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Colistin Heteroresistance: An Under-recognized and Challenging Form of Antibiotic Resistance

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Colistin Heteroresistance: An Under-recognized and Challenging Form of Antibiotic Resistance

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Abstract

Colistin Heteroresistance: An Under-recognized and Challenging Form of Antibiotic Resistance
By Victor I. Band

Antibiotic resistance threatens the delivery of safe and effective healthcare resulting in 2 million infections and 23,000 deaths annually in the United States[1,2]. Further complicating this epidemic are unexplained antibiotic treatment failures caused by bacteria that appear susceptible to an antibiotic[3]. The phenomenon of heteroresistance occurs when a minor resistant subpopulation exists within a majority susceptible strain. We describe here several instances of heteroresistance to the last-line peptide antibiotic colistin. First, we observed colistin heteroresistant isolates of *Enterobacter cloacae* that were able to mediate lethal infection during colistin treatment. Interestingly, we observed that the resistant subpopulation was augmented by drug treatment as well as host immune pressure, through macrophages and host antimicrobial compounds. We additionally show that these heteroresistant strains can be misclassified as susceptible by routine susceptibility testing. This could conceivably lead to incorrect treatment with colistin which may lead to unexplained treatment failure. We additionally chacterized strains of colistin heteroresistant *Klebsiella pneumoniae*, which were additionally resistant to the last line carbapenems. Thus, these isolates would rely on a last line drug such as colistin and could also lead to colistin treatment failure. To assess the extent of heteroresistance in clinical isolates, we conducted a study of heteroresistance in a wide-ranging pool of carbapenem resistant Enterobacteriaceae in the United States. We observed a rate of heteroresistance of over 10% in these isolates. Over 90% of these isolates were misclassified as colistin susceptible, thus underestimating total colistin non-susceptibility by over 2-fold. These findings highlight a largely unappreciated phenomenon that could have a significant impact upon antibiotic treatment outcome, exacerbating the menace of antibiotic resistance.

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Chapter 1: Introduction

History of Antibiotic Therapy

Antibiotic therapy has been one of the most impactful healthcare developments of the past century, allowing for great advancement in medicine. For many centuries before the development of antibiotics, there were few ways to treat infections with pathogens from bacterial, viral, fungal and parasitic sources. With the development of the first purified antibiotic, the anti-syphilitic salvarsan, humanity entered a new era of treatment possibilities.

However, there is significant evidence of humans using natural antibiotics for centuries before this discovery. Traditional medicines have taken advantage of sources of antibiotic compounds all over the world. Chinese traditional medicine has used the Artemsia plant as a remedy for a variety of illnesses, which contains the active antimalarial compound artemisinin that was discovered in the 1970s[4]. Red soils used to treat skin infections in Jordan as a historical therapy have recently been shown to contain actinomycete bacteria that produce potent antibiotics. There is even direct evidence of the use of tetracycline in ancient Sudanese and Egyptian skeletons, which is seen in the incorporation of tetracycline in the bones and teeth of these fossils [5,6].

The idea for synthesizing an antibiotic therapy to treat infections is generally credited to Paul Ehrlich. He considered targeted dyes that could specifically label certain microbes, hypothesizing that some type of toxic substance could target microbes in a similar fashion while leaving the host unharmed. This ‘magic bullet’ (salvarsan) as he termed it was discovered after a 5 year search for a drug to target syphilis, for which there were no treatment options at the time.

Salvarsan became the first antibiotic developed and was the sole therapy available from its discovery in 1909 until the development of penicillin in the 1940s[7]. Penicillin was discovered a decade earlier by Alexander Fleming, by observing an area of inhibition in a lawn of bacteria contaminated with Penicillium mold. Years later, due to the efforts of Howard Florey and Ernst Chain, penicillin was isolated and manufactured, showing phenomenal efficacy against a variety of infections[8]. Previously devastating bacterial diseases could be cured in several days, and risky procedures such as invasive surgeries would soon become feasible.

This discovery, along with the simple screening technique used to discover penicillin, led to a golden age in antibiotic development. Over half of the antibiotics commonly in use today were discovered in the 1950s and 1960s[9]. In the 1970s, four more classes of antibiotics were discovered. In the following 40 years, however, only one new class of antibiotic has been discovered, in 1987[10]. While there are now many classes of antibiotics available, this 'discovery void' of antibiotics after 1987 has become a significant problem when considering antibiotic resistance[11].

Antibiotic Resistance

Soon after the introduction of penicillin, mutants were described that showed decreased susceptibility to this wonder drug[12]. Indeed, after every new antibiotic has been introduced in the clinic, resistant mutants follow shortly thereafter, sometimes even before the antibiotic is introduced clinically[13]. This phenomenon, in which bacteria harbor genes or generate mutations that confer resistance to certain antibiotics is known as antibiotic resistance, and threatens the efficacy of all antibiotic therapy. Overuse and misuse of antibiotics lead to selection and

dissemination of bacteria harboring these resistance genes. This is further complicated by the discovery that bacteria can exchange and transfer resistance genes located on mobile plasmids, allowing resistance genes to spread through populations of bacteria.

Today, antibiotic resistance is recognized as a severe public health crisis by many organizations. The US Centers for Disease Control estimated in 2013 that there were over 2 million infections due to antibiotic resistant bacteria in the United States, causing 23,000 deaths and $35 billion in increased healthcare and opportunity costs[14]. Antibiotic resistance was described as "one of our most serious healthcare threats" and the CDC warned of "potentially catastrophic consequences of inaction"[14]. In 2014, the World Health Organization released a report on antimicrobial resistance that claimed we could soon be facing a "post-antibiotic era", in which antibiotic therapy was no longer effective due to widespread resistance. This possibility was a threat "so serious that it threatens the achievements of modern medicine"[15].

When you combine increasing antibiotic resistance with the reduced discovery of new antibiotics, many pathogens with multiple forms of antibiotic resistance become difficult to treat. These multi-drug resistant (MDR) organisms have become increasingly common in recent years and can manifest in a variety of bacterial species. One of the most urgent MDR threats are the carbapenem resistant Enterobacteriaceae (CRE), a family of Gram-negative bacteria that display resistance to a plethora of antibiotics and cause a range of infections in the hospital environment[16]. For pathogens such as these, the most widely used antibiotics are no longer an option, and clinicians must turn to "last-line antibiotics".

Last-line antibiotics, or drugs of last resort, are a group of antibiotics which are not used unless a pathogen displays resistance to more commonly used first line therapy. These drugs are

often less efficacious and more toxic than first line antibiotics, which is why they are used much less often. Based on global consumption numbers, first line drug classes such as penicillins and cephalosporins are the most commonly used antibiotics, accounting for 55% of all units used [17]. In contrast, the last line antibiotics, such as aminoglycosides, carbapenems and polymyxins are used very infrequently. Due to rising resistance to first line drugs, the last line drugs are increasing in usage, with carbapenem and polymyxin use increasing between 2000 and 2010 at 45% and 13%, respectively[17].

Polymyxins and antimicrobial peptide resistance

Polymyxins are an important class of last-line antibiotics, that are increasingly needed in the treatment of MDR organisms. Interestingly, they were discovered early in the history of antibiotics, in 1947. Isolated from the Gram-positive bacterium *Paenibacillus polymyxa*, 2 forms of polymyxin were discovered[18] and put into clinical use: polymyxin B and colistin (polymyxin E). However, signs of nephrotoxicity and neurotoxicity lead to polymyxins falling out of favor[19]. In recent years, with the rates of resistance to other antibiotics rising, polymyxins have been increasingly used as a last line option for MDR organisms.

Polymyxins belong to the class of molecules known as the cationic antimicrobial peptides (CAMPs). CAMPs have microbicidal properties towards a variety of pathogens including bacteria, viruses, fungi and parasites. They are a large and varied group of peptides produced by many organisms ranging from prokaryotes to vertebrates (over 1,200 have been identified thus far)[20]. These peptides contain little consensus in their amino acid sequences, though they largely maintain certain key features: they are cationic, amphipathic and relatively hydrophobic[21]. These

attributes are thought to allow polymyxins and other CAMPs to interact with bacterial membranes which contain anionic head groups and hydrophobic fatty acid chains. The CAMPs then destabilize bacterial membranes, which can involve pore formation, leading to cell lysis[22]. Other CAMPs may also have intracellular targets whose inhibition can lead to disruption of cell wall, protein and nucleic acid synthesis, as well as the direct induction of cell death[23].

While CAMPs can kill a variety of pathogens, Gram-negative bacteria represent a major target, and are the main target for polymyxins clinically. Due to the intense pressure that CAMP-mediated killing exerts on bacteria, some species have evolved ways to resist the action of these antimicrobials. Resistance to CAMPs is thought to have a significant negative effect on the ability of the host to prevent and fight bacterial infections, and also threatens the utility of polymyxins in the clinic. Unfortunately, due to their recent increased use, resistance to polymyxins is already on the rise[19].

Resistance to host cationic antimicrobials may even be facilitated by resistance to polymyxins. Clinical strains of *A. baumannii* that are polymyxin resistant are significantly more resistant to both LL-37 and lysozyme[24]. This has also been demonstrated in *Enterobacter cloacae*, where colistin heteroresistant strains are resistant to lysozyme after initial treatment with colistin[25]. This cross resistance between polymyxins and host antimicrobials may interfere with the use of CAMPs as therapeutic tools, including not only polymyxins but many other CAMPs still in development[26]. The development of CAMP resistance likely allows Gram-negative bacteria to avoid killing by both the host immune system and polymyxin antibiotics. Bacteria have developed a variety of ways to resist killing by CAMPs, which can be split into 5 basic mechanisms: surface remodeling, biofilm production, efflux pumps, binding and sequestration, and proteolytic degradation.

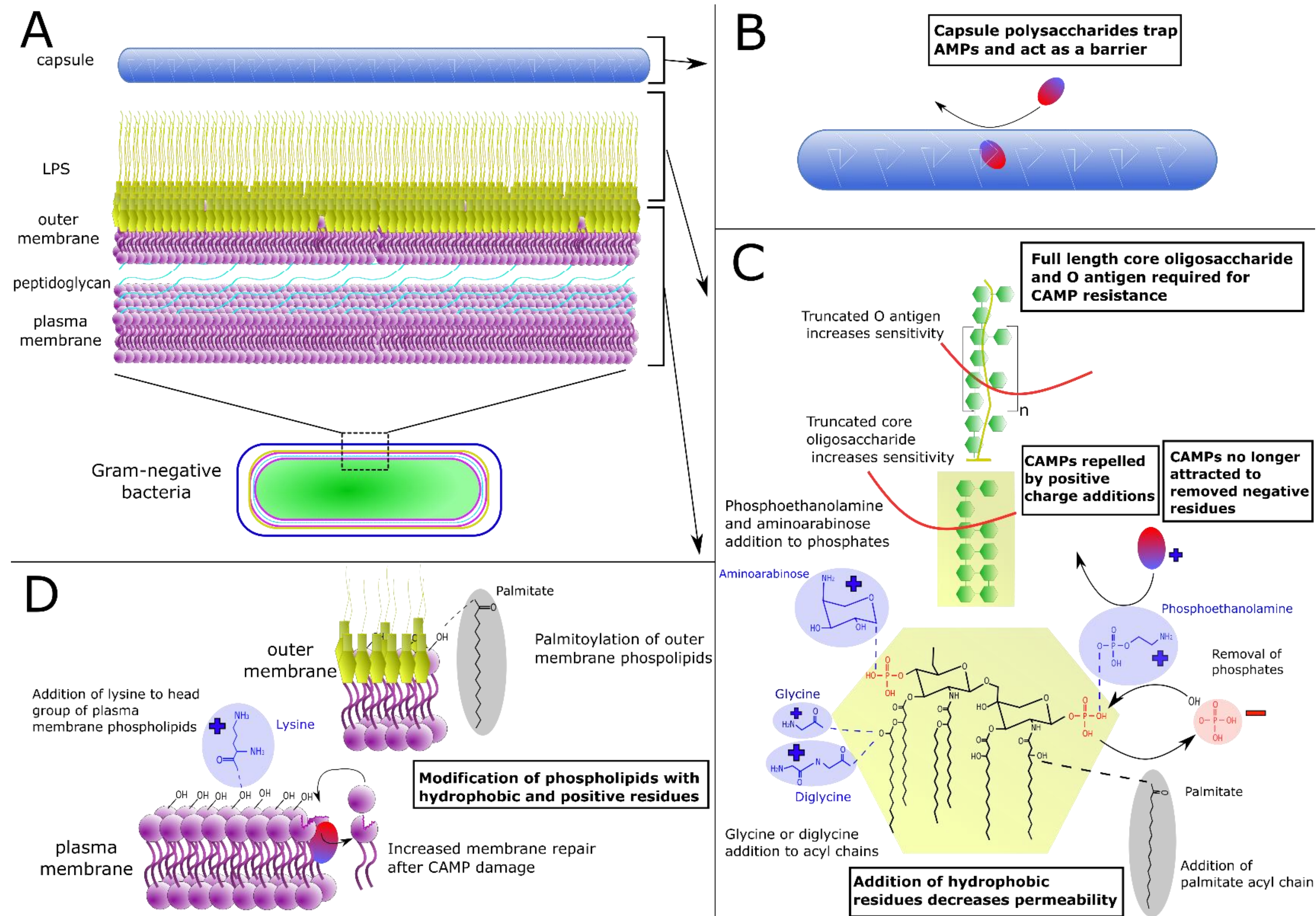

Figure 1.1. Bacterial surface modifications that enhance CAMP resistance. Gram-negative bacterial cell wall structure (A), with magnification of (B) capsule (C) lipopolysaccharide and (D) outer and plasma membranes. LPS structure varies greatly across species, depicted is a representative *E. coli* LPS structure, with modifications from various other species.

Surface remodeling

The outer surface of Gram-negative bacteria is the first barrier to CAMPs and is therefore often modified to enhance resistance **(Figure 1.1)**. One of the main ways in which killing by CAMPs can be avoided is through an increase in bacterial surface charge. Host CAMPs contain a region of highly positive charge and are attracted to negatively charged molecules, such as the surface of many bacteria. Thus, increasing surface charge prevents access of CAMPs to the vulnerable bacterial outer membrane[23].

The surface of Gram-negative bacteria is largely composed of the glycolipid lipopolysaccharide (LPS), serving as one of the initial barriers against extracellular stresses. Specifically, LPS is a major constituent of the outer leaflet of the outer membrane phospholipid bilayer, which envelops the peptidoglycan containing periplasm and the inner membrane **(Figure 1.1A)**. It is comprised of the hydrophobic lipid A whose acyl chains insert into the membrane, the diverse core oligosaccharide, and the O-antigen comprised of repeating subunits **(Figure 1.1C)**. In particular, the lipid A and core oligosaccharide often contain multiple negatively charged residues, such as phosphate groups.

To mitigate the negative charge of LPS, numerous species of bacteria add positive residues to this structure, often to the lipid A. Common positively charged additions to LPS are cationic sugars. For example, the amine containing sugar aminoarabinose is added to a lipid A phosphate group in *P. aeruginosa* as well as *Salmonella typhimurium*, resulting in increased survival of both bacteria in the presence of polymyxin B[27,28]. Aminoarabinose is also present on the LPS of *Burkholderia* species[29]. However, this addition seems to be required for *Burkholderia* survival and so has not been directly linked to CAMP resistance. *Francisella novicida* adds another

amine containing sugar, galactosamine, to its single lipid A phosphate group, similarly promoting polymyxin B resistance. Demonstrating the contribution of this modification to pathogenesis, deletion mutants lacking the galactosamine modification are highly attenuated, with a 5 log decrease in virulence in murine infections[30,31]. Increased CAMP resistance is also linked to cationic sugar addition in *Bordetella pertussis* and *Bordetella bronchiseptica,* in which glucosamine groups are added to both lipid A phosphates[30,32].

Other amine containing moieties, such as amino acids, are also added to the lipid A component of LPS to counteract its negative charge. For example, specific strains of *Vibrio cholerae,* the causative agent of the human disease cholera, add glycines to their lipid A[33]. While most strains of *Vibrio cholerae* are sensitive to CAMPs, the O1 El Tor strain, responsible for the current cholera pandemic[34], has a much higher level of resistance. Hankins et al have shown that this strain of *V. cholerae* adds glycine and diglycine amino acid residues to lipid A acyl chains, increasing the net positive charge of LPS and the bacterial cell surface[33].

Phosphoethanolamine is another amine containing group that can be added to lipid A, as is the case in *Neisseria gonorrhoeae,* the causative agent of gonorrhea. This phosphoethanolamine residue is added to one of the phosphate groups of lipid A, under the control of the *lptA* gene that is required for its ability to resist CAMP-mediated killing[35]. Importantly, this increased resistance to CAMPs facilitates the establishment of a more severe disseminated form of gonorrheal infection[36]. Phosphoethanolamine addition to LPS also occurs in *Salmonella typhimurium* and in colistin-resistant strains of *A. baumannii*[37]**,** where it increases resistance to polymyxin B[38].

In addition to adding positive charge to counteract the negative residues on LPS, some bacteria remove negative residues as an alternative mechanism of mitigating overall negative charge. The anionic phosphate groups of lipid A are major negative residues on LPS and are thus targets for removal. In *F. tularensis,* the 4' lipid A phosphate is removed by the phosphatase LpxF, leaving only one phosphate group on lipid A[39]. In *lpxF* deletion mutants that cannot remove the 4' phosphate, there is greatly increased susceptibility to polymyxin B, as well as the loss of lethality in a mouse intradermal infection[39,40]. Interestingly, the *lpxF* gene was exogenously expressed in *E. coli* whose lipid A normally has two phosphate groups. These modified *E. coli* lack a 4' lipid A phosphate and consequently display a >15-fold increase in polymyxin MIC[41]. The negatively charged phosphate groups on lipid A are a target for removal in many other pathogenic bacterial species, including *Porphyromonas gingivalis*[42], *Bacteroides fragilis*[43] and *Helicobacter pylori*[44]. Together, these data clearly demonstrate that CAMP resistance can be induced by removal of negatively charged lipid A residues.

Distinct from the alteration of charge, another strategy for generating CAMP resistance is to increase the hydrophobicity of LPS. Hydrophobic lipid chains, added to lipid A phosphates, the glucosamine backbone or existing acyl chains, serve to increase LPS saturation and decrease overall permeability, preventing CAMPs from inserting into the membrane[45]. In *Salmonella*, acyl chains are added to the glucosamine backbone and phosphates of lipid A by PagP[46]. *pagP* deletion mutants exhibited increased membrane permeability[46] and were nearly 4 times more susceptible to the antimicrobial peptide C18G (a synthetic CAMP derived from human platelet factor IV)[47]. Enhanced acylation of lipid A also occurs in *E. coli* and *Yersinia enterocolitica*[46].

Many of the LPS modifications described above are tightly regulated and induced upon exposure to CAMPs. The well characterized PhoPQ two component regulatory system of

Salmonella typhimurium controls several modifications that lead to CAMP resistance[48]. It plays a major role in pathogenesis, since deletion mutants lacking this system had over a 4 log virulence defect during murine infection[49]. The fact that this regulatory system contributes to CAMP resistance suggests that avoiding killing by these antimicrobials is critical for virulence. The sensor kinase PhoQ senses environmental stresses, such as low Mg^{2+} and Ca^{2+}, as well as those encountered by bacteria within macrophage phagosomes, even directly detecting the presence of the CAMPs LL-37 and C18G[50] leading to activation of the response regulator PhoP[51]. PhoP subsequently activates the PmrAB two component system[51]. PmrAB signaling leads to modification of lipid A phosphates with aminoarabinose, increasing charge, and 2-hydroxy myristate, increasing hydrophobicity and decreasing permeability[52]. The PhoPQ and PmrAB systems play similar roles in *Pseudomonas aeruginosa* [53] and *Serratia marcescens*[54], while PmrAB functions in lipid A modification in *Acinetobacter baumannii* in the absence of PhoPQ[55]. These data highlight distinct ways that bacteria inducibly modify lipid A to resist CAMPs, but lipid A is not the only portion of LPS that is a target for modification.

In addition to lipid A modifications, the O-antigen and core sugars have also been implicated in CAMP resistance. In *Brucella abortus*, transposon mutants that lack O-antigen showed significantly decreased survival in polymyxin B and were attenuated in a mouse model[56]. Mutants in *Burkholderia cenocepacia* with a truncated core were unable to grow in high concentrations of polymyxin B as did the wild type strain, and they were additionally outcompeted in a lung infection model[57]. Full length core and O-antigen thus can significantly contribute to CAMP resistance and have an important impact on virulence.

It is important to note that many CAMP resistant bacteria use several of the strategies listed above to mitigate the negative charge of their LPS. For example, *Helicobacter pylori* not only

decreases negative charge by removing a phosphate group, it also adds in its place a positively charged phosphoethanolamine[44], further increasing the charge of its lipid A. This results in extensive resistance to polymyxin B, with an MIC 25x higher than that of a deletion strain lacking these modifications. Many other Gram-negative bacteria use multiple strategies to mitigate the negative charge of LPS, and also modify other membrane components as well to further enhance CAMP resistance[58].

In addition to LPS, phospholipids are the other major component of the Gram-negative outer membrane. Similar to LPS, phospholipids in the outer membrane can also be modified to increase CAMP resistance **(Figure 1.1D)**. *S. typhimurium* uses its PhoPQ system to not only modify LPS, but also to modify phospholipids that reside in the outer membrane. PhoPQ-activated PagP adds palmitoyl groups to phospholipids, similar to its modification of lipid A described above. This leads to an increase in the levels of palmitoylated phosphatidylglycerols within the outer leaflet of the outer membrane, which are less polar and more hydrophobic than many other phospholipids in the outer membrane. Increased hydrophobicity in the outer membrane may decrease permeability, similar to the effect in lipid A palmityolation [59]. Therefore, localizing these modified phospholipids to the outer leaflet of the membrane results in increased CAMP resistance.

In addition, the inner membrane may be modified to increase CAMP resistance. Addition of lysine to phospholipids (lysylation) within the plasma membrane increases the charge of anionic phosphatidylglycerol to a cationic form, and thus is able to help repel cationic CAMPs and reduce their binding to the membrane. Though best studied in Staphylococcus aureus, these lysylated phospholipids are also present in Gram-negative species[60] including *Rhizobium tropici*[61] and *Caulobacter crescentus*[62].

It has also been suggested that the PagP protein mentioned above may act as part of an acute membrane repair response, facilitating rapid membrane repair after damage caused by CAMPs[59]. In addition, it has been hypothesized that one of the reasons that CAMPs do not efficiently damage eukaryotic host membranes is that eukaryotic cells have a much more robust form of membrane repair than bacteria[63]. Thus it is possible that bacteria with increased membrane repair capacity could survive higher concentrations of CAMPs, simply repairing the membrane as it is damaged. Dorschner et al suggest that proteins involved in membrane repair are prime candidates for investigation of microbial resistance[64]. There is still a lack of concrete evidence demonstrating that this occurs, but membrane repair may be an important facet of CAMP resistance and warrants further investigation.

Beyond alterations to the bacterial membranes, the outer surface of bacteria can be further modified to protect against CAMPs. The bacterial capsule is a protective layer external to the outer membrane that acts as an additional barrier and is comprised primarily of long chained repeating polysaccharides[65]. *Klebsiella pneumoniae* capsule provides increased resistance against cationic defensins, lactoferrins and polymyxins. Furthermore, there is a direct correlation between higher amounts of capsular polysaccharide, decreased levels of CAMPs binding to the outer membrane, and increased resistance to polymyxins[66]. Capsule-mediated resistance to CAMPs is likely critical for bacterial virulence during *in vivo* infection as an acapsular mutant was unable to cause pneumonia in a mouse model[67]. It should be noted, however, that the capsule can provide resistance to other immune pressures in addition to CAMPs, such as complement and phagocytosis, and thus the attenuation of the acapsular mutant is not necessarily due to a decrease in CAMP resistance.

In *Neisseria meningitidis,* capsule production was shown to increase resistance to the human CAMP LL-37[68]. Survival in the presence of LL-37 was 100-fold lower in a deletion strain lacking capsule compared to wild-type. Furthermore, upon exposure to sublethal levels of LL-37, the capsule biosynthetic genes *siaC* and *siaD* were upregulated and contributed to increased capsule production[68].

In addition to those mentioned above, numerous Gram-negative species express a polysaccharide capsule. Further, *P. aeruginosa* has also been shown to use its capsule to resist CAMPs[69]. Taken together, the data described here illustrate how Gram-negative pathogens can use numerous modifications to LPS, phospholipids, and the production of a polysaccharide capsule to resist CAMPs and protect their membranes.

Biofilm production

Bacteria can further resist CAMPs through their organization into specialized structures known as biofilms. In addition to free floating, planktonic bacterial populations, bacteria can form biofilms on diverse surfaces. These structures consist of sessile bacteria adhering to a surface in a highly organized manner that allows for circulation of nutrients[70]. Bacteria in a biofilm often secrete a slimy extracellular matrix that both aids in adherence to surfaces and acts as a barrier to outside stressors. This extracellular matrix can be composed of various compounds including cellulose, teichoic acids, proteins, lipids and nucleic acids[71]. Biofilms can form on environmental surfaces such as hospital equipment, allowing these populations to persist, and likely contributing to the growing problem of hospital-acquired infections. They can even form on ventilators and catheters, giving them access to mucosal sites in patients and further

promoting their infectivity. Biofilms are also able to form on biological surfaces such as teeth or the respiratory tract, often facilitating the establishment of chronic infections[70].

The general organization of the bacteria and extracellular components contributes to the protection offered by the biofilm structure. As a biofilm matures, it progresses from a thin homogeneous structure to a thicker, more heterogeneous form that contains many substructures. These can include stacks of bacteria forming "mushroom" shaped structures[72]. This is observed in *Pseudomonas aeruginosa,* which forms biofilms that display exceptional resistance to CAMPs and antibiotics, in some cases over 1,000 times as great as their planktonic form[73]. *Pseudomonas* biofilms contain a high level of the polysaccharide alginate, which is known to cause significant alterations to biofilm structure. A strain that overproduces alginate formed biofilms that were much thicker and more structurally heterogeneous, an architecture that acts as a more effective diffusion barrier to CAMPs[74]. Additionally, expression of *Pseudomonas* biofilm genes in *E. coli,* whose biofilms are normally flat and unstructured, resulted in the formation of biofilms with more complex architecture, correlating with increased resistance to the polymyxin antibiotic colistin. This increased resistance was not observed against other antibiotics such as ciprofloxacin, indicating that this protection may be specific to CAMPs[75]. Biofilm structure can vary greatly across different species and strains, which may account for some of the differences in CAMP susceptibility in various biofilms.

Specific components of the extracellular matrix have been shown to be critical for resistance to CAMPs. Anionic alginate in *P. aeruginosa* not only contributes to biofilm structure but can also bind to and induce conformational changes in invading CAMPs[76]. The CAMP-alginate complexes then oligomerize, hindering their ability to enter the biofilm[77]. Further, polysaccharides from biofilms of *K. pneumoniae* and *Burkholderia pyrrocinia* are able to bind

and sequester CAMPs[78]. Adding these polysaccharides to *E. coli* increased its MIC to CAMPs LL-37, human beta defensin 3, and Bac7(1-35). Extracellular DNA also forms an integral component of *P. aeruginosa*[79] and *S. typhimurium*[80] biofilms, and can also contribute to CAMP resistance. The negative charge of DNA allows it to bind and sequester cations from the surrounding environment. This results in an environment with a low concentration of cations, which is an activating signal for the previously mentioned PhoPQ system. This therefore results in the activation of CAMP resistance genes via PhoPQ that lead to LPS and other modifications[79].

In addition to signaling by PhoPQ, biofilms have several other inducible defenses against CAMPs. *P. aeruginosa* encodes the inducible biofilm gene *psrA*, which has been linked with greatly increased levels of CAMP resistance[81]. This gene was upregulated 3-fold in the presence of the CAMP indolicidin. Deletion mutants lacking *psrA* were less able to form biofilms, and showed significantly increased killing when challenged with indolicidin or polymyxin B. Pamp et al have shown that tolerance to colistin in *Pseudomonas* biofilms is due to metabolically active cells within the biofilm. While the less metabolically active cells in the biofilm were killed by colistin, a spatially distinct subset of more active cells were able to resist killing. These cells were able to upregulate PmrAB-regulated resistance genes responsible for lipid A modification[82]. Overall, biofilms confer bacteria with the ability to form a hardy structure that can withstand and resist destruction by high concentrations of CAMPs, as well as many other types of antimicrobials.

Efflux Pumps

Efflux pumps are complexes of mostly membrane bound proteins that move toxic compounds out of cells. Bacterial efflux pumps are active transporters, either directly requiring ATP or using an existing electrochemical potential gradient. These complexes play important roles in antibiotic resistance, as many bacteria use them to resist major classes of antibiotics, including fluoroquinolones, macrolides, tetracyclines, glycylcyclines, beta lactams and aminoglycosides[83]. In addition, bacterial efflux pumps contribute to colonization and persistence, likely in part by defending against host antimicrobials such as CAMPs[84]. Indeed, there are many examples of Gram-negative bacteria that use efflux pumps to increase survival and virulence *in vivo* even in the absence of antibiotics including *Salmonella typhimurium*[85,86], *Salmonella enteritidis*[87], *Enterobacter cloacae*[88], *Borrelia burgdorferi*[89], *P. aeruginosa*[90], *K. pneumoniae*[91], *V. cholerae*[92] and *N. gonnorhoeae*[93].

In addition to other resistance mechanisms described above, *K. pneumoniae* uses the AcrAB-TolC efflux pump system, known to mediate resistance against fluoroquinolones, to resist CAMPs. When the AcrB component of the efflux pump system was knocked out, mutant bacteria exhibited increased sensitivity to fluoroquinolones as well as polymyxin B[91]. The *acrB* mutant also exhibited 10-fold lower survival in bronchoalveolar lavage fluid, which contains many CAMPs, and specifically displayed increased sensitivity to the human alpha defensin HNP-1 as well as human beta defensins HBD-1 and HBD-2. Importantly, this increased susceptibility correlated with a 1-3 log attenuation of the mutant in a mouse pneumonia model[91].

