REVIEW ARTICLE

Clinical importance and antibiotic resistance of *Acinetobacter* spp.

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Members of the genus *Acinetobacter*, particularly multiresistant strains of *A. baumannii*, are implicated in a wide spectrum of nosocomial infections, including bacteraemia, secondary meningitis and urinary tract infection, but have now assumed a particularly important role as agents of nosocomial pneumonia in intensive care units (ICUs). Rapid genotyping methods for the identification and typing of these organisms have allowed a better appreciation of the epidemiology and survival of these organisms in the hospital environment. Their emergence as significant pathogens seems to be related partly to their survival ability and partly to their ability to develop resistance rapidly to the major groups of antibiotics, resulting in a considerable selective advantage in environments (such as ICUs) with widespread and heavy use of antibiotics. Molecular and biochemical mechanisms of resistance to the major β -lactam, aminoglycoside and quinolone groups of antibiotics have now been elucidated in some detail for these organisms, and experimental models, including a mouse model of *A. baumannii* pneumonia, have been developed to examine the efficacy of different therapeutic regimens for difficult-to-treat infections caused by these bacteria. 'Non-classic' antibiotic combinations—such as ticarcillin with clavulanic acid and sulbactam—seem to show promise for treating systemic infections caused by otherwise multiresistant strains, but revised screening procedures in the pharmaceutical industry may be required in the near future to select novel compounds with activity against multiresistant *Acinetobacter* spp. and other emerging gram-negative, non-fermentative bacilli in general.

Introduction

Members of the genus *Acinetobacter* are ubiquitous free-living gram-negative saprophytic bacilli than can be obtained easily from soil, water, food and sewage with appropriate enrichment techniques [1]. In the field of biotechnology, the metabolic versatility of these organisms means that they are of value in several commercially important industrial processes, as well as in the degradation of a wide range of toxic environmental pollutants [1]. They are also ubiquitous organisms in the clinical environment, where they can be isolated readily as commensals from the skin of hospital staff and patients, and certain members of the genus are now recognised as important nosocomial pathogens that play a significant role in the colonisation

and infection of patients admitted to hospitals [2]. Unfortunately, the taxonomic confusion that has long surrounded these organisms makes it difficult to interpret the older medical and scientific literature and, even today, many reports of infection do not include the necessary tests for specific rather than presumptive identification of individual species [2]. Nevertheless, it is clear that *Acinetobacter* spp., particularly *A. baumannii*, are implicated in a wide spectrum of nosocomial infections, including bacteraemia, secondary meningitis and urinary tract infection [2], but their most important role seems to be as agents of nosocomial pneumonia, particularly ventilator-associated pneumonia in patients confined to hospital intensive care units (ICUs). Outbreaks of acinetobacter infections have often been shown by sensitive typing techniques to be associated with the spread of a particular 'local' strain within an ICU, and it seems that these bacteria have a significant capacity for long-term survival on human skin, equipment and inanimate materials in the hospital environment, with correspond-

ing enhanced opportunities for transmission between individual patients [2].

These difficulties are compounded by the widespread resistance of most hospital isolates to the major groups of antibiotics. Members of the genus seem to have a remarkable ability to develop resistance to even the most potent antimicrobial agents. Many isolates are resistant to broad-spectrum cephalosporins and other β -lactam agents, aminoglycosides, quinolones and, to a certain extent, the carbapenems, so that combination therapy is normally required for effective treatment of nosocomial acinetobacter infections [2]. In addition, the extensive and increasing use of broad-spectrum compounds in hospitals has served to eliminate competing bacteria and create a vacant ecological niche that has enhanced the ability of resistant acinetobacters to colonise and subsequently cause infection in susceptible patients.

The widespread and increasing interest in the genus *Acinetobacter*, shared by microbiologists of many different persuasions, has resulted in a series of interdisciplinary workshops and symposia devoted to various aspects of these organisms. The most recent meeting was held at Eilat, Israel, during November 1996, and included several sessions focused on the developing clinical significance of *Acinetobacter* spp. The following extended abstracts serve to bring together the key features of the clinical importance of *Acinetobacter* spp. and the resistance of these organisms to the major groups of antibiotics as they are understood at the present time.

ACINETOBACTER: OVERVIEW OF DEVELOPING CLINICAL IMPORTANCE

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Introduction

It was many years before clinical microbiologists realised that the aerobic gram-negative diplobacilli isolated originally only from rare cases of bacteraemia, pneumonia or urinary tract infection (UTI) were true pathogenic organisms. The identification of these oxidase-negative, non-motile diplobacilli was difficult, partly because the morphology of these coccobacilli often mimicked *Neisseria* spp. Their taxonomy was also confusing, with designations that included *Bacterium antiratum*, *Herellea vagincola*, *Micrococcus calcoaceticus*, etc. [2]. In France, these bacteria were classified by workers at the Institut Pasteur in a common group – named ‘*Moraxella*’ – that comprised oxidase-positive (*Moraxella* spp.) and oxidase-negative coccobacillary diploid organisms [3]. An earlier group

of French workers had conceived a genus named *Acinetobacter* [4], and it was proposed subsequently that the genus *Acinetobacter* should comprise the oxidase-negative strains [5, 6]. In the early 1970s, microbiologists in hospitals discovered progressively that these organisms were pathogenic and involved in various nosocomial infections, but many clinicians remained sceptical of the role of acinetobacters in infections. The first extensive data on acinetobacter infections in France appeared at meetings in the early 1980s, and infectious diseases specialists suddenly became aware of the role of *Acinetobacter* spp. in nosocomial infections, particularly in ICUs. Simultaneously, the importance of acinetobacter infections in other countries started to attract attention [7–9].

Major changes responsible for the emergence of acinetobacter infections between 1970 and 1990

Several factors have contributed to the emergence and recognition of acinetobacters as nosocomial pathogens in the period between 1970 and 1990.

Microbiological factors. Laboratory techniques evolved considerably during this period, with major changes and improvements in identification procedures and taxonomic methods, particularly those resulting from developments in molecular biology. Transformation has provided genetic evidence for the relatedness of all gram-negative, oxidase-negative, non-motile, aerobic coccobacilli; dispersed previously in numerous different genera, these organisms were all included in the genus *Acinetobacter* by the mid-1980s [6]. Subsequently, molecular techniques have allowed the subdivision of the genus into at least 19 different genomic species (L. Dijkshoorn, this review). Improved laboratory techniques have permitted the use of phenotypic characters for routine identification of acinetobacters in clinical microbiology laboratories. Several species of clinical importance have been identified, but *A. baumannii* appears to be the major species implicated in outbreaks of nosocomial infections [2]. Increased awareness of the pathogenic role of *Acinetobacter* spp. in hospital infections has been enhanced further by the development of various typing systems, based initially on phenotypic characters (biotyping, serotyping, phage typing, etc.) [2], but followed by genotyping systems that are now perceived as being more reproducible, stable, and reliable (L. Dijkshoorn, this review). These ‘molecular’ methods have been extremely valuable for epidemiological studies in hospitals of sporadic cases and outbreaks of acinetobacter infections [10, 11].

Host factors and patient's condition. Significant improvements in resuscitation techniques have occurred during the last 20 years; invasive procedures, sophisticated surgery and pulmonary ventilation have certainly contributed to save patients' lives. However, increased use of all these procedures, together with the

impairment of immune defences in patients of advanced age or chronic severe disease, has been responsible for the development and increased importance of nosocomial infections in ICUs [12–14].

Environmental factors. *Acinetobacter* spp. are ubiquitous organisms that are normally present in soil and water, and also as commensals on the skin of patients and staff in hospitals. They have been isolated from a wide range of materials (e.g., bed linen, sinks, ventilators, mattresses, etc.) [2]. Well-known risk factors have permitted their spread and persistence in ICUs, burn units, surgical wards and pulmonary units [15, 16]. Moreover, with the increased use of broad-spectrum antibiotics, including 'third-generation' cephalosporins, ureidopenicillins, aminoglycosides and fluoroquinolones, many *acinetobacters* have become resistant to the major groups of antibiotics [16, 17]. These organisms seem to develop antibiotic resistance very rapidly and have been selected by the widespread use of potent antibiotics in ICUs, resulting in the current situation where only a limited number of antibiotics (e.g., imipenem, ticarcillin combined with sulbactam, certain combinations of β -lactam agents with aminoglycosides) can be considered for control of *acinetobacter* infections.

Clinical importance of Acinetobacter spp.

Acinetobacters have been isolated from various types of opportunistic infections, including skin and wound sepsis, septicaemia, pneumonia, endocarditis, meningitis, and UTI [18]. The distribution by site of *acinetobacter* infections does not differ from that of other nosocomial gram-negative bacteria. The main sites of infection are the lower respiratory tract and the urinary tract (Table 1) [19, 20].

Respiratory infection: nosocomial pneumonia. Nosocomial respiratory tract infection with *Acinetobacter* spp. occurs frequently in hospitals, especially in ICUs [2, 15, 21–23]. Large outbreaks of *acinetobacter*-associated pneumonia have been described in which all patients had severe underlying disease requiring assisted ventilation and most had tracheostomies or were intubated. *Acinetobacters* represent 15.6% of the total gram-negative bacilli involved in nosocomial pneumonia in France.

UTI. *Acinetobacters* have been associated with 2–61% of nosocomial UTIs [2, 7, 18]. A recent investigation in France implicated *acinetobacters* in 30.5% of nosocomial UTIs [19]. Factors predisposing patients to this infection do not seem to differ from those for other nosocomial bacteria. Occasionally, *Acinetobacter* spp. may cause community-acquired UTIs in the absence of known predisposing factors.

Meningitis. Nosocomial meningitis is an infrequent manifestation of *acinetobacter* infection. The first case report appeared in 1908 (the responsible organism, then designated *Diplococcus mucosus*, was later renamed *Mima* because of its resemblance to the meningococcus following Gram's stain). Cases of meningitis caused by *acinetobacters* have been reported after neurosurgical procedures, but rare cases of primary meningitis, especially in children, have also occurred. *Acinetobacter* meningitis may result from the direct introduction of the organism into the central nervous system during intracranial surgery, but has also been associated with the presence of indwelling ventriculostomy tubes or cerebrospinal fluid (CSF) fistulae in patients receiving antimicrobial therapy [24].

Other miscellaneous infections. *Acinetobacter* skin and wound infections, abscesses, septicaemia, endocarditis, peritonitis and burn wound superinfections have all been described in the literature [18]. Their rates vary according to the hospital, the ward and the type of patients involved.

Sporadic cases of *acinetobacter* infections are seen in many hospitals, with periodic outbreaks in ICUs, in burn units, or in paediatric wards. Strains of multi-resistant *Acinetobacter* spp., particularly *A. baumannii*, can survive in the hospital environment and may persist for 10 days or more on dry surfaces. They also persist on the skin, in the throat and in the gut of colonised patients. All of these multiple sources may contribute to the periodic outbreaks of infection that are often observed [25].

