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Carbapenem-Resistant *Klebsiella pneumoniae* Exhibiting Clinically Undetected Colistin Heteroresistance Leads to Treatment Failure in a Murine Model of Infection

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ABSTRACT Antibiotic resistance is a growing crisis and a grave threat to human health. It is projected that antibiotic-resistant infections will lead to 10 million annual deaths worldwide by the year 2050. Among the most significant threats are carbapenem-resistant *Enterobacteriaceae* (CRE), including carbapenem-resistant *Klebsiella pneumoniae* (CRKP), which lead to mortality rates as high as 40 to 50%. Few treatment options are available to treat CRKP, and the polymyxin antibiotic colistin is often the "last-line" therapy. However, resistance to colistin is increasing. Here, we identify multidrug-resistant, carbapenemase-positive CRKP isolates that were classified as susceptible to colistin by clinical diagnostics yet harbored a minor subpopulation of phenotypically resistant cells. Within these isolates, the resistant subpopulation became predominant after growth in the presence of colistin but returned to baseline levels after subsequent culture in antibiotic-free media. This indicates that the resistance was phenotypic, rather than due to a genetic mutation, consistent with heteroresistance. Importantly, colistin therapy was unable to rescue mice infected with the heteroresistant strains. These findings demonstrate that colistin heteroresistance may cause *in vivo* treatment failure during *K. pneumoniae* infection, threatening the use of colistin as a last-line treatment for CRKP. Furthermore, these data sound the alarm for use of caution in interpreting colistin susceptibility test results, as isolates identified as susceptible may in fact resist antibiotic therapy and lead to unexplained treatment failures.

IMPORTANCE This is the first report of colistin-heteroresistant *K. pneumoniae* in the United States. Two distinct isolates each led to colistin treatment failure in an *in vivo* model of infection. The data are worrisome, especially since the colistin heteroresistance was not detected by current diagnostic tests. As these isolates were carbapenem resistant, clinicians might turn to colistin as a last-line therapy for infections caused by such strains, not knowing that they in fact harbor a resistant subpopulation of cells, potentially leading to treatment failure. Our findings warn that colistin susceptibility testing results may be unreliable due to undetected heteroresistance and highlight the need for more accurate and sensitive diagnostics.

KEYWORDS *Klebsiella*, antibiotic resistance, clonal heteroresistance, colistin, heteroresistance

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Antibiotic resistance is an increasingly urgent problem, predicted to cause 10 million annual deaths worldwide by the year 2050 (1). *Klebsiella* spp., including *K. pneumoniae*, are responsible for ~10% of nosocomial infections in the United States (2),

including urinary tract, bloodstream, and soft tissue infections (3). Carbapenem-resistant *Klebsiella pneumoniae* (CRKP) is one of the carbapenem-resistant *Enterobacteriaceae* (CRE), an emerging cause of antibiotic-resistant, health care-associated infections. CRE were listed as one of the most urgent antibiotic resistance threats by the CDC and WHO (4, 5). In part due to the difficulty of effectively treating infections with CRE, mortality rates can be as high as 40 to 50% (6). These infections are a worldwide problem, with recent reports indicating that CRE are widespread in the United States (7), Europe (8), and China (9). Unfortunately, resistance to "last-line" drugs, such as colistin, is emerging in CRKP strains, and in some cases, isolates are resistant to all antibiotics tested (10). Here, we describe the identification of two multidrug-resistant CRKP isolates exhibiting colistin heteroresistance, a phenomenon in which only a minor subpopulation of genetically identical cells is phenotypically resistant. Since the frequency of the resistant subpopulation is exceedingly low in these isolates, they are not detected as being colistin resistant by clinical diagnostic tests.