Another pathogen that expresses efflux pumps to increase CAMP resistance is *Yersinia enterocolitica.* A human gut pathogen, *Y. enterocolitica* has a high level of resistance to human CAMPs, at least in part due to the action of the RosAB efflux pump system. A *rosAB* deletion mutant was more sensitive than wild-type to the CAMPs polymyxin B, cecropin P1 (produced in

pig bladders) and melittin (found in bee venom)[94]. This pump acts as a potassium antiporter, using a potassium gradient that pumps K^+ ions into the cell as it pumps out harmful CAMPs. Interestingly, the RosAB pump is activated at 37ºC and in the presence of CAMPs, similar to conditions encountered within the host during infection[94]. Under these conditions, pathogenic *Y. enterocolitica* strains are more resistant to CAMPs than non-pathogenic strains or a control *E. coli* strain[95]. While this was not explicitly shown to be due to the RosAB pump and could be due to another temperature regulated system, the data suggest that RosAB-mediated CAMP resistance is likely important for maintaining pathogenicity in *Y. enterocolitica*.

N. gonorrhoeae possess the Mtr (multiple transferrable resistance) efflux pump which facilitates resistance to numerous antimicrobials. This three protein system has been shown to pump out various hydrophobic compounds, such as bile salts and fatty acids, which can cause membrane damage. This pump also confers resistance to CAMPs as well. *mtr* deletion mutants had significant growth defects in the presence of PG-1, a protegrin produced by porcine macrophages[96], and the MIC of the human CAMP LL-37 and horseshoe crab-derived tachyplesin-1 were also reduced in the *mtr* deletion mutant. Thus, the Mtr efflux pump is able to recognize a variety of CAMP structures and remove them from the bacterial cell [96]. This efflux pump is highly relevant for *in vivo* survival; gonococci lacking *mtr* were completely outcompeted by the wild type strain in a competitive infection of the mouse genital tract[93] and this was correlated with the levels of CAMP resistance *in vitro*[97]. The closely related *Neisseria meningitidis*, which can cause meningitis in humans, also expresses the *mtr* efflux pump and it was similarly shown to contribute to CAMP resistance[98].

The RND family of efflux pumps in *Vibrio* species has a similar activity in mediating resistance to polymyxins and bile acids. *V. cholerae* has at least six loci that encode RND family

proteins, including the VexB protein which can mediate CAMP resistance. When this protein is deleted from a virulent strain, the mutant bacteria exhibit increased susceptibility to polymyxin B as well as bile acids, which are found in the GI tract that *V. cholerae* infects. Further, this deletion mutant was unable to effectively colonize the gut of mice when compared to the wild-type strain[99]. The closely related *Vibrio vulnificus*, which can cause wound infections and sepsis, encodes a different efflux pump, TykA, which is responsible for resistance to the CAMPs protamine and polymyxin B [100].

Efflux pumps have been shown to be important for resistance to a wide range of antibiotics and there has been much interest in using efflux pump inhibitors to enhance antibiotic treatment[101]. However, the extensive evidence that these pumps can enhance CAMP resistance and play a role in virulence suggests that efflux pump inhibitors may also be used therapeutically to sensitize bacteria to innate immune defenses. Inactivating bacterial efflux pumps responsible for CAMP resistance could enhance the ability of the host CAMPs to clear infections, while at the same time increasing sensitivity to antibiotics.

Binding and sequestering CAMPs

When confronted with a large concentration of CAMPs, some bacteria are able to bind and sequester these peptides so they cannot reach the bacterial membrane. One method for binding external CAMPs is through the release of negatively charged molecules that will attract these amphipathic antimicrobials. Negatively charged proteoglycans are found in abundance on the surface of fibroblasts and epithelial cells, and can be cleaved and released by bacterial enzymes at rates that exceed that of baseline release. For example, the connective tissue proteoglycan decorin is one of the major secreted products of human fibroblasts[102], and when incubated with

P. aeruginosa or *P. mirabilis,* it is cleaved to release several products, including dermatan sulphate. This degradation occurs in the presence of bacteria conditioned media, purified *P. aeruginosa* elastase, or alkaline proteinase, even in the absence of fibroblast enzymes. This released dermatan sulphate was able to efficiently bind neutrophil derived α-defensin unlike the full length uncleaved decorin molecule. This free and soluble dermatan sulphate was able to nearly completely inhibit killing by defensins at concentrations 10 times above the MIC for *P. aeruginosa*[103].

Similarly, *P. aeruginosa* takes advantage of the release of the cell surface heparin sulfate proteoglycan syndecan-1. This proteoglycan is found on the surface of epithelial cells, and is shed during tissue injury as a soluble ectodomain. Incubating epithelial cells with cell culture supernatants from *P. aeruginosa* led to cleavage of syndecan-1 and release of its soluble ectodomain[104]. This activity was found to be dependent on the *P. aeruginosa* protein LasA, which is a known virulence factor and has been previously shown to modify other proteins. Shedded ectodomains of syndecan-1 are able to bind and interfere with the antimicrobial activity of CAMPs, specifically those that are Pro/Arg-rich like cathelicidins[104], likely due to charge based interactions. This was also demonstrated *in vivo*, with increased syndecan-1 shedding from epithelial cells during *P. aeruginosa* lung infection in a mouse model[105]. The virulence of the pathogen was dependent on syndecan-1 shedding, as there was a 3 log decrease in virulence if syndecan-1 was absent or rendered resistant to shedding[105]. Syndecan-1 ectodomains not only bind to CAMPs but can also bind and interfere with a range of other immune signaling molecules [104] such as cytokines and matrix metalloproteases[106]. It is not yet known the downstream effect that this binding would have on the greater immune response, but immune modulation in

addition to direct interference with CAMPs may together account for the observed virulence decrease[105].

The fact that proteoglycans are able to interfere with host CAMP activity suggests that the bacterial capsule, which is rich in polysaccharides, may also be able to capture and sequester CAMPs[23]. Acapsular mutants often have decreased virulence *in vivo*, and *K. pneumoniae* acapsular mutants are more susceptible to α- and β-defensins[66]. This idea was further strengthened by evidence from Llobet et al, showing that the anionic polysaccharide component (CPS) of the bacterial capsule is able to impart CAMP resistance in *K. pneumoniae* and *P. aeruginosa*[69]. Purified CPS was able to increase the resistance of acapsular mutants, and was shown to bind to soluble CAMPs in a charge-dependent manner. This resulted in fewer peptides reaching the surface of the bacteria. After exposure to CAMPs, these anionic polysaccharides are released by the bacteria to bind and sequester the antimicrobials[69]. It is possible that other encapsulated bacteria can use this mechanism to enhance CAMP resistance as well.

Another component that can be released to trap CAMPs is part of the bacterial cell membrane itself, in the form of enclosed vesicles budding off from the surface known as outer membrane vesicles (OMVs). OMV release is a normal part of bacterial cell growth[107] and may be used for a variety of processes such as toxin delivery[108]. In *E. coli*, membrane stress, especially from accumulation of proteins in the outer membrane, induces an increase in OMV formation. As the targets of CAMPs are bacterial membranes, CAMPs can be bound and sequestered in these vesicles, diverting them from the membranes of living bacteria. This notion is supported by the fact that mutants that over produce OMVs are 6-fold more resistant to killing by polymyxin B, while a mutant lacking vesicle release was 10-fold more susceptible[109]. *Vibrio cholerae* has adapted its OMV response to aid in CAMP resistance as well. In the presence of sublethal

concentrations of polymyxin B, it was noted that OMVs released from the bacteria were larger and had altered protein content[110]. These OMVs were better able to protect against CAMPs, as co-incubating bacteria with them doubled the level of protection against LL-37 when compared to OMVs produced by bacteria in the absence of polymyxin B. The polymyxin B induced OMVs contained elevated levels of the protein Bap1, which was shown to mediate the increased CAMP protection by binding to but not degrading LL-37. Thus, OMV release can act as an inducible defense against CAMPs that can significantly increase levels of resistance.

Proteolytic Degradation

In addition to mechanisms to block access of CAMPs to bacteria, or pump them out of the cell, direct inactivation of these antimicrobials offers another means by which bacteria can combat them. As summarized below, diverse bacteria produce proteases that degrade CAMPs, an activity that is highly reliant upon the structural motifs of the target peptide. The human CAMP LL-37 is a linear, alpha helical peptide and is thus more susceptible to degradation by proteases than CAMPs with non-linear structures containing disulfide bonds such as defensins[111]. *P. aeruginosa* produces an elastase that is capable of rapidly degrading LL-37 *in vitro*, with its bactericidal activity completely inactivated within 1 hour[112]. Structural analysis showed that cleavage occurred at 4 peptide bonds all located within the regions of LL-37 that have bactericidal activity. Further, a *Proteus mirabilis* proteinase and *E. faecalis* gelatinase degrade and inactivate LL-37 *in vitro*, allowing for survival of bacteria in the presence of otherwise lethal doses of this antimicrobial[112]. In *S. typhimurium,* the omptin family protease PgtE degrades various alpha-helical CAMPs, including human LL-37 and its murine ortholog CRAMP[113]. Strains with deletions of this gene had 2-fold lower MICs to both CAMPs, while overexpression of *pgtE* increased the MIC by 8-fold. Interestingly, *pgtE* expression is regulated by PhoPQ,

highlighting another example of the many CAMP resistance mechanisms controlled by this two-component system.

Even though linear CAMPs are quite sensitive to degradation by proteases, they can be shielded and protected by binding to proteins such as extracellular actin. *In vivo*, LL-37 can bind to released actin molecules, preventing the access of degradative proteases while still maintaining its antimicrobial activity[114]. High levels of extracellular actin were found in areas of cell necrosis, which often occurs at sites of infection [114]. Thus, linear CAMPs like LL-37 can be protected and rendered much less vulnerable to proteolytic degradation *in vivo* due to their complexing with other proteins.

Many non-linear CAMPs are more resistant to degradation than linear CAMPs. This is due at least in part to intramolecular disulfide bonds, such as those found in the defensins, which contain a canonical array of 6 disulfide linked cysteines[115]. These linkages create non-linear tertiary structure that is much more stable in the environment and resistant to protease degradation[116,117]. However, some bacteria have evolved proteases to degrade even these CAMPs with increased stability. The protein OmpT is another omptin family protease and contributes to CAMP resistance in *E. coli*. Stumpe et al have shown that this outer membrane protein degrades the CAMP protamine[118] which is thought to conform to a nonlinear structure involving three disulfide bonds[119]. Both the OmpT and PgtE omptin proteins are present in *Shigella flexneri* and *Yersinia pestis*, suggesting that these pathogens may also use omptins to degrade CAMPs[120].

B. cenocepacia has two zinc dependent metalloproteases that have been shown to degrade CAMPs, ZmpA and ZmpB. Each of these proteins can degrade a variety of peptides, including a wide range of CAMPs. These proteins have distinct substrates, as only ZmpA can degrade linear

LL-37, while only ZmpB degrades non-linear human beta defensin-1[121]. Other CAMPs like protamine, elafin and SLPI (all of which are non-linear with disulfide bonds) were degraded by both, but they seemed to be digested into different fragments by each protease. These proteases are additionally important for the virulence of *B. cenocepacia,* since deletion mutants of each protease individually results in decreased lung pathology in a mouse infection model[122,123].

Along with its previously mentioned proteinase, *P. mirabilis* encodes the virulence factor ZapA that is involved in CAMP degradation. The ZapA protein is a secreted extracellular metalloprotease that is able to degrade a wide variety of targets, including host defense proteins such as immunoglobulins and complement components[124]. It is also able to target host CAMPs, including not only LL-37, but also disulfide bond containing defensin HBD-1. Proteolysis of LL-37 and HBD-1 by ZapA resulted in 6 and 9 fragments respectively, completely inactivating both proteins[124]. Importantly, absence of ZapA in *P. mirabilis* results in a 4 log decrease in bacteria in a mouse urinary tract infection model, suggesting that the degradation of host antimicrobials is vital to the virulence of this pathogen[125]. Many additional examples of bacteria directly degrading (or causing the degradation of) CAMPs likely exist, and presumably make important contributions to *in vivo* virulence.

Bacteria can also take advantage of host enzymes with CAMP-degrading activity. Reduced killing of *P. aeruginosa* by beta defensins in the broncho-alveolar fluid of cystic fibrosis patients has been shown to be due to the release of proteolytic cathepsins from macrophages, which are able to degrade host beta defensins. The release of cathepsins is due at least in part to the release of inflammatory mediators such as IL-13 and IFN-γ, which has been suggested to result from immune activation by LPS from *P. aeruginosa* and other commensal Gram negative bacteria[126].

Thus, *P. aeruginosa* is able to take advantage of the host immune response, facilitating the release of CAMP degrading enzymes.

Gram-negative pathogens use many diverse mechanisms to resist killing by cationic antimicrobial peptides. Bacteria can alter surface structures to repel CAMPs, establish biofilms to increase resistance, use efflux pumps to pump them out, sequester them, produce proteases to degrade them, or alter immune responses to prevent their induction. Resistance to CAMPs is thought to have a significant negative effect on the ability of the host to prevent and fight bacterial infections, and also threatens the utility of polymyxins in the clinic. Unfortunately, due to their recent increased use, resistance to polymyxins is already on the rise[19]. Resistance to host cationic antimicrobials may even be facilitated by resistance to polymyxins. Clinical strains of *A. baumannii* that are polymyxin resistant are significantly more resistant to both LL-37 and lysozyme[24]. This has also been demonstrated in *Enterobacter cloacae*, where colistin heteroresistant strains are resistant to lysozyme after initial treatment with colistin[25]. This phenomenon of heteroresistance is a unique and intriguing form of antibiotic resistance yet there is much that is unknown about this important phenotype.

Antibiotic Heteroresistance

Antibiotic resistance is often thought of as an increase in resistance that is homogenous across a population of genetically identical cells. However, resistance to antibiotics can phenotypically vary within isogenic populations.

Heteroresistance as a Form of Phenotypic Resistance

Studies on mechanisms of antibiotic resistance have typically focused on genetic mutations or acquisition of antibiotic resistance genes in distinct clinical isolates of bacteria. Beyond genotypic resistance, there also exist variations in phenotypic resistance among isogenic bacterial cells. Perhaps the best known phenotypic resistance phenomenon is that of persistence, in which a small subpopulation of bacteria that are temporarily quiescent or very slow-growing display increased resistance to a wide range of antibiotics[127]. It is thought that persistence can mediate the relapse of infection after cessation of antibiotic therapy.

A distinct phenomenon is heteroresistance, in which a subpopulation of resistant bacteria **(Figure 1.2A)** can rapidly replicate in the presence of an antibiotic **(Figure 1.2C)**, while genetically identical susceptible cells are killed). It should be noted that the term "heteroresistance" has also been used to describe mixed populations of genetically distinct bacteria. In some cases these are closely related bacteria exhibiting a point mutation[128], and in others the term has been used to describe co-infection with two unrelated strains[129]. A more stringent definition is required, and as such we refer here to heteroresistance exhibited by genetically identical subpopulations.

The relevance of heteroresistance has been debated; can the minor subpopulations of resistant bacteria affect treatment outcome? There are numerous examples of vancomycin heteroresistance in *Staphylococcus aureus* and it has been suggested that this may cause vancomycin treatment failure[130]. While some reports suggest that vancomycin heteroresistance can alter treatment outcomes[131] others have found that vancomycin is still effective in treating such strains[132]. Beyond *S. aureus*, few studies have investigated the impact of heteroresistance on the outcome of *in vivo* antibiotic therapy.

Heteroresistance in the Clinic

Heteroresistance has been observed in clinics across the world, in a variety of pathogens and to numerous classes of drugs. In addition to *E. cloacae* and *S. aureus*, heteroresistance has been detected in *Klebsiella, E. coli*, *Acinetobacter*, *Pseudomonas* and other species[133]. Heteroresistance has been demonstrated not only to colistin and vancomyin, but also to beta-lactams including carbapenems[133]. In a study of *E. coli* isolates in southwest China, 3.9% were heteroresistant to meropenem, 17.2% to ertapenem, and 25.0% to imipenem[134]. Additionally, *A. baumannii* isolates from a cohort of Spanish hospitals displayed imipenem heteroresistance (20%) and meropenem heteroresistance (24%)[135]. Overall, however, the prevalence of cHR to numerous antibiotics and in many pathogens has not been thoroughly studied. This is in part due to the fact that it can be difficult to detect cHR by conventional diagnostics, which are not optimized to detect heteroresistance. Often, resistant subpopulations will be present at a very low level, resulting in a misclassification of the isolate as susceptible in these tests **(Figure 1.2b, d)**. If these isolates are then treated with that antibiotic, this may lead to treatment failure due to the unidentified resistant subpopulation.

Mechanisms of Heteroresistance

Often the sources of the resistant subpopulations in heteroresistant isolates are the same genes that confer resistance in conventionally resistant strains. However the mechanism by which a strain maintains both susceptible and resistant subpopulations remains more elusive. In non-clonal forms of heteroresistance, this is often explained by a point mutation within the resistant subpopulation that confers this resistance. In clonal heteroresistance, it is possible that a mechanism of bistability is controlling the variation in resistance across the population. It has

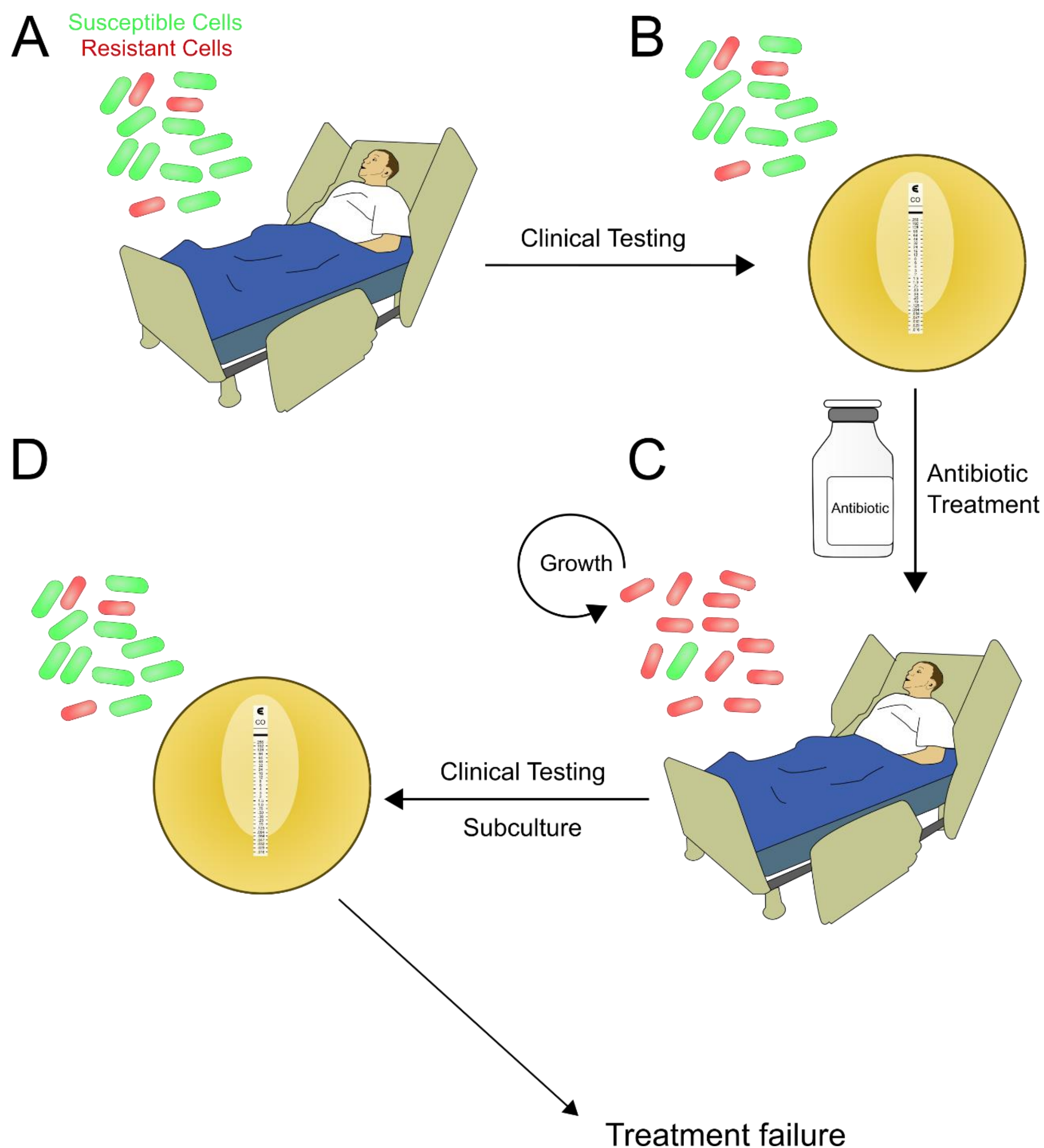

Figure 1.2. Characteristics of heteroresistance to antibiotics suggest possible clinical impact. (A) Infection with heteroresistant strain, green represent susceptible cells, red represents resistant cells. (B) Clinical testing of infecting heteroresistant pathogen is unable to detect resistant subpopulation. (C) Antibiotic treatment occurs without detection of resistant subpopulation. The resistant subpopulation is augmented by antibiotic treatment. (D) Subsequent testing results in decrease in resistant subpopulation due to subculture, and testing does not detect resistant subpopulation. Continued antibiotic therapy will lead to treatment failure.

been shown in an engineered strain of beta-lactam heteroresistant *E. coli* that a growth centric feedback loop allowed for cells within the population to display varying levels of beta-lacmase and thus varying levels of resistance[136]. Additionally, gene duplication may play a role in this variation in resistance across a population. Bacteria can modify their genome by increasing the number of copies of a specific gene; if this happens to be a resistance gene it could lead to a form of heteroresistance where the level of resistance is dependent on the number of copies within each bacterium[137].

Beyond Antibacterials: Heteroresistance in Other Fields

While heteroresistance has been best characterized in bacteria, this phenomenon exists in other pathogens as well. Infectious species of *Candida* can display heteroresistance to the antifungals amphotericin B and fluconazole[138]. Pathogenic *Cryptococcus* species have been shown to develop heteroresistance to itraconazole, while simultaneously gaining increased virulence and altered morphology[139]. While parasitic pathogens have not been reported to display heteroresistance to antiparasitics, there is one report of heteroresistance to human serum. In *Trypanosoma rhodesiense*, the pathogen responsible for African sleeping sickness, some cells exhibit resistance to serum due to differential expression of a surface glycoprotein, facilitating disease in humans[140].

Phenotypic heterogeneity to chemotherapeutics can also occur in cancer[141]. Some cancers have been observed to harbor a small population of phenotypically resistant cells exhibiting chromatin modifications, allowing the cells to resist 500 times greater concentrations of chemotherapeutic tyrosine kinase inhibitors. Thus, heteroresistance may explain the resistance of

some cancers to chemotherapeutics when the majority of tumor cells appear to respond to therapy[142].

Taken together, it is clear that heteroresistance is exhibited by both prokaryotic and eukaryotic populations of cells. Evidence suggests that this phenomenon can have a major impact on the outcome of drug therapy, and warrants much greater study. It is of the utmost importance that we investigate the prevalence of this form of resistance across numerous pathogens and cancers, and redefine our diagnostic and treatment methods to adapt to these resistant subpopulations.

In this study, we investigate the phenomenon of heteroresistance to the last line, cationic antimicrobial peptide, colistin. It is concerning that this type of resistance, which is poorly understood, occurs to a last line drug such as colistin. Thus, we sought to determine the possible clinical impact of colistin heteroresistance.

Chapter 2:

Antibiotic failure mediated by resistant subpopulation in *Enterobacter cloacae*

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Abstract

Antibiotic resistance threatens the delivery of safe and effective healthcare[11] and is projected to lead to 10 million annual deaths worldwide by 2050[2]. Further complicating this epidemic are unexplained antibiotic treatment failures caused by bacteria that appear susceptible to an antibiotic[3]. We describe an *Enterobacter cloacae* isolate harboring a minor subpopulation highly resistant to the last-line antibiotic colistin. This subpopulation is distinct from persisters and became predominant in the presence of colistin, yet returned to baseline after removal of the drug. During murine infection, but in the absence of colistin therapy, specific host innate immune defenses led to an increased frequency of the colistin-resistant subpopulation. This initial pressure by host defenses led to subsequent inefficacy of colistin therapy, which was unable to prevent a lethal infection. Presence of the resistant subpopulation was dependent on the histidine kinase PhoQ, which was required for modification of the outer membrane component lipid A. A genetically distinct *Enterobacter* clinical isolate displayed an even lower frequency colistin-resistant subpopulation that similarly increased during infection. Importantly, this colistin-resistant subpopulation was undetectable by current diagnostic methods once cultured outside the host. These data demonstrate the ability of low frequency bacterial subpopulations to contribute to clinically relevant antibiotic resistance, elucidating an enigmatic cause of antibiotic treatment failure and highlighting the critical need for more sensitive diagnostics.

Results

Multi-drug resistant *Enterobacter* spp. have emerged as an increasing cause of hospital acquired infections[143-145], with the drug colistin being relied on as a last line treatment[146,147]. Even colistin resistant strains have emerged recently, further limiting treatment options[148]. A

strain of *Enterobacter cloacae* was isolated from a renal transplant recipient[25] and was observed to harbor a distinct subpopulation with resistance to colistin, visualized as numerous colonies within the zone of inhibition upon testing by colistin Etest (we refer to the strain as "R/S", to indicate the presence of both resistant and susceptible subpopulations) **(Figure 2.1a)**. This was not observed with either colistin susceptible or resistant **(Figure S2.1)** clinical strains. Population analysis profile (PAP) of R/S, in which a strain is assayed for survival on agar plates with increasing amounts of antibiotics, revealed a major proportion of bacteria (>90%) susceptible to 1 µg/mL colistin, and a highly resistant subpopulation, able to withstand at least 500 µg/mL colistin **(Figure 2.1b)**. This was in contrast to the susceptible strain that was uniformly killed by 1 µg/mL colistin, and the resistant strain that was uniformly killed by 200 µg/mL colistin. The proportion of the R/S colistin resistant subpopulation was increased to upwards of 80% upon exposure to colistin **(Figure 2.1c)**. Further analysis revealed that this increase was due to an initial selection against the colistin susceptible population over the first 2 hours of antibiotic exposure, followed by robust replication and expansion of the resistant population in the presence of the drug **(Figure 2.1d)**. Importantly, this suggests that the resistant cells are not persisters, which do not significantly expand in number during antibiotic treatment[149-151]. The increase in the resistant subpopulation was reversible, as subsequent growth after subculture in antibiotic free media led to a return of these cells to pre-treatment levels **(Figure 2.1c)**. This suggests that the resistant subpopulation is not the result of a stable mutation. Furthermore, bacteria from within the zone of inhibition (where antibiotic levels are high) and outside this region (where antibiotic is low or not present) on a colistin Etest plate **(Figure 2.1a)** exhibited identical levels of susceptible and resistant populations after serial culturing in the absence or presence of colistin **(Figure S2.2)**, suggesting that bacteria from these two growth conditions are

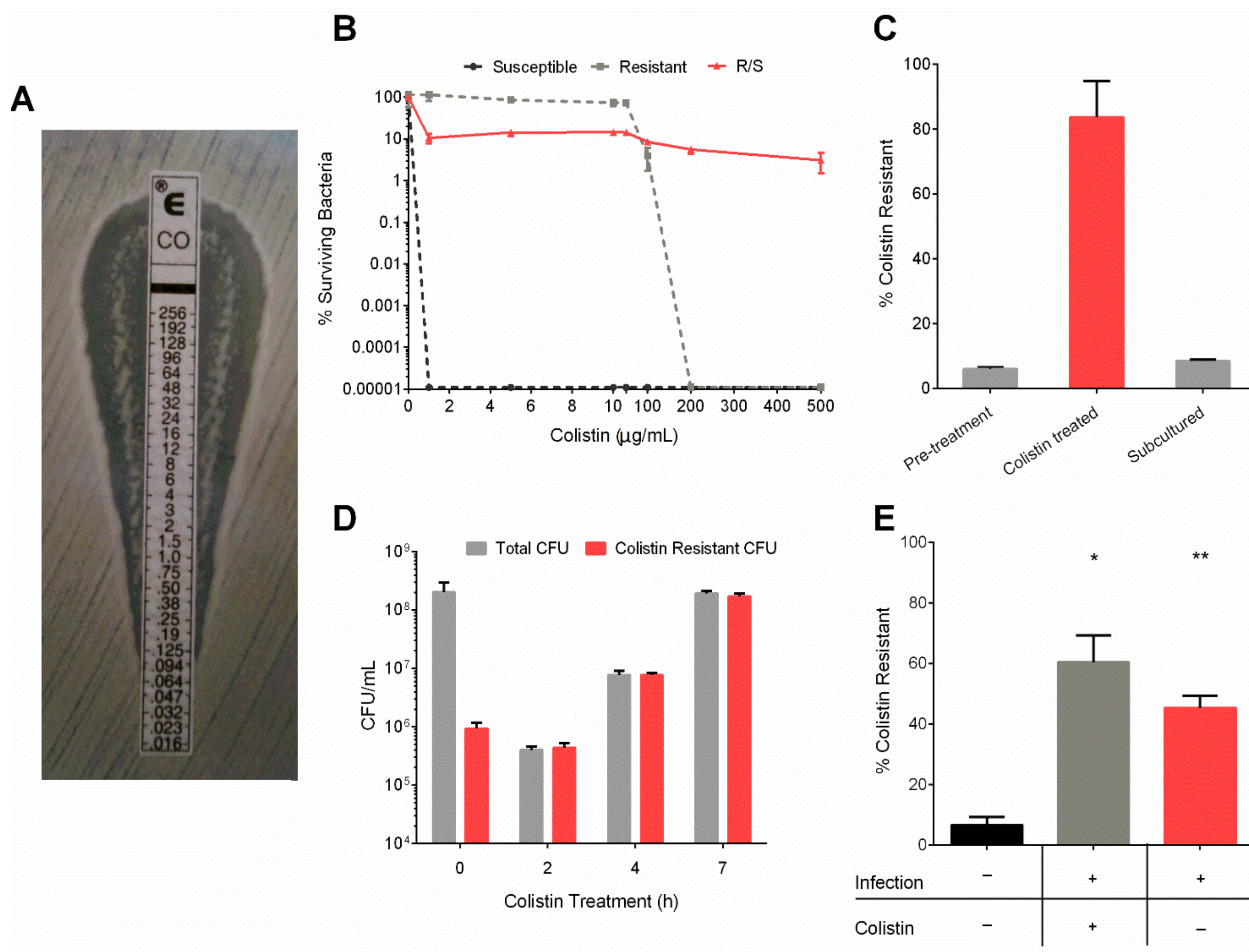


Figure 2.1. A colistin resistant subpopulation increases in frequency during *in vivo* infection. (A) Testing of *E. cloacae* clinical isolate R/S by colistin Etest. Colonies within the zone of inhibition indicate a colistin resistant subpopulation. (B) Population analysis profile of R/S as well as colistin-susceptible and -resistant *E. cloacae* clinical isolates. (C) Percentage of the colistin resistant subpopulation in R/S in antibiotic-free media, after 24 h treatment with 100 µg/mL colistin, and after 8 h subculture of the colistin treated culture in antibiotic free media. "% Colistin resistant" represents the number of CFU in each culture that can grow on media containing 100 µg/mL colistin, as a percentage of the total CFU in the culture. (D) Colistin resistant and total CFU of R/S during 7 h treatment with 100 µg/mL colistin in liquid culture. (E) Pre-infection inoculum (black bar) was used to infect mice, and peritoneal lavage was performed and harvested 24 h later and plated to calculate % colistin resistant CFU. Mice were treated at 8, 14 and 20 h with colistin (grey bar) or PBS (red bar). * $p < 0.05$, ** $p < 0.01$.

identical. Indeed, deep sequencing of R/S grown with and without colistin (conditions in which the resistant population accounted for the vast majority or minority of the total population, respectively, as summarized in **Figure S2.3** revealed identical genomes. Taken together, these data show that a minor antibiotic resistant subpopulation is capable of replicating in the presence of antibiotic, becoming predominant, and mediating resistance to high levels of drug.