The future of acinetobacter infection

As ubiquitous organisms (fortunately of low virulence), with few requirements for growth and survival, *Acinetobacter* spp. are prone to persist indefinitely in

Table 1. Distribution by site (percent of isolates) of *acinetobacter* infections in different countries and study periods

Type of specimen	USA [7] 1974–1977	France [19]		Belgium [11] 1990–1991	Germany [20] 1993
		1981	1991		
Urine	27.0	32.0	21.0	27.0	8.2
Tracheo-bronchial secretions	28.9	17.5	27.0	24.8	48.8
Pus or wound swab	21.5	34.0	27.5	22.3	16.4
Blood cultures	9.3	6.5	7.0	7.6	26.6
Catheters	0	7.0	15.5	0	0
Others*	13.3	3.0	2.0	18.3	0

*Includes specimens from the central nervous system, intra-abdominal area, burns, cardiovascular system, etc.

the hospital environment and to cause infections periodically when iatrogenic factors are present—i.e., overuse of broad spectrum antibiotics, high-risk patients and cross-infection. Are there ways to control or limit the spread of these multiresistant strains? Is it still possible to treat acinetobacter infections? First, it is necessary to improve microbiological techniques to allow earlier and more accurate identification in order to prevent inappropriate empirical treatment. Second, newer strategies for antibiotic use should be employed to reduce selection pressure, including more frequent rotation of antibiotic groups, or sequential use of antibiotic classes. The development of totally new antibiotics with novel bacterial molecular target sites may constitute therapeutic alternatives within the next few years. Nevertheless, continued surveillance of prevalent organisms in ICUs, combined with preventive measures (e.g., isolation precautions, hand disinfection, efficient sterilisation of instruments), remains absolutely essential in efforts to prevent or limit spread of acinetobacters and resulting infection.

SPECIES IDENTIFICATION, TYPING AND EPIDEMIOLOGY OF *ACINETOBACTER* SPP.

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Taxonomic history of the genus Acinetobacter

Acinetobacters were first described by Beijerinck in 1911 under the name '*Micrococcus calco-aceticus*' [26]. Other descriptions with different labels followed, but in the course of time it became clear that many of the gram-negative, non-motile, oxidase-negative coccobacillary organisms shared basic characteristics. An

extensive phenotypic study in 1968 of 106 strains by Baumann *et al.* [27] resulted in the recognition of only a single genus designated *Acinetobacter*. Clear-cut criteria for delineation of species were not found at that time, and a single species named *A. calcoaceticus* was included in the 1973 edition of Bergey's Manual of Determinative Bacteriology [28]. In 1986, a basic subdivision of the genus *Acinetobacter* was presented by Bouvet and Grimont [29] who identified 12 genomic species by DNA–DNA hybridisation. Descriptions of additional DNA hybridisation groups followed and, today, the genus is known to comprise at least 19 genomic species (Table 2) [30–33]. Seven DNA groups have species names, while the other groups are designated by numbers. Some groups were labelled independently with the same number, although the groups are apparently unrelated (Table 2). Apart from the described DNA groups, a number of strains yet to be classified were found [30], which suggests that the genus contains more genomic species than those described so far.

Identification of genomic species

The DNA–DNA hybridisation groups described by Bouvet and Grimont were accompanied by a comprehensive scheme of phenotypic tests [29]. Further investigation of this identification system with a collection of different strains showed that some groups were difficult to identify [34]. In particular, genomic species 1 (*A. calcoaceticus*) and 2 (*A. baumannii*), and the unnamed DNA groups 3 and 13 *sensu* Tjernberg and Ursing (TU), all of which have inter-group DNA–DNA homology values of 67–78% [31], could not be separated and it was proposed to combine these groups into one phenotypic group, the *A. calcoaceticus*-*A. baumannii* (Acb) complex. Another study with a

Table 2. Delineation of *Acinetobacter* genomic species (DNA groups) by different research groups

DNA relatedness group			Species name	Reference strain(s)
Bouvet <i>et al.</i> [29, 30]	Tjernberg and Ursing [31]	Nishimura <i>et al.</i> [32, 33]		
1	1	1	<i>A. calcoaceticus</i>	ATCC 23055 ^T
2	2	1	<i>A. baumannii</i>	CIP70.34 ^T = ATCC 19606 ^T
3	3	...	UN	CIP70.29 = ATCC 19004
UG	13	...	UN	CIP70.11 = ATCC 17903
4	4	4	<i>A. haemolyticus</i>	CIP64.3 ^T = ATCC 17906 ^T
5	5	...	<i>A. junii</i>	CIP64.5 ^T = ATCC 17908 ^T
6	6	4	UN	CIPA165 = ATCC 17979
7	7	3	<i>A. johnsonii</i>	CIP64.6 ^T = ATCC 17909 ^T
8	8	2	<i>A. lwoffii</i>	CIP64.10 ^T = ATCC 15309 ^T
9	8	...	UN	CIP70.31 = ATCC 9957
10	10	UG	UN	CIP70.12 = ATCC 17924
11	11	UG	UN	CIP63.46 = ATCC 11171
12	12	5	<i>A. radioresistens</i>	FO-1 ^T = IAM 13186 ^T
13	14	...	UN	CIP64.2 = ATCC 17905
14	...	...	UN	Bouvet 382
15	...	...	UN	Bouvet 240
16	UG	...	UN	ATCC 17988
17	...	...	UN	Bouvet 942
...	15	...	UN	Tjernberg 151a

^TType strain; UG, ungrouped strain; UN, unnamed DNA group (genomic species).

large number of phenotypic tests also showed that several groups were difficult to distinguish phenotypically [35]. Attempts to identify the genomic species by commercial phenotypic identification systems, including Biolog and API 20NE [36, 37], have indicated that the discriminatory capacity of these systems is not sufficient to provide separation within the Acb complex. Therefore, identification of species by such commercial systems can be considered as only presumptive. Unfortunately, the ambiguity in phenotypic species identification by these systems is frequently ignored and it is uncertain whether strains identified in some studies as (e.g.) *A. baumannii* are genuine representatives of this species.

Bouvet and Jeanjean [38] attempted to distinguish genomic species within the Acb complex by the use of multilocus enzyme electrophoresis. Electrophoretic polymorphisms of malate dehydrogenase, glutamate

dehydrogenase and catalase allowed discrimination of the environmental species *A. calcoaceticus sensu stricto* (DNA group 1) from the other DNA groups of the Acb complex that are found frequently in clinical specimens. A variety of genotypic methods for identification of acinetobacters to the genomic species level have also been explored [2, 39]. Of these methods, ribotyping has been found useful for differentiation of species belonging to the Acb complex [40]. Another method, AFLP, which is essentially a PCR-based high-resolution DNA fingerprinting method [41], was also very promising. Unpublished cluster analysis results with a large number of strains of all genomic species by this method, including those of the Acb complex, was in perfect agreement with grouping by DNA-DNA hybridisation (Fig. 1). Amplified ribosomal DNA restriction analysis (ARDRA), a simpler method with a classification scheme based on the restriction

Fig. 1. AFLP patterns of strains belonging to DNA group (DG) 1 (*A. calcoaceticus*), DG2 (*A. baumannii*) and the unnamed DGs 3 and 13TU. T, type strain; *, reference strain; C, clonal isolate from an outbreak; S, sporadic isolates. Figure courtesy of Dr P. Janssen (Ghent, Belgium).

patterns of 16S rDNA amplification products [42], correlated with most DNA groups, including those of the Acb complex. In another study, PCR analysis of tRNA spacer regions of 128 strains resulted in the identification of 17 groups [43], with strains of the Acb complex belonging to two clusters. Other promising methods include pattern analysis of PCR amplification products of the 16S–23S spacer regions [44], restriction analysis of *recA* gene amplification products [45], and direct sequencing of DNA gyrase genes [46]. However, given the heterogeneity within the genus *Acinetobacter*, the latter methods have to be validated with larger numbers of strains.

Typing of Acinetobacter strains

Typing of nosocomial pathogens to investigate their sources and mode of spread has undergone great changes as classic determinative methods are increasingly replaced by comparative typing methods. This trend is also apparent for strains of *Acinetobacter* [2, 39]. Traditional methods such as serotyping and phage typing have been found to be useful in some studies, but the necessary phages and antisera are not available widely and the typability was not always acceptable. A biotyping scheme based on the assimilation of five different carbon sources [47] has been applied in several studies as an adjunct method. Typing on the basis of a biochemical profile obtained by the use of a commercial identification system and antibiogram [48, 49] is common for primary screening of strain identity during outbreaks.

More sophisticated comparative methods for strain characterisation, such as plasmid typing, cell envelope protein typing, PCR-fingerprinting, pulsed-field gel electrophoresis (PFGE) and AFLP have all been applied to acinetobacters [2, 39]. Plasmid typing is a simple and cost-effective method for typing *Acinetobacter* spp., although some strains do not harbour plasmids, making them untypable by this method [50]. Cell envelope protein electrophoresis and ribotyping have been found to agree with the known epidemiology of isolates in several studies, but these methods are not applied on a wide scale. It is likely that PCR-fingerprinting and PFGE will be used more frequently, as these methods can also be used for other organisms. New developments in PCR-fingerprinting include the use of commercially available reagents, e.g., 'Ready-to-go' reagents (Pharmacia), and detection of subsequent PCR products by rapid automated laser fluorescence [49], both of which may help to overcome variations within and between laboratories. The banding patterns obtained by AFLP can be used for typing [51] as well as species identification. Fig. 1 shows strain patterns of the Acb complex, including sporadic and outbreak strains of *A. baumannii*. The reproducibility of AFLP is very high and a database of patterns for longitudinal comparison and strain identification can be constructed. However, successful

application of this method requires rigorous standardisation, which may limit the use of this technique to reference laboratories.

Rapid typing versus polyphasic characterisation in hospital epidemiology

There is currently no general agreement on a strategy for typing acinetobacters and, considering the rapid advances in DNA technology and automated data capture, such an agreement is unlikely, as new methods may emerge in the near future. In daily practice, rapid discrimination of epidemic and sporadic isolates can be made by methods such as biotyping with commercial systems, antibiogram typing or PCR-fingerprinting. In taxonomy, precise delineation of biologically relevant groups of organisms is being made increasingly on the basis of a combination of characters reflecting different levels of genetic expression. Such a polyphasic approach is also becoming current practice in hospital epidemiology for detailed characterisation of strains [48, 49], and application of a panel of genotypic and phenotypic methods showed recently that strains of *Acinetobacter* from outbreaks in Northwestern Europe were so similar that a clonal relatedness was assumed [52].

Conclusions

Establishment of the biology of the different genomic species of *Acinetobacter* is a challenging task, but has long been hampered by difficulties in correct identification of these groups. In particular, DNA groups 2, 3 and 13TU (which are frequently encountered in clinical specimens) and DNA group 1 (an environmental species) are difficult to distinguish by phenotypic tests. Practical genotypic methods for identification of the genomic species are presently being developed and may soon be applied in many laboratories. Various typing methods, including relatively simple methods like PCR-fingerprinting and sophisticated methods such as AFLP, have also become available. New issues in hospital epidemiology include the standardisation and quality control of methods, automated data acquisition and exchange of data by computer networks. Given the rapid advances in this field, it is expected that important questions on the epidemiology of *Acinetobacter* genomic species will be studied intensively in the near future.

OVERVIEW OF ANTIBIOTIC RESISTANCE PROBLEMS IN ACINETOBACTER SPP.

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Antibiotic resistance problems of *Acinetobacter* spp. in the period to 1990 were reviewed by Bergogne-Bérézin and Joly-Guillou [53]. A general trend to decreased

susceptibility of acinetobacters as a function of time was demonstrated, with strains of *A. baumannii* usually being more resistant than strains of *A. lwoffii*. More recently, a comprehensive review by Seifert *et al.* [20] correlated resistance to 16 different antibiotics relevant in therapy of acinetobacter infections with the precise species and clinical source of the isolates. It was demonstrated that: (i) susceptibility of acinetobacters varies according to species; (ii) *A. baumannii* is usually the most resistant species, with isolates resistant to all compounds except imipenem identified in the study; (iii) carbapenems are the most active compounds, with resistant isolates found in this study only amongst *Acinetobacter* genomic species 10 and a few ungrouped strains; and (iv) ampicillin and first- and second-generation cephalosporins are the least active compounds.

