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Nick Su April 3, 2023

Extracellular Beta-lactamase Secretion in Heteroresistant Bacteria Confer Resistance Against Piperacillin-Tazobactam

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Abstract

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By Nick Su

Heteroresistance (HR) is a form of resistance where a phenotypically unstable minority resistance subpopulation coexists with a susceptible population; it is an emerging public health threat in that it further complicates antibiotic resistance. The mechanism of HR is an area of ongoing study. This study investigates the role of beta-lactamase secretion in conferring antibiotic resistance against piperacillin-tazobactam in heteroresistant bacteria. We observed extracellular secretion of beta-lactamase in HR isolates rescues nearby susceptible populations and enable it to survive at otherwise lethal antibiotic concentrations. The presence of secreted beta-lactamase in media causes an increase in minimum inhibitory concentrations (MIC) against bacterial isolates and the increase in MIC can exceed clinical breakpoint concentration for antibiotics. We also observed detoxification of agar plates by HR isolates secreting beta-lactamase. This work demonstrates the role of extracellular beta-lactamase secretion in enabling the growth of susceptible strains under antibiotic exposure and elucidate another mechanism for heteroresistance.

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Introduction

Antibiotic resistance against beta-lactams

Antibiotic resistance is a growing global health threat that has the potential to undermine our ability to treat infectious diseases. According to the Center for Disease Control and Prevention (CDC), antibiotic resistance caused more than 1.2 million deaths worldwide and is associated with approximately 5 million deaths in 2019. In the United States, more then 2.8 million infections are resistant contributing to more than 35,000 deaths(1). Antibiotic resistance is a significant threat to public health and adds a large burden on healthcare providers. It also incurs large economics cost that is projected to grow to US$ 100 trillion worldwide by 2050 if significant measures are not taken to address the issue(2).

Beta-Lactams is a major class of antibiotics that include penicillins, cephalosporins, and carbapenems which have been a cornerstone of antibiotic treatments and accounts for more than half of the worldwide antibiotic market(3). However, beta-Lactams resistance have existed since its introduction and its increasing presence is a major threat to future antibiotic developments. Resistance to beta-Lactams is primarily mediated by two mechanisms: the production of beta-Lactamases and the production of altered penicillin-binding protein (PBP) with lower affinity for beta -Lactams.

Beta-Lactamase production is a common resistance mechanism for gram-negative bacteria(3) and it works by hydrolyzing the beta-Lactam amide(4). Beta-Lactamase from multiple families of bacteria including Enterobacteriaceae have the capability to expand their spectrum of activity due to their dissemination on plasmids and other mobile genetic elements where the acquisition of point mutations has led to the presence of extended spectrum beta-Lactamases (ESBLs) which threatens the effectiveness of a large group of antibiotics that include penicillins and cephalosporins(5). The presence of ESBL renders commonly-used antibiotics ineffective and complicates the treatment of otherwise common infections. They restrict the number of options available to treat these infections and complicate the treatment process where hospitalization may be required instead of taking oral antibiotics.

To combat the activity of beta-Lactamase, beta-Lactamase inhibitors are employed in combination with beta-Lactam antibiotics. Beta-Lactamase inhibitors has one of two common form of actions: 1) reversibly bind the beta-Lactamase with high-affinity and form unfavorable steric interactions as the acyl-enzyme or 2) irreversibly binds to the enzyme and permanently inactivate the beta-lactamase secondary reactions at the active site(6). Newly approved inhibitors such as Avibactam are demonstrated to be effective against ESBLs produced by *K. pneumoniae* and *E. coli*. Avibactam functions by covalently binding the beta-Lactamase in a reversible reaction and it is not susceptible to hydrolysis once bound to the enzyme. Resistance against beta-Lactamase inhibitors may arise but is typically associated with an increased susceptibility to beta-Lactam antibiotics, representing an inherently constrained mutation(7). This inherent trade-off does not completely preclude the possibility of mutations that increase resistance to both the inhibitor and the beta-Lactam antibiotic, however, this phenomenon is less characterized given that even with a trade-off, a mutation may provide increased fitness by providing strong resistance to one compound at the cost of mild susceptibility to the other.

Piperacillin-tazobactam

Piperacillin-tazobactam is a broad-spectrum beta-Lactam antibiotic combination that is commonly used for the treatment of skin and soft tissue infections, pneumonia, intra-abdominal infections, and appendicitis(6). The combination is one of the most used medication among all prescribed drugs in hospitals in the United States(8). Piperacillin is a broad-spectrum bactericidal penicillin and tazobactam is a beta-lactamase inhibitor. Tazobactam extends piperacillin's activity against beta-lactamase producing strains such as those in *Enterobacteriaceae* and has the potential to lower the minimum inhibitory concentrations against ESBL producing strains(6). However, resistance to piperacillin-tazobactam has been reported in a variety of bacteria, including *Enterobacterales, Pseudomonas aeruginosa*, and *Acinetobacter baumannii*. Resistance to piperacillin-tazobactam is often associated with the production of beta-lactamases, such as ESBLs and AmpC beta-lactamases, that can hydrolyze the beta-lactam ring of piperacillin and render it inactive(9).

Heteroresistance

Heteroresistance (HR) is a form of antibiotic resistance where a minority subpopulation of resistant bacterial cells coexists with a majority susceptible population. The subpopulations, however, are genetic clones of each other. Treatment with an antibiotic will neutralize the majority susceptible population while the minority resistant population rapidly replicates and becomes the dominate population. Upon discontinuation of treatment, the resistant subpopulation reverts to the original resistant-susceptible composition. It should be noted that heteroresistance have been used to describe populations that consists of two genetically distinct bacterial populations where the resistant population are stable and genetically different, commonly referred to as polyclonal HR(10). However, polyclonal HR is not the focus of this work.

HR is distinct from other forms of antibiotic resistance such as persistence and tolerance. Tolerance enables the bacterial cells to temporarily survive or slow down the lethal consequence of high antibiotic concentrations; it, however, does not allow the cells to grow in the presence of these antibiotics. Tolerance can be attributed to genetic mutations or environmental factors(11). Persistence is the ability of a subpopulation of bacteria to enter temporary dormancy or grow slowly in the presence of high dose of antibiotics. HR is different from persistence in that persistence populations does not rapidly replicate in the presence of antibiotics(12) and is not capable of causing acute treatment failure.

HR has been observed in a variety of bacteria including *E. cloacae, S. aureus, E. coli, A. baumannii, P. aeruginosa*, the *Klebsiella* species, and others(13). In addition, HR has been observed against diverse classes of antibiotics including aminoglycosides, carbapenems, and other beta-Lactams(13, 14). Detection of HR by conventional antibiotic susceptibility testing employed in clinical settings can be unreliable(13, 15) and can leads to misclassification of HR as susceptible due to the low frequency of resistant subpopulation, which can lead to inappropriate treatment(16).

Despite consisting only a minority of resistant subpopulation, HR can affect treatment outcomes and can lead to treatment failures(17, 18). While most of the cells in a HR population are likely to be highly susceptible to a given antibiotic, a small proportion of 1 to 10% of the population can be resistant to 1000x higher antibiotic concentrations(13). The resistant subpopulation can rapidly replicates in the presence of an antibiotic and lead to treatment failure.

Mechanism of heteroresistance

Mechanisms that lead to Heteroresistance are not well understood and is a major focus of current research in antimicrobial resistance. Heteroresistance is observed with a pure clone from which all cells arise from, thereby limiting the role of genetic differences. Furthermore, the frequency at which resistant subpopulation appear in HR is higher than that in resistance caused by genetic mutations(10). Gene amplification has been identified as one of the factors that contribute to HR. Previous research suggests that amplification of pre-existing genes that encode beta-lactamases could lead to the presence of cefiderocol resistant subpopulations and that the number of gene copies a subpopulation have is correlated to its resistance levels (19). In addition to gene amplification, regulation of gene transcription also contributes to HR. Changes in transcription level of genes that encode for membrane proteins and efflux channels can causes the presence of resistance subpopulation(20).

The stability, or lack thereof, in heteroresistance is an area of ongoing study and there is currently no general consensus regarding its mechanisms. It is, however, generally accepted that there are fitness costs associated with the resistant phenotype which makes it unfavourable in an environment without selective pressure(10, 15). Some studies have shown that HR populations possess secondary compensatory genes that can reduce the fitness cost of resistant genes while increasing its susceptibility(10). This type of HR strains do not revert to “truly susceptible” once antibiotic treatment is paused. Another mechanism that contribute to the instability of HR is the intrinsically unstable nature of the resistance gene. Tandem gene amplifications such as the beta-lactamase gene amplification aforementioned are innately unstable and costly; thus in the absence of antibiotic treatment the amplifications are lost and the cells’ susceptibility increase(10, 19, 21).

Beta-lactamase secretion

Beta-Lactamase and extended spectrum beta-Lactamase (ESBL) have been shown to be secreted extracellularly by *E. coli* and *Streptomyces*(22, 23, 24). Beta-lactamase produced by resistant bacterial cells are usually localized to the periplasmic space, where it inactivates the beta-lactams before they reach the cytoplasmic membrane that contains the protein receptors. However, enzymatically activate b-lactamase can also be secreted via the haemolysin (type 1) pathway directly into the extracellular medium, bypassing the periplasm(22). Previous study have shown that secreted beta-lactamase is functionally stable for more than 24 hours and at adequate concentrations, the beta-lactamase in media can provide protection to susceptible cells(25).

