Colistin Heteroresistance Is Largely Undetected among Carbapenem-Resistant Enterobacterales in the United States

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ABSTRACT Heteroresistance is a form of antibiotic resistance where a bacterial strain is comprised of a minor resistant subpopulation and a majority susceptible subpopulation. We showed previously that colistin heteroresistance can mediate the failure of colistin therapy in an in vivo infection model, even for isolates designated susceptible by clinical diagnostics. We sought to characterize the extent of colistin heteroresistance among the highly drug-resistant carbapenem-resistant Enterobacterales (CRE). We screened 408 isolates for colistin heteroresistance. These isolates were collected between 2012 and 2015 in eight U.S. states as part of active surveillance for CRE. Colistin heteroresistance was detected in 10.1% (41/408) of isolates, and it was more common than conventional homogenous resistance (7.1%, 29/408). Most (93.2%, 38/41) of these heteroresistant isolates were classified as colistin susceptible by standard clinical diagnostic testing. The frequency of colistin heteroresistance was greatest in 2015, the last year of the study. This was especially true among Enterobacter isolates, of which specific species had the highest rates of heteroresistance. Among Klebsiella pneumoniae isolates, which were the majority of isolates tested, there was a closely related cluster of colistin-heteroresistant ST-258 isolates found mostly in Georgia. However, cladistic analysis revealed that, overall, there was significant diversity in the genetic backgrounds of heteroresistant K. pneumoniae isolates. These findings suggest that due to being largely undetected in the clinic, colistin heteroresistance among CRE is underappreciated in the United States.

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Heteroresistance is an underappreciated phenomenon that may be the cause of some unexplained antibiotic treatment failures. Misclassification of heteroresistant isolates as susceptible may lead to inappropriate therapy. Heteroresistance to colistin was more common than conventional resistance and was overwhelmingly misclassified as susceptibility by clinical diagnostic testing. Higher proportions of colistin heteroresistance observed in certain Enterobacter species and clustering among heteroresistant Klebsiella pneumoniae strains may inform colistin treatment recommendations. Overall, the rate of colistin nonsusceptibility was more than double the level detected by clinical diagnostics, suggesting that the prevalence of colistin nonsusceptibility among CRE may be higher than currently appreciated in the United States.

**KEYWORDS** colistin, heteroresistance, CRE, antibiotic resistance, polymyxins, Enterobacterales, Enterobacteriaceae

Increasing antibiotic resistance has been recognized as a major health threat by the Centers for Disease Control and Prevention (CDC) and World Health Organization, resulting in at least 35,000 deaths and 2.8 million infections annually in the United States (1). To combat infections due to highly resistant bacteria, such as carbapenem-resistant Enterobacterales (CRE), that have up to a 40% mortality rate (2), clinicians are increasingly turning to drugs of last resort, including the polymyxin antibiotic colistin (polymyxin E) (3). However, resistance even to last-line drugs is increasing (4–6). Further complicating efforts to combat multidrug-resistant bacteria are instances of treatment failure of strains classified as susceptible to a given antibiotic. Heteroresistance is a form of resistance in which a strain harbors both an antibiotic-resistant subpopulation and a majority population of susceptible cells. We recently demonstrated that colistin heteroresistance can lead to colistin treatment failure in an in vivo mouse model of infection with multiple Enterobacter and Klebsiella clinical isolates (7, 8). Furthermore, colistin heteroresistance may not be detected by traditional clinical testing methods. Failure to identify heteroresistance in the clinical laboratory may lead to treatment failures in serious CRE infections. We performed a retrospective study among multidrug-resistant CRE isolates collected between 2012 and 2015 to determine the frequency of colistin heteroresistance within this collection.

Four hundred eight CRE isolates included in this project were collected as part of the U.S. CDC Emerging Infections Program’s Multisite Gram-Negative Surveillance Initiative (MuGSI) (9, 10), an ongoing, active, population- and laboratory-based surveillance system for CRE isolated from urine and sterile sites, such as blood. Isolates were collected from clinical laboratories in metropolitan areas in eight U.S. states and adhered to a strict definition of CRE based on susceptibility testing and species identification (9). All isolates that fit this definition within the surveillance area during the study years of 2012 to 2015 were included in this study. All isolates are summarized in Table S1 in the supplemental material and belonged to 3 genera: Klebsiella, Enterobacter, and Escherichia (Fig. 1a). They originated from various culture sources (Table S1) and displayed resistance to last-line antibiotics, such as aminoglycosides and tigecycline (Table S2).

