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Heteroresistance to Colistin in Multidrug-Resistant *Acinetobacter baumannii*

Jian Li,[1]* Craig R. Rayner,[1] Roger L. Nation,[1] Roxanne J. Owen,[1] Denis Spelman,[2] Kar Eng Tan,[1] and Lisa Liolios[2]

Facility for Anti-infective Drug Development and Innovation, Victorian College of Pharmacy, Monash University, Parkville, Victoria, Australia,[1] and Department of Microbiology and Infectious Diseases Unit, Alfred Hospital, Melbourne, Victoria, Australia[2]

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Multidrug-resistant *Acinetobacter baumannii* has emerged as a significant clinical problem worldwide and colistin is being used increasingly as "salvage" therapy. MICs of colistin against *A. baumannii* indicate its significant activity. However, resistance to colistin in *A. baumannii* has been reported recently. Clonotypes of 16 clinical *A. baumannii* isolates and ATCC 19606 were determined by pulsed-field gel electrophoresis (PFGE), and colistin MICs were measured. The time-kill kinetics of colistin against *A. baumannii* ATCC 19606 and clinical isolate 6 were investigated, and population analysis profiles (PAPs) were conducted. Resistance development was investigated by serial passaging with or without exposure to colistin. Five different PFGE banding patterns were found in the clinical isolates. MICs of colistin against all isolates were within 0.25 to 2 μg/ml. Colistin showed early concentration-dependent killing, but bacterial regrowth was observed at 24 h. PAPs revealed that heteroresistance to colistin occurred in 15 of the 16 clinical isolates. Subpopulations (<0.1% from inocula of 10^8 to 10^9 CFU/ml) of ATCC 19606, and most clinical isolates grew in the presence of colistin 3 to 10 μg/ml. Four successive passages of ATCC 19606 in broth containing colistin (up to 200 μg/ml) substantially increased the proportion of the resistant subpopulations able to grow in the presence of colistin at 10 μg/ml from 0.000023 to 100%; even after 16 passages in colistin-free broth, the proportion only decreased to 2.1%. This represents the first demonstration of heterogeneous colistin-resistant *A. baumannii* in "colistin-susceptible" clinical isolates. Our findings give a strong warning that colistin-resistant *A. baumannii* may be observed more frequently due to potential suboptimal dosage regimens recommended in the product information of some products of colistin methanesulfonate.

The emergence of multidrug-resistant gram-negative bacteria (*Pseudomonas*, *Acinetobacter*, *Klebsiella*, *Enterobacter*, and *Salmonella* species) is a threat to modern medicine and has been recognized worldwide (15, 17, 26, 37). Unfortunately, there simply are not enough new drugs in the pharmaceutical pipeline to keep pace with drug-resistant bacterial infections, the so-called "superbugs" (15). In particular, resistance to almost all antibiotics in some species of the genera *Acinetobacter* and *Pseudomonas* is a critical challenge (27). The number of multidrug-resistant *Acinetobacter baumannii*, which is an opportunistic pathogen that may cause pneumonia, bacteremia, infection in burn wounds, meningitis, and urinary tract infections, has been on the increase globally in the past 5 years, and it is now regarded as one of the most difficult nosocomially acquired gram-negative pathogens to treat and control (16, 23, 26, 27). Furthermore, patients infected with *A. baumannii* are often already critically ill or immunocompromised, and the associated mortality is significant (3, 9).

In the past 5 years, interest has been rekindled in the "old" polymyxin antibiotic, colistin (polymyxin E), for the treatment of infections caused by multidrug-resistant gram-negative bacteria owing to its favorable properties of rapid bacterial killing, a narrow spectrum of activity, and an associated slow development of resistance (7, 8, 23). Recent reports suggest that the resistance to carbapenems is increasing (5, 12, 28) and, since tigecycline has not been registered in many countries, colistin is often the only effective antibiotic against multidrug-resistant *A. baumannii*, particularly in intensive care units (18, 23, 29, 30, 35). In a recent study, different biotypes of *A. baumannii* displayed statistically different MICs against imipenem and meropenem; however, colistin has demonstrated apparent significant activities against all of the tested isolates, with MICs at which 50 and 90% of the isolates are inhibited of 0.5 and 1 μg/ml, respectively (6). Unfortunately, our knowledge on the pharmacokinetics and pharmacodynamics of colistin is limited, and the current dosage regimens used clinically are based on experience acquired up to 30 years ago (23). It is crucial to administer colistin in dosage regimens such that it exhibits maximal activity and with minimal potential for the development of resistance. Very worryingly, resistance to colistin in *A. baumannii* has been reported recently (1; J. Gilad, S. Eskira, K. Riesenberg, F. Schlaeffer, E. Hyam, and A. Borer, Abstr. 45th Intersci. Conf. Antimicrob. Agents Chemother., abstr. K-1292, 2005). We report here on the heteroresistance to colistin in *A. baumannii* clinical isolates that were susceptible to colistin on the basis of the MICs.