Fass *et al.* [54] compared resistance of *A. baumannii* to imipenem and ciprofloxacin within observation periods of 10 and 8 years, respectively. Only 2% of strains were resistant to imipenem in 1993, but ciprofloxacin resistance had increased from 4% in 1986 to 34% in 1993, indicating selection pressure in favour of quinolone-resistant *A. baumannii*. The incidence and level of resistance to quinolones in *A. baumannii* was also followed for an investigational period of 5 years in an ICU by Kljucar *et al.* [55]. Ciprofloxacin was used at various dosages for the treatment of secondary pneumonia (Table 3). There was a trend towards an increased incidence and level of resistance to quinolones within the observation period (Table 4) that indicated the effectiveness of

ciprofloxacin selection pressure in favour of quinolone-resistant strains of *A. baumannii*.

Acinetobacter is one of the few pathogens against which the β -lactamase inhibitor sulbactam has bactericidal activity [56]. In a pharmacodynamic model [57] it was possible to simulate human serum pharmacokinetics of sulbactam, alone and in combination with ampicillin, against cultures of *Acinetobacter*. Bactericidal activity was demonstrated with a thrice-daily dose of ampicillin 2 g plus sulbactam 1 g. A $\log_{10}2$ (99%) reduction in the cell counts of *A. baumannii* was achieved with an MIC of up to 0.5 mg/L for sulbactam alone, and 1 mg/L for ampicillin plus sulbactam (Fig. 2). Thus the combination of sulbactam with ampicillin presents a possible therapeutic alternative for acinetobacter infections.

Infections with *Acinetobacter* have shown a moderate increase in cystic fibrosis patients (50 of 480 patients, 10.4%) over an investigational period of 10 years. However, there is a remarkable difference between the various species of *Acinetobacter* in terms of their persistence in an individual patient. Thus persistence in more than three consecutive specimens was observed with *A. lwoffii*, *Acinetobacter* genomic species 3, *A. calcoaceticus* and *A. junii*, but not with *A. baumannii*, the species with the highest overall incidence (personal unpublished results). The colonisation of cystic fibrosis patients with *Acinetobacter* appears to correlate with the use of oral cephalosporins and macrolides.

In conclusion, review data from the literature and our own findings can be summarised as follows:

1. Isolates of *Acinetobacter* from infected patients may be resistant to any of the therapeutically relevant antibiotics.
2. The incidence of resistance to specific antibiotics varies according to the species and site of infection, and is influenced by selection pressure resulting from use of the preferred antibiotic.
3. In general, *A. baumannii* is the most frequent species of *Acinetobacter* causing infections and is also species in which resistance is most widespread.
4. Most acinetobacters remain susceptible to carbapenems, whereas the percentage of strains susceptible to quinolones has decreased at many locations.
5. Sulbactam used in combination with β -lactam agents enhances killing of *Acinetobacter*, as demonstrated by serum killing kinetics and in a pharmacodynamic model.
6. *Acinetobacter* spp. can be detected in c. 10% of cystic fibrosis patients.

Overall, infections caused by *Acinetobacter* spp. provide an impressive demonstration of the increasing importance of this genus as a human pathogen. This importance stems largely from the high potential of

Table 3. Consecutive antibiotic regimens used in an ICU for the treatment of secondary pneumonia

Time period	Number of patients	Antibiotic regimen
Oct. 1987–July 1988	46	Ciprofloxacin 2 $\times$ 400 mg or 2 $\times$ 600 mg
Dec. 1988–June 1989	22	Ciprofloxacin 1 $\times$ 200 mg or 2 $\times$ 200 mg
Sep. 1989–April 1991	71	Ciprofloxacin 2 $\times$ 200 mg or 2 $\times$ 400 mg

Table 4. Incidence and level of ciprofloxacin resistance in *A. baumannii* isolates from an ICU

Time period	Patients infected*/ total number of patients	(%)	Ciprofloxacin MIC ₉₀ (mg/L)
Oct. 1987–July 1988	16/46	(35)	0.5
Dec. 1988–June 1989	11/22	(50)	1
Sep. 1989–April 1991	45/71	(63)	4

*Patients infected with ciprofloxacin-resistant strains of *A. baumannii*.

Fig. 2. Bactericidal kinetics of sulbactam alone and combined with ampicillin against *A. baumannii* strain BK HH732 (MICs of ampicillin, 16 mg/L; sulbactam, 0.5 mg/L; ampicillin + sulbactam, 0.5 mg/L): □, control; △, ampicillin 2 g × 3 daily; ○, sulbactam 1 g × 3 daily; ●, ampicillin 2 g plus sulbactam 1 g × 3 daily.

this genus to develop antibiotic resistance, leading to a considerable selective advantage in environments with widespread and heavy use of antibiotics.

β-LACTAM RESISTANCE AND THE USE OF INHIBITOR COMBINATIONS

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Introduction

A. baumannii and related species have a predisposition for the acquisition of multiple antibiotic resistance rather than being inherently resistant. When these species were first implicated as human pathogens, many strains were susceptible to ampicillin and most were sensitive to the cephalosporins. In 1975, < 20% of these strains were resistant to ticarcillin [58]. However, these bacteria showed rapid acquisition of

resistance to second-generation cephalosporins in the 1970s and early 1980s, when these agents were being used extensively to control nosocomial infections. When the third-generation cephalosporins were introduced in the early 1980s, *A. baumannii* developed resistance to cefotaxime rapidly, but was much slower to develop resistance to ceftazidime [58]. However, it has been a characteristic of these bacteria that there is a progressive acquisition of resistance to β-lactam agents.

Mechanisms of resistance to β-lactam agents

Resistance to β-lactam agents could result from the production of a β-lactamase, alterations in the penicillin-binding proteins, or restriction of the passage of the β-lactam agent through the porins of the outer membrane [59]. β-Lactamase production is the easiest to quantify, and early reports of ampicillin resistance identified the ubiquitous plasmid-encoded TEM-1 and TEM-2 as the enzymes responsible [60]. However, these β-lactamases were not considered to be the sole mechanism of resistance when 76 ticarcillin-resistant

(MIC > 256 mg/L) strains of *A. calcoaceticus* were analysed for their β -lactamase content [61]. Less than 50% of the strains produced a TEM-like β -lactamase, but a few possessed an enzyme with a pI of 6.3, which was identified as the CARB-5 β -lactamase. All of these class A β -lactamases hydrolyse penicillins, but are ineffective against cephalosporins.

The rapid emergence of cephalosporin resistance suggested that other resistance mechanisms or β -lactamases must be responsible. The presence of chromosomal β -lactamases was first shown by isoelectric focusing (IEF) [62] when an enzyme with a pI around 8.6 was isolated from a strain of *A. lwoffii*. β -Lactamases with pIs ranging from 7.5 to > 10 were identified [63–65], but many of these enzymes focused poorly on conventional polyacrylamide IEF gels, and this difficulty in distinguishing β -lactamases by IEF left considerable uncertainty about the number of enzymes present in any particular strain. Morohoshi and Saito [66] concluded that the main β -lactamase activity in *Acinetobacter* spp. was a cephalosporinase, an enzyme of 30 kDa. Hikida *et al.* [65] probably examined the same enzyme and demonstrated that it was insensitive to clavulanic acid. On the basis of these observations, the β -lactamase examined by both groups would be classified as an Ambler class C enzyme.

Hood and Amyes [67] solubilised the β -lactamases with urea, Triton X-100 and β -mercaptoethanol, and separated the enzymes in agarose 1% IEF gels containing sorbitol, β -mercaptoethanol, Triton X-100 2.5% and 4 M urea. Four chromosomal β -lactamases were readily recognised in *Acinetobacter* spp. by these agarose, urea and sorbitol (AUS) IEF gels. They were designated *Acinetobacter* chromosomal enzymes (ACE) 1–4. The ACE-1 β -lactamase has two pI bands of almost equal intensity at 8.6 and 8.8. The ACE-2 β -lactamase has a distinctive single band with a pI of 8.7. The ACE-3 β -lactamase originated from a strain of *A. junii* and has two bands of almost equal

intensity with pIs of 8.1 and 8.2. Finally, ACE-4 came from a strain of *A. lwoffii* and also has two bands with pIs of 7.3 and 7.7. A similar diversity of β -lactamases has recently been reported by Perilli *et al.* [68], and these enzymes are all thought to be class C β -lactamases.

The properties of the ACE β -lactamases (Table 5) suggest that all four are cephalosporinases, although some possess a little activity against penicillins. None of the enzymes has detectable hydrolysing activity against aztreonam or the third-generation cephalosporins ceftazidime or cefotaxime. All the enzymes showed their maximum activity against cephaloridine and, except for ACE-4, good activity against cephradine. ACE-1 β -lactamases showed the broadest spectrum (Table 5). All four ACE β -lactamases described by Hood and Amyes [69] were highly susceptible to inhibition by cloxacillin as all of them were inhibited by 50% (ID₅₀) with < 0.2 μ M cloxacillin. They were all highly resistant to clavulanic acid, with ID₅₀ values of > 100 μ M (Table 5). There was little difference in the response of these enzymes to these conventional β -lactamase inhibitors. However, this was not the case when aztreonam was used. Although there had been no detectable hydrolysis of this β -lactam agent by any of the enzymes, ACE-1, ACE-2 and ACE-3 were all inhibited by it. ACE-3 was the most sensitive, but both ACE-1 and ACE-2 were inhibited by < 30 μ M. ACE-4 was virtually insensitive to the presence of aztreonam. All the original host strains had been aztreonam resistant and, although their β -lactamases did not hydrolyse the monobactam, three of them were able to bind it and this may partially account for their resistance.

The enzymes were not inducible, and this follows the views of others that the chromosomal β -lactamases of *Acinetobacter* spp. are non-inducible. The contribution of these chromosomal β -lactamases appears to be important in the expression of β -lactam resistance, but it seems likely that they are working in concert with a

Table 5. Properties of the *Acinetobacter* chromosomal β -lactamases

Property	<i>Acinetobacter</i> chromosomal β -lactamases			
	ACE-1	ACE-2	ACE-3	ACE-4
Percentage hydrolysis (nitrocephin = 100%)				
Nitrocephin	100	100	100	100
Penicillin	0.1	0.2	0.6	ND
Cephaloridine	7.9	3.6	1.6	1.8
Cephradine	1.3	0.7	0.3	ND
Cefuroxime	ND	ND	ND	ND
Cefotaxime	ND	ND	ND	ND
Ceftazidime	ND	ND	ND	ND
Aztreonam	ND	ND	ND	ND
Dose required for 50% inhibition – ID ₅₀ (μ M)				
Clavulanic acid	> 120	> 120	> 120	> 120
Aztreonam	8	23	0.08	> 120
Cloxacillin	0.005	0.022	0.003	0.18

ND, no activity could be detected.

reduction in permeability, with altered penicillin-binding proteins, or both, which may already confer some inherent resistance.