While no previous study investigated the clinical relevance of beta-lactamase secreted extracellularly via type 1 pathway, previous research have demonstrated that beta-lactamase secreted via outer membrane vesicles (OMVs) could contribute to clinical treatment failure against streptococcus pyogenes Pharyngotonsillitis (23).

Beta-lactamase secretion in HR isolates

Beta-lactamase secretion in HR isolates is an understudied area. There are no previous studies that investigated extracellular beta-lactamase secretion in HR bacteria, its mechanisms, and its role in the generation of heteroresistance. This work seeks to pave the foundation in closing this knowledge gap by demonstrating that enzymatically active beta-lactamase can be extracellularly secreted by HR bacteria and that this secreted beta-lactamase can enable susceptible subpopulations to survive under otherwise lethal antibiotic exposure. To investigate this phenomenon, a collection of HR isolates is curated from

the ALLIUM collection of clinical isolates. The ALLIUM collection is selected due the extensive characterisation with regard to heteroresistance. Bacterial species contained in the ALLIUM collection includes E*scherichia coli, Klebsiella pneumoniae, Proteus mirabillis*, and *Enterobacter cloacae*.

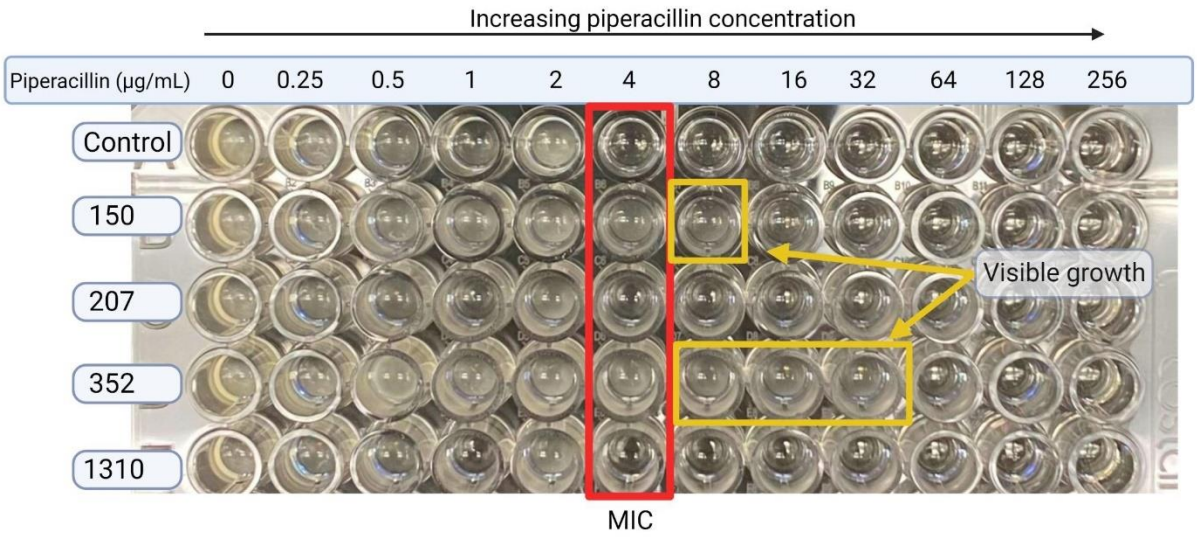
Results

Spent Media from HR strains enabled susceptible strains to grow under antibiotic exposure

Screening of the ALLIUM collection by population analysis profile (PAP) reveals that 15.7% of the strains are HR. To investigate whether HR strains are producing and extracellularly secreting enzymatically active beta-lactamases that are rescuing susceptible cells, we performed broth microdilution test using spent media from HR strains. Strain S207 were used as the benchmark strain due to its susceptibility against the piperacillin/tazobactam combination.

Spent media was collected from HR isolates and filtered to removed bacterial cells. The media was then concentrated and added to an otherwise standard broth microdilution assay for S207. Both visual inspection and data from absorbance-based microplate reader confirms that spent media from HR strains enabled the growth of a susceptible strain at antibiotic concentrations that would otherwise be lethal (Figure 1). Spent media from 4 HR strains resulted in an increase of the minimum inhibitory concentration (MIC) for the susceptible strain to above the clinical breakpoint concentration of piperacillin-tazobactam (32 μg/mL piperacillin, 4 μg/mL tazobactam)(26), spent media from 11 HR strains resulted in an subthreshold increase in MIC for the susceptible strain that did not exceed the clinical breakpoint. Concentration of tazobactam was held constant in the broth microdilution assay while piperacillin was varied from 64-fold concentrations below the breakpoint (0.25μg/mL) to 16-fold above the clinical breakpoint (256μg/mL). In wells that have an upshift in susceptible growth after the addition of spent media concentrates, there is a negative correlation between optical density and piperacillin-tazobactam density. The susceptible strain's growth in spent media decreased as the concentration of piperacillin increased.

a



b

MIC Increase	No Change	2x	4x	8x	16x	128x
Number of Isolates	32	4	7	0	3	1

Figure 1. Broth microdilution with spent supernatant shows MIC changes. (a) MIC of the same susceptible strain (S207) is tested in broth microdilution with different spent supernatants. Row labels represent the strain from which the supernatant is taken from. Control group contains only susceptible strain (S207) with no supernatant. Isolate 207 has the supernatant from the same susceptible strain added, which produces no effect on MIC as expected. The MIC of susceptible is highlighted in red. Visible growth susceptible above the MIC due to the addition of HR spent supernatant is highlighted in yellow. **(b)** Table showing the increase in MIC as a result of HR supernatant and the number of isolates observed for each magnitude of increase.

Beta-lactamase is secreted extracellularly

To investigate the presence of extracellular beta-lactamase in the spent supernatant, 5 HR from the ALLIUM collection that are associated with MIC shift are selected and avibactam is added to the broth microdilution assay. Spent supernatant from HR isolates are purified to remove any bacterial cells and outer membrane vesicles (OMVs) that could potentially carry intracellular/periplasmic beta-lactamase. Out of consideration for logistical and time constraints, only 5 strain are selected. These strains are each representative of a category of phenotype that we are interested in.

Avibactam is a recently approved non-beta-lactam beta-lactamase inhibitor that is commonly used in combination with ceftazidime to treat complicated infections. Avibactam has no antimicrobial activity itself, it restores the activity of beta-lactams by degrading a variety of serine beta-lactamases(27). Avibactam has expanded spectrum of activity against ambler class A, C , and D beta-lactamases and is found to restore antibiotic activity of beta-lactams against ESBL, AmpC, *Klebsiella pnuemoniae* carbapenemase (KPC) and OXA-48 producing *Enterobacteriaceae* and MDR *P. aeruginosa*(27). Avibactam is one of the most potent broad-spectrum beta-lactamase inhibitor currently approved for clinical use.

We expect that avibactam will inhibit all beta-lactamase produced by strains from this collection with the exception of metallo-beta-lactamase, for which currently there is no approved inhibitor(27).

Addition of avibactam to spent HR supernatant restored the antimicrobial activity against S207 and resulted in multi-fold increase in killing compared to the assay without avibactam. Spent media from HR352 produced robust growth of up to 256 µg/mL piperacillin in assay without avibactam (Figure 2a). This number is reduced to 4 µg/mL when avibactam (at 4 µg/mL) is added to enhance the beta-lactamase inhibition (Figure 2b). Similar pattern is also observed for HR150, HR32, and HR226. Noticeably, HR8, a strain of *P. mirabilis*, did not enable enhanced susceptible growth with or without avibactam compared to the control group.

