Heteroresistance to Meropenem in Carbapenem-Susceptible Acinetobacter baumannii

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The characteristics of carbapenem heteroresistance were studied in 14 apparently carbapenem-susceptible Acinetobacter baumannii isolates. The MICs for carbapenems were determined, and the isolates were genotyped by pulsed-field gel electrophoresis (PFGE) and sequence typing (ST). Population analysis, testing of the stability of the heteroresistant subpopulations, and time-killing assays were performed. The agar dilution MICs of both imipenem and meropenem for the native isolates ranged from 0.25 to 4 mg/liter. The isolates belonged to nine PFGE types and exhibited seven ST allelic profiles. Population analysis revealed subpopulations that grew in the presence of imipenem at concentrations of up to 8 mg/liter and meropenem at concentrations of up to 32 mg/liter. The meropenem-heteroresistant subpopulations of 11 isolates exhibited stable resistance with MICs that ranged from 16 to >32 mg/liter; their PFGE profiles were identical to those of the native isolates. Time-killing assays with meropenem revealed less pronounced killing for 10 isolates. These findings indicate that meropenem pressure can produce meropenem-heteroresistant subpopulations that might subsequently select for highly resistant strains.

Acinetobacter baumannii is an opportunistic pathogen associated with severe hospital infections (1, 16), which often need the use of carbapenems as the treatment of last resort. However, reduced susceptibility or resistance to carbapenems is increasingly being observed among A. baumannii clinical isolates (2, 5, 25). In a preliminary work, we reported on the growth of distinct colonies within the inhibition halo around carbapenem disks or Etest strips and assessed these colonies as having the phenotypic manifestation of carbapenem heteroresistance (17). Likewise, a recent study (12) has described colistin-heteroresistant subpopulations among apparently colistin-susceptible A. baumannii clinical isolates, implying an intrinsic potential of the species to overcome drug pressure. These observations suggest that the use of carbapenems or colistin to treat severe multidrug-resistant A. baumannii infections may lead to the development of resistance. The present study aimed to evaluate in vitro the incidence of carbapenem heteroresistance, to characterize the traits of the heteroresistant subpopulations, and to assess the efficacies of carbapenems against heteroresistant A. baumannii isolates. The study included carbapenem-susceptible A. baumannii clinical isolates that cause infections; such infections are commonly treated with carbapenems, although it is recognized that the heterogeneity of resistance might affect the outcome. The study also determined how the heterogeneity of resistance might affect the treatment outcome.

Materials and Methods

Study population and susceptibility testing. All single A. baumannii clinical isolates that were recovered in the University Hospital of Larissa (Larissa, Greece), a tertiary-care hospital with 700 beds, over a 6-month period from September 2007 to March 2008 were recorded. The study included all isolates that were initially identified as carbapenem susceptible by use of the Vitek 2 system (bioMérieux, Marcy l’Etoile, France) to investigate their putative heteroresistant phenotype. Imipenem and meropenem MICs were also tested by agar dilution (4). The susceptibilities of the native isolates and their meropenem-heteroresistant subpopulations to other β-lactams, aminoglycosides, quinolones, and colistin were determined by disk diffusion. Pseudomonas aeruginosa ATCC 27853 was used as a control.

PFGE and ST. The clinical isolates as well as their meropenem-heteroresistant subpopulations were tested by pulsed-field gel electrophoresis (PFGE) of Apal-digested genomic DNA. The banding patterns were compared by using the criteria proposed by Tenover et al. (23). Sequence typing (ST) was performed by use of a previously described scheme (27). The sequences of both strands were determined at Lark Technologies, Inc. (Takeley, United Kingdom), and were analyzed by using DNASTar software (version 5.07; Lasergene, Madison, WI). STs were assigned by using the scheme that has recently been developed by J. F. Turton and R. Meyers (http://www.hpa-bioinformatics.org.uk/ABhome.php).

PCR assays. PCR assays for genes coding for known class B and D carbapenemases (blaIMP, blaVIM, blaGES, blaTEM, and IS128 IC, blaOKA38, blaOKA23, blaOKA24, and blaOKA58) were performed as described previously (3, 11, 14, 18). Amplification of the intrinsic blaOKA58 allele (28) was also done to confirm the phenotypic identification of the isolates as A. baumannii. Insertion sequences IS481 and IS482 were amplified, and screening for their possible location upstream of the class D carbapenemase genes blaOKA58 and blaOKA58 was performed by PCR mapping with primers IS481 forward and OKA58 reverse and primers IS482 forward and OKA58 reverse, respectively (24). The nucleotide sequences of both strands of the PCR products were determined at Lark Technologies, Inc., and sequence analysis was carried out with DNASTar software (version 5.07; Lasergene, Madison, WI).