Carbapenem resistance

The final defence against multiresistant *Acinetobacter* spp. has been the carbapenems. However, carbapenem resistance has been emerging and, in some areas, causing significant problems for treatment [70]. It was first studied in detail in an imipenem-resistant strain of *A. baumannii* isolated from a blood culture in Edinburgh in 1985 [71]. This strain had an imipenem MIC of 16 mg/L, and this resistance was caused by a β -lactamase because separation of a cell-free extract by IEF revealed two bands: one with considerable activity at pI 6.65, and a second, weaker band focusing at higher pI. Purification of both β -lactamases demonstrated that the pI 6.65 enzyme was 23 kDa in size, whereas the high pI enzyme was 58 kDa and was identified finally as the ACE-2 cephalosporinase described by Hood and Amyes [69]. Enzyme kinetic data showed that the pI 6.65 enzyme had a high affinity for penicillins and cephaloridine, but that hydrolysis of these substrates was slow. No hydrolysis could be demonstrated by conventional spectrophotometric assays when imipenem, cefuroxime, cefotaxime and ceftazidime were used as substrates. Paton *et al.* [71] developed a plate assay that demonstrated the physical presence of the β -lactamase and provided a more sensitive technique for determining hydrolytic activity. This showed clearly that the pI 6.65 enzyme hydrolysed imipenem and it was designated ARI-1 (*Acinetobacter* Resistant to Imipenem). This enzyme was inhibited by clavulanic acid [72], with an ID₅₀ *c.* 200-fold greater than TEM-1, but not by pCMB or EDTA [71], suggesting that it was *not* a metallo- β -lactamase. Furthermore, when imipenem or meropenem was used as inhibitor rather than substrate, each inhibited the action of ARI-1; they were without effect on the TEM-1 β -lactamase, demonstrating that the carbapenems were bound much more strongly by ARI-1. The inhibitor BRL42715 was a powerful inhibitor of ARI-1, with an ID₅₀ of 1 nM, and these results suggested that ARI-1 is an unusual carbapenemase in that it is serine-based, not a metallo- β -lactamase, and probably a class A β -lactamase. This is very rare for carbapenem resistance and never found previously outside Japan. Recently, it has been demonstrated that the ARI-1 β -lactamase gene can transfer into *A. junii*. The *bla*_{ARI-1} gene is located on a plasmid of *c.* 45 kb, the first time that a gene of this class of carbapenem-hydrolysing β -lactamase has been shown to be transferable [73].

In the last year, it has been shown that similar resistance genes are associated with extensive carbapenem resistance in Buenos Aires hospitals [72]. In 11 strains of *A. baumannii*, isolated from related resistance clusters, seven different resistance phenotypes and five different biotypes were found. Imipe-

nem resistance in this part of Argentina does not appear to result from the clonal spread of a single strain. Brown *et al.* [72] examined these strains in detail and identified a second class A carbapenemase. This enzyme, designated ARI-2, is not inducible and has a pI different from ARI-1; however, it is also inhibited by clavulanic acid and BRL42715, although resistant to EDTA. The two carbapenemases, ARI-1 and ARI-2, cannot be identical, but they do seem to have closely related properties. It is very worrying that such similar resistance mechanisms can emerge on opposite sides of the world.

There has been one report of a metallo- β -lactamase in a strain of *A. calcoaceticus* from Cuba [74]. This strain produces a β -lactamase that hydrolyses imipenem rapidly when measured spectrophotometrically. This hydrolysis is inhibited by EDTA and it was concluded that the enzyme was a metallo- β -lactamase. These properties demonstrate that this β -lactamase is different from both ARI enzymes.

Treatment options

If *A. baumannii* has acquired resistance to cephalosporins, 4-quinolones and carbapenems, what are the available treatment options? Unexpectedly, these may include the β -lactamase-inhibitors. In-vitro studies with sulbactam, in particular, have shown that it is very effective in combination with conventional β -lactam antibiotics; however, many of these studies have been performed in France where the convention is to test β -lactamase-inhibitors at fixed concentrations (at 8 mg/L for sulbactam). Studies showed that 99% of isolates in an *A. baumannii* population were susceptible to the combination of cefepime and sulbactam (8 mg/L); however, 95% of the same population were *sensitive to sulbactam alone*, when tested at the same concentration [75]. Sulbactam is considered traditionally to be an adjunct to other β -lactam agents, potentiating their activity because of its preferential binding to class A β -lactamases. However, in the case of *Acinetobacter* spp., it has its own antibacterial properties as it binds to PBP2 and has some affinity to PBP1. Tazobactam has the same effect, but only at much higher concentrations, which are unlikely to be within the therapeutic range. Clavulanic acid seems to have no significant activity against *Acinetobacter* PBPs. The MIC₅₀ of *A. calcoaceticus* subsp. *anitratus* has been shown to be around 1 mg/L, with an MIC₉₀ around 4 mg/L (Fig. 3) [76]. These concentrations are achievable under most therapeutic conditions. Unlike most β -lactam antibiotics, sulbactam is not bactericidal at these concentrations and never demonstrates a rapid bactericidal effect, even at concentrations up to 50 mg/L [77]. In the treatment of nosocomial infections, a bactericidal antibiotic is to be preferred but, with the emergence of carbapenem resistance, it may not be possible to make a choice. Sulbactam is a viable antibiotic, on its own, as treatment for acinetobacter

Fig. 3. Cumulative inhibition percentages for 40 clinical isolates of *A. calcoaceticus* subsp. *anitratus* (data taken from Obana and Nishino [76]). ○ sulbactam; ▲ tazobactam; □ clavulanic acid; ● piperacillin.

infections; however, its use in combination with another β -lactam agent may be preferred to delay the emergence of resistance, for it is certain that resistance will eventually develop.

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MOLECULAR BASIS OF AMINOGLYCOSIDE RESISTANCE IN *ACINETOBACTER* SPP.

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Introduction

Despite the development of new β -lactam agents and fluoroquinolones, aminoglycosides are still used for treatment of infections caused by *Acinetobacter* spp. Aminoglycosides are bactericidal antibiotics that can display synergy when combined with β -lactams. Since the introduction of aminoglycosides into clinical use, resistance to these drugs has been reported. Bacteria can become resistant to aminoglycosides by three distinct mechanisms: (i) alteration of the ribosomal target; (ii) reduction of uptake; and (ii) enzymic modification of the drug.

The first mechanism is not significant in therapy as it affects only streptomycin and spectinomycin. The

second, which involves the proton electrochemical gradient, the respiratory chain and lipopolysaccharide changes, confers cross-resistance to all aminoglycosides and is common in *Acinetobacter*. However, it is the third mechanism that accounts for the majority of resistant *Acinetobacter* clinical isolates. There are three classes of aminoglycoside-modifying enzymes: *O*-phosphotransferases (APH) and *O*-nucleotidyltransferases (ANT), which catalyse phosphorylation and nucleotidylation of hydroxyl groups, respectively, and *N*-acetyltransferases (AAC) which catalyse acetylation of amino groups. In each class, the enzymes are designated by: the position of the carbon atom that carries the modified group (arabic number); the substrate range (roman number); the sequence of the corresponding gene. Subclasses are based on the site on the antibiotic molecule that is modified. Every enzyme has a substrate range that confers a resistance profile on the host bacterium. There is a relative specificity in the distribution of these enzymes among gram-positive and gram-negative bacteria. Most of the genes encoding these enzymes have been sequenced, which allows their detection by DNA-DNA hybridisation or by PCR. Aminoglycoside resistance by synthesis of modifying enzymes is mainly mediated by plasmids or transposons which play a role in the dissemination of the resistance.

Resistance phenotype

The resistance profile can be determined by testing a large number of aminoglycosides, including 5'-epiisomicin, 2'- and 6'-*N*-ethylnetilmicin, fortimicin and apramycin in addition to the aminoglycosides in clinical use. The resistance phenotype can allow the

putative enzyme content to be inferred. However, this analysis is often difficult in *Acinetobacter* because of the high prevalence of enzyme combinations (see below). The correlation between the aminoglycoside resistance phenotype and the modifying enzymes is summarised in Table 6.

Prevalence of aminoglycoside resistance in *acinetobacters*

Resistance to aminoglycosides is very common in clinical isolates of *Acinetobacter* and results from the ability of this organism to acquire resistance determinants. Two studies on aminoglycoside resistance in *Acinetobacter* were performed in France. The first was on 195 *Acinetobacter* isolates at the Henri Mondor hospital, Créteil [78]. The second comprised 322 *Acinetobacter* isolates from French ICUs in 1994 and was supervised by the Schering-Plough laboratory [79]. Resistance rates in the first study were: amikacin, 71%; gentamicin and kanamycin, 87%; netilmicin, 73% and tobramycin, 62%; and in the second study: amikacin, 66%; isepamicin, 62%; gentamicin, 74.5%; kanamycin, 83%; netilmicin, 60% and tobramycin, 65%. The study

Table 6. Correlation between the aminoglycoside resistance phenotype and the modifying enzyme content in *Acinetobacter*

Resistance phenotype	Compatible enzyme content
Gen	AAC(3)-I
Gen, Tob	ANT(2'') + [AAC(3)-I]*
Gen, Tob, Net	AAC(3)-II or AAC(3)-IV
Ami, Ise	APH(3')-VI
Ami, Gen, Ise	AAC(3)-I + APH(3')-VI
Ami, Gen, Ise, Tob	ANT(2'') + APH(3')-VI
Ami, Ise†, Tob, Net	AAC(6')-I
Ami, Gen, Ise†, Net, Tob	AAC(6')-I + AAC(3)-I or AAC(3)-IV or AAC(3)-II or ANT(2'')

Gen, gentamicin; Tob, tobramycin; Net, netilmicin; Ami, amikacin; Ise, isepamicin.

*An enzyme that could be present but remain undetected.

†The MIC of isepamicin could be altered slightly by the AAC(6')-I enzyme.

of aminoglycoside resistance mechanisms was performed by: (i) inference of the enzyme content from the resistance phenotype by disk diffusion with a large number of aminoglycosides, and (ii) analysis of the resistance genes by DNA-DNA hybridisation with specific probes [79]. The mechanisms found among 1189 aminoglycoside-resistant *Acinetobacter* isolates were complex; many isolates (74.5%) had multiple mechanisms. Four enzymes were very common: AAC(3)-I, AAC(6')-I, ANT(2'') and APH(3')-VI (Fig. 4).

Acquired aminoglycoside resistance in *acinetobacters*

APH(3')-I is common in *Acinetobacter*; however, this enzyme confers resistance to kanamycin only and is thus not important in therapy. APH(3')-VI confers resistance to kanamycin, amikacin and isepamicin and was found to be mediated by the 63-kb plasmid pIP1848 in *A. baumannii* strain BM2580 [80]. This plasmid is transferable by conjugation to other *Acinetobacter* spp., but not to *Escherichia coli*. Study of the distribution of amikacin resistance in *Acinetobacter* showed that phosphorylation is more widely spread than 6'-acetylation and more often plasmid-mediated than chromosomally mediated. Moreover, the *aph(3')-VIa* gene was flanked by direct copies of IS15Δ and was part of a Tn1528-like transposon [81]. Therefore, it appears that IS15Δ is likely to be responsible for dissemination of the *aph(3')-VIa* gene, which remains rare outside of the genus *Acinetobacter*.

AAC(3)-I, AAC(3)-II and ANT(2'') are common and plasmid-mediated in *Acinetobacter* (personal unpublished results). AAC(6')-Ib is of clinical significance because this enzyme affects amikacin and isepamicin and is known to be part of a class 1 integron [82]; such an integron has been found recently in *Acinetobacter*. AAC(6')-Ih is, to date, confined to *Acinetobacter* and is approximately twice as frequent as AAC(6')-Ib. The *aac(6')-Ih* gene was found to be carried by the 13.7-kb plasmid pIP1858 in *A.*

Fig. 4. Distribution of resistance mechanisms amongst aminoglycoside-resistant strains of *Acinetobacter*. ▨ single mechanism; □ combined mechanisms; IMP, impermeability.

baumannii strain BM2686 and was transferable by transformation to other *Acinetobacter* spp., but not to *E. coli* [83].