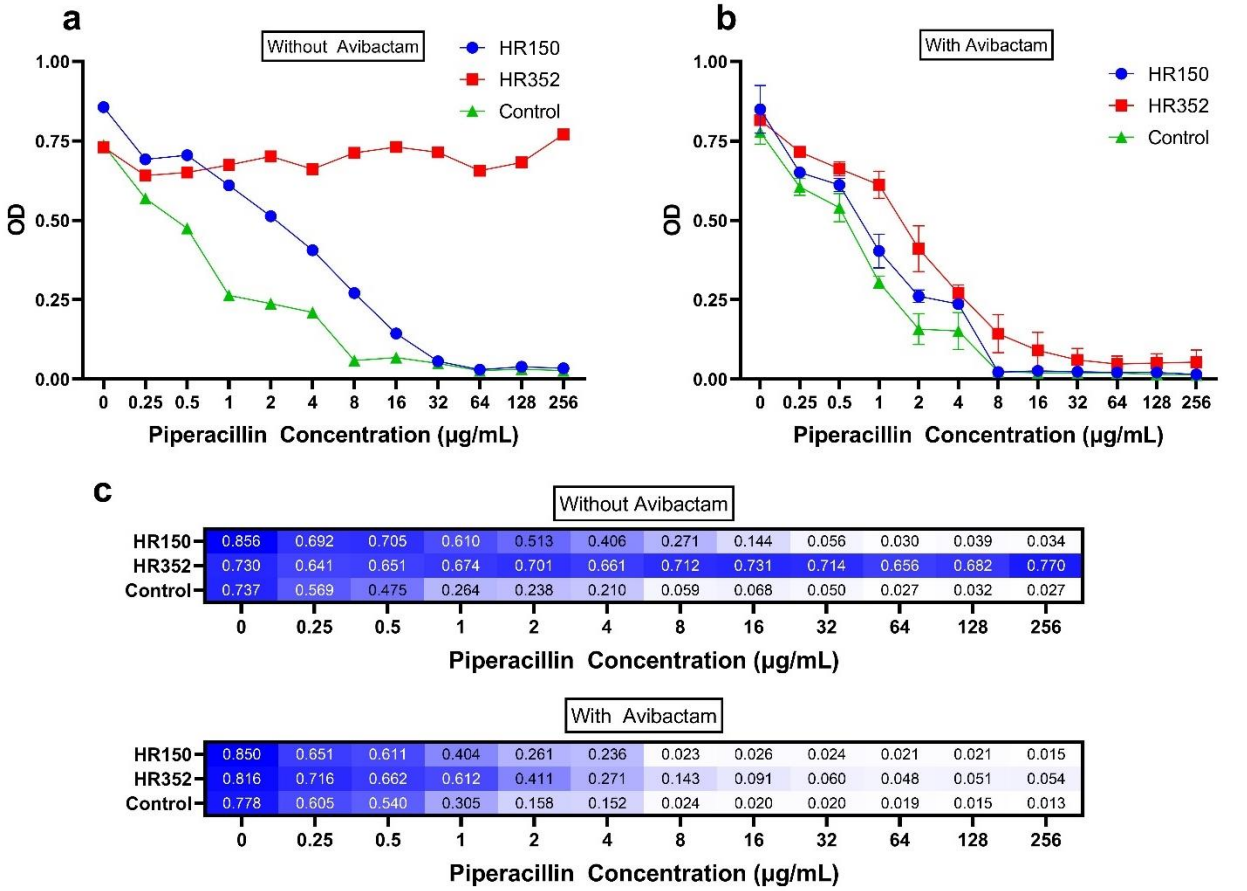


Figure 2. Bacterial growth represented by optical density (OD) at varying piperacillin concentrations. Avibactam inhibits secreted beta-lactamase and restores piperacillin activity. **(a-b)** Normalized optical density of susceptible strain's growth in HR supernatant at increasing antibiotic concentrations with or without avibactam. Susceptible is grown in HR supernatant for 20 hours; data represents a snapshot of the growth dynamic at hour 20. Legend represents the HR strain from which the supernatant is taken from. Control group contains only susceptible strain in antibiotic solution without HR supernatant. Error bar represents standard error of mean (n=3). **(a)** Broth microdilution with piperacillin-tazobactam only show susceptible grown in supernatant of HR352 has significant increase in minimum inhibitory concentration (from 8-µg/mL to 256 µg/mL) compared to control. HR150 also enables an increase in MIC (from 8 µg/mL to 32 µg/mL). **(b)** Addition of avibactam reverts the MIC increase seen in (**a**). HR150 does not cause increase in MIC compared to control (MIC at 8 µg/mL) when avibactam is added; the MIC increase for HR352 is significantly lower with avibactam. **(c)** heatmap showing susceptible growth in HR

supernatant compared to control. Value inside the heatmap represents optical density value obtained at 600nm. MIC change as a result of HR supernatant with and without avibactam is illustrated.

Since avibactam itself has no antimicrobial property, the restored piperacillin activity against the susceptible strain suggests that avibactam inhibited beta-lactamase activity in the spent HR media. Compared to tazobactam, avibactam has additional inhibitory activity against AmpC beta-lactamase expressed in Enterobacteriaceae and KPC and OXA-48 carbapenemase expressed in K. pneumoniae (Table 1) (28). This observation confirms the presence of secreted beta-lactamase in HR supernatant and the fact that this beta-lactamase is able to rescue susceptible cells, although at various magnitude, from otherwise lethal antibiotic concentrations.

Beta-Lactamase	Class	Substrates	Inhibited by tazobactam	Inhibited by avibactam
OXA-48	D	Carbapenems	No	Yes
E. coli AmpC	C	Cephalosporins	No at 4 μg/mL	Yes
KPC-2 KPC-3	A	Broad spectrum	No	Yes
TEM-1 TEM-2 SHV-1	A	Penicillin	Yes	yes
IMP-1 NDM-1 VIM-1	B	Broad spectrum	No	No

Table 1. Comparison of tazobactam and avibactam's activities against classes beta-lactamases. Note that clinical breakpoint for tazobactam is 4 μg/mL and this is the commonly used concentration at which tazobactam is administered with piperacillin in clinical treatment(26). However, at 4 μg/mL, *E. coli* AmpC is not effectively inhibited by tazobactam.

Enrichment under antibiotic exposure is correlated with beta-lactamase secretion

Previous study by Choby et al confirms the role of beta-lactamase gene-amplification as a mechanism that drives HR in E. cloacae(19). Antibiotic enrichment stability assay is used to investigate the proportion of subpopulation in a HR isolate that are resistant. It is an important experiment used to determine whether a strain can revert to original homeostatic frequency after antibiotic exposure and therefore used to determine the stability of HR. It was expected that once a HR isolate is exposed to a given antibiotic, the resistant subpopulation will rapidly replicate and its proportion as a percentage to the entire population will increase (enrichment). However, we have observed HR isolates that do not enrich under antibiotic exposure, inconsistent with what previous studies suggest. Based on this, we hypothesized that beta-lactamase secretion may be another factor along with gene-amplification that is contributing to HR.

To investigate the correlation between beta-lactamase secretion and enrichment under antibiotic exposure, we enriched HR150 (*E. coli*) and HR352 (*K. pneumoniae*) in piperacillin/tazobactam and compared the subpopulation profile to unenriched samples. After enrichment, the average percentage of resistant subpopulation in HR150 was 0.00067%, a 11-fold increase compared to the baseline unenriched population where 0.000063% of the population was resistant (Figure 3a). The proportion of resistant subpopulation in HR352 increased from 0.00119% in unenriched to an average of 42.5% after enrichment, an increase of more than 35,000-fold (Figure 3b).

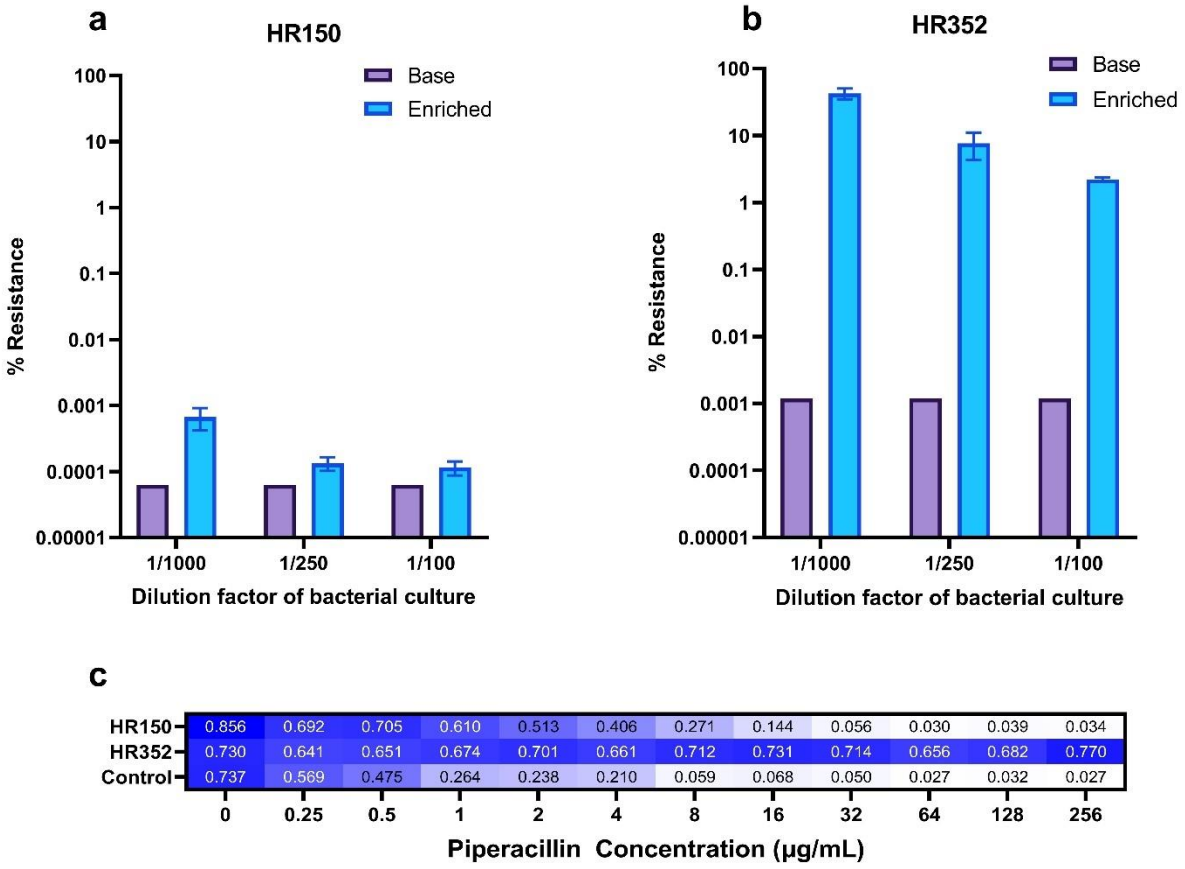


Figure 3. Enrichment under antibiotic exposure is correlated with magnitude of beta-lactamase secretion. (a-b) HR150 failed to achieve noticeable enrichment under piperacillin/tazobactam at breakpoint concentration (32/4 µg/mL) whereas HR352 contained 42.5% resistant subpopulation after enrichment in the same concentrations. Dilution factor represents the final concentration of bacteria in the enrichment assay from the initial culture. Error bar presents standard error of the mean. **(c)** piperacillin/tazobactam broth microdilution results with susceptible in HR supernatant show differences in MIC increase compared to control. Susceptible MIC in HR150 supernatant is 32 µg/mL. HR352 has MIC greater than 256 µg/mL. Value inside heatmap represents optical density reading.

