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Colistin Heteroresistance in *Acinetobacter* and Its Association with Previous Colistin Therapy▽

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Colistin heteroresistance has been reported among *Acinetobacter* isolates; however, its association with prior colistin therapy has not been not described. A population analysis profile identified resistant *Acinetobacter* subpopulations from colistin-susceptible clinical isolates. The proportion of cells exhibiting heteroresistance was significantly higher among isolates recovered from patients treated with colistin.

Therapeutic options for serious infections caused by *Acinetobacter baumannii-calcoaceticus* complex (ABC) are limited by intrinsic and acquired resistance. In a population of casualties returning from Iraq and Afghanistan, the most effective agents were colistin and minocycline (6). In the United States, minocycline is not available in an intravenous formulation, leaving colistin as the only agent available for some seriously ill patients. A recent report of heteroresistance to colistin among ABC isolates from Australia is of concern due to the potential implications for therapeutic failures (7). Whether previous colistin exposure contributed to the development of ABC heteroresistance was not addressed in that report. In the present study, we assessed multidrug-resistant (MDR) ABC isolates for heteroresistance to colistin from patients with or without prior colistin exposure.

We selected a convenience sample of 19 different patient's MDR ABC isolates with previously determined susceptibility to colistin (MIC of ≤1 μg/ml) from among ABC isolates retained by our clinical microbiology laboratory from October 2003 to November 2005. MDR was defined as susceptibility to two or fewer antimicrobial agent classes. Seven of the isolates (study isolates) were from patients who had previously been treated with colistin (colistimethate sodium) for their ABC infection. Twelve control isolates were selected from patients without any prior colistin exposure. Isolates were identified as ABC using the Vitek system (bioMerieux, Inc., Durham, NC). Broth microdilution MIC testing using in-lab prepared frozen panels was performed according to the Clinical and Laboratory Standards Institute reference susceptibility method with cation-adjusted Mueller-Hinton broth, a standard inoculum density of 5×10^5 CFU/ml, and incubation at 35°C for 20 to 24 h (3). Panels contained colistin sulfate, polymyxin B, tigecycline, minocycline, doxycycline, ceftazidime, ticarcillin-clavulanate, ampicillin-sulbactam, sulbactam, imipenem, gentamicin, and amikacin. The quality control organisms *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 were tested simultaneously with patient isolates and always provided MICs within acceptable limits (4).

Colistin sulfate (Sigma, St. Louis, MO) was added to molten Mueller-Hinton agar (Difco/BD, Sparks, MD) to produce plates with colistin concentrations of 1, 2, and 8 μg/ml. Frozen ABC isolates were subcultured twice on sheep blood agar prior to preparation of a suspension in 0.9% saline equivalent to approximately a no. 5 McFarland standard. An aliquot of 100 μl of the adjusted inoculum suspension of each isolate was used to inoculate each of two 1-, 2-, and 8-μg/ml colistin plates. To determine the actual inoculum delivered to each plate, 100 μl of a $1{:}10^6$ dilution of the inoculum suspension was spread onto duplicate sheep blood agar plates to determine colony counts. To exclude bias due to high inoculum, 2-, 4-, and 8-μg/ml colistin plates were subsequently inoculated using 1:10 and 1:100 dilutions of the initial inoculum, and colony counts were compared to the undiluted inoculum. Plates were incubated at 35°C for 20 to 24 h prior to counting colonies. Colonies recovered from the 8-μg/ml colistin plates were subcultured onto sheep blood agar plates and passaged twice prior to again determining the colistin MIC. Heteroresistance was defined by growth of ABC colonies on plates containing 8 μg of colistin/ml, with confirmation of an MIC of >8 μg/ml by subsequent broth microdilution (BMD) testing. Statistical analysis was performed by using mean colony count values from the duplicate plates for each isolate. The colistin-exposed and nonexposed groups were compared with the Mann-Whitney U test using SPSS 11.5.1 (SPSS, Inc., Chicago, IL).