Intrinsic aminoglycoside resistance in acinetobacters

The *aac(6')-I_g* gene was found originally in the chromosome of *A. haemolyticus* strain BM2685 and has been detected in all strains of *A. haemolyticus* tested subsequently [84]. This gene has not been detected in other species of *Acinetobacter* and appears to be species-specific. Similarly, the *aac(6')-I_j* gene was found originally in the chromosome of *Acinetobacter* sp. 13 strain BM2689, and in all other strains tested belonging to this species, but not in other species of *Acinetobacter*. The *aac(6')-I_k* gene was found in the chromosome of *Acinetobacter* sp. 6 strain CIP A165. This gene has only been detected in the two other strains of *Acinetobacter* sp. 6 available and thus may also be species-specific [85].

Phylogeny of the aac(6')-I genes from Acinetobacter

We have recently characterised seven related *aac(6')-I* genes in strains 382, 79, 1011 and 942, belonging to *Acinetobacter* spp. 14, 15, 16 and 17, respectively, and in ungrouped strains 631, 640 and BM2722 (personal unpublished results). A phylogenetic tree was established from the amino-acid sequences deduced from these genes. Interestingly, these acinetobacters belong to a cluster of proteolytic strains. To establish whether the *aac(6')-I* genes were located on plasmids or in the chromosome, PFGE was performed with genomic *Acinetobacter* DNA digested with *Sfi*I [50]. Separated DNA fragments from the strains mentioned above, as well as *A. haemolyticus* strain BM2685, *Acinetobacter* sp. 6 strain CIP A165 and *Acinetobacter* sp. 13 strain BM2689, were transferred to a Nytran membrane and hybridised with an rRNA probe [86]. After recording the results, the membrane was stripped and then hybridised with probes specific for the various *aac(6')-I* genes found in *Acinetobacter*. In nine strains, the respective *aac(6')-I* genes were located in large size DNA fragments (> 400 kb); these co-hybridised with the rRNA probe in five strains (personal unpublished results). In contrast, the *aac(6')-I* gene was located on a 15-kb fragment in *Acinetobacter* sp. 15, but attempts to transfer the resistance determinant by conjugation or transformation with the plasmid DNA content of this strain were unsuccessful. These data indicate that these *aac(6')-I* genes are probably located in the chromosome and that they may derive from a common ancestor in a parental *Acinetobacter*. In this scheme, the presence of the *aac(6')-I_h* gene (which is closely related to the other *aac(6')-I* genes) on a plasmid in *A. baumannii* strain BM2586 may be the result of a transposition event from the *Acinetobacter* chromosome into an indigenous replicon. It has been reported

that plasmids from *Acinetobacter* spp. do not replicate in members of the family Enterobacteriaceae [80]. This observation could account for the fact that the *aac(6')-I_h* gene is confined, so far, to *Acinetobacter* spp. Detection of these species-specific *aac(6')-I* genes by hybridisation or by PCR may be useful in distinguishing proteolytic species of *Acinetobacter*.

Aminoglycoside resistance in A. haemolyticus

Study of the activity of various aminoglycosides against 96 *A. haemolyticus* strains from the collection of the Unité des Enterobactéries at the Institut Pasteur indicated that three strains were susceptible to tobramycin whereas the 93 remaining strains exhibited an AAC(6')-I resistance and grew on agar containing tobramycin 4 mg/L. In contrast, *A. haemolyticus* strains BM2713, BM2714 and BM2715 were susceptible to tobramycin; the MICs of amikacin, gentamicin, netilmicin and tobramycin for these strains were ≤ 1 mg/L and extracts of these strains were devoid of aminoglycoside acetyltransferase activity.

Distribution of aac(6')-I_g. Dot-blot hybridisation with an *aac(6')-I_g*-specific probe [84] detected this gene in the 96 strains of *A. haemolyticus*, but not in *Acinetobacter* strains belonging to other genomic species. PCR with oligonucleotides AL (5'-ATGAATATTAAACCTGCAT-3') and AR (5'-TTAATCTATTTTCTACT-3') yielded a 438-bp fragment corresponding to the *aac(6')-I_g* gene [87]. This PCR product was obtained for all *A. haemolyticus* isolates, except strain BM2715 which gave rise to a slightly larger fragment and strain BM2714 which did not yield any product. The *aac(6')-I_g*-like genes obtained by amplification of DNA from strains BM2713 and BM2715 were cloned under the control of the *lac* promoter into expression vector ddT-pUC19. The MICs of amikacin, netilmicin and tobramycin for *E. coli* strain JM83 (pUC19Ω*aac*_{BM2713}) and (pUC19Ω*aac*_{BM2715}) were similar to those of the parent *E. coli* JM83, and 2'-N-ethylnetilmicin and 6'-N-ethylnetilmicin displayed identical activity against all three strains. Resistant mutants of *E. coli* JM83 (pUC19Ω*aac*_{BM2713}) and *E. coli* JM83 (pUC19Ω*aac*_{BM2715}) were obtained by plating on agar containing tobramycin 5 mg/L. Clones with an AAC(6')-I phenotype, designated *E. coli* JM83 (pUC19Ω*aac*_{BM2713-1} and -2) and (pUC19Ω*aac*_{BM2715-1} and -2) were found to contain aminoglycoside-6'-acetyltransferase activity, whereas no acetyltransferase activity was detected in *E. coli* JM83 (pUC19Ω*aac*_{BM2713}) and *E. coli* JM83 (pUC19Ω*aac*_{BM2715}) extracts.

Characterisation of the silent aac(6')-I_g-like genes. The sequences of the *aac*_{BM2713} and *aac*_{BM2715} genes, and the derivative mutants, have been determined (personal unpublished results). Comparison of the sequences of *aac*_{BM2713} and *aac*_{BM2685} showed 11 nucleotide substitutions, resulting in six amino acid changes in the AAC(6')-I_g protein. The sequence of

the *aac*_{BM2713-1} and -2 genes was identical to that of *aac*_{BM2713}, except for point mutations at position 167 or 168 (Fig. 5). Thus, the Met56→Arg resulting from the G to T transversion at position 167 is responsible for loss of enzymic activity. An Arg56→Ser substitution re-established enzymic activity.

When compared to *aac*_{BM2685}, the sequence of *aac*_{BM2715} displayed an insertion of a stretch of 19 thymidines, resulting in a frame shift that probably accounts for lack of acetyltransferase activity in *A. haemolyticus* BM2715 and in *E. coli* JM83 (pUC19Ω*aac*_{BM2715}). Comparison of the *aac*_{BM2715-1} and -2 genes (encoding the AAC(6')-I activity) with *aac*_{BM2715} indicated that deletion of 19 thymidines in *aac*_{BM2715-1} restored the wild sequence, whereas *aac*_{BM2715-2} had suffered a deletion of 15 bp [TGGGT₍₁₁₎]. Surprisingly, although *aac*_{BM2715-2} had a frame shift compared to *aac*_{BM2685}, *E. coli* JM83 (pUC19Ω*aac*_{BM2715-2}) was resistant to tobramycin and directed synthesis of an AAC(6')-I. Production of an active enzyme may result from the RNA polymerase slippage that occurs during elongation at runs of adenine or thymine in *E. coli* [88].

PCR with BM2714 DNA and a primer pair complementary to regions upstream from and internal to *aac*_{BM2685} amplified a 1.4-kb fragment which has been cloned and sequenced. Comparison of the sequence obtained with that of *aac*_{BM2685} showed a 1049-bp insertion in *aac*_{BM2714} starting 50 bp after the initiation codon of the resistant gene (personal unpublished results). Further analysis indicated that the acquired element was a 1040-bp insertion sequence, IS17, with perfectly conserved 16-bp terminal inverted repeats that generated a direct duplication of 9 bp of target DNA upon insertion. Significant similarity (39%) was detected between the amino-acid sequence of the putative transposases deduced from the large open reading frames (ORFs) of IS17 and IS903, indicating that these sequences have diverged from a common ancestor.

Conclusions

Inactivation of an AAC(6')-I enzyme following a point mutation or an insertion in the structural gene has not been reported before, and loss of resistance was attributed previously to an alteration in gene regulation

Fig. 5. Comparison of the *aac*(6')-I genes from strains of *A. haemolyticus*. The nucleotide sequence of *aac*(6')-I_g from the tobramycin-resistant strain BM2685 is shown in a schematic fashion. Boxes indicate polymorphic sites that account for the tobramycin susceptibility of strains BM2713, BM2714 and BM2715. At **A**, transposition of IS17 into the *aac*(6')-I_g-like gene of BM2714 generated a 9-bp duplication. At **B**, the *aac*(6')-I_g-like gene of BM2713 contained a G to T transversion at position 167 (boxed triplet) that led to a methionine to arginine substitution. Subsequent resistant derivatives, BM2713-1 and BM2713-2, displayed ATG (M) or ACG (S) codons, respectively, at position 166–168 (boxed triplet). At **C**, the *aac*(6')-I_g-like gene of BM2715 carried an insertion of 19 thymine residues at position 225. Subsequent mutations to resistance either restored the wild-type sequence (BM2715-1) or deleted the 15-bp sequence TGGGT₍₁₁₎ (BM2715-2). In addition to the changes shown, the sequences of strains BM2713, BM2714 and BM2715 differed from the type sequence of strain BM2685 at nine additional positions (not shown), but these did not appear to influence the phenotype that was expressed.

[89]. In favour of the latter mechanism was the low-level 6'-acetylating activity observed in a 'susceptible' strain of *Serratia marcescens* with undetectable *aac(6')-Ic* mRNA [90]. Our results indicate that *aac(6')-I* gene inactivation can occur by three different mechanisms. This observation indicates that this enzyme may not be essential for the host bacterium, as opposed to AAC(2')-Ia in *Providencia stuartii* which contributes to O-acetylation of peptidoglycan [91]. Recently, the *aac(6')-Ic* gene has been reported to be genomic species-specific and has been proposed as a useful means for discriminating between *S. marcescens* and *S. liquefaciens* [92]. The 96 *A. haemolyticus* and two *Acinetobacter* sp. 6 strains studied were identified accurately by carbon source utilisation tests. Eleven strains were identified at the species level by thermal stability of DNA hybrids [29, 30]. Therefore, it can be concluded that *aac(6')-Ig* and *aac(6')-Ik* are also species-specific. Despite the high degree of identity (83%) between these genes, primers can be designed that allow differential amplification, whereas hybridisation requires stringent conditions to avoid cross-reactions. The use of these tools allowed us to identify a strain of *A. haemolyticus* that failed to utilise DL- γ -4 aminobutyrate and had been previously assigned (incorrectly) to *Acinetobacter* sp. 6.

MOLECULAR BASIS OF QUINOLONE RESISTANCE IN *A. BAUMANNII*

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Introduction

A. baumannii has been isolated with increasing frequency from clinical specimens and the hospital environment and is recognised as an important opportunist pathogen associated with various types of nosocomial infections [15, 24, 93]. Multiple antibiotic-resistant strains of this micro-organism have been implicated in outbreaks occurring in ICUs [9, 10, 14, 22, 94–96]. Until 1988, the new fluorinated quinolones showed very good activity against *Acinetobacter* strains [13, 97–99]. However, reports of a high prevalence of fluoroquinolone resistance among *Acinetobacter* isolates have now appeared [100–102]. Therefore, understanding the mechanism(s) by which this phenomenon occurs in this pathogen is important.