To determine whether the increase in the proportion of the resistant subpopulation occurs during antibiotic treatment *in vivo*, we infected mice with R/S and treated with colistin or PBS. In colistin treated mice, we observed a significant increase in the frequency of the resistant subpopulation of bacteria isolated from the peritoneum **(Figure 2.1e)** and liver **(Figure S2.4)**. Surprisingly, there was also a robust increase in the resistant subpopulation during *in vivo* infection in the absence of colistin treatment **(Figure 2.1e, Figure S2.4)**. By 48 hours, the percentage of the resistant subpopulation increased from <10% to >80% **(Figure S2.5)**. These results highlight the process of infection as leading to a significant increase in the frequency of an antibiotic resistant subpopulation of bacteria.

Various host pressures could be responsible for the increase in the colistin resistant subpopulation during infection. As macrophages are a major component of the early immune response[152], we tested their role by depleting these cells with clodronate liposomes[153] **(Figure S2.6)** and subsequently infecting mice with R/S. In contrast to bacteria recovered from mice treated with control liposomes, which demonstrated a robust increase in the frequency of the resistant subpopulation, those recovered from macrophage-depleted mice showed no such increase **(Figure 2.2a)**. Based on these results, we next determined whether macrophages were sufficient to cause the increase in the resistant subpopulation, by infecting them *in vitro*. During

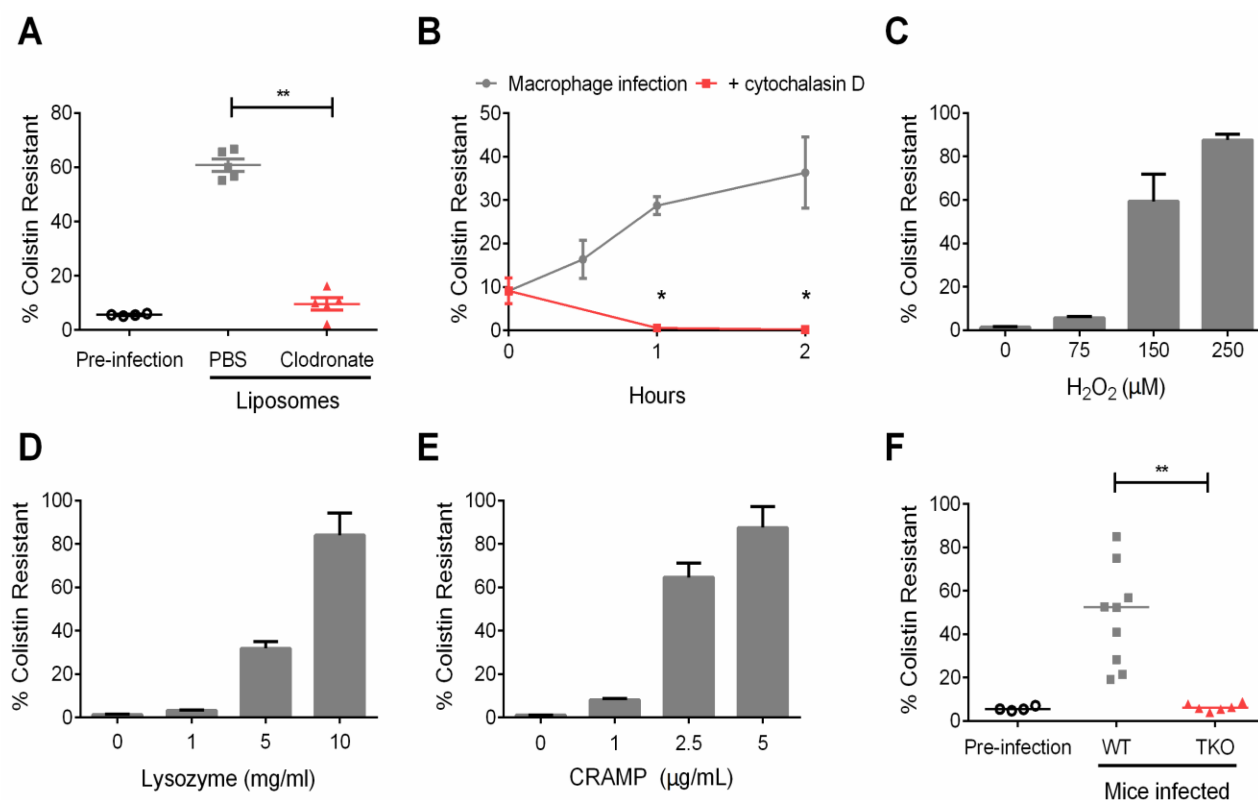


Figure 2.2. Innate immune host defenses are required for the increased frequency of the colistin resistant subpopulation during infection. (A) Mice pre-treated with PBS liposomes (as a control; grey) or clodronate liposomes (to deplete macrophages; red) were infected with R/S (pre-infection; black). After 8 h, peritoneal lavage fluid was harvested and plated to calculate % colistin resistance. (B) Murine bone marrow-derived macrophages were untreated or pretreated with cytochalasin D, infected with R/S, and % colistin resistance was calculated at the indicated timepoints. (C-E) R/S was either untreated or treated with the indicated amounts of (C) H_2O_2, (D) lysozyme, or (E) CRAMP for 5 h, and % colistin resistance was calculated. (F) Wild-type (WT; grey) or triple knockout (TKO; red) mice lacking the gp91 subunit of the NADPH oxidase, lysozyme, and CRAMP were infected with R/S (pre-infection; black). At 8 h postinfection, peritoneal lavage fluid was harvested and plated to calculate % colistin resistance. Data is compiled from two independent experiments. * $p < 0.05$, ** $p < 0.01$.

macrophage infection, the colistin resistant subpopulation increased to 40% within only 2 hours **(Figure 2.2b)**. Furthermore, this rise was dependent on internalization of the bacteria, since preventing phagocytosis with cytochalasin D abrogated this phenomenon **(Figure 2.2b)**. Therefore, macrophages are both required and sufficient for the increased frequency of the resistant subpopulation during infection, underlining a role for a specific innate immune cell type in this process.

Macrophages possess many antibacterials[154] and we hypothesized that specific components would be required for the increase in the frequency of the resistant subpopulation, testing reactive oxygen species (formed after treatment with hydrogen peroxide), lysozyme, and the murine cationic antimicrobial peptide CRAMP. All of these antibacterials resulted in a dose-dependent increase in the frequency of the colistin resistant subpopulation *in vitro* **(Figure 2.2c-e)**, as did LL-37, the human ortholog of CRAMP **(Figure S2.7)**. These results led us to test whether the antibacterials were responsible for the increase in the resistant subpopulation during *in vivo* infection. We infected wild-type and triple knockout (TKO) mice lacking a functional NADPH oxidase (which leads to the production of reactive oxygen species[155]), lysozyme, and CRAMP. TKO mice were more susceptible to infection by R/S as they harbored over 10-fold more bacteria compared to WT **(Figure S2.8)**, demonstrating the importance of these antimicrobials in host defense. While a robust increase in the frequency of the resistant subpopulation was observed in wild-type mice, this was abrogated in TKO mice **(Figure 2.2f)**. The frequency of the resistant subpopulation in mice lacking one of these three antimicrobials was not significantly different from that in wild-type mice, while it was decreased in double KO mice lacking the NADPH oxidase and CRAMP or lysozyme **(Figure S2.9)**. These data identify a

role for specific host innate immune antibacterials in the increase of an antibiotic resistant subpopulation during *in vivo* infection.

To determine the relevance of the increase in frequency of the resistant subpopulation during *in vivo* infection, we tested whether the R/S strain was able to resist colistin treatment. We infected mice with either R/S or a colistin susceptible strain, and treated the mice with PBS (as a control) or high doses of colistin after establishment of infection to simulate the progression of infection and treatment in the clinic. The levels of the susceptible strain in the peritoneum **(Figure 2.3a)** and liver **(Figure 2.3b)** were significantly reduced by colistin treatment. In contrast, the R/S strain was refractory to treatment with colistin as its levels were unchanged between the treated and untreated groups **(Figure 2.3a-b)**. In a timecourse experiment, the level of the susceptible strain was reduced by 3 logs at 42 hours, whereas the level of the R/S strain was not diminished by colistin treatment, but instead increased by roughly 10-fold **(Figure S2.10)**. These data demonstrate that the presence of the resistant subpopulation results in inefficacy of colistin to reduce bacterial levels *in vivo*. Further, these results provide *in vivo* evidence that the resistant subpopulation does not behave like persisters which do not significantly expand in number during antibiotic treatment.

We next tested whether the role of the host immune system in the increase of the resistant subpopulation was directly responsible for the inefficacy of antibiotic therapy. We first found that colistin treatment of R/S-infected mice could cause a significant reduction in bacterial levels if initiated at the time of infection (prior to the increase in the frequency of the resistant subpopulation), but not if it was delayed until only 4 hours after infection **(Figure 2.3c)**. However, in macrophage-depleted mice, treatment with colistin at 4 hours became effective, leading to a reduction in bacterial levels **(Figure 2.3c)** and indicating that the host-driven

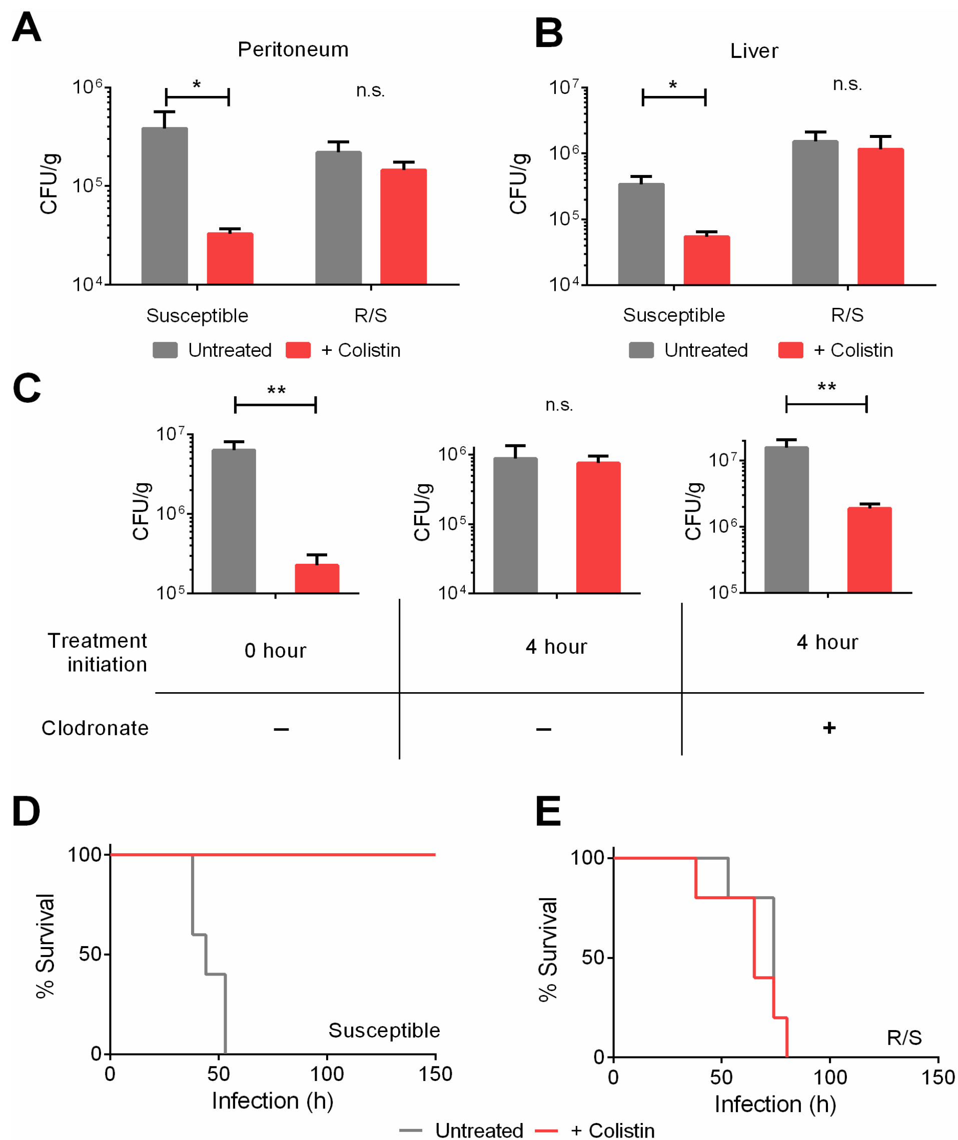


Figure 2.3. R/S is refractory to colistin during infection and leads to colistin treatment failure. (A,B) Mice infected with R/S or the susceptible isolate were treated with colistin at 8, 14 and 20 h. CFU were quantified at 24 h in the (A) peritoneal lavage fluid and (B) liver. (C) Mice pretreated with PBS (first and second panels) or clodronate (third panel) liposomes were infected with R/S and treated with colistin at 0 h (first panel) or 4 h (second and third panels). A second dose of colistin was administered 2 h after the first, and 2 h later peritoneal lavage fluid was plated to enumerate CFU. (D,E) Survival of mice infected with R/S or the colistin susceptible isolate. Mice were treated with colistin or PBS starting at 12 h post infection, with additional doses given every 6 h thereafter. Surviving mice were monitored until day 24. * $p < 0.05$, ** $p < 0.01$, n.s. = not significant.

increase in the frequency of the resistant subpopulation is responsible for the inefficacy of antibiotic treatment.

To further test the relevance of this *in vivo* phenomenon, we infected mice with a lethal dose of bacteria and treated with either PBS or colistin after 12 hours. Both the susceptible and R/S strains led to lethal infections in the absence of colistin **(Figure 2.3d,e)**. In the presence of colistin, only mice infected with the susceptible strain were rescued **(Figure 2.3d)**, whereas those infected with R/S still succumbed to infection within 100 hours **(Figure 2.3e)**. These data demonstrate the impact of an antibiotic resistant subpopulation in mediating a lethal infection in the presence of high dose antibiotic treatment.

We next set out to determine the molecular mechanism underlying the phenotype of the resistant subpopulation. RNAseq analysis was conducted **(Figure S2.3)** to determine whether there were transcriptional differences between the susceptible and resistant subpopulations of R/S. In total this analysis revealed 325 genes upregulated and 360 genes downregulated in the resistant subpopulation as compared to the susceptible subpopulation (see online supplemental material[156]). Among the upregulated genes, we noticed a signature associated with the two-component histidine kinase PhoQ[54,157-163], which has previously been implicated in polymyxin resistance, in part through its role in modification of the lipid A portion of lipopolysaccharide[164]. To validate the RNAseq data, we confirmed that the resistant subpopulation expressed higher levels of the predicted lipid A modification genes *arnB* and *eptA*[58] **(Figure S2.11)**. These data suggested that R/S displayed a modified lipid A profile, which we confirmed by thin layer chromatography (TLC) **(Figure S2.12)**. Further, modified lipid A species increased in abundance during growth of R/S in the presence of colistin, consistent with their expression by the resistant subpopulation **(Figure S2.12)**. To test whether the lipid A modifications were dependent on

PhoQ, we constructed an R/S deletion mutant lacking *phoQ* (Δ*phoQ*). Indeed, lipid A from the Δ*phoQ* strain lacked the specific lipid A modifications observed in wild-type R/S that were enhanced in the presence of colistin, which were restored in a *phoQ* complemented strain **(Figure S2.12)**. Thus, the R/S resistant subpopulation exhibits PhoQ-dependent lipid A modifications and is transcriptionally distinct when compared to the susceptible subpopulation.

To interrogate the potential contribution of PhoQ to the R/S resistance phenotype, we examined the colistin resistance profile of Δ*phoQ*. Strikingly, the Δ*phoQ* strain exhibited a complete absence of the resistant subpopulation by Etest, while the susceptible subpopulation remained unaffected, as the border of the zone of clearing was unaltered from that of wild-type R/S **(Figure 2.4a)**. Complementation with *phoQ* restored the presence of the resistant subpopulation **(Figure 2.4a)**. This was also confirmed by PAP, where Δ*phoQ* lacked the resistant subpopulation present in R/S and behaved similar to the susceptible strain **(Figure 2.4b)**. Importantly, R/S and Δ*phoQ* harbored equivalent levels of persisters, clearly indicating that the colistin resistant subpopulation (which depends on PhoQ) is not made up of persister cells **(Figure S2.13)**. The *phoQ* mutant additionally exhibited no colistin resistant subpopulation after exposure to host antimicrobials **(Figure S2.14a)**, during macrophage infection **(Figure S2.14b)**, or during *in vivo* infection **(Figure S2.14c)**. Without the presence of the resistant subpopulation, Δ*phoQ* was susceptible to colistin treatment *in vivo*, exhibiting a significantly decreased bacterial load **(Figure 2.4c)**. Furthermore, the ability of colistin to rescue mice from an otherwise lethal inoculum was restored during infection with Δ*phoQ* **(Figure 2.4d)**. Thus, the presence of the colistin resistant subpopulation is dependent on PhoQ, which is required for a lethal drug resistant infection.

The size of the resistant subpopulation can vary greatly between strains, as exemplified

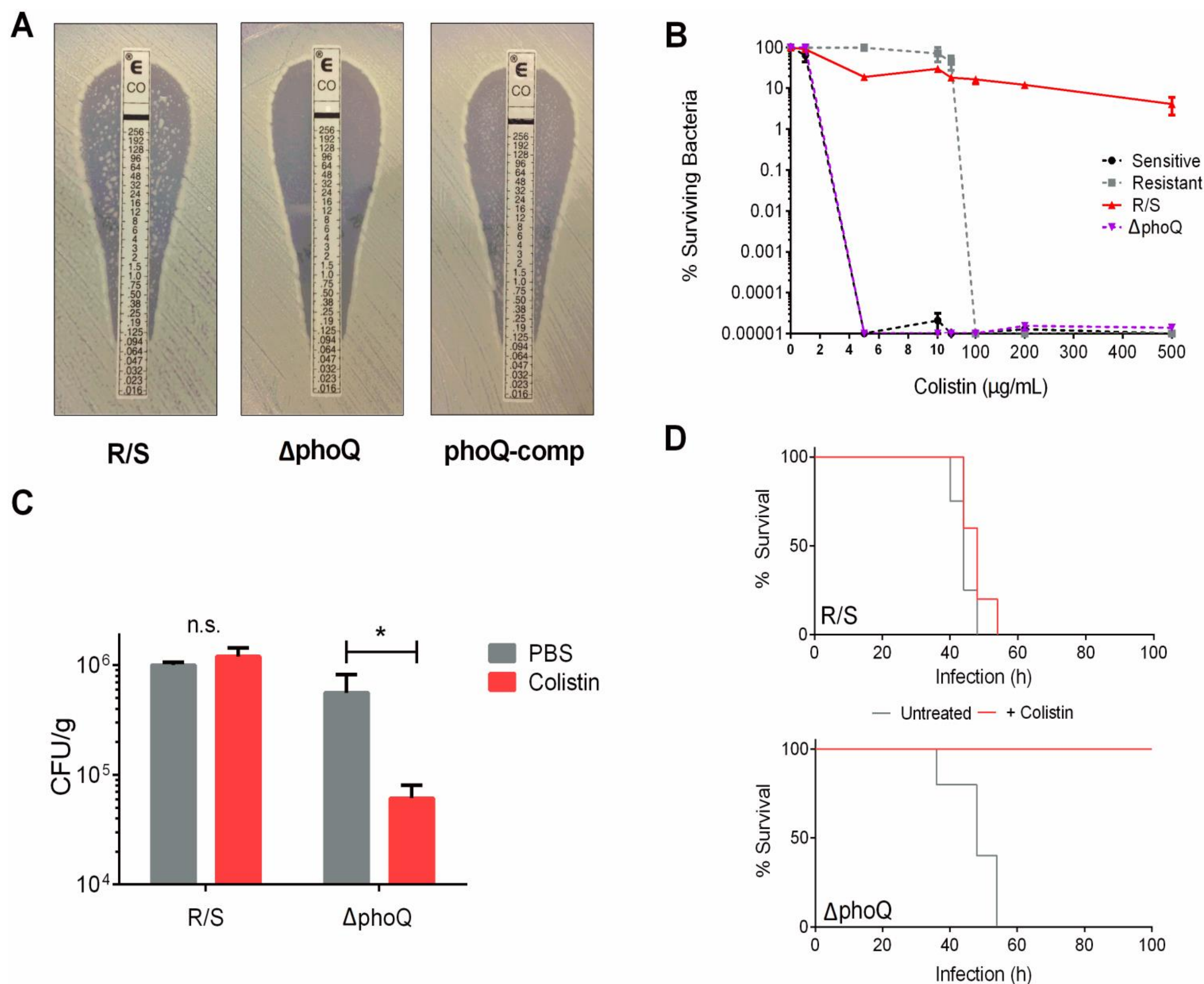
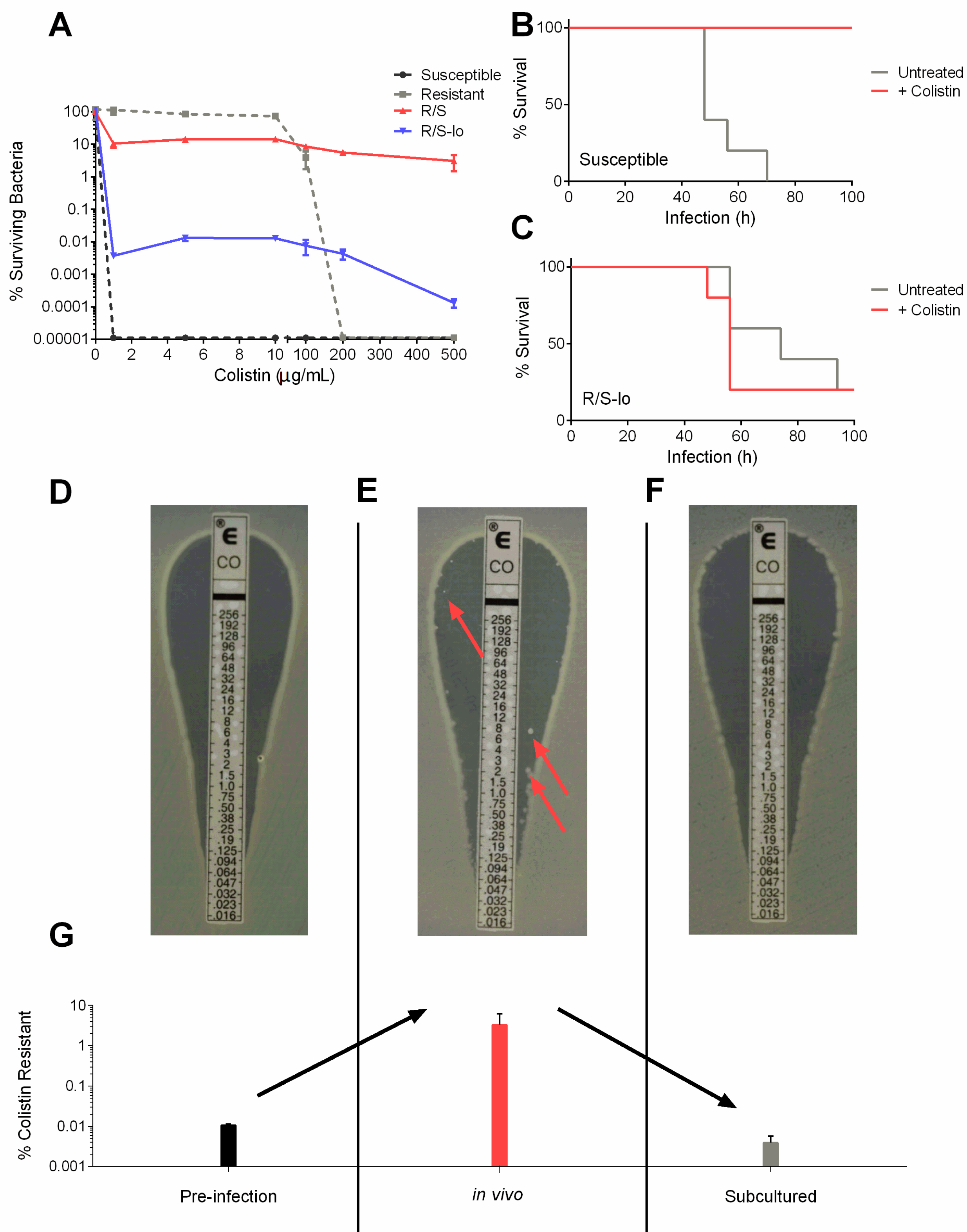


Figure 2.4. PhoQ is required for the colistin resistant subpopulation and treatment failure. (A) Colistin Etests of R/S, Δ*phoQ*, and the complement (phoQ-comp) strains. Colonies within the zone of inhibition indicate a colistin resistant subpopulation**.** (B) Population analysis profile of R/S, Δ*phoQ,* and colistin susceptible and resistant *E. cloacae* strains. (C) Mice infected with R/S or Δ*phoQ* were treated with colistin at 8, 14 and 20 h. CFU were quantified at 24 h in the peritoneal lavage fluid. (D) Survival of mice infected with R/S or the colistin susceptible isolate. Mice were treated with colistin or PBS starting at 12 h post infection, with additional doses given every 6 h thereafter. * $p < 0.05$.

by a distinct *E. cloacae* clinical isolate (termed R/S-lo) which harbors a colistin resistant subpopulation between 0.01 and 0.001% of the total population **(Figure 2.5a)**, over 1,000-fold less prevalent than that of R/S when grown in media without antibiotic. Similar to R/S, the increase of the R/S-lo resistant subpopulation in the presence of colistin **(Figure S2.15)** was due to initial selection against the susceptible subpopulation followed by expansion of the resistant subpopulation **(Figure S2.16)**. The frequency of the resistant subpopulation was likewise increased by treatment with H_2O_2, lysozyme, CRAMP, and LL-37 **(Figure S2.17)**, during macrophage infection **(Figure S2.18)**, and during *in vivo* infection of mice **(Figure S2.19)**, and was greatly diminished in macrophage-depleted **(Figure S2.20)** and TKO mice **(Figure S2.21)**. These data revealed that similar to R/S, the frequency of the resistant subpopulation of R/S-lo is increased by colistin as well as the activity of specific host innate immune components. During *in vivo* infection, while the levels of a susceptible strain were significantly reduced by colistin treatment, the levels of R/S-lo were unaffected **(Figure S2.22)**. These data directly correlated with a failure of colistin therapy to rescue R/S-lo infected mice from a lethal infection **(Figure 2.5c)**, whereas mice infected with a susceptible strain were completely rescued **(Figure 2.5b)**. Importantly, unlike R/S, R/S-lo was clinically classified as being susceptible to colistin, as the resistant subpopulation (present at a frequency of only 1 in 10,000 CFU) was not detected by Etest **(Figure 2.5d)**. Therefore, this seemingly colistin susceptible strain, harboring an undetected resistant subpopulation, is capable of causing an antibiotic resistant and lethal infection *in vivo*.

It is worrisome that R/S-lo was not identified as colistin resistant, and we wondered whether the resistant population could be detected by diagnostic testing when it is more frequent during host infection. We directly plated peritoneal lavage samples from infected mice in the


A
Susceptible
Resistant
R/S
R/S-lo
% Surviving Bacteria
100
10
1
0.1
0.01
0.001
0.0001
0.00001
0
2
4
6
8
10
100
200
300
400
500
Colistin (µg/mL)
B
% Survival
Untreated
+ Colistin
Susceptible
Infection (h)
C
R/S-lo
D
E
F
CO
G
% Colistin Resistant
Pre-infection
in vivo
Subcultured

(legend for figure on previous page)

Figure 5. Clinical isolate harboring an undetected colistin resistant subpopulation causes a lethal, antibiotic resistant infection. (A) Population analysis profile of *E. cloacae* clinical isolate R/S-lo compared to R/S and the colistin susceptible and resistant isolates. (B-C) Infection of mice with (B) the colistin susceptible isolate or (C) R/S-lo with or without colistin treatment every 6 h and initiated 12 h post infection. Surviving mice were monitored until day 24. (D-F) Colistin Etest of R/S-lo from (D) pre-infection inoculum, (E) peritoneal lavage sample from a mouse infected for 8 h and (F) the peritoneal lavage sample subcultured overnight in drug-free media. Colonies in the zone of inhibition (e, red arrows) indicate resistant bacteria. Images representative of 5 individual samples are shown. (G) The samples from (D-F) were plated to determine % colistin resistance.

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absence of subculture, and were able to detect the R/S-lo resistant subpopulation by Etest, as indicated by colonies within the zone of inhibition (Figure 2.5e, g). In contrast, when these samples were processed by the clinical microbiology laboratory (as would occur with a sample from a human patient, and including a critical subculture step), Etest could no longer detect the diminished resistant subpopulation (Figure 2.5f, g). Strikingly, these data reveal how and when detection of the resistant subpopulation can be missed during routine diagnostic testing, and how this can translate into an unexplained failure of antibiotic therapy.