To detect heteroresistance to colistin, all isolates were tested via the population analysis profile (PAP) method. This consists of plating overnight cultures of each isolate onto solid Muller-Hinton (MH) agar with or without colistin concentrations of 0.5, 1, 2, 4, 16, 32, and 100 μg/ml. Surviving colonies were enumerated and used to detect colistin-resistant subpopulations characteristic of heteroresistance. Overall, PAP revealed a proportion of 10.1% (41/408 isolates) colistin heteroresistance (Fig. 1a), which was found among all genera tested. Compared to the proportion of isolates demonstrating heteroresistance, there was a lower proportion of isolates demonstrating “conventional” colistin resistance (7.1%, 29/408), in which all cells within the population exhibited a resistant phenotype. Additionally, the proportion of isolates with colistin
heteroresistance was highest in the most recent year of the study (2015, 15.8%, 24/152, $P = 0.0039$, odds ratio $= 2.636$, 95% confidence interval [CI] = 1.366 to 5.087), and proportions were significantly higher than in all previous years (9.8% [4/41] in 2012, 6.2% [5/81] in 2013, 6.0% [8/134] in 2014) (Fig. 1b). Although only four sites collected isolates during all 4 years, including only those three sites, the rate of colistin heteroresistance in 2015 was still significantly higher than in prior years (17/118, 14.4%, $P = 0.0135$, odds ratio $= 2.665$, 95% CI = 1.225 to 5.799).

Of the 41 colistin-heteroresistant isolates detected by PAP, only 7.3% (3/41) were classified as colistin resistant by broth microdilution (BMD), while the vast majority (92.7%, 38/41) were classified as colistin susceptible (Fig. 1c). Overall, BMD classified 32 of the 408 CRE isolates as colistin nonsusceptible, a 7.2% rate of nonsusceptibility (including 3 heteroresistant isolates and 29 resistant isolates). However, testing by PAP revealed that the proportion of colistin-nonsusceptible isolates was actually 17.1% (70/408)!
Additionally, the proportion of heteroresistance in only 5 states, with the majority (66.7%, 16/24) originating in the state of Georgia. Enterobacter cloacae species (Fig. 1a). The less-common species (designated here as Enterobacter kobei), and E. cloacae, E. kobei, and E. ludwigii. Of these, E. cloacae (82.4%, 61/74) was the most common (Fig. 1a). The less-common species (designated here as “minority Enterobacter species”), E. kobei (8.1%, 6/74), E. asburiae (6.8%, 5/74), and E. ludwigii (2.7%, 2/74), accounted for the remaining Enterobacter isolates. The rate of colistin heteroresistance was highest among these minority Enterobacter species, with heteroresistance observed in 69.2% (9/13, P < 0.0001, odds ratio = 17.357, 95% CI = 4.209 to 71.576) of these isolates, compared to 11.5% (7/61) of E. cloacae isolates (Fig. 1a). Potentially in agreement with these findings, a previous report described variation in colistin heteroresistance rates among isolates from different Enterobacter cloacae complex genomic clusters (11). At least one colistin-heteroresistant Enterobacter isolate was identified from each of the eight study sites, indicating that Enterobacter isolates exhibiting this resistance phenotype were present in a wide distribution of geographic sites within the United States.

In contrast, Klebsiella isolates that were heteroresistant to colistin were found in only 5 states, with the majority (66.7%, 16/24) originating in the state of Georgia. Additionally, the proportion of heteroresistance in Klebsiella isolates was significantly higher in Georgia (15.0%, 16/107, P = 0.0034, odds ratio = 3.758, 95% CI = 1.550 to 9.115) than the proportion among isolates from all other states combined (4.5%, 8/179) (Table S4). The predominant Klebsiella species was K. pneumoniae, making up 90.9% of isolates, while other species included K. aerogenes and K. oxytoca. The higher rate of colistin heteroresistance among Klebsiella isolates in Georgia led us to consider whether this might be due to the presence of a predominant strain. To address this, we used cladistic analysis to determine the genetic relatedness of the colistin-heteroresistant K. pneumoniae isolates based on their whole-genome sequences (Fig. 2). This analysis revealed that there was a cluster of 15 closely related isolates within a 0.05 P distance (Fig. 2a, blue box) that were all sequence type 258 (ST-258) and contained similar antibiotic resistance genes. This genetic branch included isolates from all 4 years of the study, which did not cluster together temporally. The cluster of isolates consisted of 14 from Georgia and one from Minnesota, indicating that there was a highly related cluster of colistin-heteroresistant Klebsiella isolates present in Georgia. Among all the K. pneumoniae strains in the study, however, there was no association between whole-genome sequence and colistin susceptibility status (Fig. 2b). Taken together, the data indicate that colistin heteroresistance is a widespread phenomenon and that the presence of a cluster of heteroresistant K. pneumoniae isolates in Georgia may explain the high rate of colistin heteroresistance in this state.