In *E. coli*, the mechanism of resistance to fluoroquinolones has been linked mainly to mutations in the quinolone resistance-determining region of *gyrA* [103] and to a decrease in quinolone accumulation by decreased uptake or increased efflux [104–109]. Of the mutations in the *gyrA* gene, those at Ser-83 and Asp-87 have been found with a high frequency in

quinolone-resistant *E. coli* clinical isolates. Specific mutations in the *gyrB* gene also seem to be associated with quinolone resistance [110–112], although their frequency in quinolone-resistant clinical isolates of *E. coli* is very low [110, 113, 114]. It has also been shown that mutations at residues Ser-80 and Glu-84 of ParC (topoisomerase IV) from *E. coli* may contribute to decreasing fluoroquinolone susceptibility [115–117]. This report focuses on the molecular mechanisms of quinolone resistance in *A. baumannii*.

Mechanisms of quinolone resistance

Mutations associated with quinolone resistance in the *gyrA* and *parC* genes were determined in 21 clinical isolates of *A. baumannii*. These isolates had ciprofloxacin MICs of 0.125–128 mg/L and nalidixic acid MICs of 2–1024 mg/L. The clinical isolates analysed had been characterised previously by low frequency restriction analysis of chromosomal DNA, which showed that all were epidemiologically unrelated [118]. A fragment of the *gyrA* gene that included the quinolone-resistance determining region was analysed by PCR and automated direct DNA sequencing. Conserved amino-acid sequence motifs found in diverse GyrA proteins were used to design oligonucleotide primers. The PCR product had the expected size of 343 bp. The nucleotide sequence of this fragment had *c.* 71% identity with the same region in the *gyrA* gene of *E. coli*, and 69% identity with that of *Pseudomonas aeruginosa*. The derived amino-acid sequence of this portion of the *A. baumannii* GyrA protein showed 82% identity with that of *E. coli*, and 83% with that of *P. aeruginosa*. These data highlight the sequence conservation of the quinolone resistance-determining region of the *gyrA* gene of *A. baumannii* and other bacteria.

The different combinations of mutations in the *gyrA* and *parC* genes leading to amino-acid changes are shown in Table 7. All clinical isolates for which the MIC of ciprofloxacin was <1 mg/L had identical *gyrA* sequences, except for one strain (ciprofloxacin MIC 0.125 mg/L) which had a mutation at amino-acid codon Ala-119 that changed to Ser. Two other resistant strains with ciprofloxacin MICs of 4 and 8 mg/L, respectively, also had this mutation. Although it is very close to the active site, tyrosine 122, this mutation does not seem to play a particularly important role in the acquisition of resistance, as it is found in both susceptible and resistant strains. Those clinical isolates for which the ciprofloxacin MIC was <1 mg/L did not show any change at Ser-83, although a strain for which the ciprofloxacin MIC was 1 mg/L exhibited a change at Gly-81 to Val. All 15 isolates for which the ciprofloxacin MIC was >4 mg/L had a change at Ser-83 to Leu. A strain for which the ciprofloxacin MIC was 64 mg/L showed a double change of Ser-83 to Leu and Ala-84 to Pro. It has been found that the amino-acid residue that is

Table 7. Amino-acid substitutions in the DNA gyrase A and topoisomerase IV A proteins from *A. baumannii* isolates with reduced susceptibility to quinolones

Number of strains	MIC mg/L		Amino-acid changes*	
	CIP	NAL	GyrA	ParC
5	0.125–1	2–8		
1	1	8	Gly-81 Val	
5	4–16	64–256	Ser-83 Leu	
1	64	> 1024	Ser-83 Leu	Glu-84 Lys
8	32–128	> 1024	Ser-83 Leu	Ser-80 Leu
1	64	> 1024	Ser-83 – Leu/Ala-84 – Pro	Ser-80 Leu

CIP, ciprofloxacin; NAL, nalidixic acid.

*Equivalent amino-acid position in *E. coli*.

mutated most frequently in spontaneous *gyrA* mutations and in clinical isolates of *E. coli* is Ser-83. Similar mutations have been found in *A. baumannii*. Other mutations affecting Gly-81, Ala-84 and Gln-106 residues, for which substitutions are also associated with quinolone resistance in *E. coli*, are all conserved in *A. baumannii gyrA*.

The high level of variability in MICs among strains showing the same change could be a result of mutation(s) in the *parC* gene. Mutations in the *parC* region equivalent to the quinolone resistance-determining region in *gyrA* were investigated to determine their possible role in the acquisition of quinolone resistance by clinical isolates of *A. baumannii*. The same set of 21 epidemiologically unrelated quinolone-susceptible and -resistant isolates of *A. baumannii* were examined. The same strategies—PCR and DNA sequencing—were used to identify mutations in the *parC* gene. The PCR product was 327 bp long. The nucleotide sequence of this fragment had 72% identity with the same region in the *parC* gene of *E. coli*, but only 49% identity with the *gyrA* gene. The derived amino-acid sequence of the *A. baumannii* ParC protein had 89% identity with that of *E. coli*. This result highlights the sequence conservation of the quinolone resistance-determining region of the *parC* gene of *A. baumannii* and other bacteria.

Of the 21 clinical isolates analysed, 10 with a ciprofloxacin MIC > 32 mg/L showed a change in Ser-80 to Leu, except for one strain which showed a change of Glu-84 to Lys, whereas 11 clinical isolates with a ciprofloxacin MIC < 16 mg/L did not show any change. The 12 strains with no mutations at amino-acid codon 80 had three different codons for this amino acid: TCA (one strain), TCG (eight strains) and TCT (three strains). However, all the strains with a change at Ser-80 had a CT transversion from codon TCG, leading to a Ser-80 Leu substitution. There was no evidence for a *parC* mutation without the simultaneous presence of a mutation in the *gyrA* gene, suggesting that DNA topoisomerase IV could be a secondary target for quinolones in *A. baumannii*. These results are in agreement with those found in *E. coli* [115–117], but in contrast with those reported for

Staphylococcus aureus where topoisomerase IV could be a primary target for quinolones, as mutations in *parC* (*grlA* in *S. aureus*) were found without mutations in *gyrA* [119].

Specific mutations in the *gyrB* and *parE* genes do not seem to be associated frequently with acquisition of quinolone resistance in clinical isolates of *E. coli* [110, 113, 114, 120]. Although these genes have not been investigated in *A. baumannii*, extrapolation of the findings from *E. coli* suggests that mutations in *gyrB* and *parE* probably do not play an important role in the acquisition of quinolone resistance in clinical isolates of *A. baumannii*.

In most studies, mutations in either the *gyrA* or *parC* genes are analysed by conventional cloning and DNA sequencing or, more recently, by PCR amplification of the quinolone-resistance determining region and sequencing, either after cloning the PCR product or directly. However, this technique is too cumbersome and time-consuming for routine use. Instead, PCR-RFLP analysis with *HinfI* can be used to detect specific mutations affecting Ser-83 in GyrA and Ser-80 in ParC. The *A. baumannii* GyrA protein carries conserved Asp–Ser residues at positions equivalent to 82 and 83 in the protein from *E. coli*; both codons combine to form a *HinfI* restriction site (GANTC). *HinfI* digestion of the PCR product from a quinolone-susceptible *A. baumannii* strain generates two fragments of 291 and 52 bp, respectively. The *HinfI* restriction site in isolates carrying a mutation at codon 83 is abolished, resulting in no digestion of the fragment containing the full-length PCR product (Fig. 6). Similarly, the *A. baumannii* ParC protein has Asp–Ser residues at positions equivalent to 79 and 80 in the protein from *E. coli*, forming a *HinfI* restriction site. Therefore, *HinfI* digestion of the PCR product from a clinical isolate which does not carry a mutation at amino-acid codon Ser-80 generates two fragments of 206 and 121 bp, respectively (Fig. 6), whereas the *HinfI* restriction site in isolates carrying a mutation at codon Ser-80 is abolished, resulting in no digestion of the fragment. Therefore, the presence or absence of *gyrA* mutations at codon 83 and *parC* mutations at codon Ser-80 can be determined by digestion of PCR products with *HinfI*, and this

Fig. 6. Schematic representation and analysis of mutations in the *gyrA* and *parC* genes of quinolone-resistant clinical isolates of *A. baumannii* following restriction fragment length polymorphism analysis of PCR products. Lanes: **M**, DNA molecular size marker VI (Boehringer); **1**, undigested *gyrA* PCR product from a quinolone-susceptible strain; **2**, *Hinf*I-digested *gyrA* product from a susceptible strain; **3**, *Hinf*I-digested *gyrA* product from a strain with a Gly-81 Val substitution; **4**, *Hinf*I-digested *gyrA* product from a strain with Ser-83 Leu and Ala-119 Ser substitutions; **5**, *Hinf*I-digested *gyrA* product from a strain with a Ser-83 Leu substitution; **6**, *Hinf*I-digested *gyrA* product from a strain with a Ser-83 Leu and Ala-84 Pro substitutions; **7**, undigested *parC* PCR product from a quinolone-susceptible strain; **8**, *Hinf*I-digested *parC* product from a strain with a TCA codon for Ser-83; **9**, *Hinf*I-digested *parC* product from a strain with a TCG codon for Ser-83; **10**, *Hinf*I-digested *parC* product from a strain with a TCT codon for Ser-83; **11**, *Hinf*I-digested *parC* product from a strain with a Glu-84 Lys substitution; **12**, *Hinf*I-digested *parC* product from a strain with a Ser-80 Leu substitution.

procedure may be used for rapid screening of a large number of clinical isolates. Unfortunately, the technique cannot recognise a mutation at other sites in the *gyrA* or *parC* genes. Single-strand conformational polymorphism analysis has been used to examine *gyrA* mutations in *E. coli* [114] and this method could probably be used to detect other mutations in *gyrA* or *parC* in *A. baumannii*.

A possible mechanism of quinolone resistance, other than *gyrA* and *parC* mutations, is reduced accumulation of quinolones, either by a decrease in the permeability of the outer membrane or an increased efflux from the cell. Such mechanisms have not been studied in depth in *A. baumannii*. It has been shown that the intrinsic antibiotic resistance of acinetobacters may be attributable to the presence of a small number of small-size porins, resulting in only 1% of the permeability shown by *E. coli* and less permeability than that of *P. aeruginosa* [121]. The outer membrane of *P. aeruginosa* has a very low non-specific permeability to small, hydrophilic molecules [122, 123],

and this has generally been thought to be the main cause of the intrinsic resistance of this micro-organism. However, recent evidence has suggested that the *mexAB-oprM* operon, encoding an active efflux system, probably contributes to the intrinsic resistance of *P. aeruginosa* to tetracycline, chloramphenicol and fluoroquinolones [124]. A mechanism similar to the efflux system found in *P. aeruginosa* may, in combination with the low outer-membrane permeability, contribute to the intrinsic resistance of *A. baumannii*.

Conclusions

Although alterations in drug permeability or drug efflux could also contribute to the quinolone resistance of *A. baumannii* clinical isolates, the investigations performed to date suggest that a specific substitution at the Ser-83 codon in GyrA is associated with a moderate level of resistance to quinolones, whereas a double substitution at both Ser-83 from GyrA and Ser-80 or Glu-84 from ParC render *A. baumannii* highly

resistant to quinolones. The results also suggest that GyrA acts as a primary target and ParC as a secondary target for quinolones in this organism.