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MATERIALS AND METHODS

Bacterial strains. ATCC 19606 was purchased from the American Type Culture Collection (Manassas, VA). Sixteen clinical isolates from 16 patients at the Alfred Hospital (Prahran, Victoria, Australia) were used. These isolates were from sputum, nasal aspirate, wound, blood, urine, and bronchoalveolar lavage samples and were randomly selected from the collection between 12 December 2002 and 17 November

* Corresponding author. Mailing address: Facility for Anti-infective Drug Development and Innovation, Victorian College of Pharmacy, Monash University, 381 Royal Parade, Parkville, Victoria 3052, Australia. Phone: 61 3 9903 9702. Fax: 61 3 9903 9629. E-mail: Jian.Li@vcp.monash.edu.au.

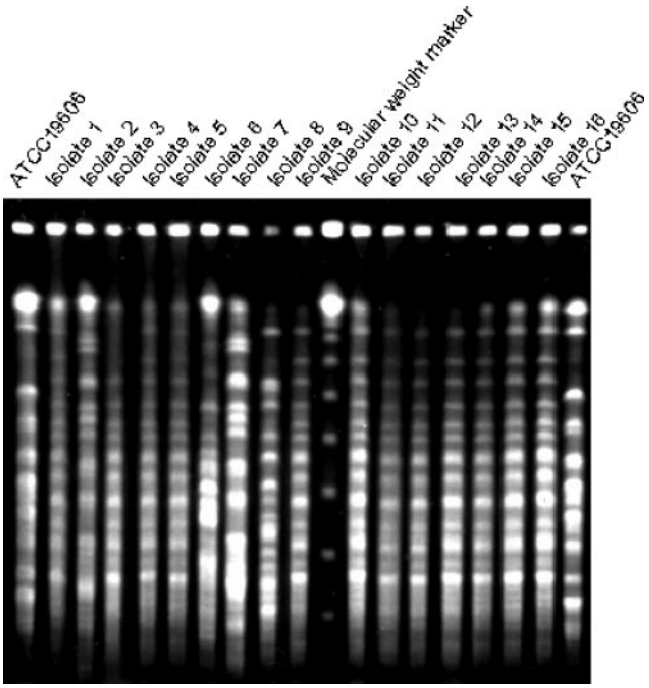

FIG. 1. PFGE profiles of ATCC 19606 and the clinical isolates digested with ApaI.

2004. Of the 16 isolates, 14 were from patients after admission to the intensive care unit. *A. baumannii* was identified by VITEK GNI+ cards (bioMérieux). Using the VITEK card (GNS-424; bioMérieux), the following susceptibilities to antibiotics were determined: 100% were resistant to amoxicillin, cephalothin, and amoxicillin-clavulanic acid; 75% were resistant to cefotaxime; 68.8% were resistant to gentamicin; 68.8% were intermediate resistant to meropenem; and 62.5% were resistant to cotrimoxazole and ticarcillin-clavulanic acid. Isolates were stored at −80°C before the investigations described here.

Genotyping. Clonotypes of the clinical isolates and ATCC 19606 were investigated by pulsed-field gel electrophoresis (PFGE) (34) at the Microbiological Diagnosis Unit, University of Melbourne (Parkville, Australia). The band patterns were interpreted according to the criteria suggested by Tenover et al. (36).

Susceptibility tests. MICs were determined using colistin sulfate (Lot123K1382; Sigma-Aldrich, Castle Hill, New South Wales, Australia) by broth microdilution in cation-adjusted Mueller-Hinton broth (CAMHB; Oxoid Australia, West Heidelberg, Victoria, Australia) according to Clinical and Laboratory Standards Institute standards (4, 31).

