Investigation of *Klebsiella pneumoniae* inner colonies during fosfomycin susceptibility testing

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Dedication

This dissertation is dedicated to my family, friends, and my dog Fern. Shortly after rescuing Fern as a puppy in the summer of 2022, she showed great promise as a gluten detection dog and has been doing her best to keep me healthy ever since.

Abstract

Fosfomycin is a broad-spectrum antibiotic indicated for first-line treatment of uncomplicated urinary tract infection (UTI) caused by *Escherichia coli*. However, it is often used for non-*E. coli* Enterobacterales when limited oral antibiotics are available to treat UTI caused by increasingly resistant urinary pathogens. Both agar dilution (AD) and disk diffusion (DD) are approved methods for fosfomycin susceptibility testing, though DD is used more often in clinical settings. During DD testing, visually distinct inner colony (IC) sub-populations frequently develop. A collection of *K. pneumoniae* (n = 262), obtained from five US locations in 2013-2023 and their corresponding IC (n = 116; 44.3%) underwent susceptibility testing. Median minimal inhibitory concentration (MIC) values increased three, 2-fold dilutions from IC-producers to IC. IC-producers along with IC never producers (NP) underwent heteroresistance screening. IC-producers had greater odds (odds ratio: 1.71) of screening positive for heteroresistance. Whole genome sequencing among selected IC-producer/IC pairs revealed variations in several known fosfomycin resistance genes within IC isolates not found in their IC-producers or NP. Further *in vitro* fitness testing including growth curve and generation time analysis demonstrated no loss of fitness for IC in monoculture. Among a 72-hour competition time growth between IC-producers and IC, prolonged generation times were observed across all IC-producers and IC. In competition, some IC entered death phase as early 24-36 hours post inoculation where all IC-producers maintained logarithmic growth for all 72 hours. Finally, *in vivo* fitness testing using an ascending UTI mouse model, showed varying colonizing abilities of IC isolates when in competition—up to 50% for IC isolates. This work reveals that specific genetic variations did not

alter *in vivo* fitness of *K. pneumoniae* when colonizing the mouse urinary tract. This indicates that fosfomycin IC should be considered relevant, and not ignored during DD testing.

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List of Abbreviations

AD	Agar dilution susceptibility testing
BMD	Broth microdilution susceptibility testing
CFU	Colony forming units
CLSI	Clinical and Laboratory Standards Institute (United States' guiding body for development of antimicrobial breakpoints)
DD	Disk diffusion susceptibility testing
ESBL	Extended-spectrum beta-lactamase
EUCAST	European Committee on Antimicrobial Susceptibility Testing (European Union's guiding body for development of antimicrobial breakpoints)
G3P	Glycerol-3-phosphate
G6P	Glucose-6-phosphate
IC	Inner colony isolate that arose from disk diffusion
IC-producer	Isolate which produces inner colonies during disk diffusion testing
MDR	Multi-drug resistant
MHA	Mueller-Hinton agar
MHB	Mueller-Hinton broth
MIC	Minimal inhibitory concentration
NP	Isolate which never produces an inner colony "never producer"`
UTI	Urinary tract infection
uUTI	Uncomplicated urinary tract infection
cUTI	Complicated urinary tract infection

1. Introduction

Antibiotic resistance is a worldwide problem threatening the ability to effectively treat bacterial infections, thus requiring a need to prevent further resistance and prolong their ability to be used. Urinary tract infections (UTI) are one of the most common causes for antibiotic prescriptions in the United States, and fosfomycin is recommended as first-line treatment of uncomplicated UTI (uUTI) (1, 2).

Fosfomycin is a broad spectrum antibiotic first developed in the 1960s and approved by the Food and Drug administration in 1996 (3, 4). It the only drug in its class of drugs and has a chemical structure unrelated to other known antibiotics (5–7). Fosfomycin has broad spectrum activity against both Gram-positive and Gram-negative organisms, this includes both methicillin-resistant and -susceptible *Staphylococcus aureus* (MSSA and MRSA) as well as extended spectrum beta-lactamase (ESBL) and *Klebsiella pneumoniae* carbapenemase (KPC) producing organisms (8–10). It is indicated as a first-line agent for uUTI caused by *Escherichia coli* and *Enterococcus faecalis;* however, it is often used to treat UTI caused by non-*E. coli* organisms as there are limited oral antibiotics available to treat UTI caused by increasingly resistant urinary pathogens (11–19). Because of its activity against many multidrug-resistant pathogens, fosfomycin has undergone revival, but concerns regarding susceptibility testing procedures have recently been highlighted (20–23).