EXPERIMENTAL MODELS OF ACINETOBACTER INFECTION

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Introduction

A. baumannii is now recognised as an increasingly important nosocomial pathogen. Difficult-to-treat infections with *A. baumannii* are a consequence of the widespread dissemination of strains in hospitals and their increasing resistance to antibiotics [125]. Animal models represent an essential step between in-vitro susceptibility testing and clinical studies. The World Health Organization has stated that 'the testing of antimicrobial agents in animal models of infection is recognised as an essential prerequisite for acceptance of a new agent or mode of therapy, either by the medical/scientific community or by government authorities'. The increasing resistance of *A. baumannii* to many antibiotics means that an experimental model is needed to evaluate the efficacy of new antibiotic regimens. Experimental models for acute systemic infection and UTI with *Acinetobacter* spp. were first developed in mice in 1985 [76, 126] to examine the virulence factors of *Acinetobacter* spp. and the efficacy of therapy with tetracyclines and aminoglycosides. More recently, in response to the increasing role of *A. baumannii* in nosocomial pneumonia associated with mechanical ventilation, a mouse model of pneumonia caused by *A. baumannii* has been developed (see below) that has similarities to the human disease.

Virulence of *Acinetobacter*

The virulence of clinical isolates of *Acinetobacter* in mice inoculated intraperitoneally (i.p.) or intravenously (i.v.) was studied by Obana [126]. LD50 values were generally $\geq 10^6$ cells/mouse by either the i.p. or i.v. route. Virulence was enhanced to give LD50 values of between 10^3 and $\geq 10^6$ cells/mouse (depending on the particular strain) if cells were inoculated as suspensions containing hog gastric mucin 3% v/v [126]. It was also demonstrated that 14% of *Acinetobacter* clinical isolates had slime-producing ability, and that the slime also enhanced the virulence of strains of *E. coli*, *S. marcescens* and *P. aeruginosa*. This study emphasised the potential role of *Acinetobacter* in the enhancement of virulence in the mixed infections that constitute c. 50% of pulmonary infections involving *Acinetobacter*.

Mouse models of infection with *Acinetobacter* and therapeutic investigations

Acute systemic infection. Acute systemic infection has been induced in male mice by i.p. injection of a suspension of *Acinetobacter* ($100 \times$ the LD50 value) in hog gastric mucin 3% v/v [76]. Antibiotics were administered either by gavage or subcutaneously 2 h after the initial infection. The therapeutic efficacy of tetracyclines, aminoglycosides and colistin was estimated by the 50% effective dose (ED50 value). The results of the protection tests in mice showed that there was a good correlation between antibiotics that were highly effective *in vitro* and the ED50 value.

Experimental UTI. Ascending UTI has been induced in female mice by transurethral inoculation with 5×10^4 cfu/mouse [76]. The external meatus was closed with a small clip for 4 h and then removed. Antibiotics were administered by gavage or subcutaneously 4 h after the initial infection. The efficacy of tetracyclines, aminoglycosides and colistin was assessed in terms of the numbers of bacteria found in the kidney. The antibiotics that were highly active against systemic infection were also active against ascending UTI.

Experimental pneumonia. The increasing role of multi-resistant strains of *A. baumannii* in nosocomial infection, particularly nosocomial pneumonia, led to the development of an experimental model of acinetobacter pneumonia for the evaluation of new therapeutic regimens [127]. Transiently neutropenic C3H/HcN female mice were anaesthetised and infected by intratracheal instillation of 50 μ l of an *A. baumannii* suspension containing 10^8 cfu/ml. The efficacy of inoculation was confirmed by quantifying the viable bacteria in the lungs removed from two infected control animals immediately after inoculation and 3 h later. In the experimental animals, the mean (SD) $\log_{10}$ cfu/g of lung homogenates were 9 (0.9), 9.4 (0.8), 8.6 (1.2) and 7.7 (1.4) on days 1, 2, 3 and 4 after inoculation, respectively. The lung pathology was characterised by pneumonitis with oedema and a patchy distribution of haemorrhage in the peribronchovascular spaces of both lungs. Abscesses formed on days 3 and 4. Subacute pneumonitis, characterised by alveolar macrophage proliferation and areas of fibrosis, was also observed on day 4. The cumulative mortality on day 4 was 85%.

This new model was used to study the effects of the following antibiotic regimens (mg/kg/dose): imipenem, 50; ticarcillin, 500; sulbactam, 100; ticarcillin plus clavulanic acid in a 25:1 ratio; and the combination of ticarcillin plus clavulanic acid plus sulbactam. To study the bactericidal effect, treatment (four i.p. doses, see above, at 3-h intervals) was initiated 3 h after infection, with the bacterial counts in the lungs determined 3 h after the last dose. To assess the effect of the various regimens on mortality, treatment commenced 8 h after infection, at which

time the clinical and histological patterns of pneumonia were already present. The bacterial counts in the lungs (3 h after the last dose of antibiotic) and the mortality rates at day 5 are shown in Table 8 and Fig. 7. The results suggest that imipenem, sulbactam and the combination of ticarcillin plus clavulanic acid plus sulbactam, all have the same bactericidal effect *in vivo* (a reduction in cfu of $\log_{10}3$) when administered soon after infection. In animals treated later, the combination of ticarcillin with clavulanic acid and sulbactam achieved the lowest mortality rate. This non-classic combination, as well as sulbactam used alone, may be considered as possible therapeutic alternatives for acinetobacter pneumonia.

Conclusions

Animal models may be useful in the investigation of virulence patterns and new antibiotic regimens. They represent an essential step between in-vitro susceptibility testing and clinical studies, providing that they show a reproducible acute course of infection and similarities to the disease in man. Thus, with the usual caveat that caution be used in transposing therapeutic lessons from animal models to a clinical setting, the animal model for pneumonia caused by *A. baumannii* has been useful in identifying possible therapeutic alternatives for 'difficult-to-treat' *A. baumannii* infections, including the use of non-classic combinations such as ticarcillin with clavulanic acid and sulbactam

Table 8. In-vivo bactericidal activity and mortality rates with different treatment regimens in a mouse model of *A. baumannii* pneumonia

Treatment regimen	Mean (SD) $\log_{10}$ cfu/g of lung homogenate (3 h after last antibiotic dose)	Mouse mortality rate (%)
None (control sample)	7.57 (0.49)*	26/27 (96)
Ticarcillin	6.69 (0.68)	14/16 (88)
Sulbactam	4.31 (0.19)	4/15 (27)
Ticarcillin + clavulanate (1:25) + sulbactam	4.25 (0.29)	1/15 (7)
Ticarcillin + clavulanate (1:15) + sulbactam	5.78 (0.81)	5/16 (31)
Ticarcillin + clavulanate (1:10) + sulbactam	4.47 (0.44)	6/15 (40)
Ticarcillin + clavulanate (1:25)	5.79 (0.25)	10/16 (62)
Ticarcillin + clavulanate (1:10)	5.60 (0.30)	16/18 (89)
Ticarcillin + sulbactam	5.39 (0.59)	10/16 (62)
Imipenem	4.47 (0.44)	6/15 (40)
Cefotaxime	...	12/15 (80)

*Bacteria in the lungs of the control mice were counted 3 h after the original inoculation of bacteria.

Fig. 7. Cumulative mortality rates in the mouse pneumonia model at days 0–5 (DO, ■; D1, ▨; D2, ▩; D3, ▪; D4, □; D5, ▫) post-infection with different treatment regimens (Ticar, ticarcillin; CA, clavulanic acid).

for systemic infections, and sulbactam alone for local infections.

ACINETOBACTER – WHAT'S ON A CLINICIAN'S MIND?

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Introduction

Acinetobacter spp. are gram-negative coccobacilli that contribute profoundly to the burden of modern medicine, where the term 'burden' refers to the sum of clinical and economic load. This burden can be further divided into 'expected burden', which results from the expected sporadic infections caused by these bacteria, and 'unexpected burden', which results from local epidemics. The fact that most clinical isolates of *Acinetobacter* are now multiresistant to antibiotics means that infection with these organisms prolongs hospitalisation, and this prolonged hospitalisation is the main burden resulting from acinetobacter infection. In Sheba Medical Centre, a tertiary academic facility, c. 800 isolates are cultured each year. The most difficult task for the clinician is to differentiate between colonisation and true infection. The situation is particularly confusing when *Acinetobacter* is isolated from pulmonary secretions, wound infections and urine, and unfortunately is exacerbated precisely in those patients where differentiation is particularly important.

Acinetobacter as a pathogenic bacterium in the hospital

The fact that the genus *Acinetobacter* has undergone extensive taxonomic changes over the years is often used as an excuse for the fact that these bacteria have not been well studied previously. However, the lack of interest is also associated with the fact that most clinicians would not previously have regarded these organisms as a cause of major infections; indeed most isolates would have been disregarded as 'non-pathogenic contaminants'. Only 30 years ago, it was stated authoritatively that 'members of the genus *Mima* or *Herellea*, including a species originally named *Bacterium anitratum*, are tiny gram-negative aerobic coccobacilli that are ... considered non-pathogenic species of *Neisseria*' [128]. The situation has now changed considerably, and acinetobacters have assumed increasing importance in nosocomial infections, especially in ICUs. The principal sites and types of infection include the respiratory tract, bacteraemia, peritoneum, surgical wounds and meningitis. Nevertheless, the expected clinical burden of *Acinetobacter* is relatively low, and proven nosocomial infections caused by these organisms generally account for no more than 1–2% of all

nosocomial infections (the prevalence of bacteraemia as an indication of nosocomial infection is 1.0–1.4% in different hospitals and is higher for other gram-negative and gram-positive bacteria). On the other hand, the fact that *Acinetobacter* infects the very seriously ill, is multi-drug resistant, prolongs hospitalisation and causes epidemics leads to a very profound and unexpected burden on the health care system.

Acinetobacter wound infection and UTI

Usually the clinician has a patient with a disease on one side and a bacterium on the other, and has to combine what is known about the two to make a therapeutic decision. *Acinetobacter* wound infection or UTI does not differ from the same infections caused by other gram-negative organisms. In wound infections, *Acinetobacter* is rarely the first and only micro-organism isolated from the wound. Other gram-negative and anaerobic bacteria are often isolated and, even if *Acinetobacter* is isolated, it is not certain that it should be treated as aggressively as other pathogens isolated from such infections, except possibly in immunocompromised and burns patients where acinetobacter wound infection might not be so benign. However, even in these latter groups of patients, *Acinetobacter* is not normally a common pathogen, accounting for <1% of infections in 1267 patients in the National Nosocomial Infection Study [129]. Most acinetobacters isolated from wounds may well be colonisers, and the clinician must decide on the probability that the organism is or is not causing the infection, and the 'price' of treating or not treating a specific infection in a specific patient in a specific environment. The same decision must be made for nosocomial acinetobacter UTI; this is also quite rare and the same difficulties in making therapeutic decisions apply, especially in the context of a bacterium that is often resistant to most available antibiotics.

Acinetobacter pneumonia in the ICU

Respiratory tract infections in the ICU have been the most common manifestation of acinetobacter infection. While sporadic nosocomial infections caused by *Acinetobacter* spp. remain relatively uncommon (accounting for 4% of pulmonary infections in patients with pneumonia enrolled in the National Nosocomial Infection Study Registry during 1990–1992 [130]), large nosocomial outbreaks of infection have been described repeatedly [130]. The infection is often transmitted through contaminated respirators and other equipment, as well as on the gloves worn by nursing and medical staff. *Acinetobacter* has even been isolated from an oxygen outlet and the petroleum grease used to lubricate various parts of a respirator (personal unpublished results). Risk factors for acquisition of acinetobacter pneumonia include age, underlying disease, prolonged ventilation and prolonged use of broad-

spectrum antibiotics. Clinically, there are no characteristic symptoms or radiological signs of acinetobacter pneumonia that differentiate it from other types of gram-negative pneumonia, but acinetobacter pneumonias are associated with considerable mortality and prolonged periods of hospitalisation.

