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Colistin Heteroresistance in *Enterobacter cloacae* Is Associated with Cross-Resistance to the Host Antimicrobial Lysozyme

Brooke A. Napier,[a]* Victor Band,[a] Eileen M. Burd,[c,d] David S. Weiss[a,b,c]

Department of Microbiology and Immunology,[a] Emory Vaccine Center,[b] Division of Infectious Diseases, Department of Medicine,[c] and Department of Pathology and Laboratory Medicine,[d] Emory University School of Medicine, Atlanta, Georgia, USA

Here, we describe the first identification of colistin-heteroresistant *Enterobacter cloacae* in the United States. Treatment of this isolate with colistin increased the frequency of the resistant subpopulation and induced cross-resistance to the host antimicrobial lysozyme. This is the first description of heteroresistance conferring cross-resistance to a host antimicrobial and suggests that clinical treatment with colistin may inadvertently select for bacteria that are resistant to components of the host innate immune system.

Antibiotic-resistant pathogens are responsible for 2 million infections and at least 23,000 deaths each year in the United States alone (1). The problem of increasing antibiotic resistance is compounded by the lack of new drugs in development, together threatening a return to the preantibiotic era. Colistin is often the only therapeutic option to treat infections caused by Gram-negative bacteria that are resistant to most or all other antibiotics (2–4). This cationic antimicrobial peptide disrupts both the outer and inner membranes of Gram-negative bacteria (5), acting similarly to several host antimicrobials, including the cationic C-terminal portion of lysozyme (6–8). Like colistin, this nonenzymatic portion of lysozyme exerts a potent antimicrobial action against a variety of Gram-negative bacteria by crossing the outer membrane via self-promoted uptake and forming pores within the inner membrane (6–8). Unfortunately, resistance to colistin has emerged, rendering infections by some strains essentially untreatable.

Several types of resistance to colistin have been identified, including heteroresistance, which has been observed in several

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Address correspondence to Eileen M. Burd, eburd@emory.edu, or David S. Weiss, david.weiss@emory.edu.

* Present address: Brooke A. Napier, Department of Microbiology and Immunology, Stanford School of Medicine, Stanford University, Stanford, California, USA.

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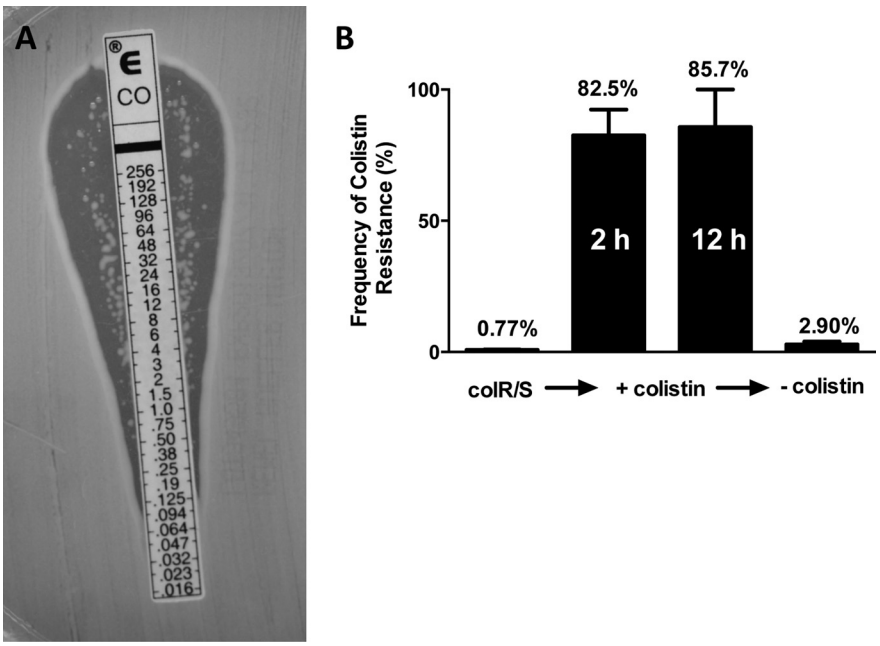

FIG 1 Frequency of the colistin-resistant subpopulation in a novel *Enterobacter cloacae* clinical isolate. (A) Image of *Enterobacter cloacae* isolate colR/S plated on MH with a colistin Etest strip. Resistant colonies are present within the zone of clearing. (B) The frequency of the colistin-resistant subpopulation of colR/S was quantified after growth in MH broth for 16 h, subsequent treatment with a sublethal dose of colistin (200 μg/ml; +colistin) for 2 h or 12 h, and subsequent passage in MH without colistin (−colistin).