The higher increase in resistant subpopulation in HR352 after antibiotic exposure corresponds to a higher MIC shift in broth microdilution with spent HR supernatant and susceptible strain (Figure 3a-c). We observed robust susceptible growth in spent supernatant at piperacillin-tazobactam concentration of 256 µg/mL, a more than 128-fold increase in MIC compared to the untreated susceptible isolate. We also observed an increase in the minimum inhibitory concentration after growing susceptible in spent supernatant from HR150; its MIC was 32 µg/mL, a 16-fold increase in MIC compared to the untreated group (Figure 3c).

The addition of avibactam to the spent supernatant resulted in a decrease in the supernatant's ability to rescue susceptible growth under antibiotic exposure. The mean MIC for susceptible strain in spent supernatant with avibactam was 4 µg/mL for HR150 and 8 µg/mL for HR352. The addition of avibactam removed beta-lactamase activity in the supernatant and restored the antimicrobial activity of piperacillin. Avibactam acted as an equalizer against beta-lactamase secretion in that the resultant susceptible MIC was not significantly different between HR352 and HR150. However, the difference in

the magnitude of shift in MIC as a result of avibactam was prominent. A 32-fold decrease in MIC was observed with supernatant from HR352 while we only observed a 2-fold decrease in MIC for HR150.

Beta-lactamase secretion enables susceptible growth on agar susceptibility testing plates

We observed satellite colonies on agar susceptibility testing plates (Figure 4a) and we suscept beta-lactamase secretion maybe an underlying mechanism contributing to this phenomenon. Agar detoxification assay was implemented to investigate the effect of beta-lactamase secretion in agar plates (Figure 4b). All 23 strains used demonstrate HR. Strains from the Georgia Multi-Site Gram-negative Surveillance Initiative (MuGSI) was used for this experiment due to its well characterisation of HR. We hypothesized the presence of a beta-lactamase secreting colony would neutralize the activity of beta-lactams in its proximity and allow higher-than-normal growth of susceptible strain. Indeed, when struck onto the surroundings of HR colonies on piperacillin-tazobactam plates, the susceptible strain was able to grow at breakpoint concentrations. With the exception of two HR isolates Mu1310 and Mu499, this was observed for all HR strains tested (Figure 4c). The susceptible Mu1197 strain only grows on piperacillin-tazobactam plates around the HR colonies, despite being inoculated to the edge of the plate (Figure 4b). We, however, did not find a correlation between whether an HR isolate detoxifies the surrounding environment and its resistant profile as revealed by PAP (see Supplementary Figure 2).

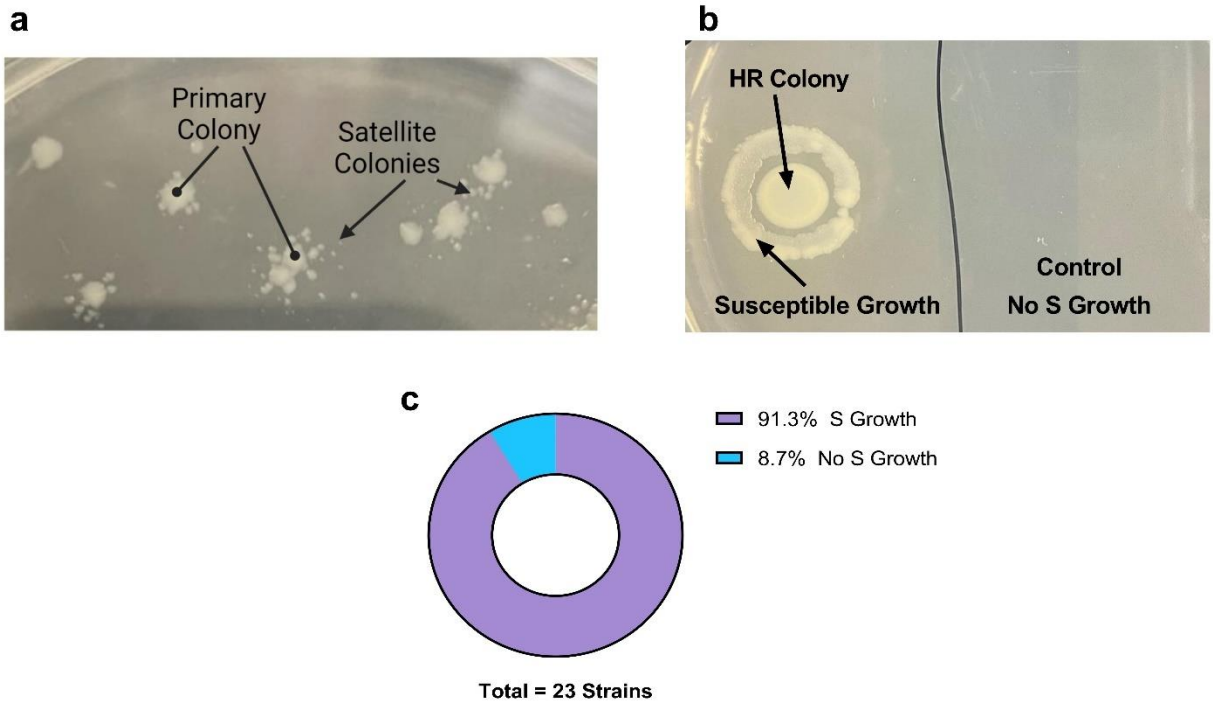


Figure 4. Beta-lactamase secretion enables susceptible population to grow under antibiotic exposure. (a) Satellite colonies are visible on agar plates. **(b)** Agar detoxification assay (32/4 µg/mL piperacillin/tazobactam) show HR colony can inhibit antibiotic activity and enable susceptible strain to grow at otherwise lethal concentrations. Control group has a susceptible isolate inoculated at the center and then around the area of inoculation; both of which failed to grow. **(c)** 91.3% of HR isolates tested enabled susceptible growth around the primary colony in agar detoxification assay.

Discussions

This work demonstrates the role of extracellular beta-lactamase secretion in enabling the growth of susceptible strains under antibiotic exposure. We found that spent media from HR strains increased the minimum inhibitory concentration (MIC) for a susceptible strain, with some cases exceeding clinical breakpoint concentrations. This finding suggests that the presence of secreted beta-lactamase in spent media from HR strains may contribute to the survival of susceptible strains in the presence of piperacillin/tazobactam. Furthermore, beta-lactamase secretion can be observed in HR isolates in not-insignificant proportions, and consistently observed across trials. The secreted beta-lactamase that leads to detoxification of antibiotic solutions are not inhibited by tazobactam. This could be due to substrate compatibility issue which makes tazobactam unable to bind or have lower affinity to the HR secreted beta-lactamases.

The addition of avibactam, a potent broad-spectrum beta-lactamase inhibitor, restored the antimicrobial activity of piperacillin against the susceptible strain. This result confirms the presence of secreted beta-lactamase in the spent media, as avibactam itself has no antimicrobial properties. Additionally, our results revealed a negative correlation between optical density and piperacillin-tazobactam concentration, indicating that the growth of the susceptible strain in spent media decreased as the concentration of the antibiotic increased.

We observed a correlation between the increased proportion of resistant subpopulations and higher beta-lactamase secretion. The higher increase in resistant subpopulations in HR352 after antibiotic selection corresponded to a higher MIC shift in the broth microdilution assay with spent HR supernatant and susceptible strain, suggesting that beta-lactamase secretion may be a contributing factor to heteroresistance along with gene amplification.

The difference in MIC shift observed from supernatant of HR352 and HR150 can be the result of different possibilities. HR352 were able to be enriched under breakpoint concentrations at higher percentage than HR150. Thus, the difference in the broth microdilution result could simply be the fact that there are more surviving cells in the HR352 isolates, and these cells were able to produce more beta-lactamase compared to HR150 isolates. Without the addition of avibactam, the high concentration of beta-lactamase produced by HR352 saturated all the available inhibitors, which means that there are free beta-lactamases to hydrolyze the piperacillin. While HR150 also secreted beta-lactamases extracellularly, it does not produce concentrations as high as HR352. Broth microdilution results show that high piperacillin concentrations (256 μg/mL and 128 μg/mL) was able to prevent the growth of the susceptible strain in HR150 spent media. This could indicate that high concentrations of piperacillin simply oversaturated the beta-lactamases and there were enough free piperacillin to prevent bacterial growth. Based on the same assumption, even at 256 μg/mL, piperacillin concentration was not adequate to saturate all the beta-lactamase produced by HR352, therefore unable to prevent the growth of susceptible isolate.

