sterile dH_2O for 10min.

The pre-wet filter paper was transferred onto the metal support screen of the vacuum apparatus and air bubbles were smoothed out by rolling a pasteur pipette over the filter paper. The pre-cut mask was centred over the Whatmann filter paper, and the nylon membrane was placed over the pre-cut mask. Care was taken to ensure that the nylon membrane was over the mask on all sides by at least 2mm and air bubbles were smoothed out using a pasteur pipette. The gel was carefully positioned over the hole in the mask and the wells were sealed off with molten agarose. The blotting apparatus was connected to a vacuum pump and set to 10.16cm (4 inches) Hg and left on for the entire transfer process.

Depurination solution was poured over the gel to cover the surface and left on for 10min. Thereafter, the excess depurination solution was removed using a pipette and transfer solution poured to about twice the depth of the gel. Transfer was allowed to take place for 45min and transfer solution was replenished often to prevent the gel from drying out. After the transfer was complete, the solution was aspirated from the gel, the vacuum turned off, and a pencil was used to mark the position of the wells on the nylon membrane to orientate it. The gel was carefully lifted off, stained with EtBr solution to confirm the efficiency of the transfer process, and discarded.

The blot was air-dried and the DNA was cross-linked to the nylon membrane by exposing it to UV light for approximately 8min. The blot was either used immediately for detection or stored in a sealed plastic bag at 4°C.

6. Preparation of complementary DNA (cDNA) probe

cDNA was prepared using the first strand cDNA synthesis kit (Boehringer Mannheim) 0,5ul of 16S and 23S rRNA (Boehringer Mannheim) was pipetted into a sterile Eppendorf tube containing 10µl sterile distilled water and heated for 5 min at 65°C to denature secondary structures in the rRNA sample. The tube was immeidately placed on ice for 5rnin.

The following were added to a microcentrifuge tube on ice:

- 4 µl Reaction buffer (10x)
- 8 µl $MgCl_2$
- 4 µl Deoxynucleotide mix
- 4 µl Random primer ($p[dN]_6$)
- 2 µl RNAse inhibitor
- 15,9µl sterile dH_2O
- 1.6µl AMV reverse transcriptase
- 0.5µl RNA sample (4µg/µl)
- Total volume: 40µl

The mixture was centrifuged briefly, incubated at 25^0C for 10min and then at 42°C for 60min. The first incubation enabled primer annealing and the second primer extension. After the reaction was complete, the AMV reverse transcriptase was denatured by incubating the reaction at 99°C in a water bath for 5 min. The reaction tube was either stored temporarily at 4°C or at -20°C for up to 2 days before proceeding with the purification procedure.

Purification of cDNA probe

TE buffer was added to 80µl cDNA reaction mixture to adjust the volume to 800µl. An equal volume of phenol:chloroform: isoamylalcohol (25:24:1) was added to the tube, vortexed for about 4s and centrifuged at 12 000rpm for 5min. The aqueous phase was carefully removed and cDNA fragments were precipitated with 0.1 vol.

NaAc (3M) and 2 volumes of prechilled 96% ethanol (EtOH). The tubes were inverted gently a few times and left to stand at -20°C for 2hr. This was followed by centrifugation at 12 000rpm for 15min to sediment the cDNA. The pellet was washed with cold 70% EtOH and resuspended in 15µl sterile distilled water.

7. Random primed labelling of cDNA probe

The purified cDNA was denatured by heating in a boiling waterbath for 5min and chilling quickly on ice. The reaction mixture consisted of the following:

15µl cDNA sample

2µl Hexanucleotide mixture

2µl dNTP labelling mixture

1µl Klenow enzyme

Total volume: 20µl

The contents of the tube were mixed gently and incubated at 37°C for 20hr. The reaction was terminated by adding 2µl EDTA solution and the labelled cDNA was precipitated with 2.5µl LiCl and 75µl pre-chilled 96% EtOH. Contents of the tube were mixed gently and left to stand at -20°C for 2hr to allow cDNA fragments to precipitate. The labelled cDNA was sedimented by centrifugation at 12 000rpm for 15min, washed twice with cold 70% EtOH (-20°C), air dried and resuspended in 50µl TE buffer.

8. Preparation of molecular weight DNA probe

The following were added to an Eppendfort tube on ice:

2µl DNA (1kb DNA ladder, 1 ug/ul)

2µl Hexanucleotide mixture

2µl dNTP mixture

The reaction was made up to a volume of 19µl with sterile distilled water and thereafter, 1µl of Klenow enzyme added. The procedure followed was the same as that for the cDNA probe labelling procedure.

9. Hybridisation and detection

Hybridisation was achieved with the DIG DNA labelling and detection kit. Blots were prehybridised in a plastic bag with at least 20ml of standard hybridisation buffer per 100 cm^2 of filter at 68°C for 3hr. A commercially available hybridisation solution (DIG Easy HYB) was tested and replaced the standard hybridisation buffer in subsequent hybridisations. Prehybridisation with DIG Easy HYB solution utilised the same volume as the standard buffer but prehybridisation was done at 42°C for 30min.

8.5µl cDNA and 1.9µl 1kb DNA probe were denatured by boiling in a water bath for 5min and then immediately cooling on ice for 5 min. The prehybridisation solution was replaced with 3.5ml per 100cm^2 filter of standard hybridisation or DIG Easy HYB solution containing the freshly denatured probes. The blot was incubated for 16hr at 68°C. Hybridisation was done in a hybridisation oven (Stuart Scientific) with the probe solution re-distributed once after 3hr.

After incubation, the excess probe was removed by washing the filter 2 x 5min in wash solution 1 (50ml/100cm^2 of filter) at room temperature, followed by 2 x 20min in wash solution 2 at 68°. The blots were either used immediately for detection of hybridisation DNA or air dried and stored in a sealed plastic bag at 4°C for later detection.

The blot was washed briefly for 3min in washing buffer and incubated for 30min in 100ml buffer 2. The anti-DIG-AP conjugate was diluted 1:5000 to 150 $mU.ml^{-1}$ in buffer 2. Blots were then incubated in 20ml of this solution for 30min and carefully transferred to a new container. Excess antibody conjugate was washed 2 X 20min with 100ml washing buffer and the blot was equilibrated for 5 min with 20ml

buffer 3. Thereafter, blots were incubated in a plastic bag, in the dark with 10ml colour solution. Washing the blot for 5min with 100ml Buffer 4 terminated the reaction. Ribotype patterns were photographed using FP4 black and white film.