Previous studies have shown that colistin heteroresistance can be mediated by PhoPQ/PmrAB-dependent cationic sugar modifications to the lipid A component of lipopolysaccharide (LPS) (12–15), resulting in an increased charge of the Gram-negative bacterial outer membrane and reduced susceptibility to colistin, a cationic antimicrobial peptide. Lipid A was analyzed using a new extraction method termed fast lipid analysis technique (FLAT) (16), as well as the previously used El Hamidi et al. method (17), on a representative sample of 12 colistin-heteroresistant isolates from this study using matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectrometry in the negative-ion mode (Table S5). After culture in the presence of colistin, which enriches for the resistant subpopulation, we observed an aminoarabinose (Δm/z 131 mass shift)
modification to the terminal phosphate in all heteroresistant isolates evaluated, as well as an additional phosphoethanolamine (Δm/z 123 mass shift) modification in the Escherichia coli isolate analyzed (Fig. S1). These data further confirm that specific lipid A modifications are strongly associated with colistin heteroresistance, in agreement with previous studies (7, 18). Interestingly, MALDI-TOF mass spectrometry was able to detect lipid A modification in 9 out of 12 heteroresistant isolates even when the cultures were not enriched for resistant cells by growth in colistin (Table S5). These data indicate that MALDI-TOF approaches, which will be investigated further in future studies, may have diagnostic utility in detecting colistin heteroresistance which was missed by other diagnostic techniques.

This is the first multisite surveillance study for colistin heteroresistance among CRE in the United States. Treatment of highly antibiotic-resistant CRE relies on last-line drugs, including colistin, and the high rate of colistin heteroresistance may threaten the effective use of this antibiotic. The proportion of heteroresistance to colistin exceeded the proportion of “conventional” homogenous resistance, which, taken together, indicates that colistin nonsusceptibility is much more common than previously appreciated. Additionally, the vast majority of colistin-heteroresistant isolates were designated susceptible by standard clinical testing. The inability to detect colistin heteroresistance in the majority of heteroresistant isolates may lead to inappropriate treatment with colistin and might be a significant cause of unexplained antibiotic treatment failure. Detection of heteroresistance in these isolates was possible
SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

REFERENCES

1. Centers for Disease Control and Prevention. 2019. Antibiotic resistance threats in the United States. Centers for Disease Control and Prevention, Atlanta, GA. https://www.cdc.gov/drugresistance/pdf/threats-report/2019-ar-threats-report-508.pdf

2. Patel G, Huprikar S, Factor SH, Jenkins SG, Calfee DP. 2008. Outcomes of carbapenem-resistant Klebsiella pneumoniae infection and the impact of antimicrobial and adjunctive therapies. Infect Control Hosp Epidemiol 29:1099–1106. https://doi.org/10.1086/592412.

3. Morrill HJ, Pogue JM, Kaye KS, LaPlante KL. 2015. Treatment options for carbapenem-resistant Enterobacteriaceae infections. Open Forum Infect Dis 2:ofv050. https://doi.org/10.1093/ofid/ofv050.

4. Halaby T, Kucukkose E, Janssen AB, Rogers MRC, Doorduijn DJ, van der Palen J, Vandenbroucke-Grauls CMJE, van Schaik W. 2016. Genomic characterization of colistin heteroresistance in Klebsiella pneumoniae during a nosocomial outbreak. Antimicrob Agents Chemother 60:6837–6843. https://doi.org/10.1128/AAC.01344-16.

5. Halaby T, Al Na‘emi N, Schaal W. 2016. Emergence of colistin resistance in Enterobacteriaceae after the introduction of selective digestive tract decontamination in an intensive care unit. Antimicrob Agents Chemother 57:3224–3229. https://doi.org/10.1128/AAC.02634-12.

6. Antoniadou A, Kontopidou F, Poulakou G, Koratzanis E, Galani I, Papadomichelakis E, Koperides P, Soulis M, Armaganidis A, Giamarellou H. 2007. Colistin-resistant isolates of Klebsiella pneumoniae exhibiting clinically undetected colistin heteroresistance leads to treatment failure in a murine model of infection. mBio 8:e00248-17. https://doi.org/10.1128/mBio.00248-17.