Time-killing kinetics. The time-killing kinetics of ATCC 19606 and one clinical isolate (isolate 6) by colistin (sulfate, MIC 1 μg/ml) were examined. The antibiotic was added to a logarithmic-phase broth culture of ca. 10^6 CFU/ml to yield concentrations of 0, 0.5, 1, 2, 4, 8, 16, 32, and 64× MIC of the isolate. Viable counting was performed on samples collected at 0, 20, 40, and 60 min and 2, 3, 4, 6, and 24 h after antibiotic addition. After appropriate dilutions with saline, samples of bacterial cell suspension (50 μl) were spirally plated on nutrient agar plates (Medium Preparation Unit, University of Melbourne) by using a Whitley automatic spiral plater (WASP; Don Whitley Scientific, West Yorkshire, United Kingdom). Colonies were counted by a ProtoCOL automated colony counter (Synbiosis, Cambridge, United Kingdom) after the incubation of subcultures for 24 h at 35°C. The lower limit of counting was 20 CFU/ml.

PAPs. Analysis of hetero-colistin-resistant subpopulations of bacteria by population analysis profiles (PAPs) was conducted in three replicates by spiral plating 50 μl of the starting bacterial cell suspension (full 24-h cultures) and/or its serial saline dilutions on Mueller-Hinton agar plates (Medium Preparation Unit) without or with various concentrations (0.5, 1, 2, 3, 4, 5, 6, 8, and 10 μg/ml) of colistin sulfate. Colonies were counted (as described above) after 48 h of incubation at 35°C. The limit of counting was 20 CFU/ml. MICs of colistin (sulfate) against these subpopulations (from PAP plates) were determined (as described above).

Serial passaging. On day 1, 10 ml of CAMHB containing colistin sulfate at 8× MIC was inoculated with ATCC 19606 (MIC = 1 μg/ml) or isolate 6 (MIC = 1 μg/ml) from a single colony (passage 1). Cultures were incubated at 37°C in a shaking water bath (100 rpm). On day 3, 0.1 ml of the culture was transferred into 10 ml of CAMHB containing colistin sulfate at 64× MIC (passage 2), and cultures were incubated (see above). On day 4, 0.1 ml of the culture of ATCC 19606 was transferred into CAMHB containing colistin sulfate at 200× MIC (passage 3), and cultures were incubated. This passaging was repeated for ATCC 19606 on day 5 (passage 4). Due to the lack of visible growth, passaging of isolate 6 was not conducted in CAMHB containing 200 μg of colistin sulfate/ml on days 4 and 5. On day 6, cultures (5 ml) of ATCC 19606 and isolate 6 were centrifuged (3,000 × *g* for 10 min at 4°C) and washed twice with 10 ml of saline before being inoculated into 10 ml of CAMHB. Daily passaging in colistin-free CAMHB (see above) continued for 16 days for both isolates. The subpopulation able to grow on PAP plates containing 10 μg of colistin sulfate/ml was determined at baseline (passage 0, before exposure to colistin) and after passaging; the subpopulation was expressed as a percentage of the original population.

RESULTS

PFGE patterns of ATCC 19606 and the clinical isolates are shown in Fig. 1. They belong to six different clonotypes: group I (ATCC 19606), group II (isolates 1, 4, 5, and 9 to 16), group III (isolate 3), group IV (isolates 2 and 7), group V (isolate 6), and group VI (isolate 8). All isolates were susceptible to colistin, with MICs ranging from 0.25 to 2 μg/ml (median = 1 μg/ml). In the time-kill studies with ATCC 19606 and isolate 6,

