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

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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

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).
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,

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.
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 ~10⁶ 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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