Agar susceptibility testing results further support the role of beta-lactamase secretion in enabling susceptible growth under antibiotic exposure. The growth of susceptible strains around HR colonies on piperacillin-tazobactam plates indicates that the presence of beta-lactamase secreting colonies could neutralize the activity of beta-lactams in their proximity, allowing for higher-than-normal growth of susceptible strains. This observation has significant clinical implications, as it suggests that beta-lactamase secretion could further implicate antibiotic susceptibility testing, which are already under-

performing in its ability to detect heteroresistance. However, it is important to note that most isolates used in the agar detoxification assay were not directly tested for beta-lactamase secretion using the combination of broth microdilution and avibactam due to time and logistical constraints. This could implicate the interpretation of the results and the detoxification of the media may be due to other factors not considered here. We made our best attempt to exclude anticipated events that may confer resistance to the susceptible strain, such as lateral gene transfer, cell motility, and contamination such that we have a high confidence that the survival of susceptible strain is due to detoxification by beta-lactamase.

SDS-PAGE was conducted to investigate the identity of secreted beta-lactamase. Supernatant from HR isolates are compared to known susceptible isolates that do not produce beta-lactamase. However, the experiment yielded inconclusive results and due to time and logistical constraints, no additional attempts were made. Future work can focus on identifying the secreted protein using a combination of SDS-PAGE and mass-spectrometry.

Despite the compelling evidence provided by our study, some limitations should be acknowledged. We only selected a small number of HR strains for analysis, which may limit the generalizability of our findings. Furthermore, the relationship between the proportion of resistant subpopulations and beta-lactamase secretion warrants further investigation, as we did not find a clear correlation between an HR isolate's ability to detoxify its environment and its resistant profile as determined by population profile analysis (PAP).

This study provides evidence that extracellular beta-lactamase secretion by HR strains can enable the growth of susceptible strains under antibiotic exposure. This finding sheds light on a potential mechanism contributing heteroresistance and highlights the importance of developing effective strategies to counteract the activity of secreted beta-lactamases. Further research is needed to confirm these findings in a larger cohort of HR strains and to explore the potential therapeutic benefits of targeting extracellular beta-lactamases to combat antibiotic resistance.

Methods

Isolate information

Escherichia coli, Klebsiella pneumoniae, Enterobacter cloacae, Proteus mirabilis, and *Enterobacter xiangfangensis* isolates were isolated from patients enrolled in the ALLIUM clinical trials. Isolates from the MuGSI were collected by the US Center for Disease Control and Prevention Emerging Infection Program and the Georgia Multi-Site Gram-negative Surveillance Initiative. MuGSI collects isolates in Georgia from 27 labs serving 184 medical facilities, representing a surveillance population of 4 million people (29).

Bacterial culture

Bacteria were struck onto Mueller Hinton agar (MHA) plates from frozen glycerol stocks. Single colonies were inoculated into 1.5mL MH broth (MHB) and incubated at 250rpm, 37°C for approximately 18 hours. Colony forming units (CFU) were determined by serial dilutions of bacteria in phosphate buffered saline (PBS) plated onto MH agar plates at 37°C.

Population analysis profile

Population analysis profile (PAP) were performed by growing bacteria to stationary phase and then plating on MHA containing various concentrations of piperacillin-tazobactam. MHA plates were made at 6 concentrations containing 0/0, 8/4, 16/4, 32/4, 64/4, and 128/4 μg/mL piperacillin/tazobactam. Breakpoint concentrations for *Enterobacteriaceae* for piperacillin-tazobactam is 32/4 μg/mL per CLSI. Single colonies for a given isolate were grown in 1.5mL MHB for 18 hours (overnight). Overnight cultures were then serially diluted 1:10 in PBS ranging from 10^{-2} to 10^{-7}. 7.5 μL of each dilution were plated on each previously indicated MHA plates. After incubation for 20 hours at 37°C, colonies were counted to determine the count of bacteria in CFU/mL at each concentration. Isolates were classified as resistant if the number of colonies that grew at the breakpoint concentration were at least 50% of those that grew on antibiotic free plates. If an isolate was not resistant, it was classified as heteroresistant if the number of colonies that grew at 2 or 4 times the breakpoint was at least 0.0001% (10^{-6}) of those that grew on antibiotic free plates. If isolates were neither classified as resistant or heteroresistant, they were classified as susceptible (see Supplementary Figure 1 for graphical illustration).

Spent supernatant

Spent supernatant from HR isolates were prepared by growing bacteria from single colonies in the presence of 32/4 μg/mL piperacillin-tazobactam in 5mL MHB at 37°C at 250 RPM for 18 hours. The enriched cultured is then concentrated to pellets by centrifugation at 4500 RPM for 20 minutes at 4°C. 4mL of the remaining supernatant is removed from the culture and filtered using 0.45 μm Celltreat syringe filters. The filtered supernatant is then concentrated using Thermofisher Pierce 10K Protein Concentrators at 4000 RPM for 10 minutes at 4°C to obtain 40-60 μL of protein concentrates (Supplementary Figure 3a).

Broth microdilution

Broth microdilution was used to determine the minimum inhibitory concentrations (MIC) of piperacillin-tazobactam for the susceptible strain (207) grown in MHB with HR supernatant and with or without avibactam by following the CLSI protocol(30). Overnight cultures in MHB were adjusted to 0.5 McFarland Standard (1.5×10^8 CFU/mL). Broth microdilution trays are prepared in 96-well Falcon Flat Bottom Clear Microplate. Equal volume of piperacillin-tazobactam solution (50 μL) at a final concentration of 0, 0.25, 0.5, 1, 2, 4, 8, 16, 32, 64, 128 μg/mL piperacillin and 4 μg/mL tazobactam and overnight solution (50 μL) were added in each of the 96 well. 2-4 μL concentrated HR supernatant is added to every well in the corresponding row. Positive control contain no spent supernatant for all 12 concentrations and negative control contain no bacteria nor supernatant. The plates were then incubated at 37°C for 20 hours and the MIC was determined both visually and using a OD600 absorbance assay. Optical density (OD) was normalized using values from the negative control. MIC was determined according to the well with the lowest concentration in which the OD is lower than 0.1 and bacterial growth was not visible. For experiments with avibactam, all procedures were identical except the antibiotic solution was prepared to include the same piperacillin-tazobactam concentrations as previously indicated as well as 4 μg/mL of avibactam in all the wells (See Supplementary Figure 3).

Antibiotic enrichment assay

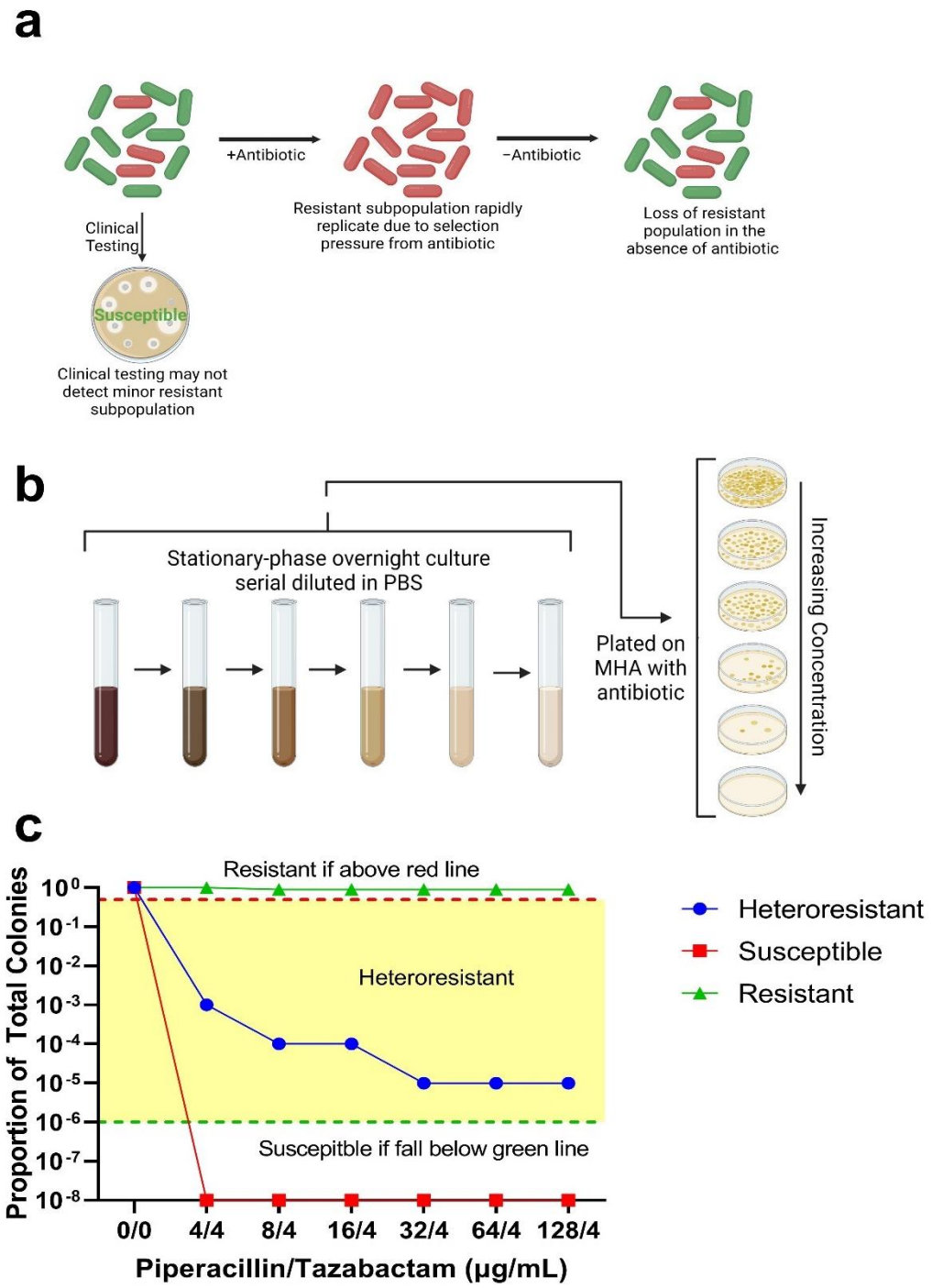
Bacteria from single colonies were used to start overnight culture in 1.5mL MHB at 37°C at 250 RPM for 18 hours. 7.5 μL bacteria overnight is plated on MHA plate with no antibiotic and 32/4 μg/mL