7. Band VI, Satola SW, Burd EM, Farley MM, Jacob JT, Weiss DS. 2018. Carbenapenem-resistant Klebsiella pneumoniae exhibiting clinically undetected colistin heteroresistance leads to treatment failure in a murine model of infection. mBio 9:e00174-18. https://doi.org/10.1128/mBio.00174-18.

8. Band VI, Satola SW, Burd EM, Farley MM, Jacob JT, Weiss DS. 2018. Carbapenem-resistant Klebsiella pneumoniae exhibiting clinically undetected colistin heteroresistance leads to treatment failure in a murine model of infection. mBio 9:e00248-17. https://doi.org/10.1128/mBio.00248-17.

9. Guh AV, Bulens SN, Mu Y, Jacob JT, Ren J, Scott J, Wilson LE, Vaeth E, Lynfield R, Shaw KM, Vagnozzi PMS, Bamburg WM, Janelle SJ, Durnyati G, Concannon C, Beldavs Z, Cunningham M, Cassidy PM, Phipps EC, Kenslow N, Travis T, Lonsway D, Rasheed JK, Limbago BM, Kallen AJ. 2015. Epidemiology of carbapenem-resistant Enterobacteriaceae in 7 US communities, 2012–2013. JAMA 314:1479–1487. https://doi.org/10.1001/jama.2015.12480.

10. Centers for Disease Control and Prevention. 2016. Multi-site Gram-negative surveillance initiative. Centers for Disease Control and Prevention, Atlanta, GA. https://www.cdc.gov/hai/eip/mugsi.html. Accessed 20 March 2020.

11. Guérin F, Isnard C, Sinel C, Morand P, Dhalluin A, Cattoir V, Giard JC. 2016. Cluster-dependent colistin hetero-resistance in Enterobacter cloacae complex. J Antimicrob Chemother 71:3058–3061. https://doi.org/10.1093/jac/dkw260.

12. Gunn JS, Lim KB, Krueger J, Kim K, Guo L, Hackett M, Miller SL. 1998. PmrA-PmrB-regulated genes necessary for 4-aminoarabinose lipid A modification and polymyxin resistance. Mol Microbiol 27:1171–1182. https://doi.org/10.1046/j.1365-2958.1998.00757.x.

13. Helander IM, Kato Y, Kilpeläinen I, Kostiainen R, Lindner B, Nummilä K, Sugiyama T, Yokochi T. 1996. Characterization of lipopolysaccharides of polymyxin-resistant and polymyxin-sensitive Klebsiella pneumoniae O3. Eur J Biochem 237:272–278. https://doi.org/10.1111/j.1432-1327.1996.tb17744.x.

14. Ernst RK, Yi EC, Guo L, Lim KB, Burns JL, Hackett M, Miller SL. 1999. Specific lipopolysaccharide found in cystic fibrosis airway Pseudomonas aeruginosa. Science 286:1561–1565. https://doi.org/10.1126/science.286.5444.1561.

15. Shi Y, Cromie MJ, Hsu F-F, Turk J, Groisman EA. 2004. PhoP-regulated
14. Salamonella resistance to the antimicrobial peptides magainin 2 and polymyxin B. Mol Microbiol 53:229–241. https://doi.org/10.1111/j.1365-2958.2004.04107.x.

15. Sorensen M, Chandler CE, Gardner FM, Ramadan S, Khot PD, Leung LM, Farrance CE, Goodlett DR, Ernst RK, Nilsson E. 2020. Rapid microbial identification and colistin resistance detection via MALDI-TOF MS using a novel on-target extraction of membrane lipids. Sci Rep 10(1):21536. https://doi.org/10.1038/s41598-020-78401-3.

16. El Hamidi A, Tirsoaga A, Novikov A, Hussein A, Caroff M. 2005. Microextraction of bacterial lipid A: easy and rapid method for mass spectrometric characterization. J Lipid Res 46:1773–1778. https://doi.org/10.1194/jlr.D500014-JLR200.

17. Kang KN, Klein DR, Kazi MI, Guérin F, Cattoir V, Brodbelt JS, Boll JM. 2019. Colistin heteroresistance in Enterobacter cloacae is regulated by PhoPQ-dependent 4-amino-4-deoxy-l-arabinose addition to lipid A. Mol Microbiol 111:1604–1616. https://doi.org/10.1111/mmi.14240.