Discussion

The findings presented here highlight the role of a minor colistin resistant bacterial subpopulation in mediating antibiotic treatment failure *in vivo*. This resistant subpopulation is genetically identical to the susceptible subpopulation, but exhibits differences in gene expression

and lipid A modification. Furthermore, the presence of this resistant subpopulation is dependent on the histidine kinase PhoQ. The data also highlight an unexpected role for specific host innate immune components (lysozyme, CRAMP and H_2O_2) in the increase of the antibiotic resistant subpopulation during infection. The increase in the frequency of the resistant subpopulation induced by host immune pressure *in vivo* was shown to be critical for eventual failure of colistin therapy.

Like bacterial persistence, the phenotypic resistance phenomenon we describe involves a resistant subpopulation, but there are important differences. Persistence involves a small subpopulation of bacteria that are tolerant to a drug due to a state of low metabolic activity, with no or limited replication[165]. Wakamoto et al showed that in some cases persisters can replicate, although it is at a very low rate, and is insufficient to cause an overall increase in the numbers of the population[149]. In contrast, we describe a resistant subpopulation that rapidly replicates both *in vitro* and *in vivo* in the presence of antibiotic, and leads to a very significant overall increase in bacterial population level **(Figure 2.1d, Figure S2.10)**. Further, we directly show that the PhoQ-dependent colistin resistant subpopulation is distinct from persisters, which are also present but independent of PhoQ **(Figure S2.13)**. Several papers have recently demonstrated the importance of persisters as a reservoir of infection during antibiotic treatment *in vivo*[150], which can continue to replicate after treatment has been stopped, leading to relapse[151]. In contrast, we demonstrate that the colistin resistant subpopulation described here facilitates bacterial growth and subsequent host lethality even in the presence of antibiotic. Persistence has also been linked to immune pressure, as bacteria within macrophages can have increased numbers of persisters[166]. We observe a similar link, as both *in vitro* and *in vivo,* specific host antimicrobials lead to an increased frequency of the resistant subpopulation. Taken together, both persisters and the

resistant subpopulation described here highlight the ability of a minority of a bacterial population to exert a striking effect on the outcome of infection and antibiotic treatment. While persisters are kept at bay by antibiotic treatment and form a reservoir that can cause relapse, the colistin resistant subpopulation described here has the ability to cause acute infection and lethality during the course of antibiotic treatment.

We propose to refer to the phenomenon described here as clonal heteroresistance. The phenomenon of heteroresistance, in which a resistant subpopulation exhibits an increased level of antibiotic resistance relative to the larger susceptible subpopulation, was described as far back as 1947[167]. However, its relevance to infection and resistance has remained unclear, and even its definition has been debated. We use the term clonal heteroresistance to distinguish the phenomenon we describe from the blanket term heteroresistance which is often used to refer to mixed populations of genetically distinct bacteria[133,168-170]. We show that clonal heteroresistance, in addition to mediating lethal infection in the presence of antibiotic, can also go undetected and cause unexplained treatment failure during *in vivo* infection **(Figure S2.23)**. Current widely used methods of antibiotic susceptibility testing rely on *in vitro* culture and analysis. Our data show that these methods can greatly alter results and present an inaccurate picture of the level of *in vivo* resistance. Our findings highlight both a need and opportunity for improved diagnostics to detect antibiotic resistant subpopulations and ultimately prevent such treatment failures.

Methods

Bacterial strains

E. cloacae strain R/S was isolated from a blood sample from a renal transplant recipient at Emory University Hospital (Atlanta, GA). *E. cloacae* R/S-lo, the colistin susceptible strain

Mu819, and the colistin resistant strain Mu117 were isolated from urine samples from patients at other Atlanta, GA hospitals.

Bacterial culture

All bacterial strains were streaked on Mueller-Hinton (MH) agar plates and grown in MH medium at 37°C in a shaking incubator from a single colony before each experiment. Colony forming units (CFU) were determined by plating dilutions on MH agar plates incubated at 37°C and then counting bacterial colonies at the lowest distinguishable dilution.

Bacterial genetics

To generate strain Δ*phoQ,* 600-700bp upstream and downstream fragments of the genomic region surrounding *phoQ* were PCR amplified with primers 81 and 118, and 82 and 119, respectively (Table S3) and fused with the hygromycin resistance cassette HmR amplified from vector pMQ310 with primers 79 and 80[171] using SOE PCR[172]. The suicide vector pEXR6K was generated by replacing the pMB1 ori from PCR linearized plasmid pEX100T[173] using primers 110 and 111 with the R6K ori amplified from plasmid pMQ310 with primers 108 and 109 using the Gibson Assembly Cloning Kit (Invitrogen). The HmR construct was inserted into SmaI (New England Biolabs) digested pEXR6K by Gibson assembly and the resulting plasmid was transformed to strain R/S by electroporation. Transformants were selected on MH agar containing 150µg/mL hygromycin (Sigma) then passaged to LB agar containing 20% sucrose and no NaCl to counterselect for vector loss. Chromosomal replacement of *phoQ* with the hygromycin marker was confirmed by Sanger sequencing. To generate strain *phoQ*-comp, the promoter region of the *phoPQ* operon was amplified with primers 142 and 143 and fused by SOE PCR to gene *phoQ* amplified with primers 144 and 145. The resulting construct was inserted to plasmid pBAV-1K-T5-GFP[174] PCR linearized with primers 146 and 147 to create the

complementation vector. The vector was transformed to strain Δ*phoQ* by electroporation and selected on MH agar containing 90 μg/mL kanamycin (Sigma).

Antibiotic susceptibility testing

Colistin susceptibility of all strains was determined using the Etest method. Briefly, the inoculum was prepared from colonies grown on a 5% sheep blood agar plate (Remel, Lenexa, KS) for 18 hours. Several colonies were suspended in 0.9% sterile saline (Remel) and adjusted to a concentration equivalent to a 0.5 McFarland turbidity standard. The suspension was used to streak a 100mm diameter MH agar plate and the Etest strip (bioMérieux, Marcy-l'Étoile, France) was placed. The plate was incubated at 35°C for 20 hours and the minimum inhibitory concentration (MIC) was read where inhibition of growth intersected the Etest strip. Small colonies that grew within the zone of inhibition were included in the MIC determination. Etest analyses of samples from mouse infections were plated directly from peritoneal lavage samples without subculturing. Population analysis profiles were performed by growing bacteria to mid-log phase, and then plating on MH agar containing various concentrations of colistin. Percentage colistin resistance was calculated as the number of bacteria that grew on 100μg/mL colistin divided by the number of bacteria that grew on MH alone.

Mice

Wild-type C57BL/6J mice were purchased from Jackson Laboratories (Bar, Harbor, ME) and used at age 8-10 weeks, all experiments using age- and sex-matched mice. Triple knockout (TKO) mice deficient in the gp91 component of the NADPH oxidase, lysozyme, and CRAMP, as well as double knockout mice lacking two of the indicated antimicrobials, were derived by crossing *cybb*$^{-/-}$ (gp91; from Jackson Laboratories), *lysM*$^{-/-}$ (lysozyme; generously provided by Dr. Daniel Portnoy, UC Berkeley), and *cnlp*$^{-/-}$ (CRAMP; Jackson Laboratories) mice. TKO mice

were investigated for health defects by histology and bacterial culture of various organs, with no overt health differences seen in uninfected TKO mice when compared to wild-type. Mice were housed under specific-pathogen free conditions in filter-top cages at Yerkes National Primate Center, Emory University, and provided food and water ad libitum. All experimental procedures were approved by the Emory University Institutional Animal Care and Use Committee.

Mouse infections

~$5x10^7$ CFU were administered per mouse for infections to quantify bacterial load; ~$2x10^8$ CFU were administered for survival experiments. Bacterial inocula were suspended in phosphate buffered saline (PBS) and 100uL was inoculated intraperitoneally (i.p.) to each mouse. Colistin methanesulfonate was injected i.p. in 100uL PBS at a dosage of 10mg/kg/dose. Mice were monitored by weight, and were sacrificed if found to be below 80% starting weight, as mandated by IACUC protocol. Mice were sacrificed and liver, spleen and peritoneal lavage samples were collected into sterile PBS. Solid organ samples were homogenized using a tissue-tearor (BioSpec, Bartlesville, OK), and then all samples were plated for CFU and % colistin resistance.

Macrophage depletion

Macrophages were depleted from mice using clodronate liposomes (clodronateliposomes.com, Haarlem, Netherlands). Mice were injected with 200µL liposomes i.p. 3 days prior to infection, and then injected again with 100µL liposomes i.v. 1 day prior to infection. Mice were infected i.p. for 8 hours before peritoneal lavage fluid was harvested and plated for CFU. Part of this sample was also used for flow cytometry to confirm macrophage depletion.

Macrophage infection

Macrophages were derived from the bone marrow of mice. Briefly, femurs from mice were removed and whole bone marrow was flushed out. The bone marrow cells were grown in media

containing Dulbecco's Modified Eagle Medium (DMEM), 10% fetal bovine serum (FBS) and macrophage colony-stimulating factor (M-CSF) which induces the differentiation and growth of macrophages. After confluent layers of macrophages were derived, cells were plated into 24 well plates at $3x10^5$ cells per well. Bacteria were added to the wells at $3x10^6$ CFU per well for a multiplicity of infection (MOI) of 10:1. Plates were centrifuged to synchronize the infection. After 30 minutes, the macrophages were washed and 100µg/mL of gentamicin was added to the media to remove and prevent growth of extracellular bacteria. At 1, 2, and 4 hours post infection, macrophages were incubated with 1% saponin in PBS for 2 minutes to lyse open cells and remove bacteria. Samples were then plated for CFU and % colistin resistance calculated. To prevent internalization of bacteria, some wells were pretreated with 1 µg/mL of cytochalasin D for 30 minutes before addition of bacteria.

Statistics

Statistical analyses were performed using Prism 5 (Graphpad Software). Significance of mouse experiments was determined with the Mann Whitney test, while all *in vitro* experiments were analyzed using the two-tailed student's t-test. All experiments were repeated at least 2-3 times (and up to 10 times).

Flow cytometry

Peritoneal lavage fluid was stained with F4/80-PE/Cy7 (BM8) (Biolegend, San Diego, CA) and CD11b-APC/A700 (M1I70) (eBioscience, San Diego, CA) antibodies for 35 minutes. Red blood cells were lysed with RBC lysis buffer (Becton Dickinson, Franklin Lakes, NJ) for 5 minutes. Cells were fixed with 1% paraformaldehyde and analyzed on an LSRII flow cytometer (BD). Macrophages were defined as F4/80+CD11b+ cells.

DNA and RNA Isolation

An overnight liquid culture of RS grown at 37°C in MH broth was back diluted in triplicate to either fresh MH broth or MH broth containing 100µg/mL colistin to enrich for susceptible or colistin resistant bacteria, respectively. Cultures were grown to exponential phase at 37°C and harvested for DNA and RNA isolation. CFU were calculated as above. DNA was isolated using the DNEasy Blood and Tissue Kit (Qiagen) following the Gram negative bacteria protocol with RNAse treatment. RNA was isolated using a modified phase extraction method[175] with initial incubation in TriReagent (Zymo) followed by phase separation with chloroform. RNA was precipitated from the aqueous phase with isopropanol and 1.2M NaCl at 4°C and further purified with the Directzol RNA Kit (Zymo) following the recommended DNase treatment step.

DNA and RNA sequencing

Sample integrities were verified with the Agilent 2100 Bioanalyzer (Agilent). DNA libraries were prepared using the NexteraXT DNA kit (Illumina). For RNA libraries, samples were first depleted of ribosomal RNAs using the Ribo-Zero rRNA Removal Kit (Illumina) and libraries prepared using the EpiCentre ScriptSeq Complete (Bacteria) Low Input kit (Illumina). Next generation short sequence reads were generated with the Illumina HiSeq 1000 platform at the Yerkes National Primate Research Center Nonhuman Primate Genomics Core (http://www.yerkes.emory.edu/nhp_genomics_core/). Long sequence reads were generated with the PacBio II platform using the P5-C3 chemistry at the Duke University Sequencing and Genomic Technologies Shared Resource.

De novo genome assembly and sequence analysis

A hybrid de novo assembly was performed using both Illumina and PacBio data using Celera Assembler version 8.2[176]. The sequence data resolved into two contigs, one representing the

chromosome and the other representing the plasmid. Quality of the assembly was confirmed by analysis using the ALE tool[177]. The assembly was automatically annotated using the NCBI prokaryotic annotation pipeline. Illumina whole shotgun sequences of the samples enriched for colistin resistance (COL) and colistin susceptibility (MH3) were aligned against the assembled genome using bwa-0.7.12[178] and visualized the samtools-1.2 mpileup function[179]. Single nucleotide polymorphisms between the assembled genome and short sequence reads were manually analyzed to determine sequence conservation between COL and MH3 samples (Table S4).

RNAseq analysis

Single end Illumina libraries from reverse-transcribed RNA were mapped against the Enterobacter de novo assembled reference using Bowtie2[180]. Differential gene expression between the three colistin-treated strains and controls was quantified by the cufflinks/cuffdiff tools in CufflinksVersion 2.2.1[181,182]. Sequences of differentially expressed genes with significant q-values were analyzed with Blast2Go software version 3.1.3 to identify the *Escherichia coli* gene ortholog and putative function[183].

Quantitative Reverse Transcriptase PCR (qRT-PCR)

RNA was harvested as above. One-step qRT-PCR was performed using the Power SYBR Green RNA-to-C_t kit (Applied Biosystems) with primers (Table S3) on a StepOnePlus Real-time PCR System (Applied Biosystems) according to the manufacturer's instructions. *rpoD* was used as the internal control gene[184]. Relative expression was calculated as $2^{-(\Delta Ct)}$ [185].

Isolation and analysis of ^{32}P Lipid A species

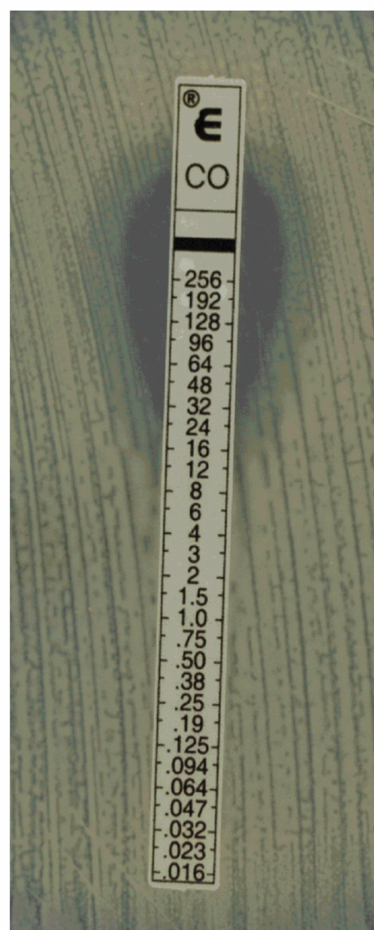
E. cloacae strains were grown overnight in MH broth, diluted 1:400 in fresh MH broth containing appropriate selective antibiotics. For induction of resistant phenotype, 100 µg/ml colistin (Sigma) was used. *Escherichia coli* W3110 and WD101 strains were grown in LB broth overnight followed by a 1:100 dilution in fresh LB medium. After dilutions, cells were immediately labeled with 2.5 µCi/ml of inorganic ^{32}P-phosphate (Perkin Elmer) and harvested at A_{600} 0.5 (*E. cloacae*) or A_{600} 1.0 (*E. coli*). Lipid A extraction, separation and visualization was performed as previously described[186]. Briefly, lipid A extraction was carried out by mild acidic hydrolysis and spotted onto silica TLC plate (10,000 cpm/lane). Labeled lipid A species were separated using a solvent mixture of chloroform, pyridine, 88% formic acid and water (50:50:16:5). TLC plate was exposed to a phosphoimager screen and visualized by phosphoimaging analysis (Bio-Rad PMI).

Supplemental Figures

A

Susceptible

B



Resistant

Figure S2.1. Etests of colistin susceptible and resistant isolates. Colistin Etest analysis of (A) susceptible or (B) resistant *E. cloacae* clinical isolates.

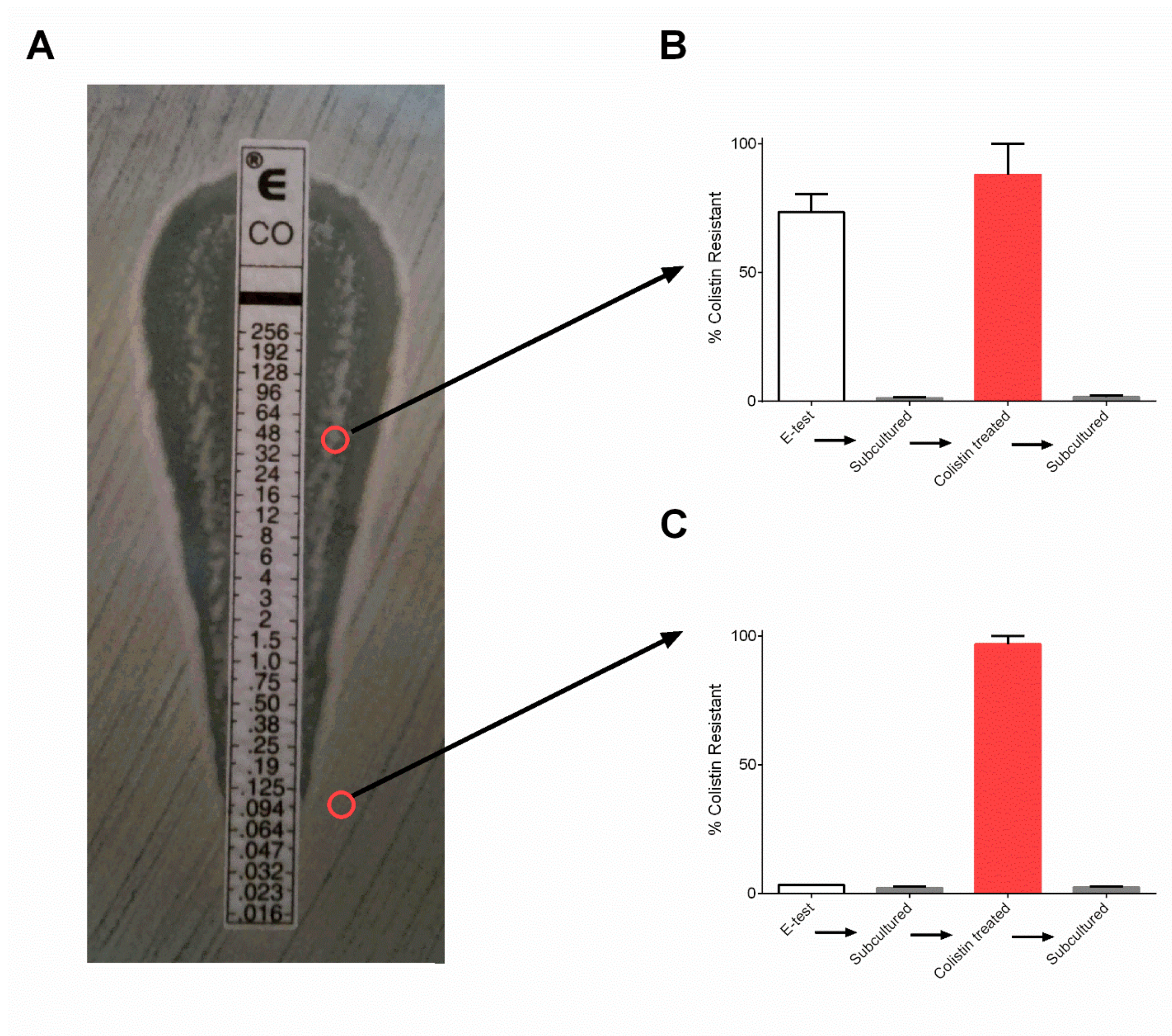


Figure S2.2. Bacteria from high and low antibiotic growth conditions behave identically after passage. (A) R/S was plated on a colistin Etest plate and bacteria (circled in red) were harvested from within or outside the zone of clearing and assayed for colistin resistant subpopulations. (B,C) Bacteria taken from (B) within the zone of inhibition, representing the colistin resistant subpopulation and (C) outside the zone of inhibition, representing the colistin susceptible subpopulation were cultured. Bacteria were first cultured in drug free media, then subcultured in 100µg/mL colistin containing media, and then subcultured in drug free media again, with samples taken from each culture to assess colistin resistant subpopulations.

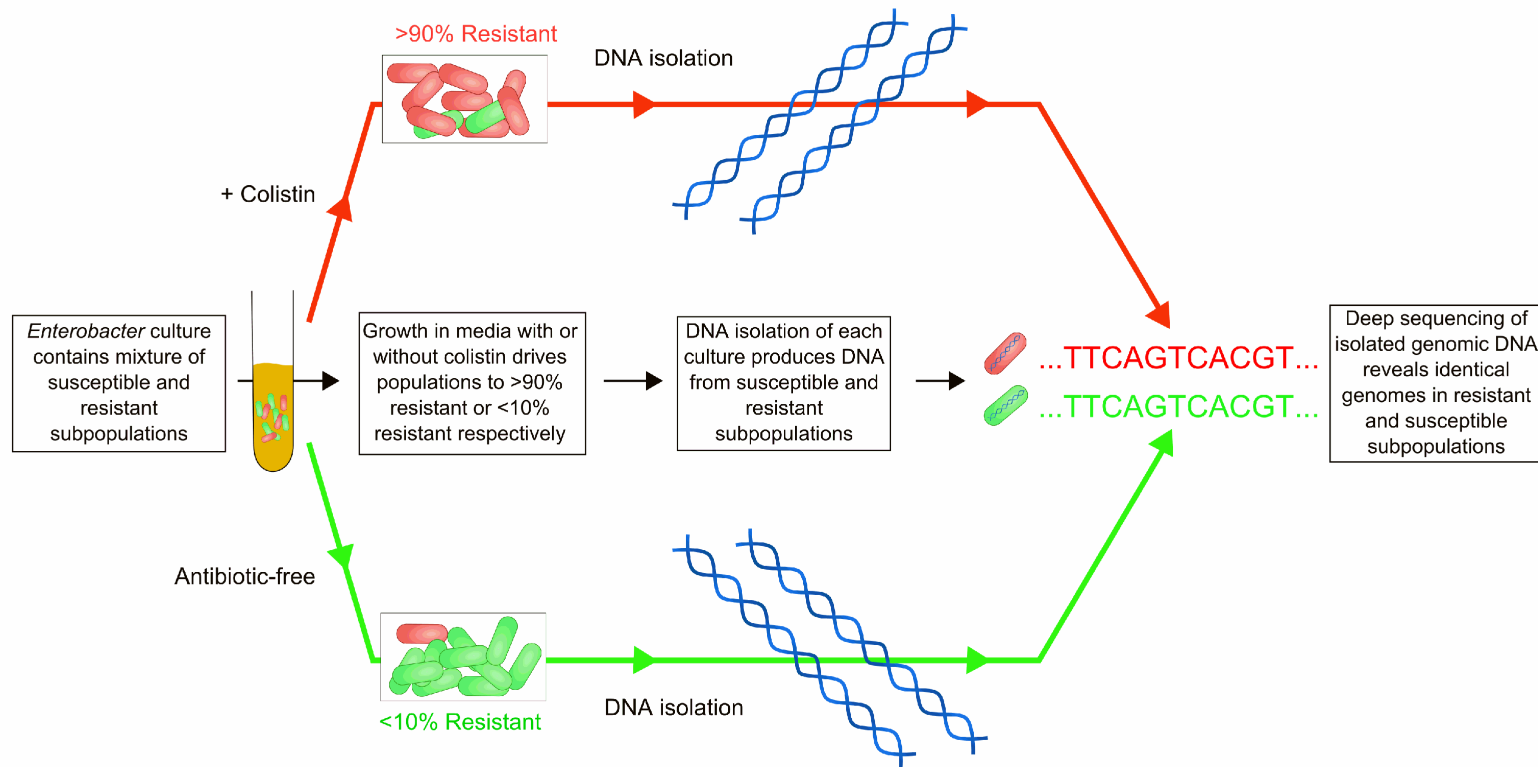


Figure S2.3. DNA sequencing of susceptible and resistant subpopulations. Flow chart of the procedure for DNA sequencing of the susceptible and resistant subpopulations of R/S. Cultures of R/S were grown in media with or without colistin to induce predominantly resistant or susceptible populations, respectively. If the DNA sequences of the two subpopulations were different, this would be detected as sequence differences when comparing the cultures in which either the susceptible or resistant subpopulation comprised the overwhelming majority of the sample. DNA was isolated from each culture and sequenced via DNAseq analysis. This analysis revealed identical genome sequences between each culture, indicating that the genome sequence of the susceptible and resistant subpopulations are identical. RNA isolated from these cultures revealed significant transcriptome differences between the two subpopulations (see online supplemental material[156]).

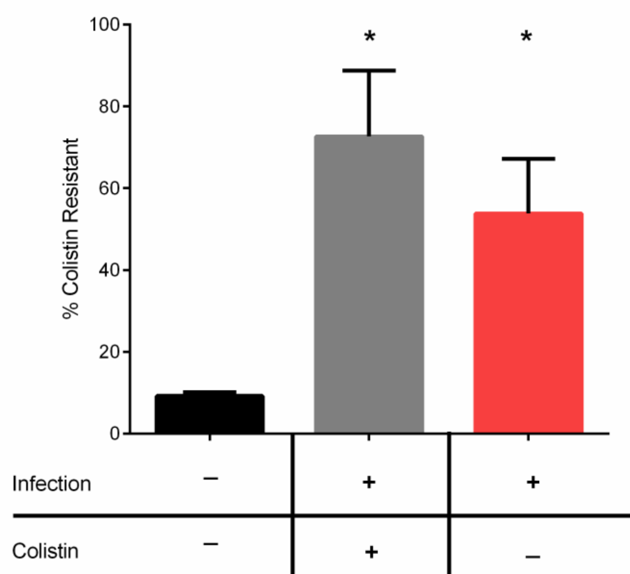


Figure S2.4. Increase in the frequency of the colistin resistant subpopulation in the liver during in vivo infection. An inoculum of strain R/S (black bar) was used to infect mice intraperitoneally. Mice were treated with colistin (grey bar) or PBS (red bar) at 8, 14 and 20 hours. At 24 hours, liver samples were harvested and plated to quantify the number of colistin-resistant and total bacteria. * $p < 0.05$.

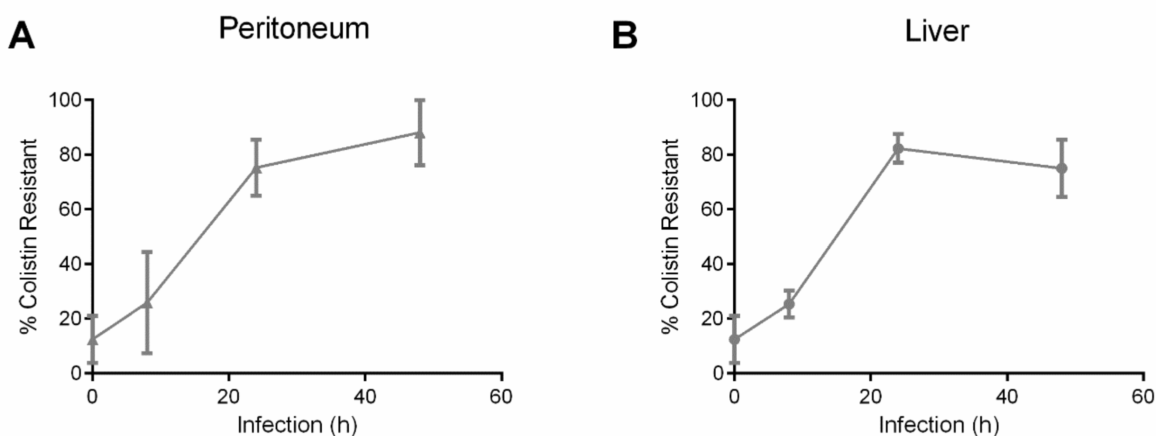


Figure S2.5. Frequency of the colistin resistant subpopulation increases during *in vivo* infection. (A,B) % colistin resistance of R/S during a 48 hour mouse infection. Bacteria were recovered at each time point from (A) peritoneal lavage or (B) liver samples.

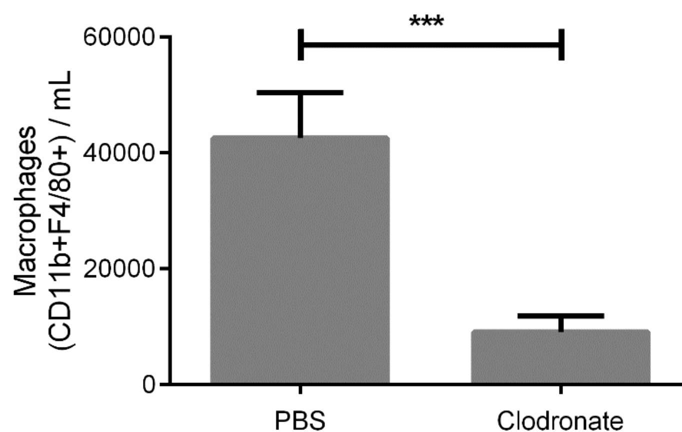


Figure S2.6. Macrophage depletion via clodronate liposomes. Number of macrophages in peritoneal lavage fluid of PBS or clodronate liposome treated mice. Macrophages were defined as $CD11b^{+}F4/80^{+}$ cells by flow cytometry. Data compiled from 4 separate experiments. ***, $p < 0.001$.

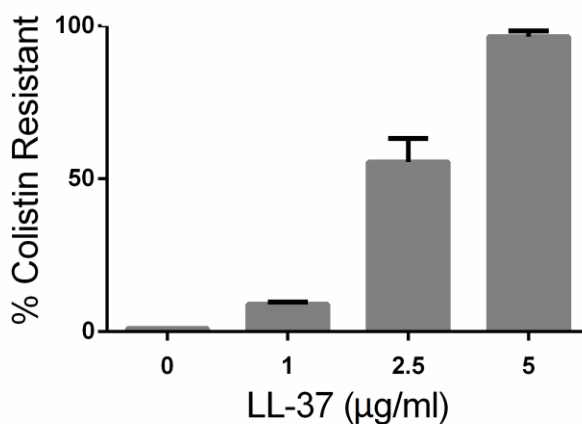


Figure S2.7. The human antimicrobial peptide LL-37 leads to an increase in frequency of the colistin resistant subpopulation. Strain R/S was treated with the indicated amounts of human LL-37 for 5 hours. Samples were plated to quantify the numbers of total and colistin-resistant bacteria and % colistin resistance was calculated.