piperacillin-tazobactam to determine the baseline proportion of resistant subpopulation. Overnight were then added 3mL of 32/4 μg/mL piperacillin-tazobactam to achieve a final dilution of 1/100, 1/250, 1/1000 bacteria. The culture were grown at 37°C at 250 RPM for 18 hours to obtain the enriched culture. The resistant profile of the enriched culture is determined by PAP. Percentage of resistant subpopulation in the culture was calculated by comparing the CFU/mL in antibiotic free MHA plates and in piperacillin-tazobactam MHA plates.

Agar detoxification assay

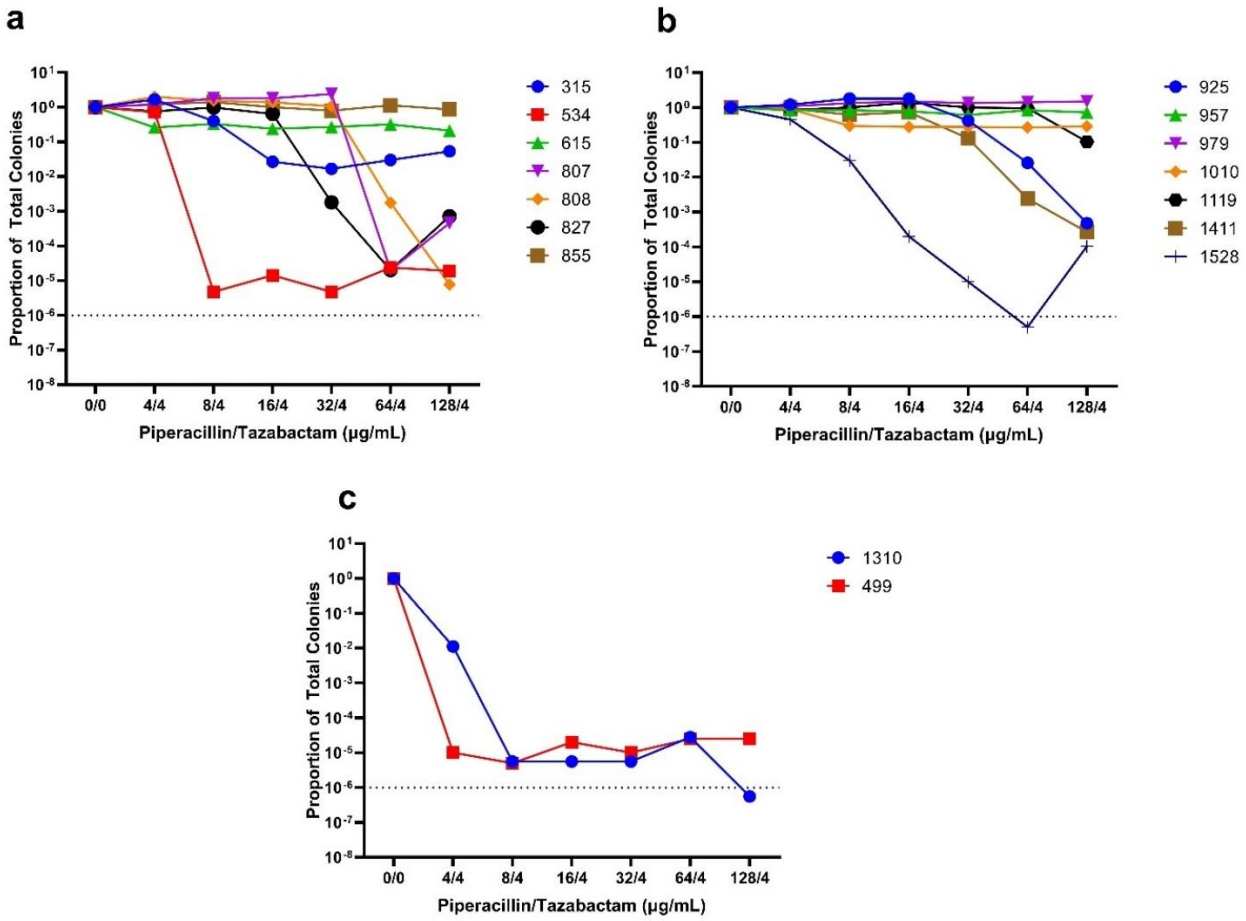
MHA plates containing no antibiotic or 32/4 μg/mL piperacillin-tazobactam were prepared. 10 μL of overnight bacteria culture were inoculated at the center of each type of plates and incubated at 37°C for 20 hours. After incubation, 10 μL of susceptible isolate 207 is streaked around the central HR colony and all the way to the edge of the plates. Positive control group had susceptible isolated inoculated as the central colony and negative control has no primary inoculation. The plates were incubated at 37°C for 20 hours and susceptible isolate growth was visually identified.

Supplementary Information

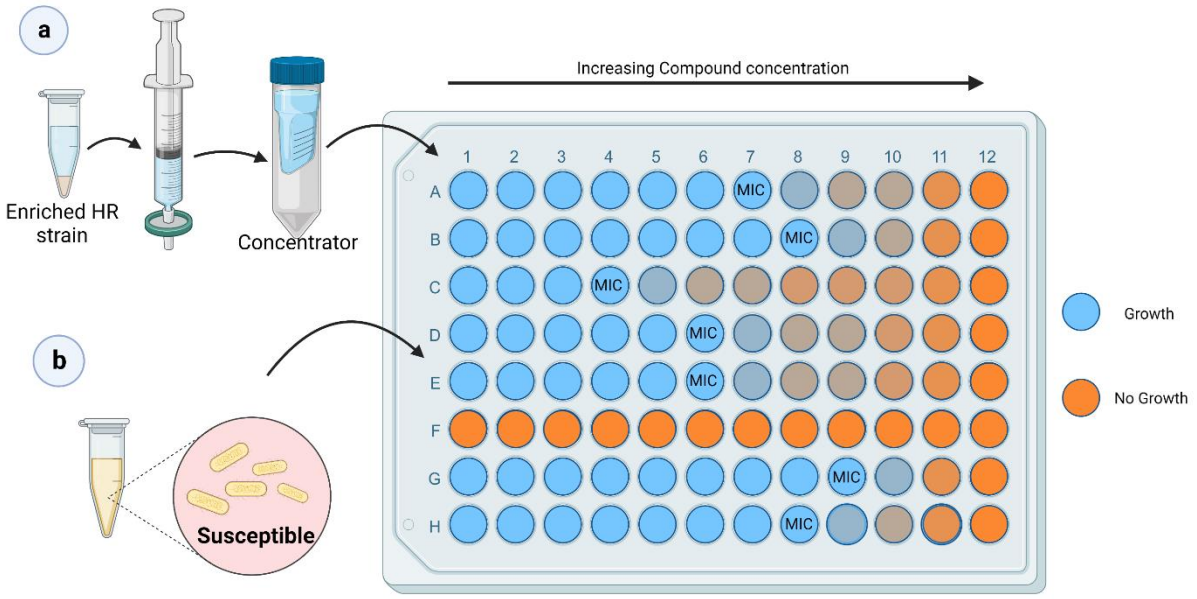


Supplementary Figure 1. Graphical overview of heteroresistance and population analysis profile (PAP). (a) Illustration of heteroresistance where a mix of minor resistant cells are present along the mostly susceptible population. The minor resistant subpopulation can sometimes escape clinical testing and lead to mistreatment. **(b)** Piperacillin-tazobactam PAP: stationary phase overnight culture from a single bacterial colony is serially diluted in PBS and each dilution is plated on increasing concentrations of piperacillin-tazobactam. After incubation, the colonies are counted. **(c)** An isolate is classified as susceptible if less than 0.0001 (10^{-6}) of the cells grow at any

concentration. An isolate is classified as heteroresistant if there is less than 50% survival at 1X breakpoint and greater than 0.0001% at 1X and 2X breakpoint. Illustration in (a-b) created with BioRender.com.