Two CRKP urine isolates (Mu9 and Mu156) were collected from different patients in Atlanta, GA, area hospitals as part of the Multi-site Gram-Negative Surveillance Initiative (MuGSI), a nationwide surveillance network for CRE hospital isolates. Isolates were grown from single colonies and frozen at −80°C prior to the study. Mu9 and Mu156 were confirmed as being genetically distinct by pulsed-field gel electrophoresis (using Xbal-digested total DNA and separated by electrophoresis on a Chef-DR III apparatus [Bio-Rad Laboratories, Hercules, CA] at 200 V [6 V/cm] with a 90-s switch time for 23 h) (data not shown). Both isolates were resistant to nearly all antibiotics tested, including all carbapenems and some aminoglycosides (see Table S1 in the supplemental material). PCR for various resistance genes revealed several beta-lactamases in each isolate, including *Klebsiella pneumoniae* carbapenemase (KPC) in both strains (Table S2). Colistin susceptibility testing by broth microdilution (11) in cation-adjusted Mueller-Hinton (MH) broth (BD Biosciences, Franklin Lakes, NJ) using colistin sulfate (Sigma-Aldrich, St. Louis, MO) (MIC of 0.5 μg/ml) and the colistin Etest (BioMérieux, Marcy-l'Étoile, France), performed on MH agar (Remel, San Diego, CA) (MIC = 0.125), classified both strains as susceptible to colistin (Fig. 1A). Subsequent examination for susceptibility was performed via population analysis profile (PAP) by plating serial dilutions of bacteria on MH agar (BD Biosciences, Franklin Lakes, NJ) containing various concentrations of colistin. PAP revealed the presence of a minor colistin-resistant subpopulation in each isolate that actively grew on antibiotic up to a concentration of 100 μg/ml. The colistin-resistant subpopulation was present at between 1 in 1,000 and 1 in 1,000,000 CFU at doses of colistin ranging from 2 to 100 μg/ml (Fig. 1B). In contrast, PAP demonstrated that all the cells of a colistin-susceptible control isolate, GA65146, were killed by 2 μg/ml of colistin (Fig. 1B).

It was concerning that broth microdilution and the Etest were unable to detect the colistin-resistant subpopulations in these isolates. The recommended incubation time for both tests is 24 h (11, 12). Extension of the incubation time to 48 h resulted in the accurate identification of resistance by broth microdilution (Fig. S1A), likely because the minor resistant subpopulation had more time to grow out. In contrast, the increased incubation time had no effect on Etest results, which remained negative (Fig. S1B).