While agar dilution (AD) is the reference method for fosfomycin, both disk diffusion (DD) and AD are approved methods for testing bacterial susceptibility to fosfomycin in the clinical

microbiology laboratory. AD is, however, both labor- and time-intensive, so DD is generally used more often in the clinical setting. However, during DD testing, the presence of visually distinct inner colony (IC) sub-populations frequently appear and can be more resistant than the IC-producer (i.e., the clinical isolate from which the IC arise) culture. One issue of great concern is that the two main organizations responsible for approving susceptibility breakpoints, the Clinical and Laboratory Standards Institute (CLSI) and European Committee on Antimicrobial Susceptibility Testing (EUCAST), have discordant recommendations for interpretation of IC present during DD testing of fosfomycin. CLSI states that the antibiotic's zone of inhibition shall be measured from the innermost colonies, whereas EUCAST states that the IC shall be ignored (24, 25). These zone of inhibition measurements determine whether a patient-specific organism is reported as susceptible or resistant to a tested antibiotic. It is unclear how large of a patient and public health issue these contradictory standards may be. Because it is unknown how often patients have misclassified fosfomycin susceptibility test results, we cannot be sure how frequently these clinical test results are leading to a less effective treatment for UTI, and potentially increasing of infections caused by undocumented fosfomycin-resistant Enterobacterales. To prevent potential misclassifications of fosfomycin susceptibility and to curb the growing prevalence of multidrug resistance, it is important to define the most accurate fosfomycin testing method and to understand whether the IC populations are clinically important.

In the most frequently used susceptibility testing method used clinically for fosfomycin, DD, IC sub-populations arise. The presence of these IC sub-populations creates three distinct phenotypes

during this DD testing, those being isolates which never produce an IC ("never producer" or NP), isolates that have IC arise during DD testing (IC-producer) and the visually distinct IC themselves. While the two major organizations responsible for determining susceptibility criteria have discordant recommendations on how to interpret these distinct IC, the most appropriate method is unknown. There is a critical need to determine the optimal interpretation of DD results for fosfomycin against common UTI pathogens as use of unreliable testing methods could lead to an increase in resistant infections.

To address this issue, a series of *in vitro* and *in vivo* experiments were performed using a collection of geographically diverse *Klebsiella pneumoniae* clinical isolates (n = 262) to determine the most effective method to interpret disk diffusion testing results for fosfomycin. In Chapter 2, I reviewed the literature on global fosfomycin susceptibility testing methods and results when used against *K. pneumoniae*, clinical outcomes of fosfomycin usage to treat *K. pneumoniae* UTI, and fosfomycin resistance mechanisms in clinical *K. pneumoniae.* In Chapter 3, I describe fosfomycin susceptibility of a *K. pneumoniae* isolate collection (n = 262) utilizing both AD and DD as testing methods. In Chapter 4, I describe the fosfomycin susceptibility of the *K. Pneumoniae* IC collection (n = 116) that arose from the primary collection during DD.I also describe the presence of heteroresistance and *fosA* genes among the collection. In Chapter 5, I describe the *in vitro* fitness of a subset of IC-producers (n = 9), IC (n = 9), and isolates which never produced IC (n = 5) and a whole genome sequence analysis of these isolates. In Chapter 6, I describe the *in vivo* fitness of four IC-producer/IC pairs using an ascending UTI model of the

mouse urinary tract. In Chapter 7, I reflect on my work and propose future directions to continue my work.

2. Chapter 2: Inconsistencies in global fosfomycin susceptibility testing of *Klebsiella* spp. and the rise of inner colony mutants observed during testing in Enterobacterales as a potential cause for poor clinical outcomes

2.1 Introduction

Both AD and DD) are approved methods for testing bacterial susceptibility to fosfomycin in the clinical microbiology laboratory. AD is both labor- and time-intensive, so other methods are often used in clinical and research laboratories (24, 26). DD is, therefore, a frequently used susceptibility testing method for fosfomycin. The presence of visually distinct IC sub-populations, which appear to be more resistant than the primary culture, frequently develop during DD testing (27–29). Additionally, there are no DD breakpoints that apply to *K. pneumoniae*; all fosfomycin DD breakpoints are extrapolated from *E. coli* (24, 26). Thus, it is necessary to understand which interpretation of inner colonies is most appropriate for *K. pneumoniae* and whether the inconsistencies in breakpoint application and the occurrence of discrete inner colonies lead to the discordance of fosfomycin susceptibility testing results and clinical outcomes when treating *K. pneumoniae.*

2.2 Identification of literature and categorization of results

PubMed was utilized to source relevant studies assessing the use of fosfomycin susceptibility testing internationally from 2006-2022, occurrence of inner colonies among Enterobacterales, and fosfomycin treatment outcomes against *K. pneumoniae.* Search terms included "fosfomycin", "susceptibility testing", "*Klebsiella*", "inner colony", "heteroresistance", "resistance", and "outcomes". Data sources were narrowed to studies published in the English language.