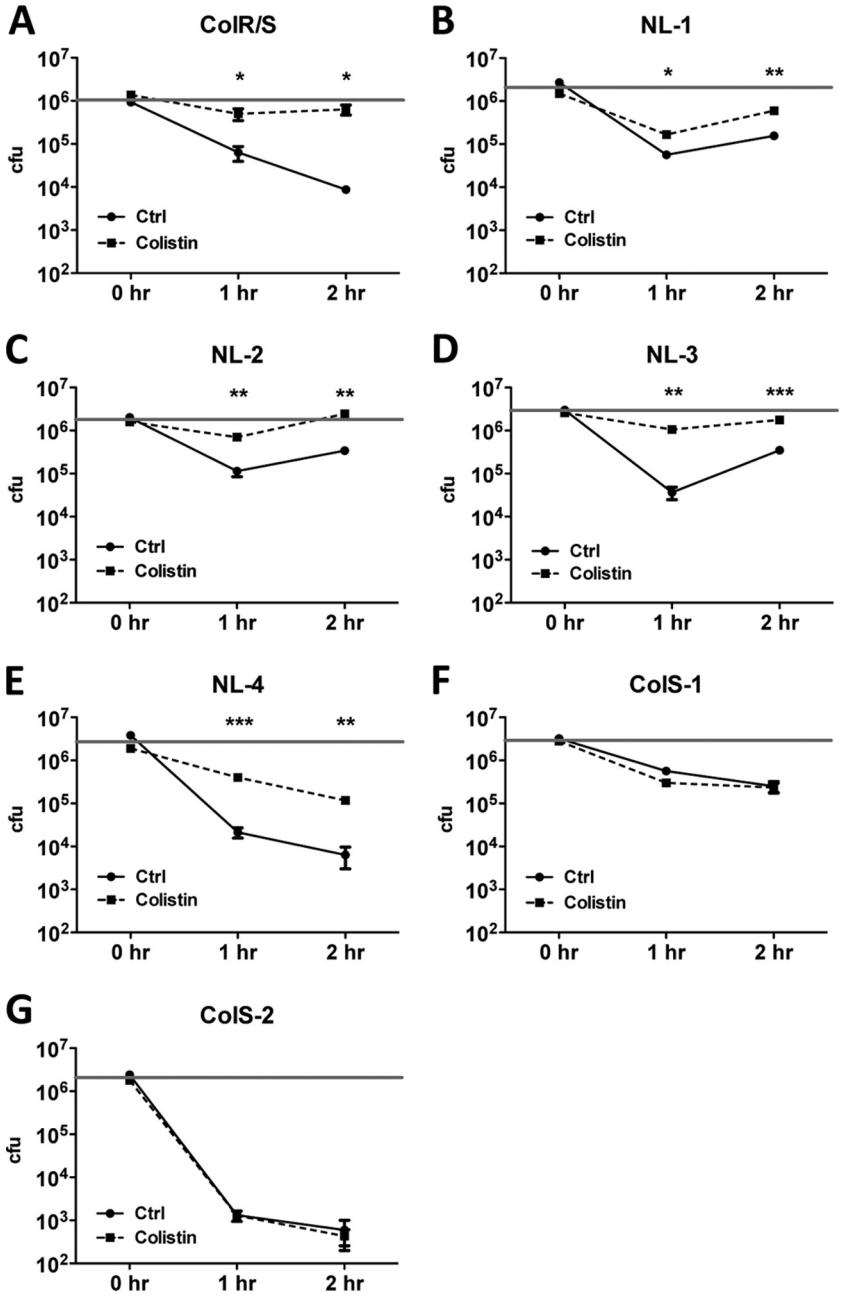

FIG 2 Colistin treatment of heteroresistant *Enterobacter cloacae* isolates induces cross-resistance to lysozyme. *E. cloacae* strains were grown in MH broth in the absence (Ctrl; solid line) or presence (dashed line) of a sub-MIC level of colistin (200 μg/ml for heteroresistant strains and 1 μg/ml for sensitive strains) for 2.5 h. Colistin-heteroresistant strains (A to E) and colistin-sensitive strains (F and G) were then treated with 5 mg/ml of lysozyme for the indicated times and plated for enumeration of CFU. Time zero CFU is indicated by the solid gray line. Data were analyzed for significance using the unpaired Student *t* test. *, $P < 0.05$; **, $P < 0.001$; ***, $P < 0.0001$. Error bars represent the standard deviations of the results determined from triplicate samples.

Gram-negative pathogens (9–11). Heteroresistance is broadly defined as the presence of an antibiotic-resistant subset of microbes within a larger population that is susceptible to the antibiotic (12–14). Heteroresistance can complicate assessment of the MIC to a specific antibiotic and may promote resistance to antibiotics *in vivo*, thereby affecting diagnostic tests and patient treatment (12).

We recently isolated a colistin-heteroresistant strain (colR/S) of *Enterobacter cloacae* from a bronchoalveolar lavage specimen from a kidney transplant patient. This is the first identification of colistin-heteroresistant *E. cloacae* in the United States and only the second description worldwide (15). *E. cloacae* is a Gram-negative intestinal commensal bacterium that colonizes 40 to 80% of the human population (16) and has previously been identified as an opportunistic nosocomial pathogen (17–19). Treatment of infection by *E. cloacae* can be complicated by its natural resistance to many antibiotics and its ability to acquire resistance to others after exposure (20, 21).

The colR/S strain was highly antibiotic resistant (see Table S1 in the supplemental material) and displayed heteroresistance to colistin as determined using Etest strips (bioMérieux, Durham,