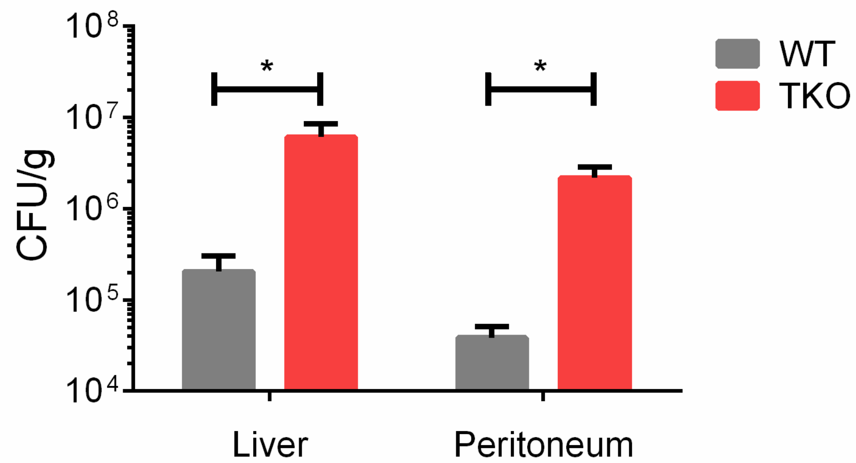


Figure S2.8. Increased CFU during infection of mice lacking host antimicrobials. Wild type or triple knockout (TKO) mice lacking LysM, CRAMP and Phox were infected with R/S, and CFU in the liver and peritoneal lavage fluid were quantified at 8 hours post infection. * $p < 0.05$

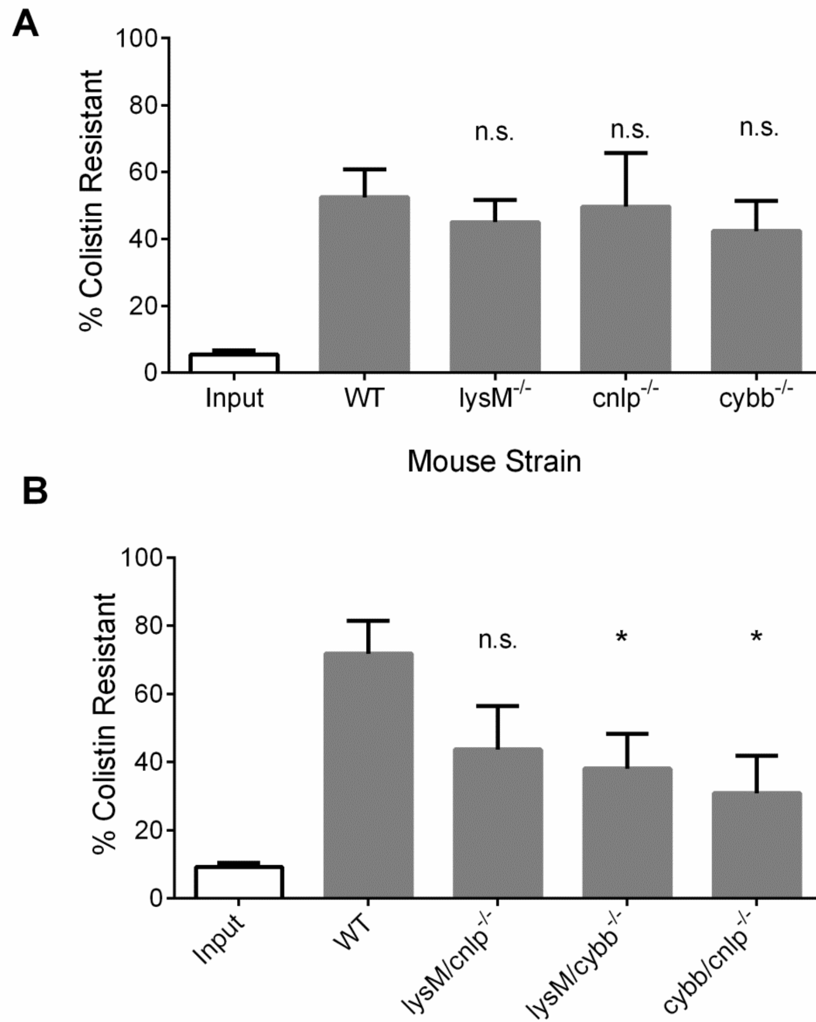


Figure S2.9. Infection of mice lacking individual host antimicrobials. The indicated strains of mice, LysM (lysM-/-), CRAMP(cnlp-/-) or Phox(cybb-/-) (A) single knockouts or (B) double knockouts, were infected with R/S for 8 hours, and the % colistin resistance was compared to that of the initial inoculum. * $p < 0.05$.

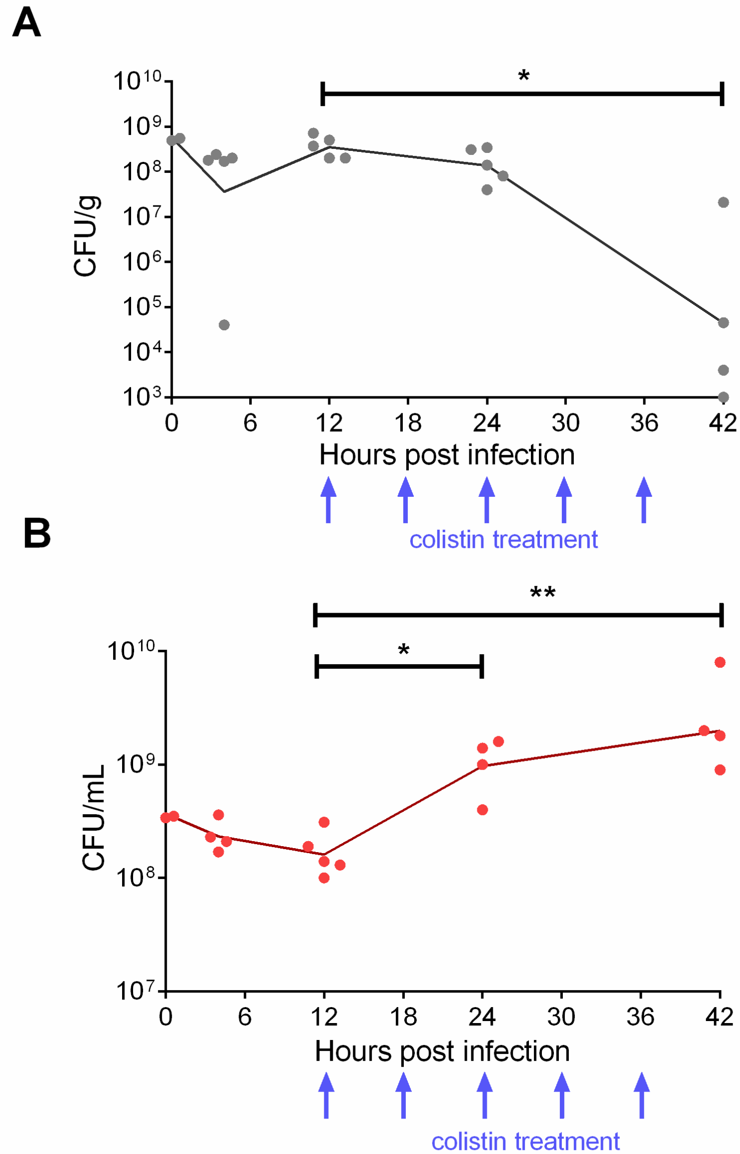


Figure S2.10. *In vivo* growth and expansion of R/S during colistin treatment of mice. Wild-type mice were infected with a lethal dose of a susceptible strain (A) or R/S (B) and then given doses of colistin every six hours starting at 12 hours post infection. Mice were sacrificed to determine peritoneal CFU counts at 0, 6, 24 and 42 hours. * $p < 0.05$, ** $p < 0.01$.

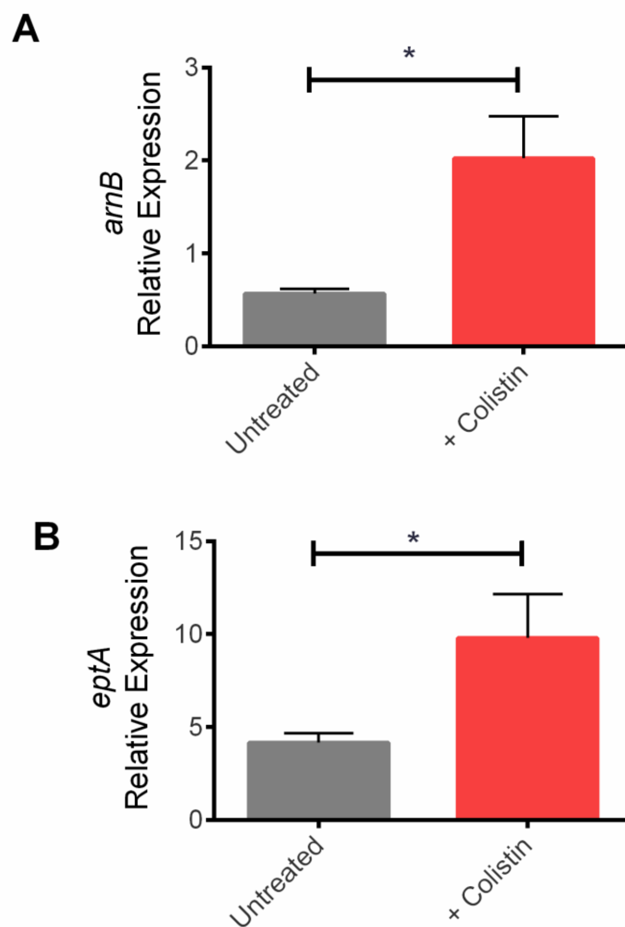


Figure S2.11. Expression of PhoQ regulated lipid A modification genes is induced by colistin.

Expression of (A) *arnB* and (B) *eptA* lipid A modification genes in R/S grown to exponential phase with or without 100 µg/ml colistin. * $p < 0.05$.

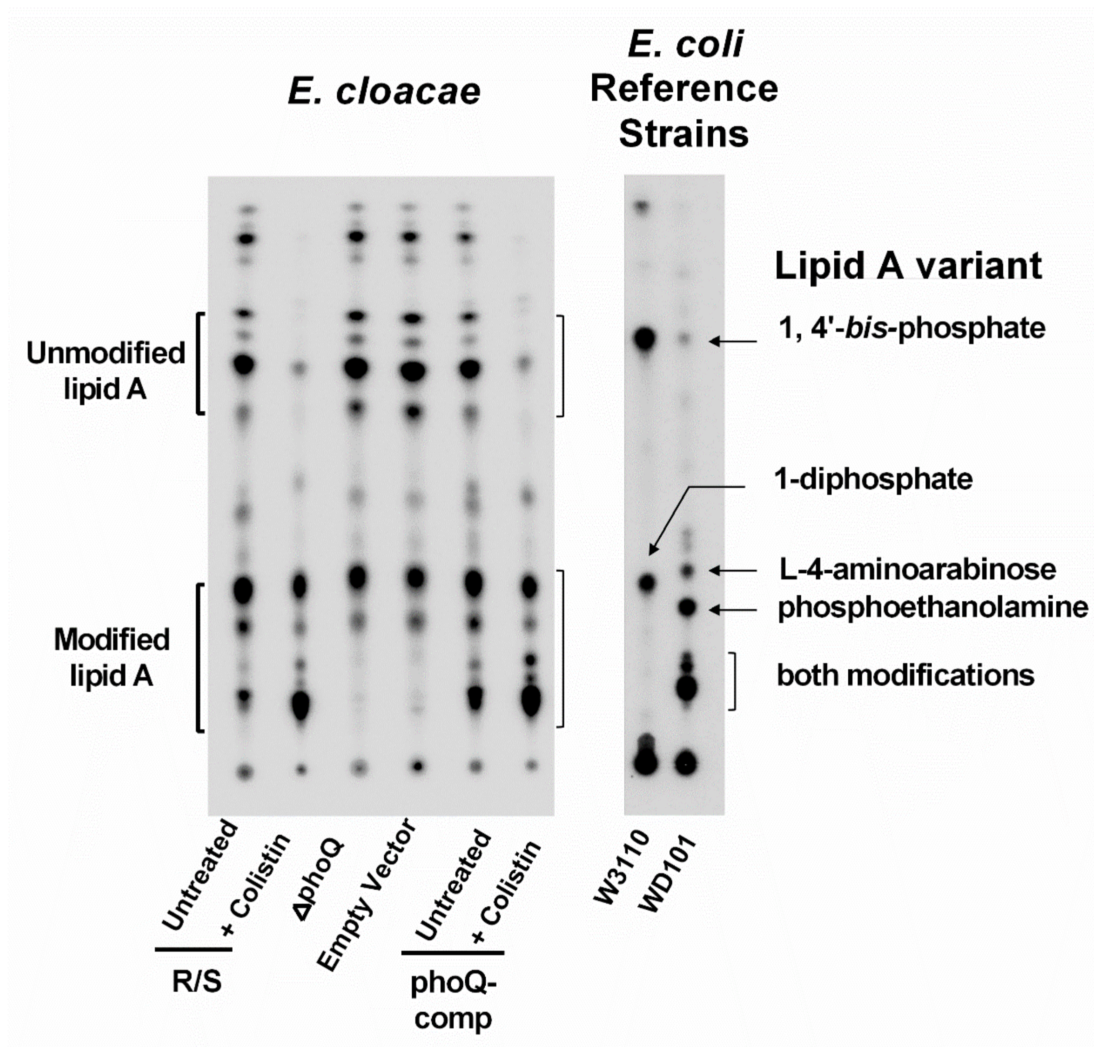


Figure S2.12. Lipid A analysis reveals modifications present in resistant subpopulation. (A) Thin layer chromatography separation of lipid A species harvested at exponential phase growth from R/S cultured without (Untreated) or with (+Colistin) colistin pretreatment, the *phoQ* deletion mutant (Δ*phoQ*), Δ*phoQ* complemented with an empty vector (Empty Vector), complemented with *phoQ* (Untreated) or complemented with *phoQ* and then treated with colistin (+Colistin). (B) Thin layer chromatography of reference *E. coli* strains W3110 (unmodified lipid A) and WD101 (modified lipid A) with known lipid A modifications were used as controls[186].

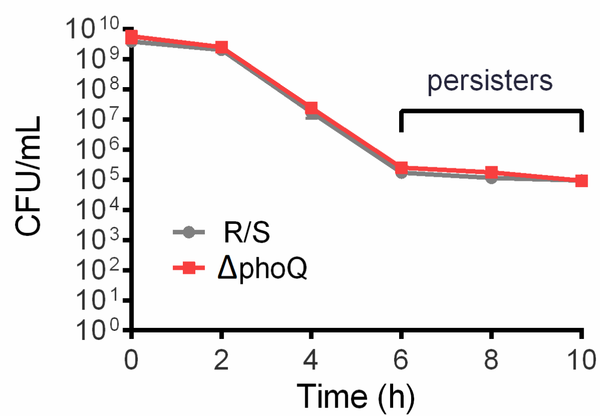


Figure S2.13. Kanamycin persisters in R/S are not dependent on PhoQ. CFU/mL of R/S and *ΔphoQ* during treatment with 900 µg/mL kanamycin. The period between 6 and 8 hours with a plateau in killing represents the population of surviving persisters.

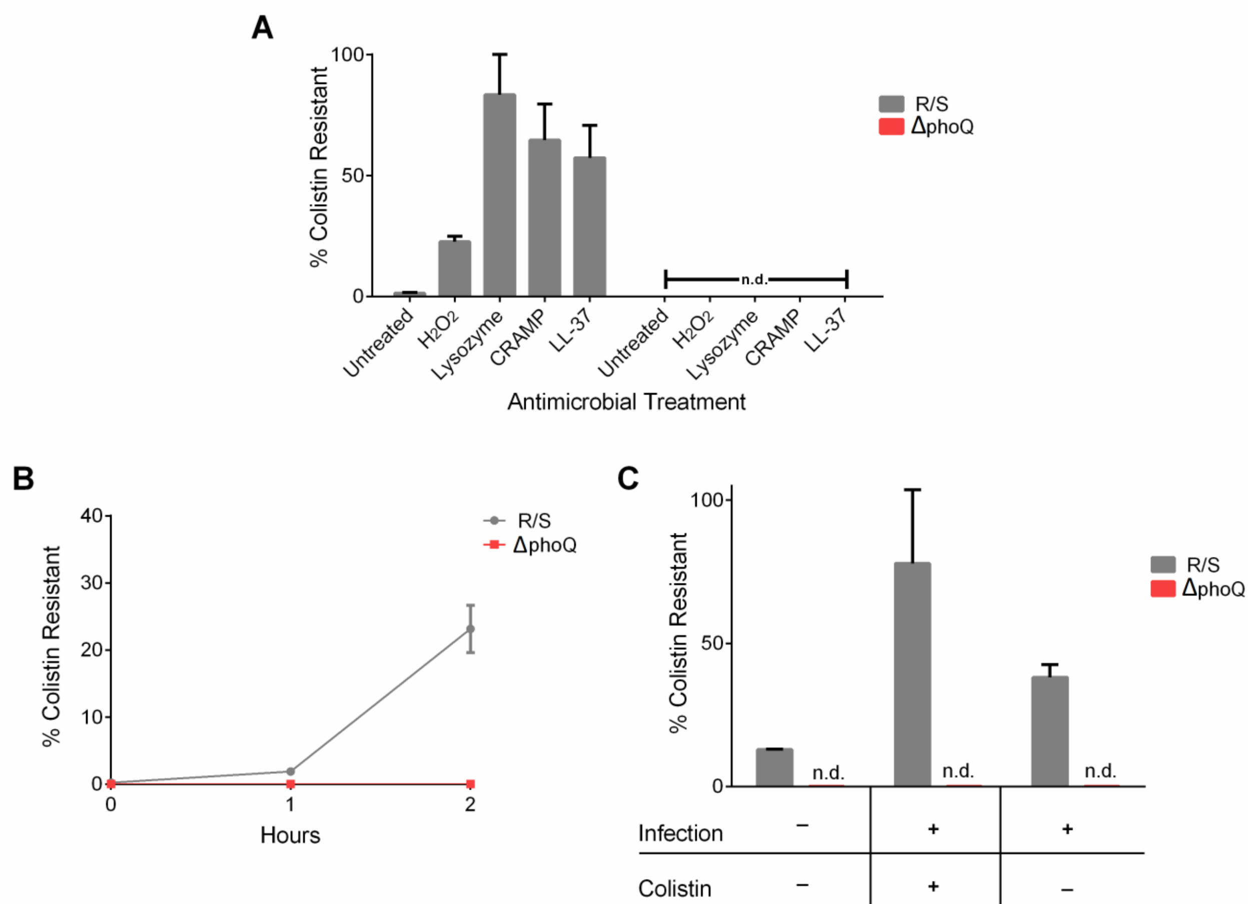


Figure S2.14. R/S deficient in PhoQ lacks ability to induce colistin resistant subpopulation. (A) % colistin resistance of R/S and Δ*phoQ* after 5 hour treatment with 100uM H_2O_2, 5 mg/mL lysozyme, 5 µg/mL CRAMP or 10 ug/mL LL-37. (B) % colistin resistance of R/S and Δ*phoQ* during macrophage infection at the indicated timepoints. (C) % colistin resistance of R/S and Δ*phoQ* after 24 hour mouse infection. No resistant colonies detected (n.d.) for all Δ*phoQ* samples.

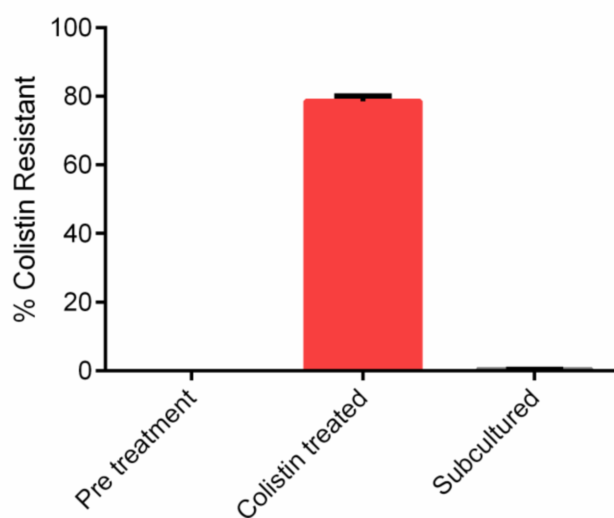


Figure S2.15. The frequency of the colistin resistant subpopulation of R/S-lo increases in the presence of drug. % colistin resistant bacteria was calculated for R/S-lo before colistin treatment, after 20 h in 100 µg/mL colistin, and after 8 h drug free subculture.

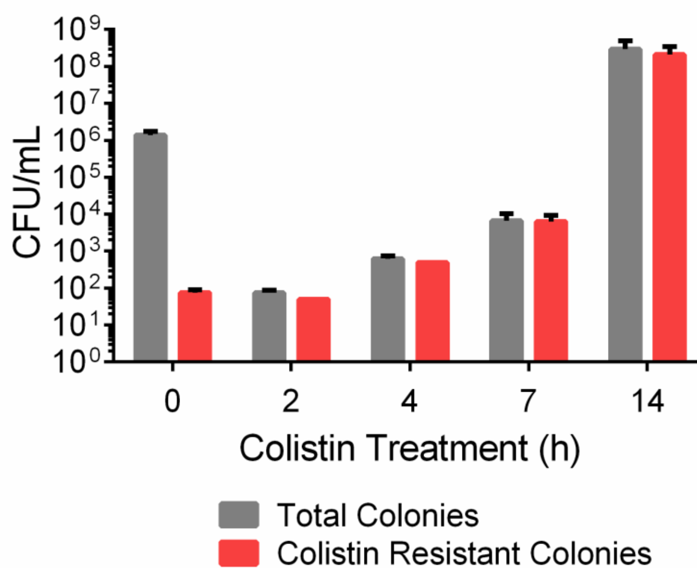


Figure S2.16. Colistin selects for the colistin resistant subpopulation of R/S-lo. Colistin resistant and total CFU of R/S-lo during 14 h treatment with 100µg/mL colistin in liquid culture.

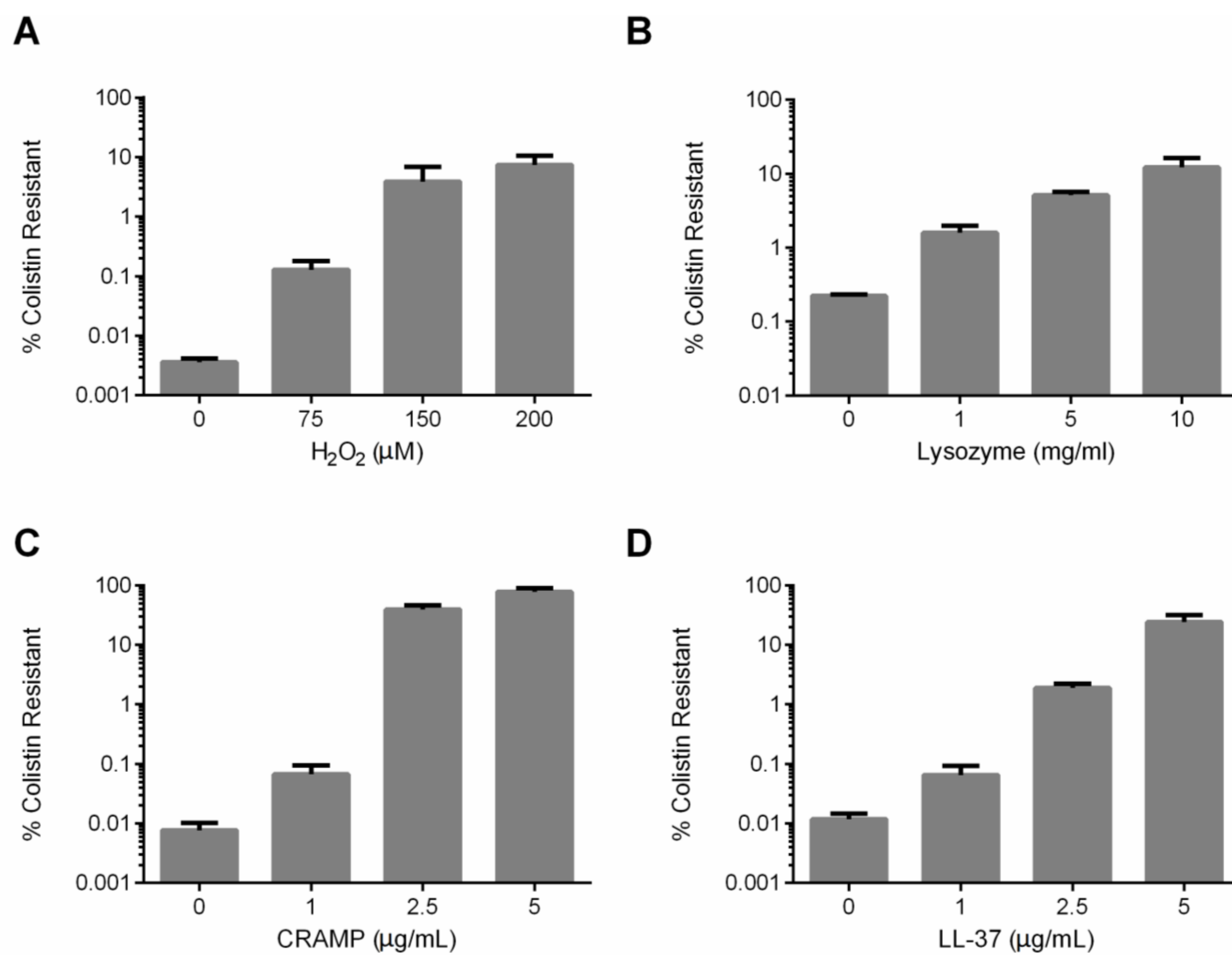


Figure S2.17. Host antimicrobials lead to an increase in the frequency of the colistin resistant subpopulation of R/S-lo. R/S-lo was treated for 5 h with the indicated concentrations of (A) H_2O_2, (B) lysozyme, (C) CRAMP or (D) LL-37 and % colistin resistance was calculated.

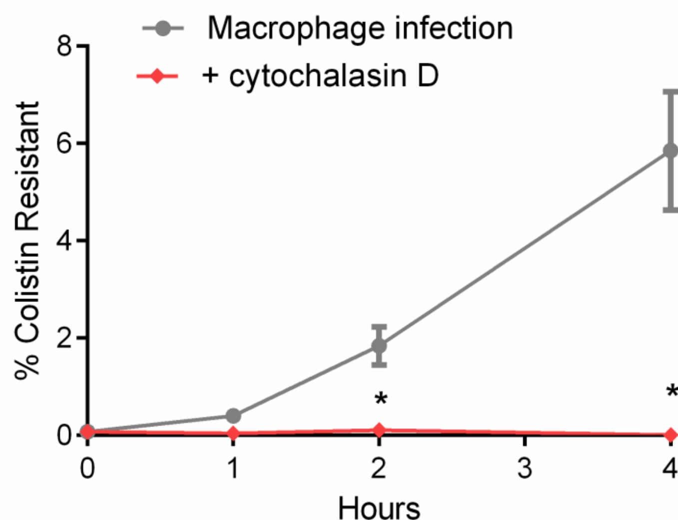


Figure S2.18. The frequency of the R/S-lo colistin resistant subpopulation increases in macrophages. Bone marrow-derived macrophages were infected with R/S-lo. % colistin resistance of R/S-lo within macrophages pretreated or untreated with cytochalasin D is shown at each timepoint. * $p<0.05$.

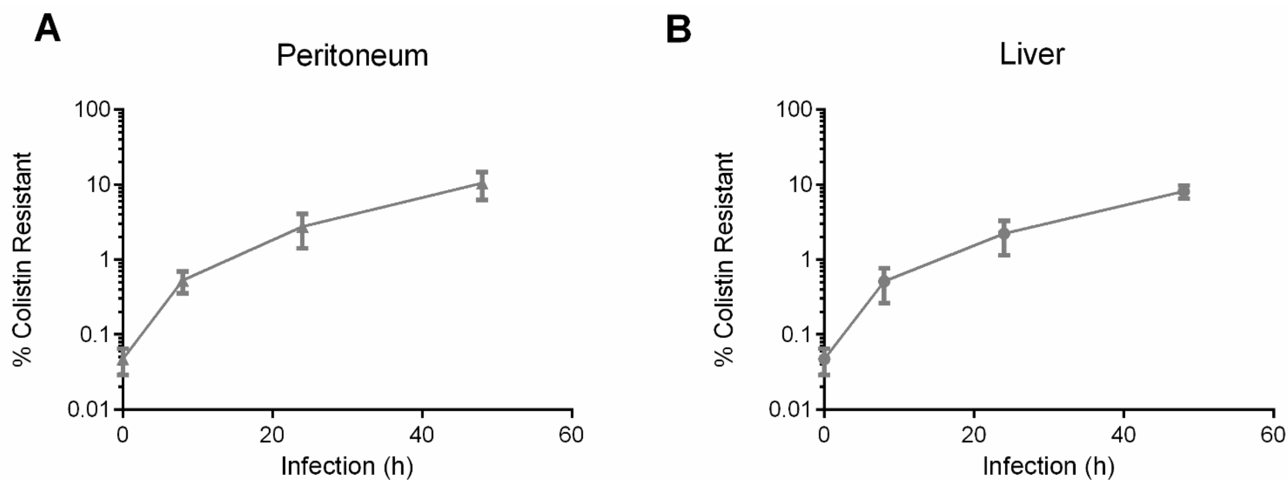


Figure S2.19. The frequency of the R/S-lo resistant subpopulation increases during mouse infection. (A,B) % colistin resistance of R/S-lo during a 48 hour mouse infection. Bacteria were recovered at each time point from (A) peritoneal lavage or (B) liver samples.