Testing data from these publications was divided broadly by use of CLSI and EUCAST breakpoints. Methods used in these studies were summarized first followed by the susceptibility interpretations produced by these methods. Studies that reported the presence of IC during fosfomycin DD testing were used to identify potential causes including: mutations in sugar-phosphate transporter genes, fosfomycin deactivating enzymes, and mutations in the fosfomycin site of action. Lastly, the discordance between fosfomycin susceptibility testing results and clinical outcomes in *K. pneumoniae* were summarized.

2.3 International utilization and interpretation of fosfomycin susceptibility testing methods

Two major breakpoint setting organizations (CLSI and EUCAST) have established breakpoints that are used for susceptibility testing globally. However, the oral breakpoints for both organizations do not apply to *K. pneumoniae* and must be extrapolated from their *E.coli* breakpoint. During the period of the included studies EUCAST had intravenous breakpoints ($EUCAST_{IV}$) which applied to *K. pneumoniae.* In clinical settings within the United States, CLSI is strictly followed while EUCAST is used exclusively in the European Union. However, outside these locations, consensus is lacking for which organization's guidelines and breakpoints are applied.

2.3.1 Collection and fosfomycin susceptibility testing practices among international studies of Klebsiella *spp.*

The most common susceptibility testing methods used were AD and DD, with a smaller number of studies utilizing ETEST, BMD, and Vitek 2 or Phoenix. Both CLSI and EUCAST approve use of AD and DD whereas CLSI explicitly recommends against use of broth microdilution and makes no recommendations on agar gradient diffusion strips (Etest, bioMérieux; MIC test strip, Liofilchem®) likely contributing to their lower use in studies (24, 30).

2.3.1 *International studies using CLSI breakpoints*

Of 29 studies included, 21 extrapolated CLSI *E. coli* breakpoints for fosfomycin against *Klebsiella* spp. (Table 1) (31–51). Only two of these studies included isolates collected and tested in the United States; both used Etest for testing of clinical *K. pnemoniae* isolates (31, 40). Endimiani *et al.*, also used AD and DD (31). Additional studies collected isolates from East Asia (2), the Middle East (6), South Asia (6), Mexico (1), Brazil (1), South Africa (1) and Spain (1).

East Asian studies took place in China and Taiwan both using *K. pneumoniae* urine isolates (39, 41). These studies used different methods for susceptibility testing for their isolates, with one using BMD and the other DD (39, 41).

Studies from the Middle East used DD as a susceptibility testing method (33, 35, 36, 38, 46, 48). Four of these studies utilized clinical *K. pneumoniae* isolates, three of which were in Iran and one in Lebanon (33, 35, 46, 48). Two additional Middle Eastern studies used *Klebsiella* spp. clinical isolates collected in either Israel and Turkey (36, 38). Peretz *et al.* utilized Vitek 2 (bioMérieux; Marcy-l ’Étoile, France) in addition to DD for their Israeli study (38).

Two south Asian studies collected and tested *K. pneumoniae* urine isolates in India (34, 44). Both studies used AD and DD for fosfomycin susceptibility testing, but Bir *et al.*, also used Etest (34, 44). In Pakistan, three studies collected and tested *Klebsiella* spp. urine isolates via DD (37, 49, 51).

The studies that took place in Brazil and South Africa utilized DD for fosfomycin susceptibility of *K. pneumoniae* and *K. oxytoca* (32, 47). Miranda-Novales *et al.*, used DD for fosfomycin susceptibility testing of *K. pneumoniae* urine isolates by DD in Mexico (43). De Cueto, *et al.*, utilized AD to test fosfomycin susceptibility against *K. pneumoniae* urine isolates from Spain (42).

2.3.2 International studies using EUCAST breakpoints

Nine of the 29 included studies either applied or extrapolated EUCAST breakpoints for fosfomycin against Enterobacterales or *E. coli* respectively. Due to the 2020 change in breakpoints for oral fosfomycin changing the susceptible breakpoint from 32 µg/mL to 8 µg/mL, some studies applied breakpoints that are no longer recommended (52).

The majority of studies that utilized EUCAST breakpoints (n/N = 6/9) have some or all of the isolates collected from countries within the European Union (53–58). Two of the studies applied $EUCAST_{IV}$ breakpoints for AD (53, 54). Tutone *et al.*, extrapolated the $EUCAST_{oral}$ breakpoints

for *E. coli* for AD and DD against *Klebsiella* spp. urine isolates collected in Belgium, United Kingdom, Italy, Spain, and Russia (55).