NC) on Mueller-Hinton (MH) agar (Difco/BD, Sparks, MD) (Fig. 1A; see also Table S2 in the supplemental material), by following the inoculation and reading instructions of the manufacturer. We determined the frequency of the resistant population by growing the colR/S strain in MH broth (Difco/BD) for 16 h and then plating on MH agar with or without colistin (100 μg/ml) (Sigma-Aldrich, St. Louis, MO). The frequency of colistin resistance was calculated by dividing the number of colonies on colistin plates by the number of colonies on the colistin-free plates and was determined to be 0.77% (Fig. 1B), a similar level seen in other examples of heteroresistance (9–11, 14). When colR/S was subsequently grown for 2 h or 12 h in the presence of 200 μg/ml of colistin, the frequency of colistin resistance increased significantly to 82.5 to 85.7% (Fig. 1B). Subsequent passage without antibiotic revealed that the frequency of resistance returned to below 3% (Fig. 1B), consistent with other examples of heteroresistance (12, 14).

We next set out to determine the frequency of the resistant population in previously identified colistin-heteroresistant strains of *E. cloacae*. We obtained four colistin-heteroresistant isolates (NL-1, NL-2, NL-3, and NL-4) (15) and measured their frequencies of baseline colistin resistance to be between 1.25% and 4.71% (see Fig. S1 in the supplemental material). When treated with colistin, similar to colR/S, these isolates displayed a robust increase in the frequency of resistance (47.4 to 62.0%) (see Fig. S1). Further, the frequency reverted to baseline upon passage in the absence of colistin (see Fig. S1). In contrast, we collected two colistin-sensitive (colistin sensitivity defined as an Etest MIC of ≤0.2 μg/ml and broth microdilution MIC of ≤8 μg/ml; see Table S2 in the supplemental material) *E. cloacae* strains (colS-1 and colS-2) and failed to observe resistant colonies after plating them on colistin (data not shown) or by Etest (see Fig. S2A and B). Taken together, these data demonstrate that the colistin-heteroresistant *E. cloacae* strains tested here have large resistant subpopulations, whose frequency is significantly increased upon colistin treatment.