We next studied the dynamics of the resistant subpopulation following colistin treatment. After treatment with 100 μg/ml colistin, the frequency of the resistant subpopulation was significantly increased in each isolate. Subsequent passage in an antibiotic-free medium greatly decreased the frequency of the resistant subpopulation (Fig. S2), suggesting that this population was phenotypically resistant and not the result of a stable genetic mutation. Additionally, this suggests that there is some disadvantage to maintaining a majority colistin-resistant subpopulation. Indeed, it has been previously shown that colistin resistance in *K. pneumoniae* confers a fitness defect (13). To directly assess whether the resistant and susceptible subpopulations were genetically homogenous, we isolated cultures with majority resistant or susceptible subpopulations by subculturing them in medium containing 16 μg/ml colistin or drug-free medium, respectively (Fig. 1C). This resulted in cultures containing >95% colistin-
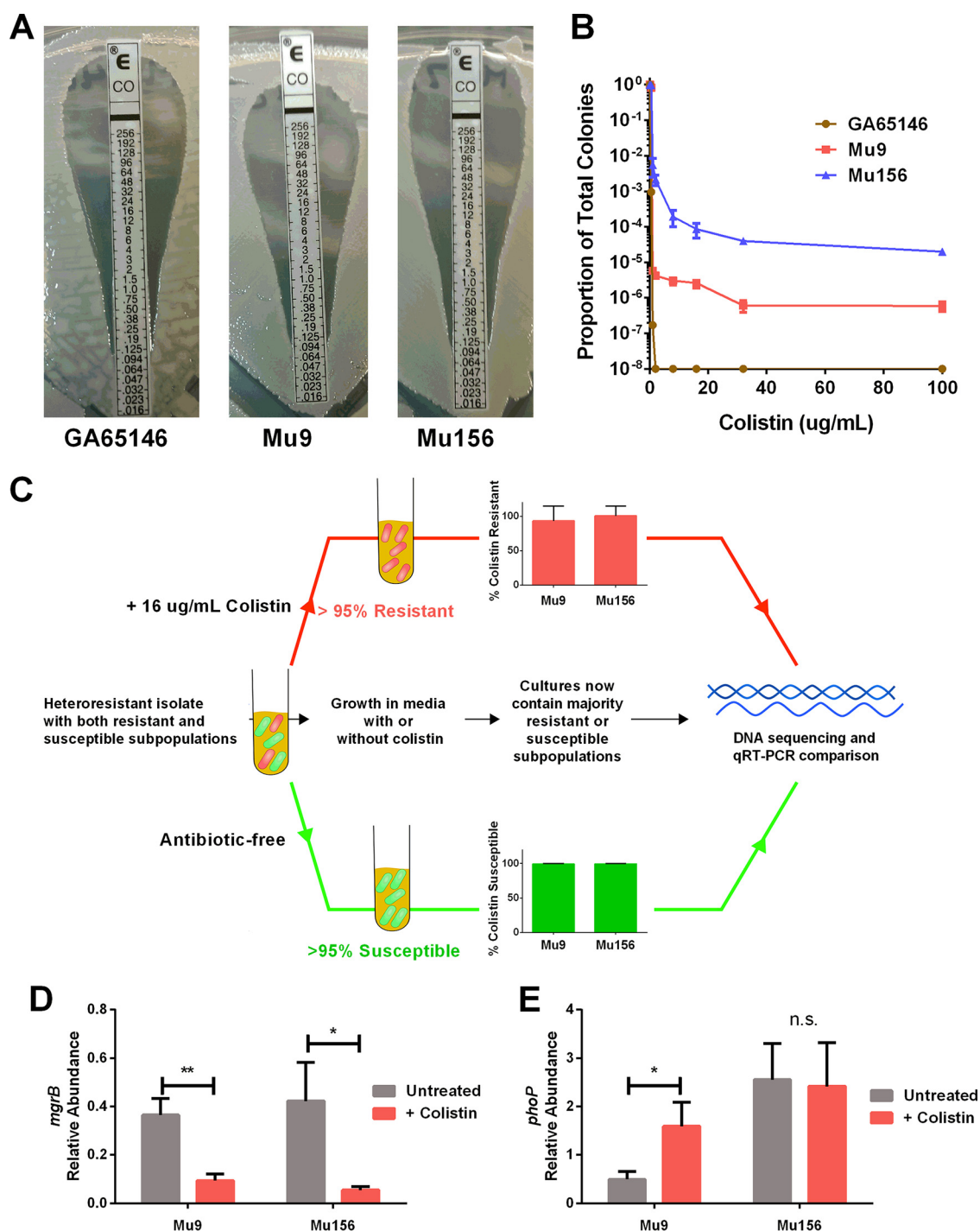
FIG 1 Carbapenem-resistant *Klebsiella pneumoniae* can harbor clinically undetected colistin-resistant subpopulations. (A) Colistin-susceptible isolate GA65146 and the colistin-heteroresistant isolates Mu9 and Mu156 were assayed for colistin resistance using the Etest (bioMérieux, Marcy-l'Étoile, France) method. The MIC is represented by the highest concentration along the strip at which bacteria grow. (B) Population analysis profile of GA65146, Mu9, and Mu156. The proportion of total colonies is the number of CFU able to grow at each concentration of colistin on solid medium divided by the number growing on medium without drug. Heteroresistant isolates exhibit a minor subpopulation that is able to grow on concentrations of colistin above 4 μg/ml. (C) Workflow for genomic and transcriptomic analysis of colistin-susceptible and -resistant subpopulations. Cultures of Mu9 or Mu156 were grown for 18 h in MH broth with or without colistin as indicated. (D and E) Quantitative real-time PCR (qRT-PCR) analysis of *mgrB* (D) and *phoP* (E) expression in resistant and susceptible subpopulations of Mu9 and Mu156. Resistant and susceptible subpopulations were enriched as shown in panel C. Relative abundance was calculated by normalizing the expression of each gene to the average expression of two housekeeping genes, *23S* and *rpsL* ($n = 6$). *, $P < 0.05$; **, $P < 0.01$; n.s., not significantly different (unpaired t test).

resistant cells or >95% colistin-susceptible cells (Fig. 1C). We then performed genomic sequencing on both populations using an Illumina HiSeq 4000 sequencer for a depth of coverage of >1,000×, revealing that the resistant and susceptible subpopulations were indeed genetically identical, consistent with heteroresistance.