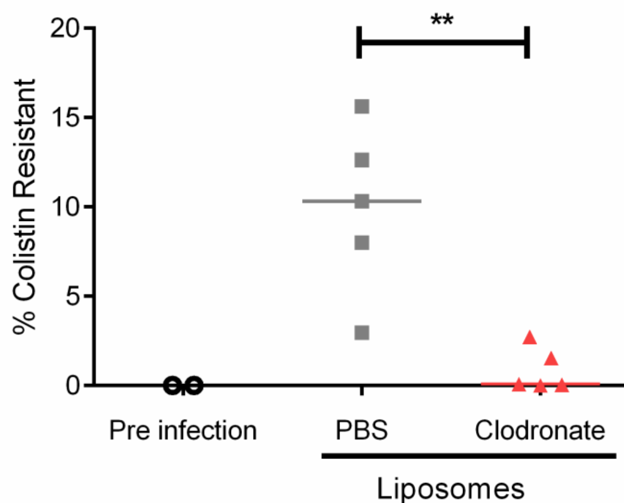


Figure S2.20. Macrophages are required for the increase in the frequency of the R/S-lo resistant subpopulation during infection. Mice pre-treated with PBS (as a control) or clodronate containing liposomes (to deplete macrophages) were infected with R/S-lo. % colistin resistance of R/S-lo recovered in peritoneal lavage fluid after 8 hour infection is shown.

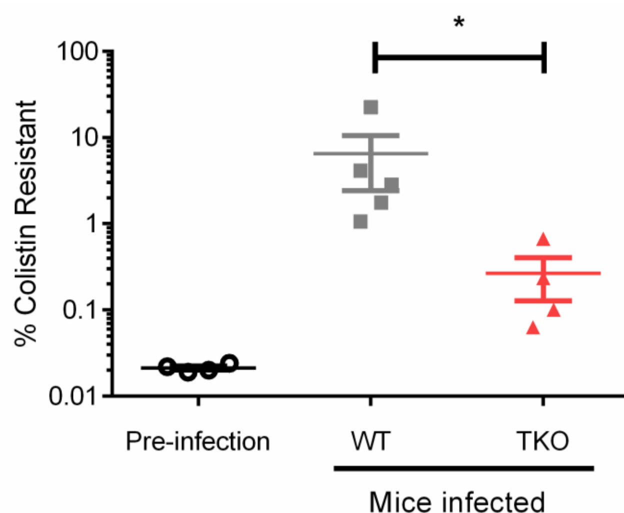


Figure S2.21. Specific host antimicrobials contribute to the increased frequency of the R/S-lo subpopulation *in vivo*. Triple knockout mice (TKO) lacking the NADPH oxidase gp91 subunit (which contributes to superoxide production), lysozyme and CRAMP were infected with R/S-lo. % colistin resistance of R/S-lo recovered in peritoneal lavage fluid after 8 hour infection is shown. * $p < 0.05$.

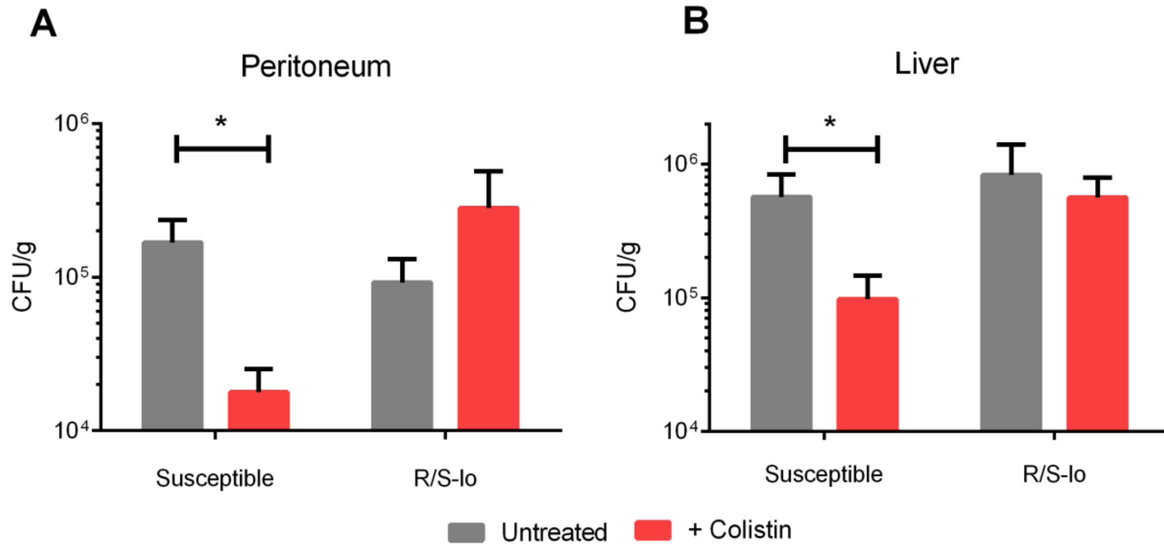


Figure S2.22. Inefficacy of colistin in reducing the levels of strain R/S-lo during *in vivo* infection. Mice infected with R/S-lo or a susceptible clinical isolate were treated with colistin at 8, 14 and 20 hours. CFU were quantified at 24 hours in the (A) peritoneal lavage fluid and (B) liver. * $p < 0.05$.

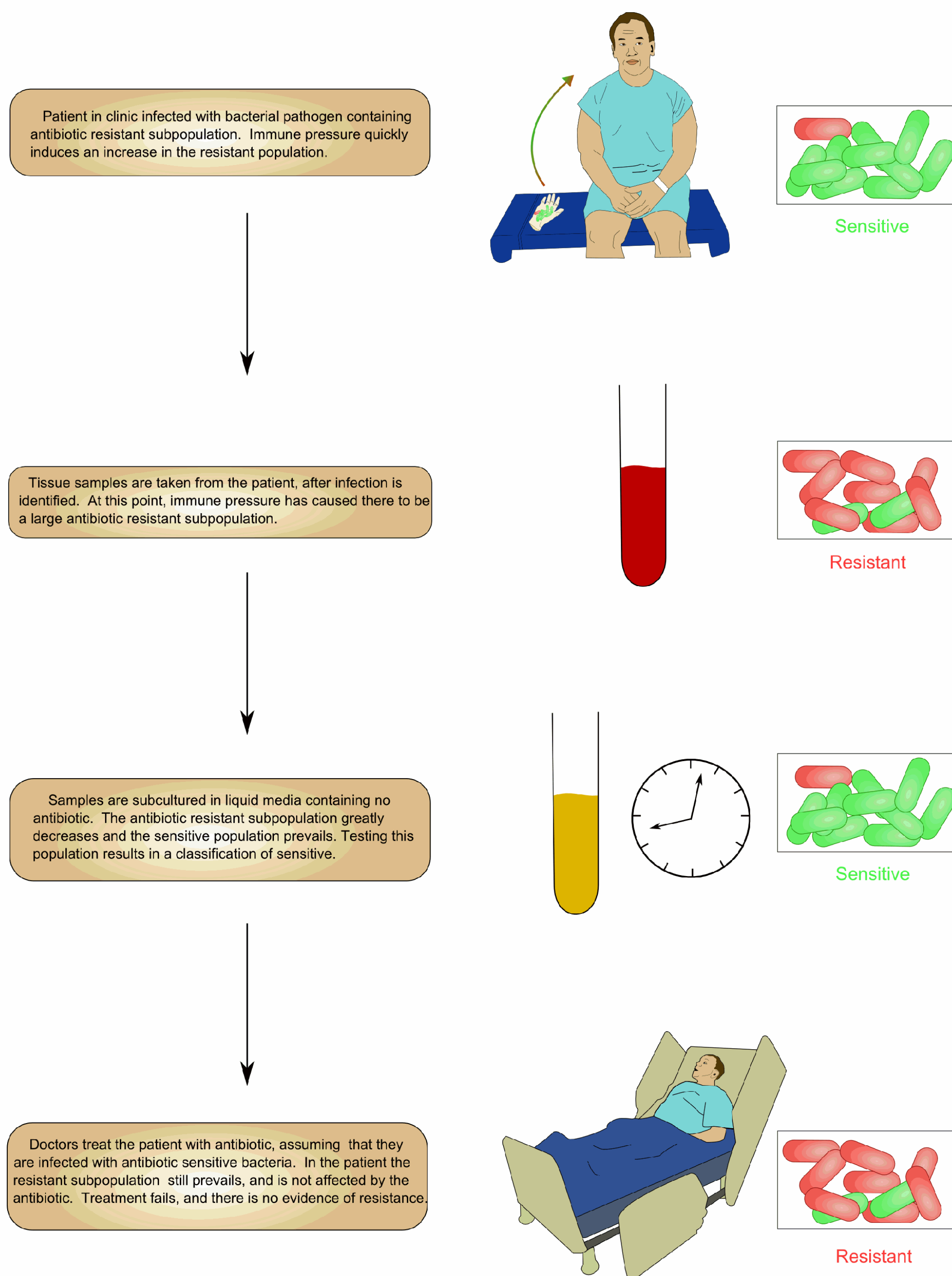


Figure S2.23. Schematic indicating how antibiotic-resistant subpopulations can lead to unexplained clinical treatment failure. Graphic showing how antibiotic resistant subpopulations that are undetected by currently used diagnostic tests, such as that described in R/S-lo, can cause unexplained antibiotic treatment failure.

Chapter 3:

Carbapenem-resistant *Klebsiella pneumoniae* exhibiting clinically undetected colistin heteroresistance lead 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 40-50%. 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 harbor 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. 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.

Introduction

Antibiotic resistance is an increasingly urgent problem, predicted to cause 10 million annual deaths worldwide by the year 2050[2]. *Klebsiella* spp. including *K. pneumoniae* are responsible for ~10% of nosocomial infections in the U.S.[187], including urinary tract, bloodstream, and soft tissue infections[188]. Carbapenem-resistant *Klebsiella pneumoniae* (CRKP) are one of the carbapenem-resistant Enterobacteriaceae (CRE), an emerging cause of antibiotic resistant healthcare-associated infections. CRE were listed as one of the most urgent antibiotic resistant threats by the CDC and WHO[1,15]. In part due to the difficulty of effectively treating infections with CRE, mortality rates can be as high as 40-50%[189]. These infections are a worldwide problem, with recent reports indicating that CRE are widespread in the United States[190], Europe[191], and China[192]. Unfortunately, resistance to "last line" drugs, such as colistin, is emerging in CRKP, and in some cases isolates are resistant to all antibiotics tested[193]. 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 are 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.

Results

Two CRKP urine isolates (Mu9 and Mu156) were collected from different patients at Atlanta, Georgia 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 study. Mu9 and Mu156 were confirmed as being genetically distinct by pulsed-field gel electrophoresis (using XbaI-digested total DNA and separated by electrophoresis on a Chef DRIII apparatus (Bio-Rad Laboratories, Hercules, CA) at 200V (6 V/cm) with a 90-s switch time for 23 hours) (data not shown). Both isolates were resistant to nearly all antibiotics tested, including all carbapenems and some aminoglycosides **(Table S3.1)**. PCR for various resistance genes revealed several beta-lactamases in each isolate, including *Klebsiella pneumoniae* carbapenemase (KPC) in both strains **(Table S3.2)**. Colistin susceptibility testing by broth microdilution[194] in cation adjusted Mueller Hinton (MH) broth (BD Biosciences, Franklin Lakes, NJ), using colistin sulfate (Sigma-Aldrich, St. Louis, MO), revealed a minimum inhibitory concentration (MIC) of 0.5 µg/ml and colistin Etest (Biomerieux, France) performed on MH Agar (Remel, San Diego, CA) indicated that both strains were susceptible to colistin **(Figure 3.1a)**. 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 varying 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 100µg/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 100µg/mL **(Figure 3.1b)**. In contrast, PAP demonstrated that all the cells of a colistin susceptible control isolate, GA65146, were killed by 2µg/mL of colistin **(Figure 3.1b)**.

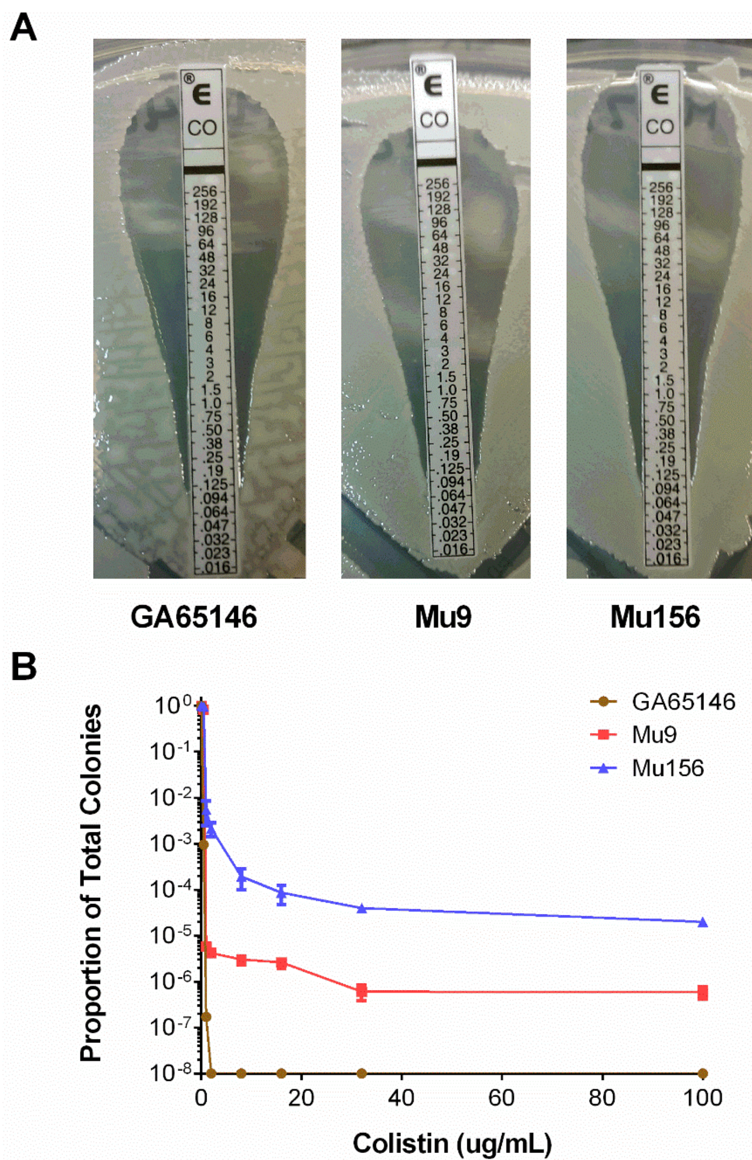

Figure 3.1. Colistin heteroresistant, carbapenem-resistant *Klebsiella pneumoniae* harbor clinically undetected resistant subpopulations. (A) Colistin susceptible isolate GA65146 and the colistin heteroresistant isolates Mu9 and Mu156 were assayed for colistin resistance using the Etest (bioMérieux, Durham, NC, USA) method. The minimum inhibitory concentration (MIC) is represented by the highest concentration along the strip at which bacteria grow. (B) Population analysis profile of GA65146, Mu9, and Mu156. Proportion of total colonies is the number of CFU able to grow at each concentration of colistin on solid media divided by the number growing on media without drug. Heteroresistant isolates exhibit a minor subpopulation that is able to grow on concentrations of colistin above 4µg/mL.

It was concerning that broth microdilution and Etest were unable to detect the colistin resistant subpopulations in these isolates. The recommended incubation time for both tests is 24 hours [194,195]. Extension of the incubation time to 48 hours resulted in the accurate identification of resistance by broth microdilution **(Figure S3.1a)**, likely because the minor resistant subpopulation had more time to grow out. In contrast, increased incubation time had no effect on Etest results, which remained negative **(Figure S3.1b)**.

We next studied the dynamics of the resistant subpopulation following colistin treatment. After treatment with 100µg/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 **(Figure S3.2)**, 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[196]. To directly assess whether the resistant and susceptible subpopulations were genetically homogenous, we isolated cultures with majority resistant or susceptible subpopulations by subculturing in media containing 16µg/mL colistin or drug-free media, respectively **(Figure S3.3)**. This resulted in cultures containing >95% colistin resistant cells or >95% colistin susceptible cells **(Figure S3.3)**. We then performed genomic sequencing on both populations using Illumina HiSeq 4000 for a depth of coverage of >1000X, 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[197] and *mgrB* expression was lower in resistant cells cultured in colistin as compared to susceptible cells grown in drug free media **(Figure S3.4a)**. Additionally, expression of *phoP*, which is autoinduced when the PhoPQ system is active[198], was increased in colistin resistant cells of Mu9 as compared to susceptible cells, although this was not observed in Mu156 **(Figure S3.4b)**. Taken together, these data are consistent with involvement of the PhoPQ pathway in the resistant subpopulation 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. We infected mice (C57BL/6J, Jackson Labs, Bar Harbor, ME) intraperitoneally with a lethal dose ($3x10^8$ CFU) of either of the heteroresistant *K. pneumoniae* isolates, subsequently leaving the mice untreated or treating with colistin after 12 hours (20mg/kg colistin methanesulfonate (Chem Impex, Wood Dale, IL), given intraperitoneally every 6 hours), to simulate infection and subsequent treatment upon clinical presentation. Interestingly, even in the absence of colistin, the frequency of the resistant subpopulation of both heteroresistant isolates increased following 24 hour *in vivo* infection compared to the inoculum **(Figure S3.5)**. This may be due to cross-resistance of these cells to host innate immune antimicrobials, as has previously been demonstrated[156]. We next assessed the impact of heteroresistance on colistin treatment outcome. Mice infected with either of the heteroresistant isolates (Mu9 or Mu156) were unable to survive the infection, even in the presence of colistin **(Figure 3.2a, b)**. In contrast, mice infected with the colistin susceptible strain (GA65146) succumbed to infection in the absence of antibiotic but were rescued by colistin treatment **(Figure 3.2c)**. These data strikingly demonstrate

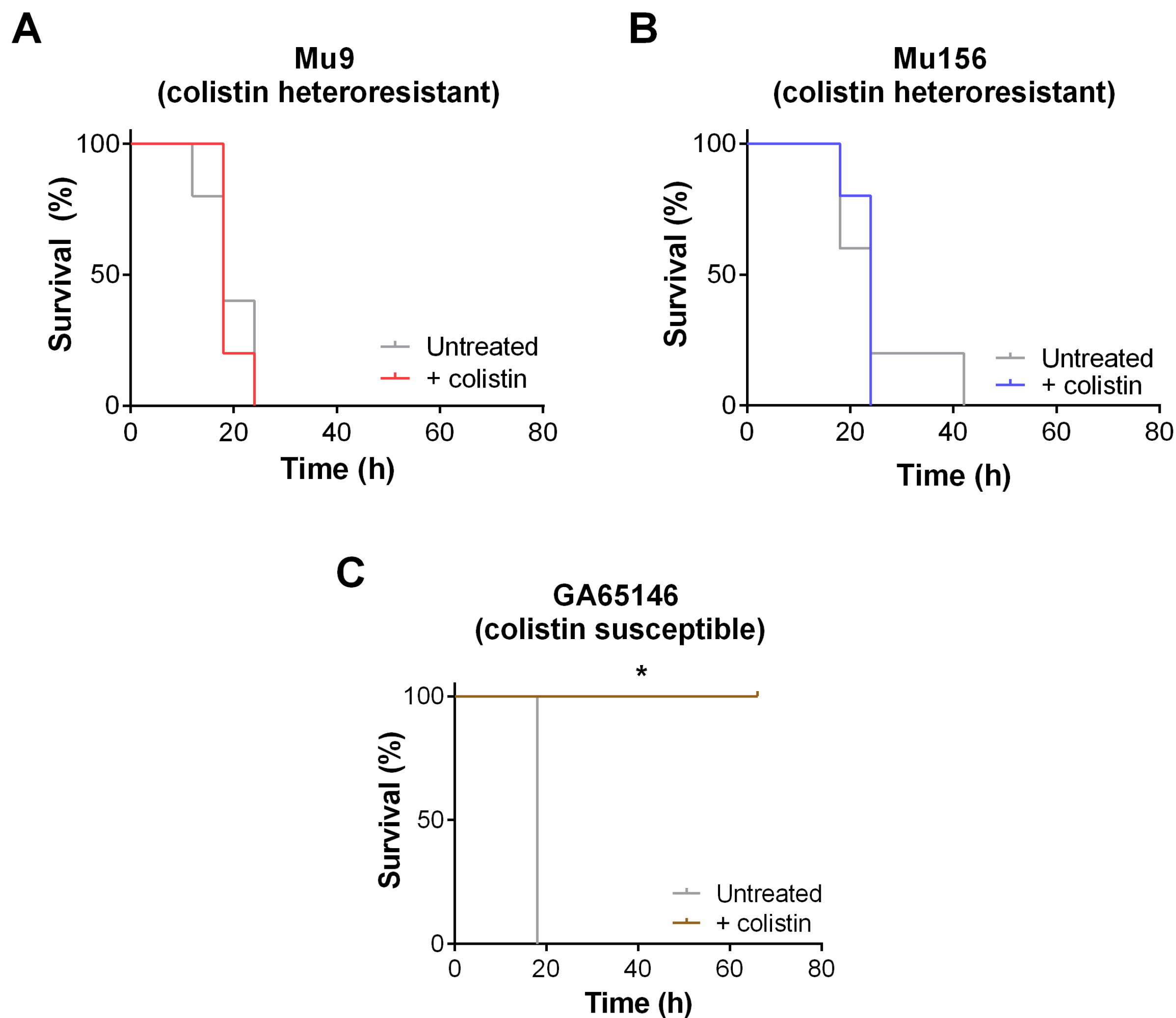

Figure 3.2. K. pneumoniae isolates lead to *in vivo* colistin treatment failure. (A-C) Mice were infected with the colistin heteroresistant isolates Mu9 (A) or Mu156 (B), or the colistin susceptible isolate GA65146 (C), and then treated with 10 mg/kg colistin every 6 hours starting at 12 hours. Mice were monitored for survival and weight loss and were sacrificed if below 80% starting weight. n = 5 * $p < 0.05$ (Gehan-Breslow-Wilcoxon test).

that colistin heteroresistance can lead to *in vivo* colistin treatment failure in CRKP.

Discussion

This is the first report of colistin heteroresistant *K. pneumoniae* in the U.S. 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 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[199]. 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 would waste critical time and resources that 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 cumbersome for most clinical laboratories to implement. Our findings suggest that broth microdilution with increased incubation time (48 hours) 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 susceptible bacteria could become contaminated or that *de novo* mutant cells would have the time necessary to grow out, leading to

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 Figures

	Mu9		Mu156	
	MIC	**S/I/R**	**MIC**	**S/I/R**
Amikacin	32	I	32	I
Amp/Sulb	>16/8	R	>16/8	R
Ampicillin	>16	R	>16	R
Aztreonam	>16	R	>16	R
Cefazolin	>16	R	>16	R
Cefepime	>16	R	>16	R
Cefotaxime	>32	R	>32	R
Cefoxitin	>16	R	>16	R
Ceftazidime	>16	R	>16	R
Ceftriaxone	>32	R	>32	R
Cefuroxime	>16	R	>16	R
Cephalothin	>16	N/A	>16	N/A
Ciprofloxacin	>2	R	>2	R
Ertapenem	>4	R	>4	R
Gentamicin	≤4	S	≤4	S
Imipenem	>8	R	>8	R
Levofloxacin	>4	R	>4	R
Meropenem	>8	R	>8	R
Nitrofurantoin	>64	N/A	>64	N/A
Pip/Tazo	>64	R	>64	R
Piperacillin	>64	R	>64	R
Tetracycline	8	I	8	I
Tigecycline	≤2	S	≤2	S
Tobramycin	>6	R	>8	R
Trimeth/Sulfa	>2/38	R	>2/38	R

Table S3.1. Antibiograms of colistin heteroresistant *K. pneumoniae* isolates. Results of Microscan (Beckman Coulter, Inc, Brea, CA, USA) antimicrobial susceptibility testing of Mu9 and Mu156. Minimum inhibitory concentration (MIC) results are listed with the interpretive category (S - susceptible, I - intermediate, R - resistant) as defined by the Clinical & Laboratory Standards Institute (CLSI). Antibiotics designated as N/A have no defined CLSI MIC breakpoints for *K. pneumoniae*.

	Mu9	Mu156
SHV	+	+
TEM	+	+
CTX	-	-
OXA	-	-
CMY	-	-
KPC	+	+
VIM	-	-
IMP	-	-
NDM	-	-

Table S3.2. Beta-lactam resistance genes in *K. pneumoniae* isolates. PCR analysis was conducted on Mu9 and Mu156 for 9 common *K. pneumoniae* resistance genes. Positive genes were those that produced a band at the expected size as assayed by PCR.

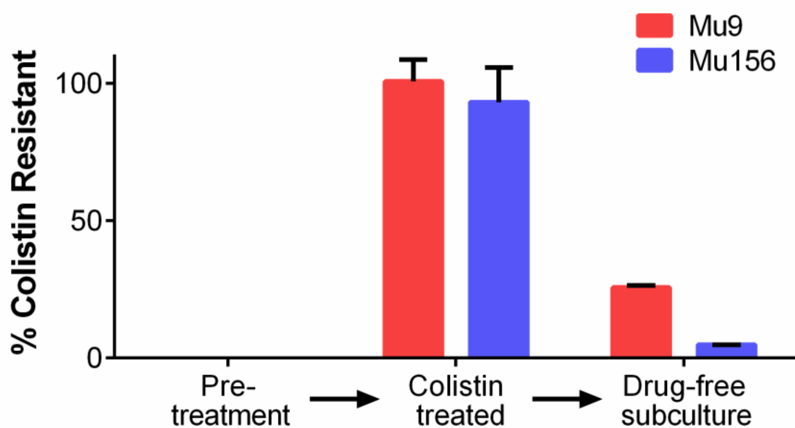

Figure S3.2. Frequency of the resistant subpopulation increases in the presence of colistin. Heteroresistant *K. pneumoniae* were grown without colistin (“pre-treatment”), then subcultured with 100µg/mL colistin (“colistin treated”), and then subcultured again without colistin (“drug-free subculture”). The frequency of the colistin resistant subpopulation was measured at each step.

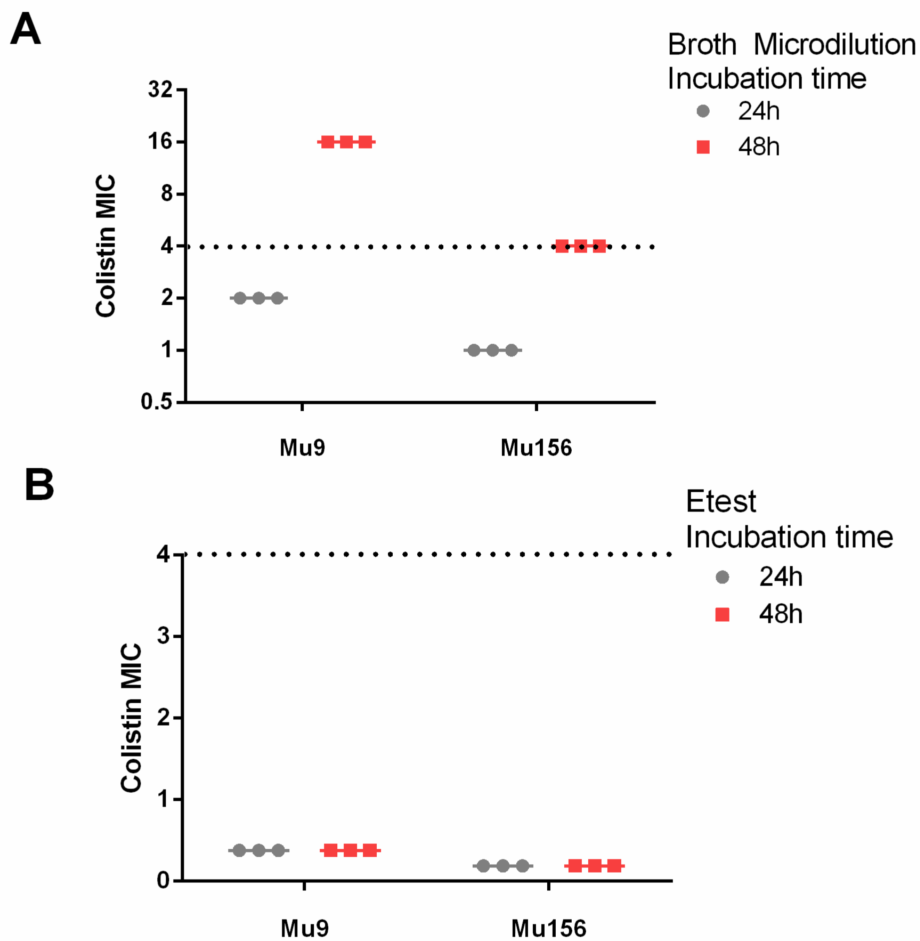

Figure S3.1. Increased broth microdilution incubation time facilitates detection of colistin heteroresistance. (A, B) The colistin MIC was determined for heteroresistant *K. pneumoniae* by both broth microdilution (A) and Etest (B) at the recommended 24 hour timepoint or after 48 hour incubation. The dashed line indicates the CLSI breakpoint for resistance to colistin of 4μg/mL(11). n=3.