Population analysis and investigation of the stability of the heterogeneous phenotype. Population analyses with imipenem and meropenem were performed by a previously described protocol for P. aeruginosa (19), with some modifications. Briefly, subpopulations were yielded by spreading approximately 10⁸ bacterial CFU (10⁹ CFU/ml) of a starting inoculum containing approximately 10⁹ CFU/ml on Mueller-Hinton agar plates with imipenem or meropenem in serial dilutions at concentrations ranging from 0.5 to 32 mg/liter and incubating the plates for 48 h. The analysis was performed three times for all isolates, and the mean numbers of viable CFU were estimated and plotted on a semilogarithmic graph.

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P. aeruginosa ATCC 27853 was used as a control for the population analysis experiments. The frequency of appearance of heteroresistant subpopulations in the presence of the highest drug concentration was calculated by dividing the number of colonies that grew on the antibiotic-containing plate by the colony counts from the same bacterial inoculum that grew on antibiotic-free plates. For each strain, three distinct colonies grown in the presence of the highest drug concentration were tested by agar dilution on Mueller-Hinton agar plates to check the stability of the heteroresistant phenotypes.

**Results**

During the study period, 142 nonrepetitive *A. baumannii* isolates were recovered from various wards and clinical samples. Among them, 14 (9.9%) were classified as carbapenem susceptible by use of the Vitek 2 automated system and were further investigated. The isolates were also susceptible to colistin and exhibited various profiles of susceptibility to other antimicrobials used to treat *A. baumannii* infections (Table 1). PFGE analysis discriminated nine distinct genotypes among the 14 study isolates. For each clinical isolate, the native and the meropenem-heteroresistant populations exhibited identical PFGE profiles. By ST, six isolates belonged to ST1, three belonged to ST2, and five exhibited various allelic profiles that were not assigned a separate ST because they have not yet been found among outbreak strains (Table 2). PCR for genes encoding known carbapenemases (except the intrinsic *bla* _OXA-51-like_ carbapenemase) was negative. Nucleotide sequencing showed that the *bla* _OXA-66_ and *bla* _OXA-68_ alleles were more common, as anticipated, according to a previous nationwide study (8). No IS _Ab_ _A1_ elements were detected upstream of the gene *bla* _OXA-51-like_ carbapenemase.

**Time-kill studies.** In time-kill studies with meropenem, four isolates (isolates AB5, AB13, AB79, and AB135) were killed in a time-dependent manner. For the remaining isolates, the ini-
tial bactericidal effect was followed by substantial regrowth, which was observed after 9 h to 12 h of incubation for three isolates (isolates AB27, AB49, and AB68) and after 24 h for seven isolates (isolates AB32, AB71, AB72, AB78, AB119, AB129, and AB133). The survival curves obtained from time-kill studies are shown in Fig. 1.

In vitro characterization of the heteroresistant subpopulations. The agar dilution MICs of imipenem for the clinical isolates ranged from 0.25 to 4 mg/liter. Population analysis assays with imipenem showed that colonies grew in the presence of 8 mg/liter for 3 isolates, whereas the 11 isolates that remained were within the susceptibility range. However, the heterogeneous growth in the presence of imipenem was not stable; after seven daily subcultures in drug-free medium, the imipenem MICs for the colonies grown in the presence of the highest imipenem concentration were similar to those for the native isolates (Table 2).

The agar dilution MICs of meropenem for the clinical isolates ranged from 0.25 to 4 mg/liter. Population analysis assays with meropenem revealed colonies that grew in the presence of 8 to 32 mg/liter for 13 isolates and in the presence of up to 2 mg/liter for isolate AB13 (Table 2; Fig. 2). The heteroresistant colonies showed stable meropenem resistance when they were retested by agar dilution after seven daily subcultures in drug-free medium but showed susceptibility to imipenem (Table 2). The frequency of meropenem-heteroresistant subpopulations, as calculated by the population analysis assays, ranged from $3 \times 10^{-7}$ to $5 \times 10^{-5}$. The antibiotic resistance profiles of most of the heteroresistant colonies did not exhibit considerable differences from the profiles of the native isolates for aminoglycosides, fluoroquinolones, and beta-lactam antibiotics; three isolates, however, acquired resistance to ampicillin-sulbactam, and one isolate also acquired resistance to piperacillin-tazobactam (Table 1).