To investigate the phenotypic differences between the resistant and susceptible subpopulations, we quantified the expression of two genes in the PhoPQ two-component system pathway, which is known to mediate colistin resistance in *K. pneumoniae*. MgrB is a negative regulator of PhoPQ signaling (14), and *mgrB* expression was lower in resistant cells cultured in colistin than in susceptible cells grown in drug-free medium (Fig. 1D). Additionally, expression of *phoP*, which is autoinduced when the PhoPQ system is active (15), was increased in colistin-resistant cells of Mu9 compared to its expression in susceptible cells, although this was not observed in Mu156 (Fig. 1E). Taken together, these data are consistent with involvement of the PhoPQ pathway in the resistant subpopulations of both Mu9 and Mu156.

It was unclear whether the minor colistin-resistant subpopulations present in these isolates would have an effect on the outcome of colistin treatment during an *in vivo* infection. To assess the *in vivo* relevance of the colistin-resistant subpopulations, we used a mouse model of peritonitis (done in accordance with IACUC protocol #4000046). We infected mice (C57BL/6J; Jackson Laboratory, Bar Harbor, ME) intraperitoneally with a lethal dose (3×10^8 CFU) of either of the heteroresistant *K. pneumoniae* isolates and subsequently left the mice untreated or treated them with colistin after 12 h (20 mg colistin methanesulfonate/kg of body weight [Chem Impex, Wood Dale, IL], given intraperitoneally every 6 h) to simulate infection and subsequent treatment upon clinical presentation. Interestingly, even in the absence of colistin, the frequency of the resistant subpopulations of both heteroresistant isolates increased following 24 h of *in vivo* infection compared to the frequency produced by the inoculum (Fig. 2A). This may be due to cross-resistance of these cells to host innate immune antimicrobials, such as antimicrobial peptides and reactive oxygen species, as has previously been demonstrated (16). We next assessed the impact of heteroresistance on colistin treatment outcome. Mice infected with the colistin-susceptible strain (GA65146) succumbed to infection in the absence of antibiotic but were rescued by colistin treatment (Fig. 2B). In contrast, mice infected with either of the heteroresistant isolates (Mu9 or Mu156) were unable to survive the infection, even in the presence of colistin (Fig. 2C and D). These data strikingly demonstrate that colistin heteroresistance can lead to *in vivo* colistin treatment failure for CRKP.

Concluding remarks. This is the first report of colistin-heteroresistant *K. pneumoniae* in the United States. In highly resistant CRE isolates, colistin is a vital last-line treatment option. We show here that in a mouse model of infection, colistin-heteroresistant CRKP isolates fail colistin therapy. This stresses the need to assess the relevance of colistin heteroresistance on the outcome of colistin therapy in human infection, which has yet to be determined.