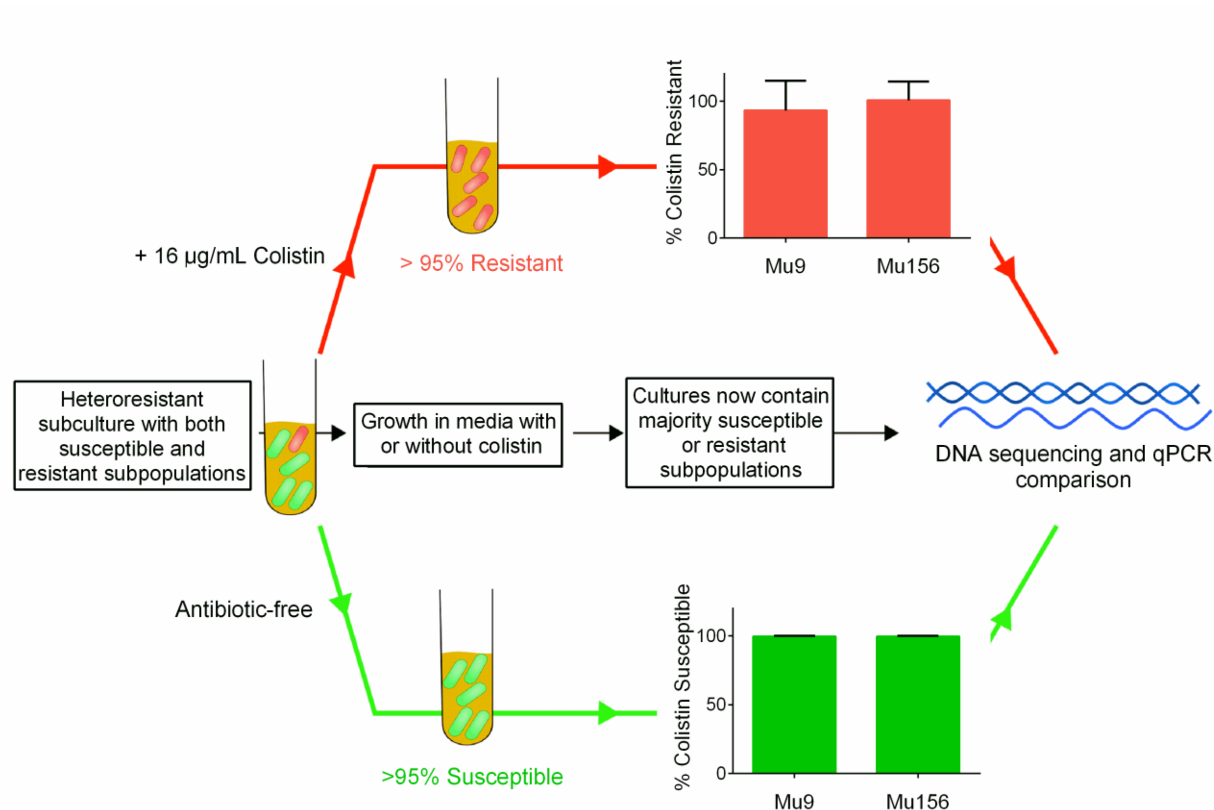

Figure S3.3. Workflow for genomic and transcriptomic analysis of colistin susceptible and resistant subpopulations. Heteroresistant *K. pneumoniae* were grown in media containing 16ug/mL colistin or drug-free media, resulting in cultures with >95% resistant or susceptible bacteria, respectively. Bacteria from the cultures were subsequently used for DNA sequence comparison and mRNA analysis by qRT-PCR.

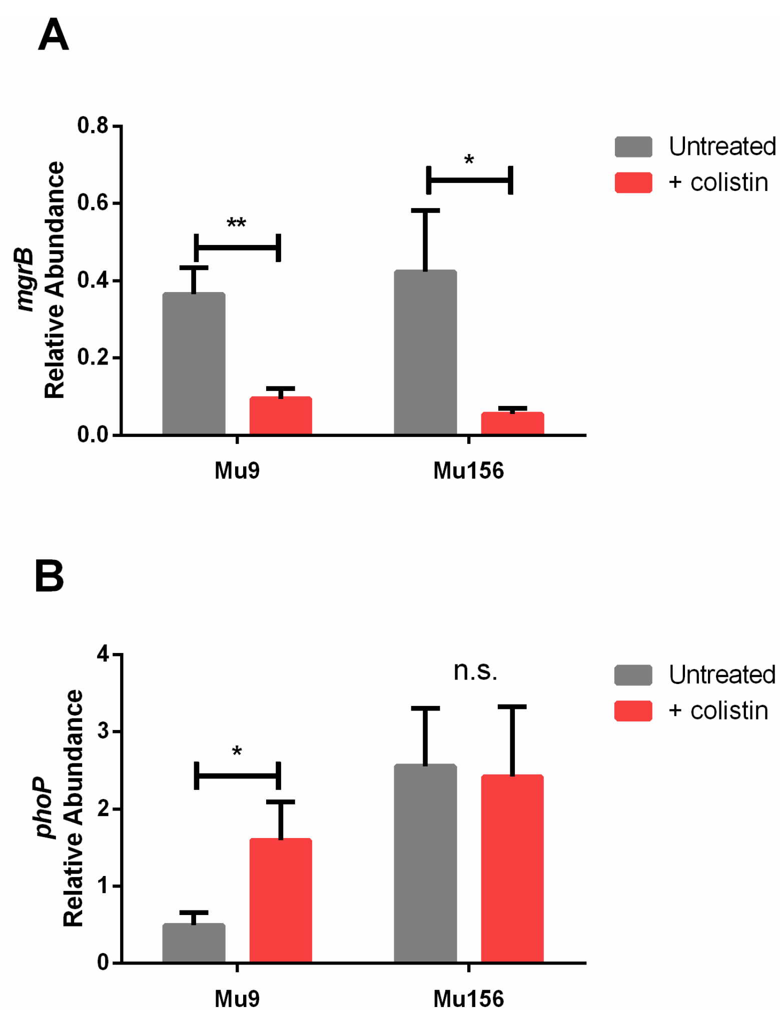

Figure S3.4. Expression of PhoPQ pathway genes in susceptible and resistant subpopulations. (A, B) Quantitative real-time PCR analysis of *mgrB* (A) and *phoP* (B) expression in resistant and susceptible subpopulations of Mu9 and Mu156. Resistant and susceptible subpopulations were enriched as shown in Figure S2. Relative abundance was calculated by normalizing expression of each gene to the average of two housekeeping genes, *23S* and *rpsL*. n=6, * $p<0.05$, ** $p<0.01$ (unpaired t-test)

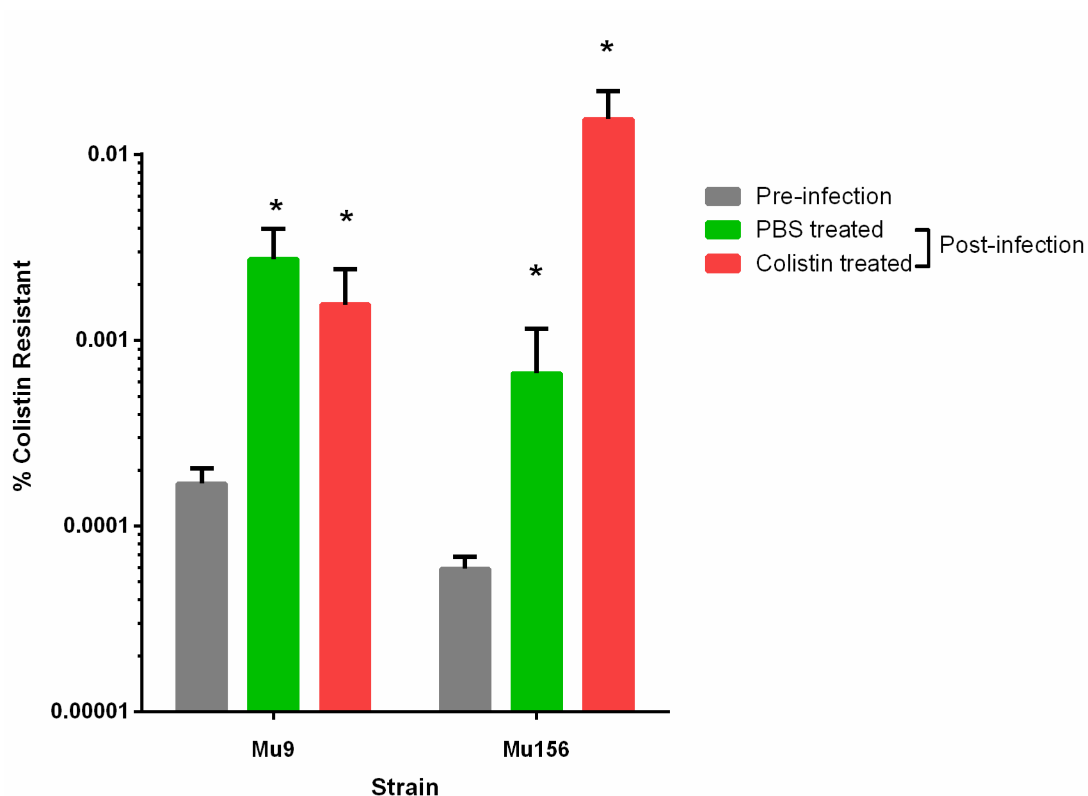

Figure S3.5. Frequency of the resistant subpopulation increases during *in vivo* infection. Mice were infected intraperitoneally with 1x10^8 CFU of Mu9 or Mu156, treated with PBS or colistin (10mg/kg) at 12 and 18 hours, and then sacrificed at 24 hours. Peritoneal lavage fluid was collected and plated onto drug free media and media containing 16 ug/mL colistin to assess % colistin resistance of the heteroresistant strains. The pre-infection inoculum (input) was plated similarly. n=5, * $p < 0.05$ (Mann-Whitney test).

Chapter 4

Heteroresistance to colistin among carbapenem-resistant Enterobacteriaceae: A Multistate Study, 2012-2015

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Abstract

Antibiotic resistance is predicted to cause 10 million annual deaths worldwide by the year 2050 and add $100 trillion to healthcare costs. This grave problem is further complicated by unexplained failures of antibiotic therapy caused by bacteria that appear susceptible to a given drug. We previously showed that heteroresistance, in which one bacterial strain harbors both a minor resistant subpopulation and a majority susceptible subpopulation, can mediate failure of antibiotic therapy in an *in vivo* infection model and go undetected by current diagnostic tests. Colistin is a critical last-line antibiotic to treat carbapenem-resistant *Enterobacteriaceae* (CRE) and we sought to characterize the extent of colistin heteroresistance within US populations in a retrospective study. We screened 408 CRE isolated between 2012-2015 from 8 US states. Colistin heteroresistance (10.1% of isolates) was more common than “conventional” resistance (7.1%) in which all the cells of a bacterial isolate are phenotypically resistant. Furthermore, among the sample of isolates tested, the frequency of colistin heteroresistance increased from 2012 to 2015, especially among *Enterobacter* isolates. We also revealed that colistin heteroresistant *Klebsiella* are much more common in Georgia than other states surveyed, where a specific strain cluster is prevalent. Alarmingly, the vast majority (93.2%) of the heteroresistant isolates were classified as colistin susceptible by clinical diagnostic testing. When this undetected resistance is taken into account, the overall rate of colistin non-susceptibility among CRE in the US is more than double the level currently detected. Taken together, these data highlight a largely unappreciated crisis in which colistin heteroresistance is prevalent among CRE, overwhelmingly undetected, and may lead to unexplained antibiotic treatment failure in clinical settings.

Introduction

Increasing antibiotic resistance has been recognized as a major health threat by the CDC and WHO[14,15]. To combat highly resistant bacteria like the carbapenem-resistant *Enterobacteriaceae* (CRE) which cause infections with a ~40% mortality rate[189], clinicians are increasingly turning to drugs of last resort such as the polymyxin antibiotic colistin[16]. However, resistance is increasing even to these last-line drugs[200-202]. Further complicating efforts to combat multidrug resistant bacteria are instances of treatment failure of strains classified as susceptible to a given antibiotic. Heteroresistance is an often unrecognized form of resistance in which a strain harbors both a minor antibiotic resistant subpopulation and a majority population of susceptible cells, which was recently shown to mediate colistin treatment failure in an *in vivo* model[156]. Furthermore, colistin heteroresistance can go undetected by clinical testing methods, highlighting the potential for clinicians to unknowingly make ineffective treatment decisions[156]. Since colistin heteroresistance may pose a significant risk in the clinic and as it is unclear how common this phenotype is in the US, we performed a retrospective study among highly resistant CRE isolates from 8 states between 2012 and 2015.

Results

Four hundred and eight CRE isolates were collected between 2012-2015. Of these, 226 (55%) isolates were from female patients, and 335 (82%) isolates were from patients 50 years of age or older **(Table 4.1)**. Isolates were most commonly derived from urine cultures (332, 81%) and blood cultures (57, 14%) **(Table 4.1)**. Of the three genera, *Klebsiella* was the most frequently observed (260, 63%) followed by *Enterobacter* (100, 24%) and *Escherichia* (48, 11%) **(Table 4.2)**. All of the isolates were resistant to third generation cephalosporins and non-

	Colistin susceptibility by PAP, No. (%)				
	Susceptible	Conventional Resistant	Heteroresistant	Total	p value[a]
Sex					
Female	194 (86)	16 (7)	16 (7)	226	
Male	143 (79)	13 (7)	24 (13)	180	0.0439
Age					
0-9	1 (25)	0 (0)	3 (75)	4	0.0033
10-19	2 (100)	0 (0)	0 (0)	2	1.0000
20-29	14 (88)	2 (13)	0	16	0.3856
30-39	17 (85)	1 (5)	2 (10)	20	1.0000
40-49	27 (93)	1 (3)	1 (3)	29	0.3391
50-59	65 (87)	3 (4)	7 (9)	75	1.0000
60-69	92 (82)	8 (7)	12 (11)	112	0.7120
70-79	72 (87)	9 (11)	2 (2)	83	0.0071
80+	47 (72)	5 (8)	13 (20)	65	0.0056
Culture source					
Urine	275 (83)	24 (7)	34 (10)	333	1.0000
Blood	47 (82)	5 (9)	5 (9)	57	1.0000
Other sterile site	13 (87)	0 (0)	2 (13)	15	0.6561
Total	338	29	41	408	

Table 4.1 Sex, Age, and Culture Source of Carbapenem Resistant Enterobacteriaceae. [a] p value for % colistin heteroresistance in each category, by odds ratio. PAP – population analysis profile

susceptible to at least one carbapenem, as part of their case inclusion criteria. Resistance to the aminoglycoside tobramycin was widespread (264/361, 73%), while lower rates of resistance were exhibited to other aminoglycosides, gentamicin and amikacin (38% and 36%, respectively). The lowest rate of resistance was to tigecycline, as only 1.5% of the isolates were non-susceptible **(Table S4.1)**.

Population analysis profile (PAP) of the 408 isolates revealed the rate of colistin heteroresistance to be 10.1% (41/408**) (Table 4.1)**. In contrast, 7.1% (29/408) of isolates were classified as exhibiting “conventional” resistance (in which all the cells of the population exhibit resistance). Among this collection of isolates, the rate of colistin heteroresistance was significantly higher in 2015 (15.8%, 24/152) (p=0.0039) than in previous years (9.8%, 4/41 in 2012, 6.2%, 5/81 in 2013, 6.0%, 8/134 in 2014) **(Figure 4.1)**. It should be noted that new surveillance sites were added in 2013 and 2014. Excluding these 4 sites, the rate of colistin heteroresistance in 2015 was still significantly increased as compared to prior years (17/118, 14.41%, p = 0.0117) Additionally, even with these sites excluded, colistin heteroresistance was present at slightly higher levels than “conventional” resistance (9.06% heteroresistance, 8.13% conventional resistance). Taken together, these data suggest that colistin heteroresistance is present at a high rate and in fact is observed more frequently than “conventional” colistin resistance among the isolates in this surveillance population.

Among the genera, *Enterobacter* sp. displayed the highest rate of heteroresistance (18%, 18/100, p = 0.0031), followed by *Klebsiella* sp. (8.5%, 22/212) and *E. coli* (2.1%, 1/47). Among *Enterobacter*, the rate of colistin heteroresistance was significantly higher in 2015 (30.0%, 12/40) (p=0.0144) as compared to prior years (1/14 or 7.1% in 2012, 0/14 or 0.0% in 2013, 5/32 or 15.6% in 2014). This is consistent even when surveillance sites added in 2013 and 2014 are

	Colistin susceptibility by PAP, No. (%)				
	Susceptible	Conventional Resistant	Heteroresistant	Total	p value[a]
Genera					
Enterobacter	79 (79)	3 (3)	18 (18)	100	0.0159
Klebsiella	212 (82)	22 (8)	26 (10)	260	0.4127
Escherichia	47 (98)	0 (0)	1 (2)	48	0.0453
State					
Colorado	35 (88)	2 (5)	3 (8)	40	0.7833
Georgia	101 (80)	8 (6)	17 (13)	126	0.1530
Maryland	104 (87)	11 (9)	5 (4)	120	0.0107
Minnesota	49 (88)	3 (5)	4 (9)	56	0.6316
New Mexico	8 (80)	0 (0)	2 (20)	10	0.2653
New York	23 (82)	1 (4)	4 (14)	28	0.5085
Oregon	11 (61)	4 (22)	3 (17)	18	0.4091
Tennessee	7 (70)	0 (0)	3 (30)	10	0.0687

Table 4.2 Genus and State of Origin of Carbapenem Resistant Enterobacteriaceae. [a] p value for % colistin heteroresistance in each category, by odds ratio. PAP – population analysis profile.

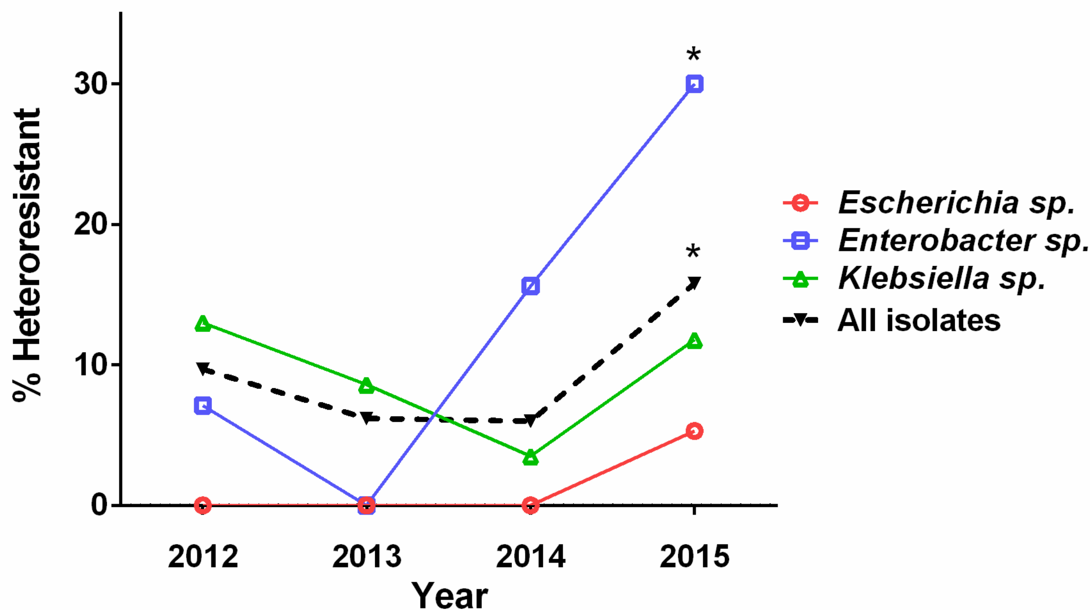

Figure 4.1 Rate of Colistin Heteroresistance by Genus, (2012-2015). Rates of total colistin heteroresistant isolates in percent, in each year from 2012-2015. * p <0.05 for positive linear trend from 2012-2015 (Cochran-Armitage Trend test).

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excluded (p = 0.0387) (data not shown).

To further examine the concerning increase in the rate of colistin heteroresistance among *Enterobacter*, we analyzed the rate in specific species (as determined by the clinical laboratory). *Enterobacter* isolates from this study could be classified into 5 species including *E. aerogenes* and the species that collectively make up the *Enterobacter cloacae* complex (ECC): *E. cloacae, E. asburiae, E. kobei*, and *E. ludwigii* [143]. Of these, *E. cloacae* and *E. aerogenes* were the most common, making up 61% (61/100) and 26% (26/100) of the total *Enterobacter* isolates, respectively **(Table 4.3)**. Less common species of *Enterobacter* (designated here as “minority ECC species”), *E. kobei* (6%, 6/100), *E. ludwigii* (2%, 2/100) and *E. asburiae* (5%, 5/100) made

	Colistin susceptibility by PAP, No. (%)				
	Susceptible	Conventional Resistant	Heteroresistant	Total	p value[a]
Enterobacter species	79 (79.0)	3 (3.0)	18 (18.0)	100	
E. aerogenes	23 (88.5)	1 (3.8)	2 (7.7)	26	0.1441
E. cloacae	53 (86.9)	1 (1.6)	7 (11.5)	61	0.0594
E. asburiae	1 (20.0)	0 (0)	4 (80.0)	5	0.0034
E. kobei	2 (33.3)	1 (16.7)	3 (50.0)	6	0.0698
E. ludwigii	0 (0)	0 (0)	2 (100.0)	2	0.0309
Enterobacter cloacae complex (ECC)[b]	56 (75.7)	2 (2.7)	16 (21.6)	74	0.1441
Minority ECC species[c]	3 (23.1)	2 (15.4)	9 (69.2)	13	0.0001

Table 4.3 *Enterobacter* Species Identification and Colistin Susceptibility. [a] p value for % colistin heteroresistance in each category, by odds ratio. [b] Includes *E. cloacae*, *E. asburaie*, *E. kobei,* and *E. ludwigii.* [c] Includes *E. asburiae, E. kobei,* and *E. ludwigii.* PAP – population analysis profile.

up the remaining 13% of isolates. Strikingly, the rate of colistin heteroresistance was highest among these minority ECC species, with heteroresistance observed in 69.2% (9/13) of these isolates, significantly higher than the rates in *E. aerogenes* (7.7%, 2/26) or *E. cloacae* (11.5%, 6/61, $p < 0.0001$) **(Table 4.3)**. In fact, despite their relatively infrequent occurrence, minority ECC species comprised 50% of the total colistin heteroresistant *Enterobacter* isolates observed in this study (9/18).

Indeed, the high rate of heteroresistance in minority ECC species may have contributed to the precipitous increase in *Enterobacter* colistin heteroresistance over the course of this study. Between 2012 and 2015, the overall rate of colistin heteroresistance among all *Enterobacter* rose significantly from 7.1% in 2012 to 30% in 2015 **(Figure 4.1)**. The incidence of minority ECC species rose from 7.1% (1/14) to 20.0% (5/40), closely mirroring the overall rise in the rate of colistin heteroresistance **(Figure S4.1a)**. Thus, these minority ECC species are a concerning source of colistin heteroresistance. In addition, the rate of heteroresistance among *E. cloacae* rose from 2012-2015 (0% to 24%) **(Figure S4.1b)**, which also contributed to the rising rate of colistin heteroresistance among the ECC (12.5% to 33.3%) **(Table 4.3)**. Importantly, at least one colistin heteroresistant *Enterobacter* isolate was identified in each of the 8 study sites, indicating that *Enterobacter* exhibiting this resistance phenotype are isolated from a wide distribution of geographic sites within the US.

While *Enterobacter* isolates exhibited the highest rate of colistin heteroresistance, *Klebsiella* sp. were the most numerous bacteria in this study and also had the highest overall number of colistin heteroresistant isolates. Of the 22 colistin heteroresistant *Klebsiella* isolates identified, 15 (68%) were from Georgia **(Table 4.3)**. While Georgia had a higher rate of *Klebsiella pneumoniae* (104/126 (82.5%), $p < 0.0001$) among its CRE isolates than the other

	Colistin susceptibility by PAP, No. (%)				
	Susceptible	Conventional Resistant	Heteroresistant	Total	p value[a]
Klebsiella sp.					
Colorado	14 (82)	2 (12)	1 (6)	17	1.0000
Georgia	82 (78)	8 (8)	15 (14)	105	0.0109
Maryland	81 (86)	10 (11)	3 (3)	94	0.0211
Minnesota	12 (80)	2 (13)	1 (7)	15	1.0000
New Mexico	3 (75)	0 (0)	1 (25)	4	0.2994
New York	15 (88)	1 (6)	1 (6)	17	1.0000
Oregon	3 (50)	3 (50)	0 (0)	6	1.0000
Tennessee	2 (100)	0 (0)	0 (0)	2	1.0000
Total	212	26	22	260	

Table 4.4 State of Origin of Carbapenem Resistant *Klebsiella* Species. [a] p value for % colistin heteroresistance in each category, by odds ratio.

PAP – population analysis profile

states (data not shown), the rate of colistin heteroresistance among *Klebsiella* isolates was also higher in Georgia (14.2%, 15/105) compared to the other states combined (4.5%, 7/155, p = 0.0109) (**Table 4.4**).

The high 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 *Klebsiella* isolates based on their genome sequences (**Figure S4.2**). We observed that among the 15 colistin heteroresistant *Klebsiella* from Georgia, 13 (86.7%) clustered very closely together compared to heteroresistant isolates from other states. This genetic branch comprised isolates from all 4 years of the study, which did not cluster together temporally. All isolates were ST-258, an epidemic strain type in the US, and had capsule type KL106. In addition, they shared many resistance genes in common, including the KPC-3 carbapenemase (**Figure S4.2**). Overall, these isolates were within 0.05 p-distance, and may indicate that colistin heteroresistant, carbapenem-resistant *Klebsiella* are endemic in the Georgia surveillance catchment area. Importantly, a single colistin heteroresistant ST-258 isolate closely related to the strains in the Georgia cluster was found in Minnesota in 2015, demonstrating that strains highly related to these endemic strains are present outside Georgia as well.

While we were able to detect 41 colistin heteroresistant isolates in this study using the labor-intensive PAP method, this is unlikely to be a feasible testing method in a clinical laboratory. Broth microdilution (BMD) is the CLSI approved method to test for colistin susceptibility, and we therefore retested all the heteroresistant isolates by BMD to assess how they would be classified in the clinic. Alarmingly, only 3/41 (7.4%) heteroresistant isolates were classified as resistant to colistin, while the other 38 (92.6%) were misclassified as colistin

	Total Isolates Detected by BMD, No. (%)			Total Isolates, No. (%)		
	Detected Conventional Resistance[a]	Detected Heteroresistance[b]	Total Lab Detected Non-susceptible[c]	Undetected Heteroresistance[d]	Total Non-Susceptible[e]	Change in Non-susceptible Rate[f]
***Enterobacter* species**	3 (3.0)	1 (1.0)	4 (4.0)	17 (17.0)	21 (21.0)	+525%
***Escherichia* species**	0 (0.0)	0 (0.0)	0 (0.0)	1 (2.1)	1 (2.1)	n/a
***Klebsiella* species**	26 (10.0)	2 (0.8)	28 (10.8)	20 (7.7)	48 (18.5)	+171%
Total	29 (7.1)	3 (0.7)	32 (7.8)	38 (9.3)	70 (17.1)	+219%

Table 4.5 Colistin Susceptibility Results of Enterobacteriaceae by Clinical and Laboratory Testing. [a] Total conventional resistant isolates detected as non-susceptible by broth-microdilution. [b] Total heteroresistant isolates detected as non-susceptible by broth microdilution. [c] Total isolates (resistant and heteroresistant) detected as non-susceptible by broth microdilition. [d] Total heteroresistant isolates detected as susceptible by broth microdilution. [e] Total non-susceptible isolates as detected by laboratory based population analysis profile. [f] Change in rate of non-susceptibility, in %, as detected by population analysis profile compared to broth-microdilution

susceptible **(Table 4.5)**. Therefore, in total, 29 isolates exhibiting "conventional" colistin resistance as well as 3 colistin heteroresistant isolates (32/408, 7.1%) were accurately identified as colistin nonsusceptible. However, when the 38 colistin heteroresistant isolates undetected by BMD are taken into account, the overall rate of colistin non-susceptibility (70/408, 17.1%) is revealed to be more than double that which is currently detected. This is a worrisome finding that indicates colistin non-susceptibility is much more widespread among highly resistant CRE than previously thought.

Discussion

This is the first multi-site surveillance study for colistin heteroresistance among CRE in the US. Treatment of highly antibiotic resistant CRE relies on last line drugs such as colistin. Our findings reveal the rate of heteroresistance to colistin to be 10.1% among CRE isolates in this surveillance population. The rate of heteroresistance to colistin exceeded the rate of "conventional" resistance (7.1%), wherein all the cells of a bacterial isolate are resistant. In total, 17.1% of isolates were classified as colistin non-susceptible. This is a surprisingly high rate as cases of colistin non-susceptible CRE in the United States were first reported in 2011[203].

Importantly, none of the bacteria in this study were positive for the recently described plasmid-borne *mcr-1* colistin resistance gene. Previous studies found the rate of *mcr-1* positive isolates to be well below 1%[204-207]**.** While there is a great deal of justified concern about the potential for *mcr-1* to spread widely, our data suggest that colistin heteroresistance is much more prevalent among highly resistant CRE in the US. Thus, colistin heteroresistance, and especially

that which is undetected, may currently represent a more urgent threat than plasmid borne colistin resistance.

The high rate of colistin heteroresistance observed in this study is compounded by the low number of isolates in which hetereoresistance was detected using standard clinical testing. By BMD, only 3/41 heteroresistant isolates were detected as being non-susceptible, indicating a major shortfall in clincal testing methods. Misidentification of a resistant isolate is considered by the FDA to be a 'very major error', and it is recommended that this rate should be under 5% for effective diagnostic tests[199]. Errors such as these could lead to treatment of colistin heteroresistant infections with colistin which could lead to treatment failure as has been shown previously in a mouse model[156]**.** Additionally, these misclassified heteroresistant strains indicate that the rate of non-susceptibility is actually 17.1%, rather than the clinically detected rate of 7.4%. Thus, colistin non-susceptible isolates are more than 2-fold more common than currently detected by clinical susceptibility testing.

While current susceptibility testing fails to correctly classify most colistin heteroresistant isolates, the results presented here suggest potential empiric guidelines that may be useful in predicting colistin heteroresistance. Among the *Enterobacter* sp. in this study, 18% were colistin heteroresistant, more than twice that of other genera. Furthermore, within *Enterobacter*, the minority species *E. asburiae*, *E. kobei*, and *E. ludwigii* were much more likely to be colistin heteroresistant (69.2% of these isolates combined). These findings may suggest that these minority species of *Enterobacter* be presumed to be colistin nonsusceptible and the use of colistin avoided when treating such infections, unless they are definitively proven to be susceptible by PAP or another highly sensitive method.

The most numerous bacteria in this study were *Klebsiella* sp., and rates of colistin heteroresistance were much higher among isolates from Georgia. Most of the heteroresistant Georgia isolates were found to be highly genetically related and shared numerous antibiotic resistance genes, suggesting that this cluster of isolates may represent a regional epidemic of colistin heteroresistant, carbapenem-resistant *Klebsiella pneumoniae* (CRKP) in GA. These isolates were all from sequence type 258 (ST-258), an epidemic form of *Klebsiella pneumoniae* that emerged as a significant clinical problem in the mid-2000s [208]. A single colistin heteroresistant ST-258 isolate within this cluster was found outside of Georgia, isolated in Minnesota in 2015, portending potential future spread of this colistin heteroresistant CRKP.