We previously demonstrated that colistin-resistant clinical isolates of the nosocomial bacterium *Acinetobacter baumannii* (resistance was due to point mutations, and these strains did not exhibit heteroresistance) displayed cross-resistance to the host antimicrobial lysozyme (22). We therefore set out to determine whether colistin heteroresistance could confer cross-resistance to lysozyme. colR/S was grown from frozen stock in MH broth at 37°C with aeration and then diluted to a final concentration of ~10^6 CFU/ml in 25% MH broth. Bacteria were either treated with a sublethal dose (200 μg/ml for heteroresistant strains and 1 μg/ml for sensitive strains) of colistin for 2.5 h or were not treated. Subsequently, bacteria were challenged with lysozyme (5 mg/ml) and incubated with aeration at 37°C, and aliquots were plated at 0 h, 1 h, and 2 h for enumeration of CFU. Colistin pretreatment of colR/S prevented killing upon subsequent exposure to lysozyme, compared to bacteria that were not pretreated and whose levels were reduced 100-fold at 2 h (Fig. 2A). Interestingly, colistin pretreatment similarly induced enhanced resistance to lysozyme treatment in the four previously described colistin-heteroresistant *E. cloacae* strains (Fig. 2B to E). In contrast, the colistin-sensitive *E. cloacae* isolates were not protected from lysozyme, whether or not they had been pretreated with colistin (Fig. 2F and G). These data indicate that, among the colistin-heteroresistant *E. cloacae* isolates tested here, pretreatment with colistin induces an increased frequency of colistin resistance as well as cross-resistance to the host antimicrobial lysozyme. This is the first demonstration that heteroresistance to an antibiotic can confer cross-resistance to a component of the host innate immune system.

The data presented here suggest that *E. cloacae* heteroresistance to colistin may impact the outcome of clinical infection, since exposure to this antibiotic can lead to increased resistance to host innate immune defenses. This is likely a phenomenon that occurs broadly and is relevant to a range of pathogens for which colistin-heteroresistant strains have been isolated, including *Acinetobacter baumannii* and *Klebsiella pneumoniae* (9–11). Use of susceptibility testing methods capable of identification of heteroresistance may be essential in guiding optimal patient treatment, to avoid unknowingly inducing resistance to the host innate immune system.

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REFERENCES

1. **CDC.** 16 September 2013, **posting date.** Antibiotic resistance threats in the United States. CDC, Atlanta, GA. http://www.cdc.gov/drugresistance/threat-report-2013/.
2. **Zavascki AP, Goldani LZ, Li J, Nation RL.** 2007. Polymyxin B for the treatment of multidrug-resistant pathogens: a critical review. J. Antimicrob. Chemother. **60:**1206–1215. http://dx.doi.org/10.1093/jac/dkm357.
3. **Tamayo M, Santiso R, Otero F, Bou G, Lepe JA, McConnell MJ, Cisneros JM, Gosálvez J, Fernández JL.** 2013. Rapid determination of colistin resistance in clinical strains of *Acinetobacter baumannii* by use of the micromax assay. J. Clin. Microbiol. **51:**3675–3682. http://dx.doi.org/10.1128/JCM.01787-13.
4. **Biswas S, Brunel JM, Dubus JC, Reynaud-Gaubert M, Rolain JM.** 2012. Colistin: an update on the antibiotic of the 21st century. Expert Rev. Anti. Infect. Ther. **10:**917–934. http://dx.doi.org/10.1586/eri.12.78.
5. **Fidai S, Farmer SW, Hancock RE.** 1997. Interaction of cationic peptides with bacterial membranes. Methods Mol. Biol. **78:**187–204.
6. **Düring K, Porsch P, Mahn A, Brinkmann O, Gieffers W.** 1999. The non-enzymatic microbicidal activity of lysozymes. FEBS Lett. **449:**93–100. http://dx.doi.org/10.1016/S0014-5793(99)00405-6.
7. **Ibrahim HR, Thomas U, Pellegrini A.** 2001. A helix-loop-helix peptide at the upper lip of the active site cleft of lysozyme confers potent antimicrobial activity with membrane permeabilization action. J. Biol. Chem. **276:**43767–43774. http://dx.doi.org/10.1074/jbc.M106317200.
8. **Laible NJ, Germaine GR.** 1985. Bactericidal activity of human lysozyme, muramidase-inactive lysozyme, and cationic polypeptides against *Streptococcus sanguis* and *Streptococcus faecalis*: inhibition by chitin oligosaccharides. Infect. Immun. **48:**720–728.
9. **Li J, Rayner CR, Nation RL, Owen RJ, Spelman D, Tan KE, Liolios L.** 2006. Heteroresistance to colistin in multidrug-resistant *Acinetobacter baumannii*. Antimicrob. Agents Chemother. **50:**2946–2950. http://dx.doi.org/10.1128/AAC.00103-06.
10. **Hawley JS, Murray CK, Jorgensen JH.** 2008. Colistin heteroresistance in acinetobacter and its association with previous colistin therapy. Antimicrob. Agents Chemother. **52:**351–352. http://dx.doi.org/10.1128/AAC.00766-07.
11. **Meletis G, Tzampaz E, Sianou E, Tzavaras I, Sofianou D.** 2011. Colistin heteroresistance in carbapenemase-producing *Klebsiella pneumoniae*. J. Antimicrob. Chemother. **66:**946–947. http://dx.doi.org/10.1093/jac/dkr007.
12. **Falagas ME, Makris GC, Dimopoulos G, Matthaiou DK.** 2008. Heteroresistance: a concern of increasing clinical significance? Clin. Microbiol. Infect. **14:**101–104. http://dx.doi.org/10.1111/j.1469-0691.2007.01912.x.
13. **Deresinski S.** 2009. Vancomycin heteroresistance and methicillin-resistant *Staphylococcus aureus*. J. Infect. Dis. **199:**605–609. http://dx.doi.org/10.1086/596630.
14. **Morand B, Mühlemann K.** 2007. Heteroresistance to penicillin in *Strep-*