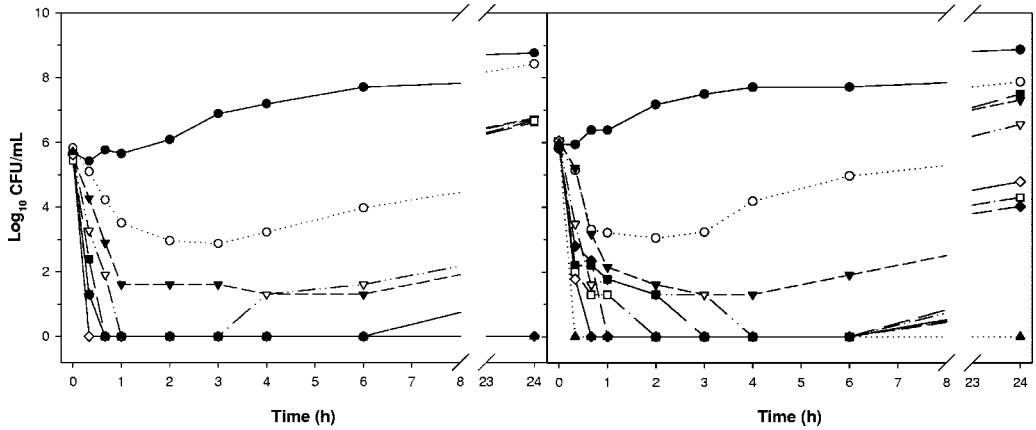

FIG. 2. Killing curves for ATCC 19606 (left panel) and isolate 6 (right panel) by colistin. Symbols: ●, control; ○, 0.5× MIC; ▼, 1× MIC; ▽, 2× MIC; ■, 4× MIC; □, 8× MIC; ◆, 16× MIC; ◇, 32× MIC; ▲, 64× MIC.

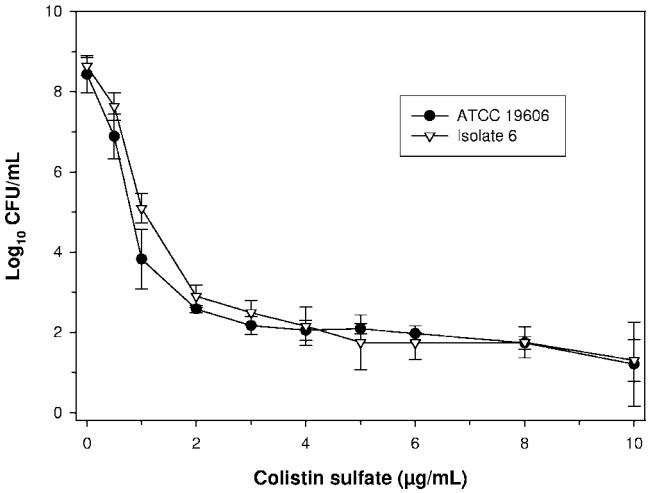

FIG. 3. PAPs of ATCC 19606 and isolate 6 (n = 3).

colistin was bactericidal in a concentration-dependent manner (Fig. 2). With colistin (MIC = 1 μg/ml for both strains), >99% of bacteria were killed in 2 h even at 0.5× MIC (Fig. 2). There was substantial regrowth at 24 h at concentrations up to 32× MIC for both isolates, but there was no regrowth at 64× MIC (Fig. 2).

Figure 3 shows the PAPs of ATCC 19606 and isolate 6 (n = 3). Both the reference strain and the clinical isolate contained resistant subpopulations that grew in the presence of up to 10 μg of colistin (sulfate)/ml, even though both had an MIC of 1.0 μg/ml. The same phenomenon was also observed in most of the other isolates (Fig. 4). The proportion of resistant subpopulations that were able to grow in the presence of 10 μg of colistin/ml was on the order of 0.00001 to 0.000001% in most isolates except for isolates 1, 5, 10, and 11 (Fig. 3 and 4). Isolate 10 had no subpopulations able to grow in the presence of colistin (sulfate) at concentrations higher than 1 μg/ml. The

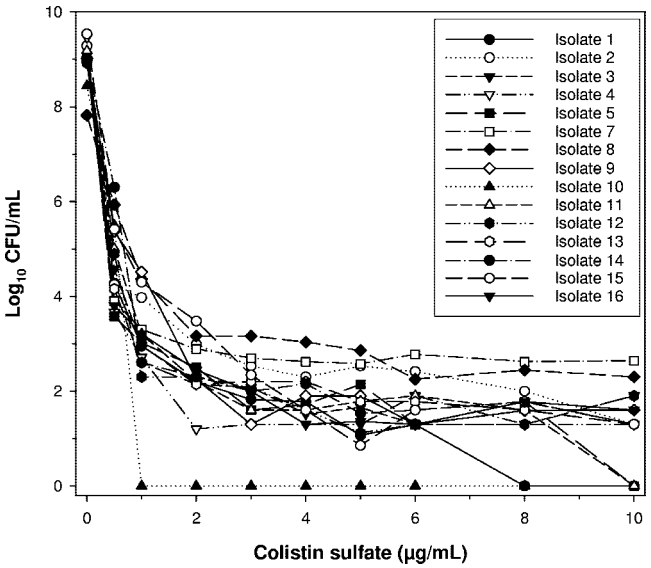

FIG. 4. PAPs of 15 clinical isolates (excluding isolate 6).