In a classic case control study, Fagon and colleagues [131] compared mortality rates and length of hospitalisation for patients with acinetobacter or pseudomonas pneumonia against matched (especially with regard to underlying disease) patients. The mortality rate for ventilated patients with pneumonia was twice that for ventilated patients without pneumonia. The mortality rate for those patients with pneumonia associated with *Acinetobacter* or *Pseudomonas* spp. was 2.5-fold greater than the mortality rate of those without pneumonia, and 1.57-fold greater than the mortality rate of patients with pneumonia caused by other organisms. In addition, the median hospitalisation period for patients with pneumonia was 21 days, compared with 15 days for patients without pneumonia. For patients who survived, the difference was even more striking (27 versus 13 days). In patients with acinetobacter or pseudomonas pneumonia, the mean length of stay was 21 days compared with 14 days for controls. In Sheba Medical Centre, all isolates of *Acinetobacter* are multidrug-resistant and are sensitive only to imipenem and amikacin; thus in direct antibiotic costs alone, an acinetobacter pneumonia increases the costs of hospitalisation by *c.* £75 (\$120)/patient/day.

The main problem for the clinician is the decision about treatment once *Acinetobacter* has been isolated from the sputum. First, the clinician has to decide whether or not pneumonia is present ('not everything which barks is a dog'); atelectasis, pulmonary emboli, pulmonary haemorrhage, etc., might cause fever, leucocytosis and lung infiltrates similar to pneumonia, especially in ventilated patients. Second, when pneumonia is present and *Acinetobacter* is isolated from the sputum, the clinician has to decide whether the *Acinetobacter* isolate is the cause of the lung pathology. *Acinetobacter* is a 'fast coloniser' of the respiratory tract—while oropharyngeal colonisation occurs in only about 7% of healthy subjects, oropharyngeal colonisation amongst mechanically ventilated patients in the ICU might reach 45%. Fibreoptic bronchoscopy with a protected specimen brush (PSB) or bronchoalveolar lavage (BAL) with quantitative assessment of the bacteria isolated is a considerable help, but does not provide a definitive answer. Third, a decision has to be made in cases when *Acinetobacter* is isolated from respiratory secretions, ample sputum is produced, but there is no pneumonia. Fagon *et al.* [132] compared the crude mortality rate observed in 52 mechanically ventilated patients who had developed nosocomial pneumonia with *Acinetobacter* or *Pseudomonas* spp. with the

crude mortality rate of 33 patients who had only tracheobronchitis caused by the same organisms. The overall mortality rate of patients without pneumonia who did not receive antibiotic treatment was 28%, compared with a rate of 75% in those patients who developed true pneumonia. In our experience, of the *c.* 200 patients/year whose sputum yielded *Acinetobacter*, it was possible to provide a definitive diagnosis to the infectious diseases physician in <12 cases.

Acinetobacter bacteraemia

As more naturally sterile tissue is reached, it might be hoped that the dilemmas would decrease, but some important questions still remain. Clinically, acinetobacter bacteraemia or sepsis does not differ from that caused by other gram-negative bacteria. The affected patients are usually debilitated, with malignancy, burns and trauma being the most common predisposing factors. In such patients, the respiratory tract is often the source of bacteraemia, but iatrogenically introduced bacteria (e.g., through transducers of pressure monitors, central lines, etc.) are also encountered. Polymicrobial bacteraemia is seen in many of these patients, which makes it even more difficult to determine the contribution of *Acinetobacter* to the clinical disease.

As an example, in Sept.–Nov. 1994, a mini-epidemic of acinetobacter bacteraemia occurred in our hospital, with 21 cases compared to a normal average of 12 in previous years. Most (17) of the cases were from the neurosurgery ICU, but four cases were from the paediatric ICU. Within the 2-week period before the onset of bacteraemia, 80% of the patients involved had vascular catheters and all had received antibiotics. The bacteraemia was considered to be clinically significant in 80% of the cases. The most common sources of *Acinetobacter* were identified as pneumonia (40%), wound infection, skin and urine (37%) and meningitis (23%). The isolate involved was multi-resistant, susceptible only to imipenem and amikacin.

Acinetobacter meningitis

At the top of the pyramid are the isolates of *Acinetobacter* obtained from CSF. The diagnostic dilemmas are usually few when there is frank meningitis with low sugar, high protein and polymorphonuclear leucocytes in the CSF, but the treatment dilemmas remain much the same. *Acinetobacter* is not a regular pathogen of the meninges and it does not normally cross the blood-brain barrier in healthy subjects. Usually, acinetobacter meningitis is a sporadic infection seen in the neurosurgery unit after surgery or head trauma, although several epidemics have been described [133]. Clinically, the most significant features of acinetobacter meningitis are mental changes and seizures; unlike other forms of bacterial meningitis, few patients have frank meningeal irritation. The infection is polymicrobial in *c.* 50% of patients. In

general, the mortality rate is *c.* 20–25%—i.e., about half that of patients with meningitis caused by other gram-negative bacteria.

The normal isolation rate of *Acinetobacter* from CSF at Sheba Medical Centre is about 7–8 isolates/year. However, in Sept. 1994, a sharp rise in the isolation rate was observed, with 17 cases, of which 11 occurred in 1 month. Of these 11 cases, nine were males, seven had severe trauma with drainage and a ventriculostome in place, three had intracerebral haemorrhage with drainage and a ventriculostome, and one had laminectomy as a result of a spinal tumour. Interestingly, all of the patients except one had a ventriculostome. An epidemiological investigation was started, but it was not possible to trace the origin of the epidemic. Each week, more cases were added to the list, and it was decided eventually to close the ICU for 1 week and transfer the infected patients to a separate unit on another floor of the building. At the same time, advice was given to change the ventriculostome site according to clinical guidelines. In the month following the investigation, the outbreak of infection ceased spontaneously.

In conclusion, from the examples given above, it can be seen that *Acinetobacter* has assumed an important role in nosocomial infection, where it now exerts a considerable clinical and economical burden on modern medicine.

NON-FERMENTATIVE GRAM-NEGATIVE BACILLI: A CHALLENGE FOR THE PHARMACEUTICAL INDUSTRY

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Overview of resistance problems

Bacteria, especially common pathogens, are able to adapt rapidly to the new weapons invented by the human brain. The discovery of the potential therapeutic uses of penicillin G against gram-positive cocci (*Staph. aureus* and *Streptococcus pyogenes*), the antituberculosis activity of streptomycin and the broad-spectrum antibacterials such as chloramphenicol and oxytetracycline, were quickly followed by the selection of resistant strains and the emergence of new pathogens, including members of the Enterobacteriaceae and *P. aeruginosa*. These problems led to a search for new compounds. The emergence of staphylococci resistant to penicillin G led to penicillin M derivatives, cephalosporins, erythromycin and other macrolides, lincosamides, vancomycin and streptogramin. The emergence of gram-negative bacteria as pathogens resulted in the development of the first penicillin A

(ampicillin), α -carboxypenicillin (carbenicillin) and aminoglycoside derivatives (gentamicin). Rapid selection of resistant strains amongst enteric pathogens and *P. aeruginosa* was followed by the preparation of novel compounds, such as *N*-acyl penicillins and 5-amino-2-thiazolyl cepheems, or the discovery of completely new entities such as carbapenems or fluoroquinolones.

Today, the epidemiological situation has changed profoundly. Since the mid-1990s, as we move towards the dawn of the 21st century, we are gradually returning to the clinical situation of the 1940s. The old 'plagues' (e.g. tuberculosis and gram-positive cocci) once again present therapeutic problems because of the resistance mechanisms that have evolved during the last 50 years. Penicillin G is less active against *Str. pneumoniae*, *Str. viridans* and *Neisseria meningitidis*. Macrolides have partly lost their activity against gram-positive cocci, especially *Str. pyogenes*. Additional resistance mechanisms, e.g., extended-spectrum- β -lactamases, are appearing continuously. In addition, new 'plagues' associated with the emergence of new pathogens, mainly consisting of gram-positive bacteria and non-fermentative gram-negative bacilli, are assuming increasing importance.

Within the gram-negative bacilli, the non-fermentative bacilli occupy an important place. The recent emergence seems to be associated with antibiotic selection pressure in the hospital community, increasing numbers of immunocompromised patients, and high-technology medicine or surgery. The main pathogen in this group is *P. aeruginosa*, which continues to pose seemingly insurmountable problems. In addition, *Stenotrophomonas maltophilia*, *Acinetobacter* spp., *Burkholderia cepacia*, *Bordetella bronchiseptica*, *Alcaligenes* spp., *Flavobacterium* spp. and, rarely, *Chryseobacterium* spp. have emerged as opportunist nosocomial pathogens. These pathogens are resistant, or only poorly susceptible, to most available antibacterial agents, in part because the original selection of these compounds was on the basis of activity against 'standard' pathogens, which include gram-positive cocci (staphylococci and streptococci), enteric pathogens and *P. aeruginosa*. Thus, new opportunist pathogens are generally not included in the 'panel' of strains for pre-selection and selection of a new compound. If the pharmaceutical industry is to develop new antibacterial agents with activity against the non-fermentative gram-negative bacilli, it is essential that well-defined strains belonging to these species, with different resistance phenotypes, are included in the test panel of strains for pre-selection of new compounds.

The problem of Acinetobacter spp.

The taxonomic problems in this genus have meant that it is difficult to compare results obtained in the past by different groups of investigators. However, it seems

that *Acinetobacter* spp. are sometimes susceptible to aminoglycosides, although many recent isolates produce inactivating enzymes. They are sometimes susceptible to α -carboxypenicillin (ticarcillin), new *N*-acyl penicillins and fluoroquinolones. They are poorly susceptible to cepheims. Some compounds show bimodal activity (e.g., ceftazidime, cefpirome and the new catechol cepheims). Most resistance is found amongst nosocomial isolates of *A. baumannii*.

How to analyse the antibacterial activity of new entities against *A. baumannii*? The best approach might be to test the new compounds against a standard collection of isolates with various resistance phenotypes (resistance to aminoglycosides, fluoroquinolones, α -carboxypenicillins) or combinations of different resistance mechanisms, and then to test in-vitro activity against recent clinical isolates from two or three different centres. Bactericidal activity should be determined by kill-kinetics, alone or in combination with other antibacterial agents. Different concentrations should be tested (MIC $\times 2$, $\times 4$ and $\times 8$). The most difficult challenge is to determine the in-vivo activity of a new compound. *Acinetobacter* spp. are usually of low virulence, so that systemic infection, in, for example, a mouse model can be induced only by a large inoculum. However, the development of experimental murine lung infections in immunodepressed mice (M. L. Joly-Guillou and colleagues, this review) may allow respiratory nosocomial infections to be simulated.

In conclusion, the selection of a new compound with activity against *Acinetobacter* spp. will occur as part of a test for activity against non-fermentative gram-negative bacilli in general. The challenge for the pharmaceutical industry is to be able to appreciate and act upon the different therapeutic needs—gram-positive bacteria, non-fermentative gram-negative bacilli, or both—and to decide how the various pharmaceutical risks can be evaluated.

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