tococcus pneumoniae. Proc. Natl. Acad. Sci. U. S. A. **104:**14098–14103. http://dx.doi.org/10.1073/pnas.0702377104.

15. **Lo-Ten-Foe JR, de Smet AM, Diederen BM, Kluytmans JA, van Keulen PH.** 2007. Comparative evaluation of the VITEK 2, disk diffusion, Etest, broth microdilution, and agar dilution susceptibility testing methods for colistin in clinical isolates, including heteroresistant *Enterobacter cloacae* and *Acinetobacter baumannii* strains. Antimicrob. Agents Chemother. **51:** 3726–3730. http://dx.doi.org/10.1128/AAC.01406-06.
16. **Gaston MA.** 1988. Enterobacter: an emerging nosocomial pathogen. J. Hosp. Infect. **11:**197–208. http://dx.doi.org/10.1016/0195-6701(88)90098-9.
17. **John JF, Sharbaugh RJ, Bannister ER.** 1982. *Enterobacter cloacae*: bacteremia, epidemiology, and antibiotic resistance. Rev. Infect. Dis. **4:**13–28. http://dx.doi.org/10.1093/clinids/4.1.13.
18. **Keller R, Pedroso MZ, Ritchmann R, Silva RM.** 1998. Occurrence of virulence-associated properties in *Enterobacter cloacae*. Infect. Immun. **66:**645–649.
19. **Sanders WE, Sanders CC.** 1997. *Enterobacter* spp.: pathogens poised to flourish at the turn of the century. Clin. Microbiol. Rev. **10:**220–241.
20. **Mezzatesta ML, Gona F, Stefani S.** 2012. *Enterobacter cloacae* complex: clinical impact and emerging antibiotic resistance. Future Microbiol. **7:**887–902. http://dx.doi.org/10.2217/fmb.12.61.
21. **Stock I, Grüger T, Wiedemann B.** 2001. Natural antibiotic susceptibility of strains of the *Enterobacter cloacae* complex. Int. J. Antimicrob. Agents **18:**537–545. http://dx.doi.org/10.1016/S0924-8579(01)00463-0.
22. **Napier BA, Burd EM, Satola SW, Cagle SM, Ray SM, McGann P, Pohl J, Lesho EP, Weiss DS.** 2013. Clinical use of colistin induces cross-resistance to host antimicrobials in *Acinetobacter baumannii*. mBio **4**(3): e00021-13. http://dx.doi.org/10.1128/mBio.00021-13.