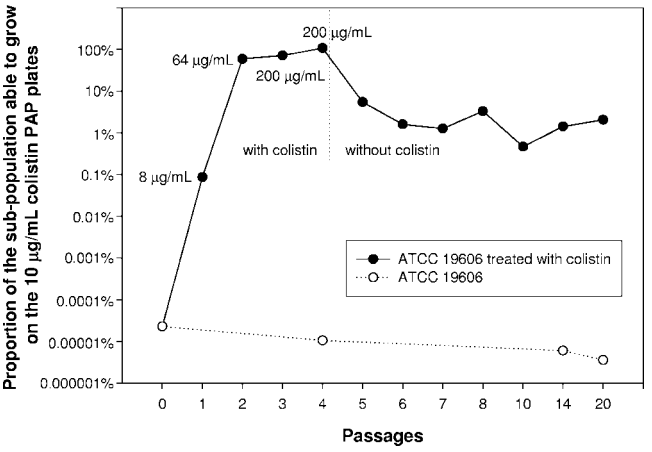

FIG. 5. Passage of ATCC 19606 in CAMHB with or without colistin.

MICs of subpopulations of ATCC 19606 and isolate 6 on colistin plates at 10 μg/ml were >128 and 32 μg/ml, respectively.

Passage of ATCC 19606 (Fig. 5) and isolate 6 in the presence of colistin (sulfate) resulted in a substantial change in the proportion of colistin-resistant subpopulations (based on the subpopulation able to grow on PAP plates with 10 μg of colistin/ml). Some subpopulations of colistin-exposed ATCC 19606 were able to grow in the presence of even 200 μg of colistin (sulfate)/ml; after only four passages in the presence of colistin (8 to 200 μg/ml), the proportion of the subpopulation able to grow on the PAP plates with 10 μg of colistin/ml increased dramatically from 0.000023 to ca. 100%. Even after 16 successive passages in colistin-free CAMHB, the proportion of the subpopulation able to grow on PAP plates with 10 μg of colistin/ml dropped to only 2.1%, far higher than the proportion (0.000023%) in ATCC 19606 without exposure to colistin (Fig. 5). However, the proportion of the subpopulation able to grow on the 10-μg/ml colistin PAP plates in the control without exposure to colistin did not increase after serial passaging (Fig. 5). With clinical isolate 6, after passage in CAMHB containing 64 μg of colistin sulfate/ml, there was no visible growth after 3 days; however, 2,720 CFU/ml were quantified. Similar to ATCC 19606, after 16 successive passages in drug-free CAMHB, the proportion of the subpopulation able to grow on PAP plates with 10 μg of colistin/ml was 3.0% compared to the original value of 0.000012% before colistin exposure.

DISCUSSION

There are two forms of colistin available commercially: colistin (sulfate) mainly for topical use and colistin methanesulfonate (sodium) for parenteral use. Colistin methanesulfonate is less toxic than colistin (23). Colistin is formed in vitro (20) and in vivo after the administration of colistin methanesulfonate (19, 21, 24). The clinical applications, pharmacokinetics, and pharmacodynamics of these two forms are different (19, 21–25), and colistin methanesulfonate is a nonactive pro-drug (2); therefore, only colistin was used in the present study.

The Clinical and Laboratory Standards Institute has selected an MIC of ≤2 μg/ml as susceptible and an MIC of ≥4 μg/ml