Total colistin exposure varied among study isolates, with total cumulative patients' doses ranging from 325 to 900 mg (Table 1). The number of days from the last dose until recovery of the study isolates ranged from 0 to 18 days. Colistin heteroresistance was demonstrated in all study and control ABC isolates. However, the mean numbers of colonies that grew on plates with colistin at 8 μg/ml were 307 for colistin-exposed isolates ($n = 7$) and 97 for nonexposed isolates ($n = 12$), values equivalent to 2.11 CFU (range, 0.72 to 2.88 [standard deviation = 0.76]) per million CFU of ABC plated (0.000211%) and 0.53 CFU (range, 0.07 to 0.96 [standard deviation = 0.34]) per million CFU of ABC plated (0.000053%), respectively ($P < 0.001$). The numbers of colonies growing on plates containing 2 and 4 μg of colistin/ml were too numerous to count for all

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TABLE 1. Prior colistin treatment of patients who contributed study isolates

Isolate	Cumulative dose (mg of colistin base activity)	No. of doses	No. of days since last dose
1	900	7	0
2	325	2	12
3	325	2	18
4	260	2	13
5	375	3	2
6	450	4	4
7	450	4	1

isolates. There was no significant difference between the proportion of heteroresistant colonies recovered using undiluted inoculum versus 1:10 and 1:100 dilutions of the inoculum suspension (data not shown), excluding inoculum effect as the cause of our findings. When colonies recovered on plates with colistin at 8 μg/ml were passaged twice on sheep blood plates and then tested for antimicrobial susceptibilities, all isolates from both control and study groups demonstrated colistin MICs of >16 μg/ml. There was no significant difference between the two isolate groups with regard to the mean MICs of the other tested antibiotics, except for sulbactam ($P = 0.002$) and gentamicin ($P = 0.001$), which demonstrated greater susceptibility in the colistin-exposed group. Pulsed-field gel electrophoresis typing after digestion with ApaI revealed seven different clones within the 19 isolates when the banding patterns were analyzed using BioNumerics software (Applied Maths). Within some of the seven unique clones, there were isolates from both patients who were and patients who were not previously exposed to colistin.

Due to the frequent finding of multidrug resistance, *Acinetobacter* is considered a pathogen of particular concern by the Infectious Diseases Society of America (10). Although the impact of ABC infection upon mortality is controversial, it remains an important cause of morbidity among seriously ill patients (1, 5). Colistin is one agent reliably active against many isolates (6). Heteroresistance to colistin among *Acinetobacter* isolates has been previously described in one report (7), and preliminary in vitro data indicate that the emergence of heteroresistant populations may be amplified by colistin exposure (11). The relationship of such heteroresistance to prior colistin exposure in patients has not yet been described. We have extended those initial observations by testing isolates from patients treated with colistin and a control group of patients without colistin exposure. We have demonstrated by using pulsed-field gel electrophoresis that these isolates were from multiple lineages and not a single clone. We found a statistically significantly higher degree of heteroresistance among isolates from patients with previous colistin treatment. The proportion of resistant subpopulations in this study (0.000211% for study isolates and 0.000053% for control isolates) was slightly higher than that reported in the previous study of heteroresistance (0.00001 to 0.000001%) (7). Due to the small number of isolates available from colistin-treated patients, we did not attempt to correlate the dose, duration, or timing of colistin exposure with the degree of heteroresistance.

It is a matter of conern that the resistant subpopulations isolated during the population analysis profile demonstrated stable colistin MICs exceeding 16 μg/ml, which is above the peak serum levels achievable at standard doses (8) and well above the current definition of colistin resistance in *Acinetobacter* (4). It is possible that the heteroresistant proportion of the bacterial population might be selected and become predominant during colistin therapy, leading to treatment failure. However, the clinical relevance of colistin heteroresistance is unproven at this time. These findings suggest that careful monitoring for emerging resistance during prolonged colistin therapy is warranted.

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