**DISCUSSION**

During the last few years, *A. baumannii* isolates that exhibit carbapenem resistance have increasingly been isolated and pose substantial therapeutic problems in many regions, including the United States (6, 20). A further worrisome observation is heteroresistance to carbapenems, which may have implications for the treatment of multiresistant *A. baumannii* infections. Heteroresistance to meropenem was first detected in...
methicillin ( meticillin)-resistant staphylococci (9). The existence of heteroresistant phenotypes in A. baumannii was observed previously (15) but was not substantiated in subsequent studies. We reported previously on clinical A. baumannii isolates that exhibited subpopulations that grew within the inhibition halos around carbapenem disks and Etest strips (17). However, that study mainly included isolates with reduced carbapenem susceptibility or resistance.

In the present study, 14 apparently carbapenem-susceptible isolates have been investigated for heteroresistance by population analysis and time-killing assays. The isolates belonged to several PFGE types; to ST1 and ST2, which are associated with European clones II and I, respectively (27) and which were also previously detected among Greek A. baumannii (7); and to other unassigned types. By standard agar dilution MIC testing, all native isolates were susceptible to both imipenem and meropenem (MICs ≤ 4 mg/liter). Population analyses revealed carbapenem-heteroresistant subpopulations that grew in the presence of concentrations that were 2- to 16-fold higher than the MICs for the respective native populations and, in most cases, that exceeded the CLSI susceptibility breakpoints. However, after seven daily subcultures in antibiotic-free medium, the colonies that grew in the presence of the highest imipenem concentration exhibited carbapenem MICs that were similar to those of the native isolates. It could be postulated that growth in the presence of higher imipenem concentrations might be attributed to the heavy bacterial inocula used in the population analyses and/or to imipenem instability (26). On the contrary, when colonies grown in the presence of the highest meropenem concentration were retested, the meropenem MICs were considerably higher than those of the native isolates. This observation implies that meropenem pressure in A. baumannii may induce resistance mechanisms that allow growth in the presence of higher concentrations. Meropenem bactericidal assays revealed a less pronounced killing that was followed by a substantial regrowth for 10 isolates. This regrowth could be due to the fact that a proportion of the meropenem-heteroresistant subpopulations survived the meropenem exposure. Nevertheless, three isolates that exhibited heteroresistant subpopulations (isolates AB5, AB79, and AB135) and the non-heteroresistant AB13 isolate did not show regrowth and were killed in a time-dependent manner. It should be noted that the meropenem heteroresistance of the study isolates observed involves many unrelated A. baumannii genotypes and is not due to the spread of one or a few A. baumannii lineages.

The aminoglycoside and fluoroquinolone resistance profiles of the heteroresistant colonies were similar to those of the native isolates, indicating that efflux pump overexpression might not contribute to the observed meropenem heteroresistance, while three isolates became resistant to beta-lactam-beta-lactamase inhibitor combinations, and this possibly may be attributed to AmpC overproduction. However, in most cases the possible mechanism responsible for meropenem heteroresistance could not be hypothesized from the antibiotic

**FIG. 2.** Population analysis assays for meropenem. The results for isolate AB13 are kept in each graph for comparison.
resistance profiles; the underlying mechanisms are currently under investigation.

The medical records of patients treated with meropenem showed that six patients were given meropenem for at least 1 week and that three of them died from an A. baumannii bloodstream infection while they were receiving meropenem (data not shown). A relevant publication by Nunez et al. described a case of fatal Acinetobacter meningitis (13). In that case, nine clonally related strains were isolated from cerebrospinal fluid; the first eight strains were carbapenem susceptible, but the ninth one was meropenem resistant (MIC > 32 mg/liter) and still imipenem susceptible. The meropenem-resistant strain was isolated after two courses of meropenem treatment. This observation is in accordance with our findings, suggesting a resistance mechanism that affects meropenem but not imipenem and that might result from meropenem pressure.

Conclusion. The observations of the present study suggest that A. baumannii isolates that are apparently meropenem susceptible by standard susceptibility testing may contain a certain amount of meropenem-resistant subpopulations, and those subpopulations could be selected for by the use of suboptimal therapeutic drug dosages. In this context, the implementation of screening techniques for the identification of heteroresistant isolates and the elucidation of the underlying molecular mechanisms would be of significant importance.

REFERENCES