Supplementary Figure 2. PAP graph for isolates from agar detoxification assays. (a-b) PAP results for isolates that enabled susceptible growth in proximity to the primary colony. **(c)** Isolate 1310 and 499 did not enable susceptible growth in agar detoxification.



Supplementary Figure 3. Illustration of broth microdilution. (a) Enriched HR isolate is pelleted through centrifugation and the top layer of supernatant is removed and filtered to remove bacterial cells. The supernatant is then concentrated using a protein concentrator; 2-4 µL of the concentrate is then added to every well. **(b)** The susceptible isolate (207) is added to every well and incubated at 37°C for 20 hours. MIC is then determined by both visual inspection and OD600 absorbance assay. Illustration created with BioRender.com.

References

1. CDC. Antibiotic Resistance Threats in the United States, 2019. Atlanta, GA: U.S. Department of Health and Human Services, CDC; 2019.
2. Resistance RoA. Antimicrobial Resistance: Tackling a Crisis for the Health and Wealth of Nations. London, UK: Review on Antimicrobial Resistance; 2014.
3. Worthington RJ, Melander C. Overcoming resistance to beta-lactam antibiotics. J Org Chem. 2013;78(9):4207-13.
4. Bush K. Past and Present Perspectives on beta-Lactamases. Antimicrob Agents Chemother. 2018;62(10).
5. Tooke CL, Hinchliffe P, Bragginton EC, Colenso CK, Hirvonen VHA, Takebayashi Y, et al. beta-Lactamases and beta-Lactamase Inhibitors in the 21st Century. J Mol Biol. 2019;431(18):3472-500.
6. Drawz SM, Bonomo RA. Three decades of beta-lactamase inhibitors. Clin Microbiol Rev. 2010;23(1):160-201.
7. Russ D, Glaser F, Shaer Tamar E, Yelin I, Baym M, Kelsic ED, et al. Escape mutations circumvent a tradeoff between resistance to a beta-lactam and resistance to a beta-lactamase inhibitor. Nat Commun. 2020;11(1):2029.
8. Schumock GT, Stubbings J, Wiest MD, Li EC, Suda KJ, Matusiak LM, et al. National trends in prescription drug expenditures and projections for 2018. Am J Health Syst Pharm. 2018;75(14):1023-38.
9. Edwards T, Heinz E, van Aartsen J, Howard A, Roberts P, Corless C, et al. Piperacillin/tazobactam-resistant, cephalosporin-susceptible Escherichia coli bloodstream infections are driven by multiple acquisition of resistance across diverse sequence types. Microb Genom. 2022;8(4).

10. Andersson DI, Nicoloff H, Hjort K. Mechanisms and clinical relevance of bacterial heteroresistance. Nat Rev Microbiol. 2019;17(8):479-96.
11. Windels EM, Michiels JE, Van den Bergh B, Fauvart M, Michiels J. Antibiotics: Combatting Tolerance To Stop Resistance. mBio. 2019;10(5).
12. Brauner A, Fridman O, Gefen O, Balaban NQ. Distinguishing between resistance, tolerance and persistence to antibiotic treatment. Nat Rev Microbiol. 2016;14(5):320-30.
13. Band VI, Weiss DS. Heteroresistance: A cause of unexplained antibiotic treatment failure? PLoS Pathog. 2019;15(6):e1007726.
14. Band VI, Hufnagel DA, Jaggavarapu S, Sherman EX, Wozniak JE, Satola SW, et al. Antibiotic combinations that exploit heteroresistance to multiple drugs effectively control infection. Nat Microbiol. 2019;4(10):1627-35.
15. Pereira C, Larsson J, Hjort K, Elf J, Andersson DI. The highly dynamic nature of bacterial heteroresistance impairs its clinical detection. Commun Biol. 2021;4(1):521.
16. Band VI, Satola SW, Smith RD, Hufnagel DA, Bower C, Conley AB, et al. Colistin Heteroresistance Is Largely Undetected among Carbapenem-Resistant Enterobacterales in the United States. mBio. 2021;12(1).
17. Band VI, Crispell EK, Napier BA, Herrera CM, Tharp GK, Vavikolanu K, et al. Antibiotic failure mediated by a resistant subpopulation in Enterobacter cloacae. Nat Microbiol. 2016;1(6):16053.
18. Band VI, Satola SW, Burd EM, Farley MM, Jacob JT, Weiss DS. Carbapenem-Resistant Klebsiella pneumoniae Exhibiting Clinically Undetected Colistin Heteroresistance Leads to Treatment Failure in a Murine Model of Infection. mBio. 2018;9(2).
19. Choby JE, Ozturk T, Abbott CN, Satola SW, Weiss DS. Heteroresistance via beta-lactamase gene amplification threatens the beta-lactam pipeline. bioRxiv. 2022:2022.06.07.494873.
20. Xu Y, Zheng X, Zeng W, Chen T, Liao W, Qian J, et al. Mechanisms of Heteroresistance and Resistance to Imipenem in Pseudomonas aeruginosa. Infect Drug Resist. 2020;13:1419-28.
21. Hjort K, Nicoloff H, Andersson DI. Unstable tandem gene amplification generates heteroresistance (variation in resistance within a population) to colistin in Salmonella enterica. Mol Microbiol. 2016;102(2):274-89.
22. Chervaux C, Sauvonnet N, Le Clainche A, Kenny B, Hung AL, Broome-Smith JK, et al. Secretion of active beta-lactamase to the medium mediated by the Escherichia coli haemolysin transport pathway. Mol Gen Genet. 1995;249(2):237-45.
23. Bielaszewska M, Daniel O, Nyc O, Mellmann A. In Vivo Secretion of beta-Lactamase-Carrying Outer Membrane Vesicles as a Mechanism of beta-Lactam Therapy Failure. Membranes (Basel). 2021;11(11).
24. Deak E, Szabo AI, Kalmaczhelyi A, Gal Z, Barabas G, Penyige A. Membrane-bound and extracellular beta-lactamase production with developmental regulation in Streptomyces griseus NRRL B-2682. Microbiology (Reading). 1998;144 (Pt 8):2169-77.
25. Rangama S, Lidbury I, Holden JM, Borsetto C, Murphy ARJ, Hawkey PM, et al. Mechanisms Involved in the Active Secretion of CTX-M-15 beta-Lactamase by Pathogenic Escherichia coli ST131. Antimicrob Agents Chemother. 2021;65(10):e0066321.
26. CLSI. Piperacillin-Tazobactam Breakpoints for Enterobacterales. Berwyn, PA2022.
27. Shirley M. Ceftazidime-Avibactam: A Review in the Treatment of Serious Gram-Negative Bacterial Infections. Drugs. 2018;78(6):675-92.
28. van Duin D, Bonomo RA. Ceftazidime/Avibactam and Ceftolozane/Tazobactam: Second-generation beta-Lactam/beta-Lactamase Inhibitor Combinations. Clin Infect Dis. 2016;63(2):234-41.
29. Prevention USCfDCa. Multi-site Gram-negative Surveillance Initiative 2023 [Available from: https://www.cdc.gov/hai/eip/mugsi.html.