as resistant for colistin (4). All clinical isolates and ATCC 19606 were susceptible to colistin based on the MICs (≤2 μg/ml). This was also demonstrated by the rapid initial killing (<2 h) in the time-kill studies (Fig. 2). In contrast to our previous report on the in vitro pharmacodynamics of colistin against multidrug-resistant *P. aeruginosa* (25), there was significant regrowth at 24 h (Fig. 2). Further investigation using PAPs led to the discovery of heteroresistance to colistin in most of the clinical *A. baumannii* isolates (Fig. 3 and 4). Considering the relative magnitude of the MICs and the total plasma concentrations of colistin typically achieved (0.5 to 3.5 μg/ml) after intravenous administration of colistin methanesulfonate in patients (19, 24), concentrations of colistin (sulfate) in the PAP studies were selected from 0.5 to 10 μg/ml. These resistant subpopulations were assumed to contribute to the significant regrowth in the time-kill studies (Fig. 2) and the rapid development of resistance in the passaging studies (Fig. 5). Case histories showed that the patients from whom the isolates in the present study were obtained had not been exposed previously to colistin, since it was only recently introduced in this hospital due to infections caused by multidrug-resistant *A. baumannii*; it is never used by inhalation or for prophylaxis. Thus, the heteroresistance observed in the present study is unlikely to be related to previous exposure to colistin.

Most knowledge on heteroresistance to antibiotics concerns vancomycin in *Staphylococcus aureus* (13, 14). The existence of aminoglycoside-resistant subpopulations has been reported in "aminoglycoside-susceptible" *P. aeruginosa* (10, 11). Very limited investigations have been conducted for other bacteria (33). Recently, by using E-test, heteroresistance to carbapenems was reported in *A. baumannii*, and it is believed to be the cause of the spreading of carbapenem-resistant *A. baumannii* in Greece (32). Hetero-colistin-resistant *A. baumannii* has not been reported previously, possibly because commercial automated systems and the disk diffusion susceptibility test, most commonly used in clinical laboratories, cannot detect these subpopulations. The present study clearly demonstrates that hetero-colistin-resistant *A. baumannii* cannot be discriminated from colistin-susceptible *A. baumannii* by MIC measurement alone.

Considering the substantially decreased proportion of the colistin-resistant subpopulations after passage in drug-free broth (Fig. 5), it is very likely that they were not stable mutators. Interestingly, the emergence of nosocomial colistin-resistant *A. baumannii* was reported recently in Israel, and there was a strong correlation between colistin methanesulfonate use and resistance development over the period 2001 to 2004 (J. Gilad, S. Eskira, K. Riesenberg, F. Schlaeffer, E. Hyam, and A. Borer, Abstr. 45th Intersci. Conf. Antimicrob. Agents Chemother., abstr. K-1292, 2005). Hetero-colistin-resistant *A. baumannii* may be a preliminary stage that leads to the proliferation of resistant subpopulations upon exposure to colistin. The substantial increase in the proportion of resistant subpopulations after exposure to colistin in the present investigations indicates the importance of eradicating the more resistant subpopulations at a very early stage of therapy. Since the achieved concentrations of colistin (0.5 to 3.5 μg/ml) in plasma in patients with the recommended dosage regimens of colistin methanesulfonate (19, 24) may be substantially lower than those required to eradicate the more resistant subpopulations, our results suggest potential risk when colistin methanesulfonate monotherapy is used for infections caused by multidrug-resistant *A. baumannii*.

In conclusion, colistin is believed to be very active against multidrug-resistant *A. baumannii* based on MICs and has been used more frequently worldwide in recent years (23). However, currently recommended dosage regimens for colistin methanesulfonate (see information on Colomycin injection [summary of product characteristics; Forest Laboratories United Kingdom, Ltd.], Coly-Mycin M parenteral package insert [Monarch Pharmaceuticals, Bristol, TN], and Coly-Mycin M parenteral package insert [Link Medical Products Pty, Ltd., Mosman, New South Wales, Australia]) are based on experience gained at least three decades ago (23). In the present study we have demonstrated, for the first time, heteroresistance to colistin in clinical isolates of *A. baumannii* that were apparently susceptible to colistin on the basis of MICs. The detection of hetero-colistin-resistant *A. baumannii* in the clinical isolates provides a strong warning that if colistin or colistin methanesulfonate, which is often the last-line antibiotic, is used inappropriately (24), there may be substantial potential for the rapid development of resistance and therapeutic failure. Moreover, our study suggests that monotherapy with colistin methanesulfonate for treatment of infections caused by hetero-colistin-resistant *A. baumannii* may be problematic. We believe that resistance to this "last resort" antibiotic should be monitored in global and local surveillance programs and that every effort should be made to increase its life span by ensuring that it is used judiciously and in appropriate dosage regimens.

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