When highly resistant CRKP strains are isolated in the clinic, testing of last-line antibiotics identifies crucial treatment options. Colistin-heteroresistant isolates, such as the ones reported here, can be misclassified as colistin susceptible, a "very major discrepancy" according to FDA susceptibility testing guidelines (17). Subsequent treatment of these isolates with colistin may then lead to unexplained treatment failure, as was demonstrated in our *in vivo* mouse model. Thus, the misclassification of colistin susceptibility status wastes critical time and resources and may lead to further infection complications and patient mortality. Clinical laboratories should consider testing for heteroresistance to colistin if this last-line antibiotic is required for treatment. Unfortunately, the current standard test for heteroresistance, the population analysis profile, is time- and labor-intensive, and it is cumbersome for most clinical laboratories to implement. Our findings suggest that broth microdilution with an increased incubation time (48 h) may detect colistin heteroresistance. However, the increased incubation time is a downside in itself, and there is also an increased chance that a culture of
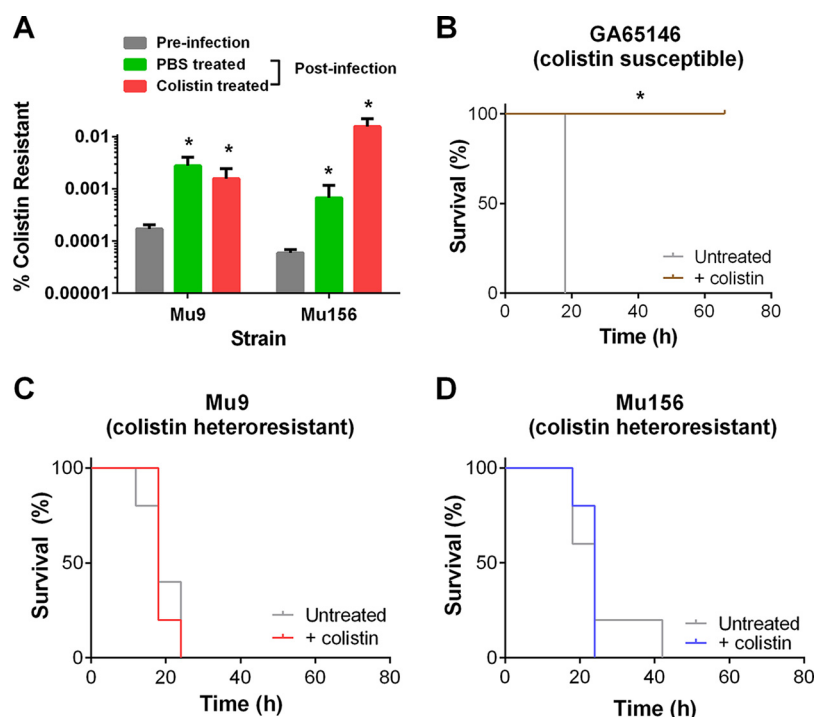
FIG 2 *K. pneumoniae* isolates lead to *in vivo* colistin treatment failure. (A) Mice were infected intraperitoneally with 3×10^8 CFU of Mu9 or Mu156, treated with phosphate-buffered saline (PBS) or colistin (20 mg/kg colistin methanesulfonate) at 12 and 18 h, and then sacrificed at 24 h. Peritoneal lavage fluid was collected and plated onto drug-free medium and medium containing 16 μg/ml colistin to assess percentages of colistin-resistant cells of the heteroresistant strains. The preinfection inoculum (input) was plated similarly ($n = 5$). *, $P < 0.05$ (Mann-Whitney test). (B to D) Mice were infected with the colistin-susceptible isolate GA65146 (B) or the colistin-heteroresistant isolate Mu9 (C) or Mu156 (D) and then treated with 20 mg/kg colistin methanesulfonate every 6 h starting at 12 h. Mice were monitored for survival and weight loss and were sacrificed if their weight fell below 80% of their starting weight ($n = 5$). *, $P < 0.05$ (Gehan-Breslow-Wilcoxon test).

susceptible bacteria will become contaminated or that *de novo* mutant cells will have the time necessary to grow out, leading to an inaccurate identification as resistant. Therefore, novel diagnostics that rapidly and accurately detect colistin heteroresistance are needed.

Taken together, these findings serve to sound the alarm about a worrisome and underappreciated phenomenon in CRKP infections and highlight the need for more sensitive and accurate diagnostics. We suggest that clinical microbiologists and clinicians alike use caution when treating CRKP infections with colistin.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/mBio .02448-17.

FIG S1, PDF file, 0.04 MB.
FIG S2, PDF file, 0.03 MB.
TABLE S1, PDF file, 0.03 MB.
TABLE S2, PDF file, 0.02 MB.

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