30. CLSI. Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically; Approved Standard—Ninth Edition. Wayne, PA2012.

Appendix A: Optical Density Data of Broth Microdilution with Spent Supernatant

Isolate ID	MHB	0.25	0.5	1	2	4	8	16	32	64	128	256
Negative C	0.985	0.694	0.461	0.276	0.206	0.124	0.119	0.105	0.095	0.09	0.058	0.227
Negative C	0.874	0.639	0.492	0.265	0.211	0.12	0.114	0.12	0.136	0.099	0.072	0.206
2	0.851	0.674	0.632	0.428	0.462	0.198	0.112	0.114	0.148	0.321	0.092	0.845
6	0.838	0.6	0.476	0.258	0.18	0.127	0.119	0.138	0.127	0.12	0.088	0.185
8	0.892	0.701	0.645	0.659	0.448	0.229	0.273	0.149	0.136	0.134	0.105	0.169
9	0.851	0.63	0.429	0.241	0.225	0.13	0.129	0.121	0.121	0.094	0.097	0.132
14	0.825	0.621	0.586	0.268	0.208	0.116	0.121	0.124	0.097	0.092	0.07	0.076
15	0.882	0.691	0.619	0.271	0.225	0.112	0.112	0.102	0.088	0.077	0.049	0.182
32	0.763	0.624	0.581	0.437	0.308	0.204	0.126	0.153	0.116	0.077	0.099	0.646
39	0.716	0.588	0.54	0.436	0.312	0.264	0.123	0.134	0.122	0.103	0.094	0.46
61	0.709	0.577	0.567	0.348	0.239	0.137	0.133	0.12	0.114	0.093	0.065	0.188
63	0.728	0.559	0.608	0.266	0.22	0.139	0.143	0.138	0.115	0.098	0.072	0.156
100	0.73	0.573	0.549	0.288	0.235	0.129	0.132	0.119	0.106	0.082	0.062	0.156
169	0.734	0.56	0.516	0.262	0.179	0.192	0.116	0.123	0.089	0.08	0.058	0.148
194	0.745	0.571	0.493	0.257	0.223	0.13	0.14	0.12	0.111	0.093	0.076	0.161
Negative C	0.6695	0.5315	0.4435	0.2135	0.135	0.1105	0.0755	0.087	0.038	0.082	0.028	0.0245
Negative C	0.6245	0.4855	0.3705	0.2175	0.139	0.1155	0.1125	0.092	0.031	0.042	0.029	0.0205
Positive Co	0.6475	0.4605	0.3395	0.2335	0.212	0.1235	0.0955	0.099	0.044	0.031	0.029	0.0205
110	0.5875	0.4005	0.2415	0.2155	0.242	0.1125	0.1245	0.075	0.036	0.03	0.031	0.0195
120	0.6325	0.6055	0.5455	0.4235	0.245	0.1205	0.1015	0.068	0.033	0.029	0.031	0.0205
134	0.6355	0.5465	0.5045	0.4665	0.483	0.1225	0.1105	0.058	0.032	0.034	0.035	0.0205
138	0.6325	0.5145	0.4685	0.2675	0.259	0.1125	0.0335	0.039	0.029	0.029	0.036	0.0205
150	0.6515	0.5265	0.5185	0.2295	0.226	0.2345	0.0795	0.028	0.03	0.03	0.025	0.0215
154	0.8145	0.6805	0.6355	0.5705	0.267	0.0855	0.0445	0.039	0.041	0.037	0.037	0.0305
162	0.7375	0.5945	0.5325	0.2405	0.26	0.1095	0.1175	0.094	0.104	0.074	0.035	0.0265
183	0.6775	0.5225	0.5115	0.2825	0.142	0.0685	0.0395	0.033	0.034	0.037	0.029	0.0235
197	0.6895	0.5095	0.4865	0.2715	0.207	0.0955	0.1045	0.101	0.084	0.057	0.037	0.0255
226	0.6625	0.6435	0.5855	0.4935	0.419	0.3535	0.3345	0.312	0.282	0.155	0.086	0.0525
238	0.6875	0.5935	0.6215	0.6055	0.615	0.6095	0.6395	0.655	0.498	0.285	0.325	0.1335
248	0.6195	0.5625	0.4595	0.2525	0.227	0.2325	0.0915	0.081	0.071	0.058	0.036	0.0225
249	0.4975	0.5105	0.5115	0.4385	0.177	0.0335	0.0305	0.029	0.031	0.035	0.031	0.0245
5	0.6725	0.6275	0.5905	0.4355	0.36	0.1155	0.0685	0.046	0.034	0.03	0.035	0.0345
350	0.6625	0.5005	0.3205	0.1955	0.234	0.1205	0.1245	0.106	0.053	0.027	0.034	0.0225
2	0.6555	0.5945	0.5545	0.5185	0.432	0.3445	0.3805	0.122	0.167	0.067	0.032	0.0285
8	0.6755	0.5575	0.5295	0.4725	0.316	0.2925	0.2595	0.108	0.113	0.054	0.031	0.0185
32	0.6345	0.5325	0.5235	0.4955	0.343	0.3235	0.3035	0.236	0.245	0.141	0.106	0.0415
39	0.6045	0.4985	0.4805	0.4745	0.42	0.3205	0.2785	0.161	0.12	0.09	0.069	0.0415
88	0.752	0.63	0.577	0.496	0.354	0.264	0.049	0.031	0.05	0.039	0.035	0.029
298	0.743	0.552	0.472	0.33	0.265	0.177	0.259	0.029	0.035	0.039	0.036	0.027
Negative C	0.664	0.399	0.37	0.234	0.225	0.091	0.08	0.059	0.037	0.034	0.03	0.022
Negative C	0.694	0.458	0.346	0.191	0.128	0.123	0.1	0.07	0.04	0.036	0.032	0.022
295	0.714	0.49	0.248	0.236	0.196	0.032	0.032	0.029	0.161	0.035	0.031	0.025
285	0.698	0.533	0.544	0.391	0.39	0.179	0.04	0.039	0.211	0.037	0.033	0.021
173	0.627	0.513	0.476	0.319	0.254	0.232	0.247	0.033	0.037	0.036	0.033	0.023
46	0.672	0.588	0.436	0.487	0.273	0.052	0.049	0.055	0.037	0.039	0.033	0.022
352	0.614	0.493	0.517	0.477	0.427	0.549	0.402	0.387	0.204	0.086	0.303	0.176
332	0.663	0.473	0.495	0.459	0.362	0.283	0.055	0.053	0.034	0.043	0.04	0.025
331	0.627	0.527	0.511	0.449	0.172	0.096	0.1	0.078	0.035	0.036	0.038	0.023
55	0.703	0.53	0.467	0.247	0.195	0.193	0.077	0.06	0.039	0.031	0.025	0.022
259	0.714	0.499	0.348	0.264	0.278	0.048	0.032	0.031	0.033	0.041	0.033	0.029
257	0.633	0.49	0.504	0.189	0.488	0.185	0.035	0.031	0.036	0.042	0.035	0.028
323	0.636	0.468	0.516	0.4	0.304	0.071	0.16	0.035	0.032	0.24	0.033	0.024
289	0.594	0.427	0.426	0.208	0.185	0.047	0.078	0.034	0.037	0.041	0.033	0.025
274	0.628	0.484	0.428	0.238	0.227	0.182	0.292	0.032	0.043	0.043	0.037	0.023
305	0.599	0.448	0.459	0.25	0.286	0.197	0.248	0.029	0.032	0.036	0.031	0.023
363	0.627	0.519	0.5	0.332	0.165	0.173	0.063	0.043	0.039	0.038	0.03	0.023
258	0.65	0.508	0.487	0.21	0.277	0.078	0.064	0.057	0.044	0.039	0.031	0.025
150	0.856	0.692	0.705	0.61	0.513	0.406	0.271	0.144	0.056	0.03	0.039	0.034
352	0.73	0.641	0.651	0.674	0.701	0.661	0.712	0.731	0.714	0.656	0.682	0.77
32	0.767	0.679	0.66	0.631	0.619	0.451	0.375	0.331	0.323	0.255	0.23	0.239
226	0.8	0.699	0.696	0.665	0.63	0.564	0.034	0.557	0.338	0.342	0.322	0.48
8	0.739	0.578	0.472	0.254	0.212	0.222	0.057	0.028	0.034	0.027	0.034	0.026
Negative C	0.731	0.545	0.443	0.259	0.224	0.197	0.032	0.06	0.064	0.027	0.027	0.027
Negative C	0.743	0.593	0.507	0.268	0.252	0.223	0.086	0.076	0.035	0.027	0.036	0.026

Appendix B: Optical Density Data of Broth Microdilution with Spent Supernatant and Avibactam

Isolate ID	MHB	0.25	0.5	1	2	4	8	16	32	64	128	256
8a	0.8680	0.7370	0.6427	0.3910	0.2780	0.2337	0.0243	0.0210	0.0207	0.0183	0.0197	0.0153
8b	0.7690	0.6210	0.5577	0.3330	0.2440	0.0367	0.0213	0.0210	0.0217	0.0203	0.0187	0.0143
8c	0.7090	0.5610	0.5967	0.2840	0.2520	0.1897	0.0223	0.0220	0.0207	0.0223	0.0187	0.0133
226a	0.7580	0.6900	0.7197	0.6760	0.4450	0.1817	0.0253	0.0220	0.0227	0.0223	0.0217	0.0153
226b	0.7550	0.7110	0.6347	0.6080	0.4200	0.1917	0.0443	0.0210	0.0207	0.0213	0.0197	0.0133
226c	0.7450	0.6400	0.6367	0.4760	0.3030	0.2377	0.0213	0.0210	0.0247	0.0233	0.0187	0.0133
150a	0.8080	0.6500	0.6417	0.4660	0.2740	0.2167	0.0233	0.0270	0.0257	0.0223	0.0227	0.0143
150b	0.7450	0.6280	0.5707	0.2980	0.2220	0.2227	0.0213	0.0220	0.0207	0.0193	0.0187	0.0143
150c	0.9960	0.6740	0.6217	0.4470	0.2880	0.2697	0.0233	0.0290	0.0247	0.0223	0.0217	0.0163
32a	0.8450	0.6810	0.6137	0.5130	0.3330	0.3267	0.2393	0.0570	0.0237	0.0203	0.0187	0.0163
32b	0.8090	0.7940	0.7297	0.5820	0.5130	0.3197	0.2773	0.1680	0.1117	0.0793	0.1257	0.0683
32c	0.8470	0.7420	0.6247	0.6120	0.5100	0.3127	0.2763	0.1560	0.0697	0.0693	0.0737	0.0153
352a	0.8070	0.7380	0.7017	0.6820	0.5290	0.3227	0.2263	0.2030	0.1337	0.0993	0.1107	0.1303
352b	0.8220	0.6870	0.6237	0.5360	0.2810	0.2467	0.0273	0.0250	0.0247	0.0243	0.0217	0.0153
352c	0.8180	0.7220	0.6617	0.6180	0.4220	0.2447	0.1753	0.0440	0.0227	0.0203	0.0207	0.0153
207a	0.8540	0.6460	0.6167	0.3400	0.2550	0.2127	0.0233	0.0230	0.0217	0.0203	0.0187	0.0183
207b	0.7410	0.6150	0.5397	0.3020	0.1030	0.2067	0.0183	0.0160	0.0177	0.0183	0.0127	0.0113
207c	0.7390	0.5550	0.4637	0.2730	0.1150	0.0357	0.0293	0.0210	0.0207	0.0193	0.0147	0.0103