This study had some limitations that call for future investigations into the rates of colistin heteroresistance in the US and worldwide. The study population consisted of patients from 8 US areas, but this population is not representative of the US as a whole. Additionally, this study analyzed a convenience sample of the total CRE isolates from each site and therefore may not be representative of the entire CRE population at each site. As such, trends in the US patient population cannot be definitively determined from these isolates. Data is only available for 4 years (2012-2015), and all 8 study sites participated in only the two latter years. Thus, we do not yet have a clear picture of the trends of colistin heteroresistance rates among CRE in the US. However, this is an ongoing surveillance initiative (MuGSI) and we will continue to collect data on the rates of colistin heteroresistance in the future, which may provide the increased numbers needed to control for confounding factors such as site differences. These isolates are also limited to the culture sources collected as part of this surveillance project. Some samples such as respiratory cultures were not obtained. Within the study areas, there was low participation of larger private laboratories that serve specialty populations, such as dialysis, long term care, and

large private reference laboratories which largely serve outpatients. Our patient population may be biased towards isolates collected from local healthcare facilities and thus may consist of sicker patients than the general population. This study is also only limited to carbapenem-resistant *Enterobacteriaceae*, and the rates of colistin heteroresistance among other groups of bacteria may be different. CRE are the most likely group of bacteria to be treated with a last-line antibiotic like colistin, but such drugs are also used to treat other highly resistant organisms such as carbapenem-resistant *Acinetobacter* and *Pseudomonas* [209]. Another shortfall of this study is that we are not able to draw conclusions as to whether colistin heteroresistance led to colistin treatment failure. In order to make such conclusions, patients would have to be treated with colistin monotherapy such that other co-administered antibiotics are not a confounding variable. Since all the isolates were susceptible to antibiotics other than colistin, and since colistin is often used in combination, dedicated clinical trials will be necessary to conclusively address this important question.

The results from this study show that colistin heteroresistance is an emerging problem among carbapenem-resistant *Enterobacteriaceae* (CRE). Over 10% of isolates were colistin heteroresistant and the vast majority were misclassified as colistin susceptible. These findings provide further warning that colistin heteroresistance should be considered and caution should be used when interpreting susceptibility testing results and making clinical treatment decisions.

Methods

Study Design and Inclusion Criteria

The carbapenem-resistant *Enterobacteriaceae* included in this study were collected as part of the Centers for Disease Control and Prevention's (CDC) Emerging Infections Program's Multi-site Gram-Negative Surveillance Initiative (MuGSI)[190,210]. MuGSI is an ongoing, active population- and laboratory-based surveillance system for carbapenem-resistant Enterobacteriaceae. Isolates were collected between 2012-2015 from clinical laboratories in metropolitan areas in 8 US states: Colorado (2013-15), Georgia (2012-15), Maryland (2012-15), Minnesota (2012-15), New Mexico (2014-15), New York (2013-15), Oregon (2012-15) and Tennessee (2014-15). The results of the primary antibiotic susceptibility testing methods (e.g. minimum inhibitory concentration (MIC), zone diameter interpretive criteria) used by participating local clinical laboratories were used to determine eligibility for the study. Isolates were included in the study if they tested non-susceptible to at least one carbapenem excluding ertapenem (doripenem, imipenem, meropenem MIC ≥2 μg/mL), and resistant to all third generation cephalosporins tested (ceftriaxone MIC ≥4 μg/mL, ceftazidime MIC ≥16 μg/mL, and cefotaxime MIC ≥4 μg/mL). Data collection was performed by trained surveillance epidemiologists at each MuGSI site. Medical records and/or laboratory reports were reviewed to obtain patient demographic data and microbiological data for analysis. Isolates from incident cases were sent to CDC for further characterization.

Colistin heteroresistance testing

Colistin heteroresistance was assessed using the population analysis profile method[211]. Briefly, each isolate was grown from a single colony and then serial dilutions were plated on solid Mueller-Hinton (MH) agar without colistin or containing concentrations of colistin from 0.5 to 100 ug/mL. The proportion of resistant colonies was calculated by dividing the number of colonies growing on each concentration of colistin by the number of total colonies growing on a

plate with no colistin. Isolates were considered heteroresistant if the proportion (percentage) of resistant colonies at 16ug/mL of colistin or greater was at least 1 in 10^6 but less than 5 in 10^1. Isolates were considered as exhibiting "conventional" resistance (wherein all cells of the population are phenotypically resistant) if the proportion of total colonies surviving at 4ug/mL colistin or greater was more than 5 in 10^1. All other isolates were considered colistin susceptible.

Broth Microdilution

Colistin susceptibility testing was performed as previously described by CLSI standards **(ref)**. 5×10^5 colony forming units (CFU) were grown in Cation-Adjusted Mueller-Hinton in the presence of potency adjusted colistin methyl sulfate at 37˚C shaking at 250 rpm for 20 hours. Turbidity was assessed for determination of the colistin minimum inhibitory concentration in biological replicates.

Genomics

Genome sequence data for the *Klebsiella* isolates analyzed here were accessed as FASTQ files from the National Center for Biotechnology Information (NCBI) Short Read Archive (SRA) (https://www.ncbi.nlm.nih.gov/sra). SRA accession numbers and isolate names are shown in Supplementary Table S2. The program FASTQC version 0.11.5 [28] was used to evaluate the quality of the FASTQ files. Low quality reads and bases (Q<20) were removed prior to assembly using the program PRINSEQ version 0.20.4 [212]. *Ab initio* genome assembly was performed using the program SPAdes version 3.10.0 [213] with default parameters. The resulting assemblies were screened for the presence of plasmids by using BLAT version 36x1 [214] sequence similarity searches against a custom database of bacterial plasmid sequences curated from the NCBI Genbank RefSeq database[215]. Plasmid size was confirmed by pulsed field gel electrophoresis

(PFGE). Antibiotic resistance genes were annotated using BLAST [216] sequence comparisons with the ARG-ANNOT [217] and BIGSdb [218] databases, respectively. Pairwise genome sequence comparisons were performed using the MUMmer version 3.23 [219] implementation of the average nucleotide identity (ANI) approach. The resulting pairwise genome sequence identities were converted to p-distances, which were used to reconstruct the isolate phylogeny with the program MEGA version 6.0 [220].

Supplemental Figures

	Colistin Susceptibility, No. Non-susceptible/Total Tested (%)				
	Susceptible	Conventional Resistant	Heteroresistant	Total	p value[a]
Amikacin Non-susceptible	107/300 (35.6)	14/25 (56.0)	10/36 (27.8)	131/361 (36.3)	0.2659
Gentamicin Non-susceptible	120/311 (38.6)	15/25 (60.0)	8/38 (21.1)	143/374 (38.2)	0.0254
Tobramycin Non-susceptible	223/302 (73.8)	19/24 (79.2)	22/35 (62.9)	264/361 (73.1)	0.1527
Tigecycline Non-susceptible	4/62 (6.5)	1/5 (20.0)	1/13 (7.7)	6/80 (7.5)	0.9771

Table S1. Susceptibility to Last-line Antibiotics by Clinical Testing. [a] p value for % colistin heteroresistance in each category, by odds ratio. PAP – population analysis profile.

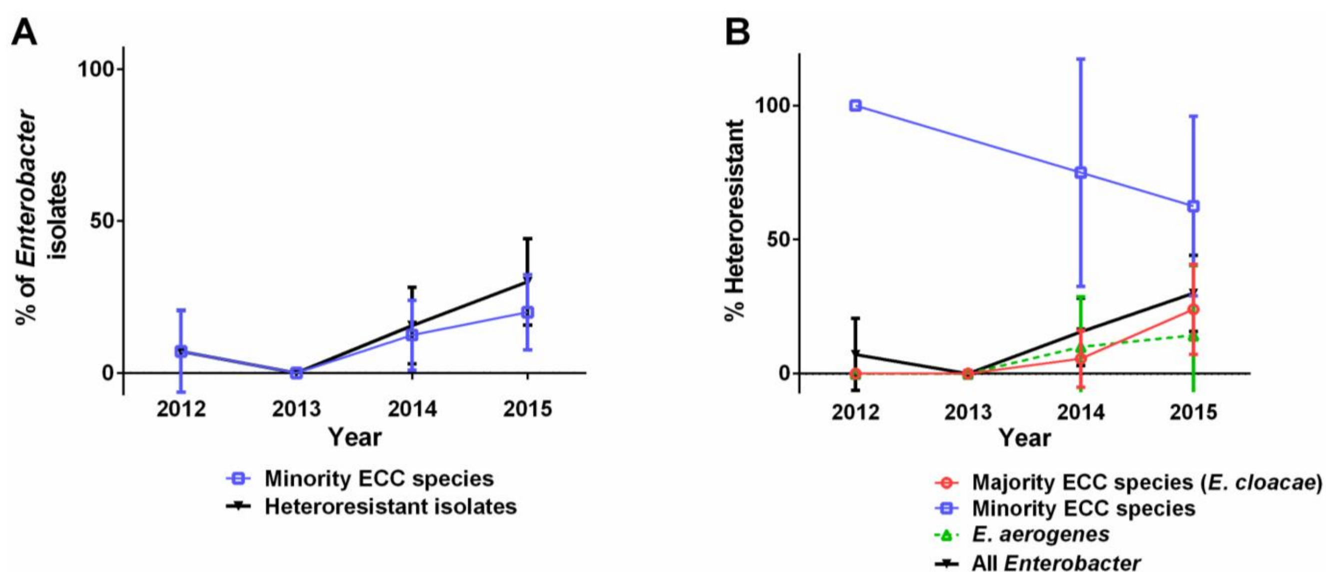
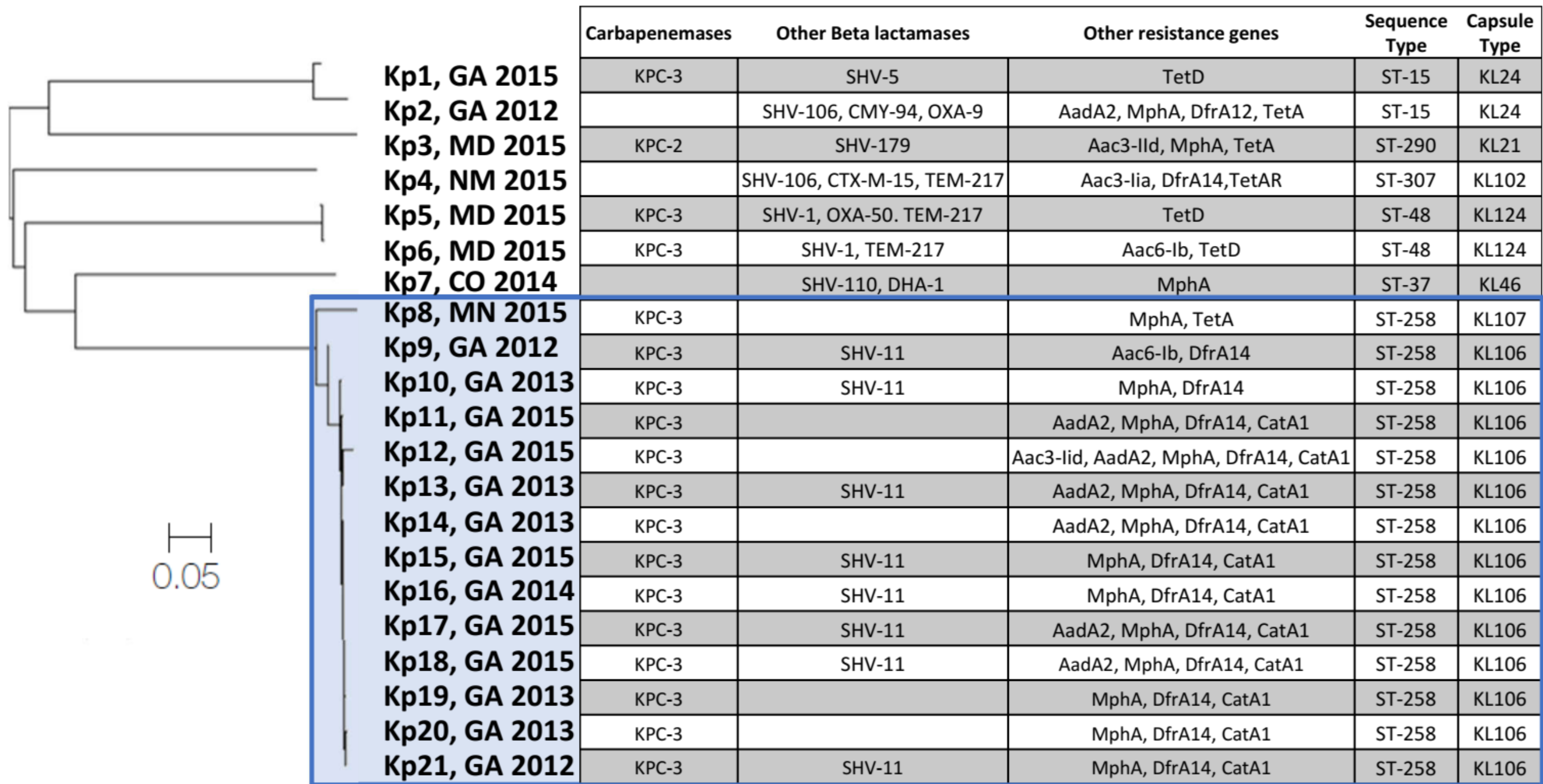


Figure S1. Rate of Enterobacter Species and Heteroresistance by Year, 2012-2015. (A) Rates of minority *Enterobacter cloacae* complex species (*E. asburiae, E. kobei, E. ludwigii)* as % of total *Enterobacter* species (blue line), and rates of heteroresistant *Enterobacter* species as % of total *Enterobacter* species (black line), by year. (B) Rates of heteroresistance for majority ECC species (*E. cloacae,* red line), minority ECC species (*E. asburiae, E. kobei, E. ludwigii*, blue line*) E. aerogenes* (green line) and all *Enterobacter* species (black line), as rate of heteroresistance in %.



Isolate	Carbapenemases	Other Beta lactamases	Other resistance genes	Sequence Type	Capsule Type
Kp1, GA 2015	KPC-3	SHV-5	TetD	ST-15	KL24
Kp2, GA 2012		SHV-106, CMY-94, OXA-9	AadA2, MphA, DfrA12, TetA	ST-15	KL24
Kp3, MD 2015	KPC-2	SHV-179	Aac3-IId, MphA, TetA	ST-290	KL21
Kp4, NM 2015		SHV-106, CTX-M-15, TEM-217	Aac3-Iia, DfrA14,TetAR	ST-307	KL102
Kp5, MD 2015	KPC-3	SHV-1, OXA-50. TEM-217	TetD	ST-48	KL124
Kp6, MD 2015	KPC-3	SHV-1, TEM-217	Aac6-Ib, TetD	ST-48	KL124
Kp7, CO 2014		SHV-110, DHA-1	MphA	ST-37	KL46
Kp8, MN 2015	KPC-3		MphA, TetA	ST-258	KL107
Kp9, GA 2012	KPC-3	SHV-11	Aac6-Ib, DfrA14	ST-258	KL106
Kp10, GA 2013	KPC-3	SHV-11	MphA, DfrA14	ST-258	KL106
Kp11, GA 2015	KPC-3		AadA2, MphA, DfrA14, CatA1	ST-258	KL106
Kp12, GA 2015	KPC-3		Aac3-Iid, AadA2, MphA, DfrA14, CatA1	ST-258	KL106
Kp13, GA 2013	KPC-3	SHV-11	AadA2, MphA, DfrA14, CatA1	ST-258	KL106
Kp14, GA 2013	KPC-3		AadA2, MphA, DfrA14, CatA1	ST-258	KL106
Kp15, GA 2015	KPC-3	SHV-11	MphA, DfrA14, CatA1	ST-258	KL106
Kp16, GA 2014	KPC-3	SHV-11	MphA, DfrA14, CatA1	ST-258	KL106
Kp17, GA 2015	KPC-3	SHV-11	AadA2, MphA, DfrA14, CatA1	ST-258	KL106
Kp18, GA 2015	KPC-3	SHV-11	AadA2, MphA, DfrA14, CatA1	ST-258	KL106
Kp19, GA 2013	KPC-3		MphA, DfrA14, CatA1	ST-258	KL106
Kp20, GA 2013	KPC-3		MphA, DfrA14, CatA1	ST-258	KL106
Kp21, GA 2012	KPC-3	SHV-11	MphA, DfrA14, CatA1	ST-258	KL106

Figure S2. Phylogenetic Tree, Resistance Genes, and Sequence Type of Colistin Heteroresistant *Klebsiella* Species. Phylogenetic tree of heteroresistant *Klebsiella* isolates (left), constructed based on whole genome sequence, with identifiers for state and year (GA-Georgia, MD-Maryland, NM-New Mexico, CO-Colorado). Table (right) of resistance found in each isolate, and the sequence and capsule type of each isolate. All isolates in the table shared AmpH and OqxAB resistance genes. Cluster highlighted in blue box represents highly related group of ST-258 isolates. OqxAB genes all shared same alleles within the highlighted cluster.

Chapter 5:

Discussion and Relevance

The findings we present here illustrate the importance of the phenomenon of colistin heteroresistance in Enterobacteriaceae. This phenomenon of a minor subpopulation being phenotypically resistant to colistin is concerning in several ways. First, we have shown that this resistant subpopulation is dynamic and its frequency is increased during infection and colistin treatment. As a result, minor subpopulations of resistant bacteria can mediate failure of colistin therapy. Additionally, the resistant subpopulation within colistin heteroresistant strains is often undetected by clinical susceptibility testing, which could lead to unexplained treatment failures. We further showed that colistin heteroresistance is a threat in highly antibiotic resistant CRKP, and finally, observed that the overall rate of colistin heteroresistance among CRE in the United States was over 10% in a surveillance sample from states across the country. Thus, colistin heteroresistance has the potential to be a widespread, difficult to detect, and clinically impactful form of antibiotic resistance that certainly warrants further study.

That heteroresistance can mediate failure of antibiotic therapy is a controversial topic in the current literature. It initially seems counterintuitive that such a small subpopulation of resistant cells would lead to the inefficacy of antibiotic therapy. Perhaps the best studied group of heteroresistant pathogens are the vancomycin heteroresistant *Staphylococcus aureus* (hVISA)[221]. While at least one group has found that these isolates are indeed more resistant to standard vancomycin therapy *in vivo*[131,222], many other groups have shown that this phenotype does not seem to impact treatment outcome[132,223,224]. These isolates are defined by a population analysis profile, but only the area under the curve is considered, rather than the presence of discrete subpopulations. Additionally, in hVISA, the resistant subpopulation seems to have only a

marginally higher MIC (within 4 fold) than the susceptible subpopulation[211]. This is in contrast to what we have shown with colistin heteroresistance, in which the resistant subpopulation can survive at many logs higher drug concentrations than the susceptible population. This may explain the discrepancy between the impact of heteroresistance in hVISA and in colistin heteroresistant Enterobacteriaceae in our animal model.

Immune pressure drives antibiotic resistance

One of the interesting results from this study was the substantial increase in the frequency of the resistant subpopulation after introduction into the host, even in the absence of colistin treatment. There was a multiple log increase in the frequency of the colistin resistant subpopulation **(Figure 2.1)**, such that the resistant cells became the majority when *in vivo*. This induction in resistance occurred very early in infection, with a significant increase by 4 hours and peaking at 24 hours after infection **(Figure S2.5)**. This phenomenon is a very important contributor to the resulting failure of antibiotic therapy, and thus further understanding of this mechanism is crucial to dealing with colistin heteroresistant infections. We show that macrophages are important contributors to this phenomenon **(Figure 2.2)**, but it is likely that other cell types could also contribute. Neutrophils are another cell type that are likely involved in this resistance increase, as they are important contributors to antibacterial host defense early in infection[225]. Additionally, we identified immune compounds that mediate the colistin resistance increase: the cathelicidin LL-37, lysozyme, and H_2O_2 **(Figure 2.2)**. There are numerous antimicrobials that the immune system produces in addition to these[64], and it is probable that many of these can also mediate the resistance increase.

The pathway involved in colistin heteroresistance is incompletely understood, but requires the PhoPQ two component system. PhoPQ can respond to stresses, able to detect changes in the extracellular environment and facilitating bacterial responses that mitigate those stresses[161]. If it were possible to inhibit this pathway or alter the environment so that it is not induced, it may be possible to prevent the increase in the colistin resistant subpopulation. This in turn could lead to efficacy of colistin against heteroresistant strains. A combination of PhoPQ pathway inhibitors and colistin may therefore be a viable option for future study as a way to counteract colistin heteroresistant infections.

There are many important implications to the increase in colistin heteroresistance *in vivo*. If the host immune system is leading to an increase in colistin resistant cells, then it could be leading to selection of antibiotic resistant organisms. This is particularly concerning in this era of increased antibiotic resistance, as decreased use of colistin may not be enough to reduce resistance due to constant selection by the immune system. Additionally, the increased resistance within the host as compared to *in vitro* may threaten the efficacy of colistin even when strains appear to be susceptible by clinical testing.

Detection of heteroresistance

In Chapter 2, we observed *E. cloacae* isolate R/S-lo mediate treatment failure while simultaneously being classified as colistin susceptible by clinical susceptibility testing. This is a very alarming result as it suggests that clinicians may be unaware of the presence of colistin heteroresistance in some isolates. As a result, they may prescribe colistin to treat a colistin heteroresistant isolate. If the outcome data from our mouse model holds true in humans, this

would then lead to treatment failure. Even if the isolate was retested after this treatment failure, it would still appear to be colistin susceptible, leading to an unexplainable failure of colistin therapy. This is even more concerning in Chapter 3, where we highlight two *K. pneumoniae* isolates that had a similar phenotype of undetected colistin heteroresistance while also being resistant to last line carbapenem drugs. These isolates are more likely to be treated with colistin, making this undetected heteroresistance even more relevant in this setting.

In Chapter 4, we investigated how often colistin heteroresistance is observed in human infections. We chose to specifically look at the carbapenem resistant Enterobacteriaceae, as this class of organisms is highly drug resistant and is among the most frequent to be treated with colistin[16]. In the US based sample set we studied, we found a rate of colistin heteroresistance that was over 10%, indicating that colistin heteroresistance is a prevalent form of resistance. Surprisingly, this rate was even higher than the rate of conventional colistin resistance. Additionally, we observed that over 90% of colistin heteroresistant isolates in our study were undetectable by the clinical standard susceptibility test of broth microdilution. These undetected heteroresistant isolates more than double the rate of colistin non-susceptible isolates, meaning it is possible that roughly half of total colistin resistance is undetected by clinical testing methods. While we show that colistin heteroresistance leads to treatment failure in a mouse model of infection, we have yet to determine its importance in human infections. Unfortunately, this study could not address that question, as it was a retrospective analysis and there was no control over antibiotic therapy prescribed. Thus, we were unable to analyze the impact of colistin heteroresistance on outcome of colistin therapy. Thus, it will be important in the future to conduct controlled studies that address whether colistin heteroresistance impacts colistin treatment outcome in human patients.

The significant failure of susceptibility testing to detect most resistant subpopulations illustrates a major shortfall. The FDA suggests that this type of error, considered a “very major error”, occur less than 10% of the time with an acceptable diagnostic[199]. This signals the need for new diagnostic methods to detect colistin heteroresistance, and there are a few options for methods that may address this problem. In our studies, we have used the population analysis profile as the gold standard method of detecting heteroresistance, which is able to find populations at a frequency of as low as 1 in 10^7 cells. Unfortunately, this is strictly a laboratory-based method, as it labor and resource intensive, as well as taking at least 24 hours to generate results. Automation of this method may lead to a more manageable test that could be used in certain clinical situations, though the test would still take a significant amount of time. Another possibility is to alter the composition of the testing media, to augment the visibility of the resistant subpopulations. We can see that many conditions, especially those experienced *in vivo*, can lead to an increase in the resistant subpopulation (**Figure 2.2**). Creating a media that can simulate this more accurately may lead to better rates of detection with conventional broth microdilution or Etest. Finally, flow cytometric methods have shown promise in leading to faster antimicrobial susceptibility testing[226], and this type of test may possibly be adapted to detect lower frequency populations that occur in heteroresistant isolates.

One of the most important implications of this study is the idea that bacterial subpopulations are clinically relevant and often overlooked. The most difficult hurdle when detecting heteroresistance is that no current clinical testing method has a designation for heteroresistance. In its current form, antimicrobial resistance testing assigns an MIC value to a strain, and it is assumed that this applies homogenously to all bacteria in the population. Whether this MIC falls above or below a certain breakpoint determines whether a treatment is likely to be

efficacious. In heteroresistance, there are subpopulations which straddle the breakpoint, one population having an MIC below the breakpoint and one above. In most testing, the MIC of a heteroresistant strain will be determined by the frequency of the resistant subpopulation. As we have shown, the misclassification of heteroresistant strains can cause major problems in the form of unexplained treatment failures. Knowledge of the presence of resistant subpopulations would allow clinicians to avoid using antibiotics that would likely fail. In addition to the presence of the resistant subpopulation, knowledge of the frequency of this subpopulation may also be important in the clinic. In this study, we have observed isolates with resistant subpopulations with frequencies as low as 1 in 10^5 cells, and all of these strains mediated treatment failure. However, it is likely that frequencies somewhere below this rate would not impact treatment outcome. The idea of heterogeneity among bacterial populations is not new [72,136,227] but is vastly underappreciated in a clinical setting. Thus, we hope this study will lead to more consideration of these types of phenomena in the hospital environment.

Heteroresistance to other drugs

One shortfall of the findings described here is that they only address heteroresistance to colistin, and not any other antibiotics. Heteroresistance to many other classes of drugs has been described[133], though it remains to be seen if the findings described here for colistin also apply to these other drugs. If heteroresistance to other antibiotics can mediate failure of antibiotic therapy, that will further increase the importance of heteroresistance. Unexplained treatment failures occur during use of numerous antibiotic classes[228-230] and heteroresistance may be a cause in many instances. Heteroresistance to other drugs may differ significantly from what is described

here for colistin, and this calls for wide ranging study into the characteristics of heteroresistance as a whole and for every antibiotic.

For one, colistin heteroresistance is augmented by passage in the host and it is possible this is true for other drugs. Polymyxins are unique in acting as a cationic antimicrobial peptide, which are also produced by the host and work in a similar charge dependent mechanism. However, other host conditions, such as oxidative stress **(Figure 2.2c)**, pH, and metal concentration (unpublished data) seem to influence colistin heteroresistance as well. Thus, there may be many host conditions that can influence the rate of heteroresistance to other antibiotics. Additionally, these forms of heteroresistance could also be under the control of stress response regulators, such as PhoPQ, which would likely be activated during an infection.

Another consideration for heteroresistance to other antibiotics is the ability of clinical testing to detect the resistant subpopulations. This will depend on many factors, including the frequency of the resistant subpopulation and how it is induced during susceptibility testing. In colistin heteroresistance, it seems that growth in media used during diagnostic testing lowers the frequency of the resistant subpopulation **(Figure 2.1),** making it harder to detect. This may be true for other drugs as well, making these forms of heteroresistance similarly elusive.

The most important consideration for heteroresistance to other antibiotics is the ability of it to mediate treatment failure. As previously mentioned in the case of hVISA, it will likely be important to consider the difference in MIC between the susceptible and resistant subpopulations. A higher MIC in the resistant subpopulation may allow the strain to better resist drug treatment. Additionally, any resistance mechanisms that can inactivate the antibiotic may provide a significant advantage to a heteroresistant strain during drug treatment. This may allow

a minor subpopulation to break down local antibiotic and protect neighboring susceptible cells. This is a well-known phenomenon in the satellite colonies observed during drug selection on beta-lactams[231]. Any heteroresistance that relies on the production of a beta-lactamase may therefore have a significant effect on treatment outcome. As beta-lactams are the most commonly used class of antibiotics in the world[17], this is a very worrying possibility.

Multiple heteroresistance

If there are multiple different antibiotics in multiple classes that bacteria can be heteroresistant to, then it follows that a single strain could harbor heteroresistance to two or more drugs at once. This possibility introduces several intriguing questions that may amplify the importance of heteroresistance.

If heteroresistance occurs to multiple different antibiotics at once, what are the dynamics of these two resistant subpopulations in relation to one another? The resistant subpopulations may actually be the same subpopulation of cells, which has multiple pathways turned on that allow it to resist several different drugs. This possibility could mean that any induction of the resistant population, by host immune response or other stresses, could greatly increase the overall antibiotic resistance of the strain to several different drugs. On the other hand, it is possible that strains could harbor multiple resistant subpopulations that are unrelated to one another. In this case, there would be a mix of single resistant cells and dual resistant cells, dependent of the frequency of each subpopulation. The dual heteroresistant cells would be very infrequent in the case of low frequency resistant subpopulations.

This type of dual heteroresistance could open a new avenue of therapy for heteroresistant strains, through the use of combination antibiotic therapy. Targeted use of two antibiotics when treating infections caused by strains that are heteroresistant to both antibiotics would ensure that most cells being targeted would be susceptible to at least one of these antibiotics, given the extreme rarity of a cell being resistant to both drugs at once. Combination therapy is currently used in cases of multi-drug resistant infections, to varying success[232,233]. However, this therapy is often empiric, or employs drugs to which the target strain is seemingly resistant[234]. In a heteroresistance targeted combination therapy, the drug combinations could be personalized to the specific infecting pathogen, which may increase the success of this type of treatment. In fact, it is even possible that the success of a number of combination therapies has been due to multiple heteroresistance. This even opens up possibilities for antibiotic therapy for pan-resistant strains, which seem to be resistant to all antibiotics[193]. If the strain were actually heteroresistant to several of these drugs, they could be useful as a targeted combination therapy. For this reason, we believe that investigation into multiple heteroresistance is the most exciting future direction for this project.

Overall, we believe this study has demonstrated the importance of heteroresistance to colistin in strains that have caused clinical infections. It is necessary to not only further characterize these organisms, but to develop better ways to detect this type of resistance in the clinical environment. Additionally, heteroresistance to colistin, and likely other antibiotics, needs to be considered when determining treatment options. Heteroresistance is clearly a phenomenon that warrants much further study, and it may hold an important key to advancing our understanding of antibiotic resistance.

Acknowledgements

Chapter 2

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Chapter 3

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Chapter 4

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