Three of the European studies utilized the pre-2020 breakpoints for their collections of *K. pneumoniae* isolates (56–58). Each used a different testing method, one extrapolating the DD breakpoints using urine isolates from the Czech Republic (56), another applying the MIC breakpoints to German isolates using AD (57), and one used Phoenix (Becton Dickinson; Franklin Lakes, New Jersey) for testing their isolates from Italy (58).

Outside the European Union, the pre-2020 EUCAST breakpoints were utilized in studies from India, Taiwan, and Turkey (44, 59, 60). The studies that took place in Taiwan and Turkey both utilized AD for testing *K. pneumoniae* (59, 60). Bir *et al.*, applied and extrapolated the breakpoints for the same *K. pneumoniae* urine isolates using AD, DD, and ETEST as was used for the CLSI breakpoints (44).

2.3.3 Fosfomycin susceptibility of international collections of Klebsiella spp. isolates

When studies extrapolated fosfomycin MIC breakpoints from CLSI against *K. pneumoniae,* using AD it often resulted in >90% of isolates being susceptible to fosfomycin (Table 2). De Cueto *et al.* reported 92.8% susceptibility (n/N = 128/138) (42). Similar results were observed across other studies: Endimiani *et al.*, reported 92.6% (n/N = 63/68), Bir *et al.*, 92.1% (n/N = 35/38), and Behera *et al.* 91.3% (n/N = 42/46) of isolates susceptible to fosfomycin (31, 34, 44).

The use of DD as a fosfomycin susceptibility testing method for extrapolating CLSI breakpoints resulted in approximately 30% fewer susceptible *Klebsiella* spp. isolates with only the collection from Mothibi *et al.*, having >90% susceptibility (32). Slightly lower rates of susceptibility were found in collections from Lima *et al.* 86.7% (n/N = 409/472) and Abdullah *et al.*, 77.5% (n/N = 1253/1617) (47, 51). Rates of susceptible isolates were as low as 57.6 (n/N = 38/66) from Liu *et al.* (41).

Bir *et al.* and Endimiani *et al.* published lower rates of susceptibility when utilizing DD as compared to AD for susceptibility testing within their isolate collections (31, 44). Bir *et al.* reported two less isolates (n/N = 32/38; 84.2%) susceptible to fosfomycin when using DD as compared to AD (44). Endimiani *et al.* observed 20 fewer susceptible isolates (n/N = 43/69; 63.2%) when using DD compared to AD (31).

When studies applied $EUCAST_{IV}$ fosfomycin breakpoints for use with AD, susceptibility ranged from 66.0% (n/N = 165/250; Kowalska-Krochmal *et al.*) to 90.6% (n/N = 29/32; Aydemir *et al.*) (53, 60). When Tutone *et al.* extrapolated the post-2020 oral MIC breakpoints, only 27.1% (n/N = 42/155) of *Klebsiella* spp. were susceptible to fosfomycin (55).

When studies extrapolated the EUCAST oral DD breakpoints ($S \geq 24mm$) between 50.0% (n/N = 19/38; Bir *et al.*) and 80.4% (n/N = 517/643; Fajfr *et al.*) of isolates were found to be susceptible (44, 56). When Tutone *et al.* extrapolated the oral breakpoints, only 1.9% (n/N = 3/155) were susceptible (55).

Both of these studies including both AD and DD, reported a reduction in interpreted susceptibility when measuring with DD versus AD (44, 55). Bir *et al.* noted 14 more susceptible isolates (n/N = 33/38; 86.9%) when using AD as opposed to DD (44). Similarly, Tutone *et al.* reported 39 more susceptible isolates (N= 155) when using AD as the susceptibility testing method (55).

2.4 Clinical outcomes of fosfomycin treatment of *Klebsiella pneumoniae* UTI

Clinical outcomes studies and their conclusions are limited for fosfomycin treatment of *Klebsiella* spp. UTIs compared to *E. coli*. Most of these studies were retrospective chart reviews which introduces limitations in interpretation of the results (11, 15, 16).

2.4.1 Treatment of urinary tract infection with fosfomycin

When studying the treatment of symptomatic UTI caused by *K. pneumoniae* (and *E. coli*) results conclude that fosfomycin susceptibility data for *K. pneumoniae* does not predict patient outcomes as it does in *E. coli* (11, 15, 16). They conclude that fosfomycin is suitable especially for the treatment of *E. coli* caused by UTI and that a standard single 3g oral dose of fosfomycin tromethamine is inadequate for infections caused by *Klebsiella* spp. (15).

Matthews *et al.*, included patients with UTI caused by either *K. pneumoniae* (n =9) or *E. coli* (n = 52) in their retrospective cohort study, 66.7% and 58.5% of which were ESBL-positive, respectively (16). In vitro susceptibility to fosfomycin (based on pre-2020 $EUCAST_{oral}$ MIC

breakpoints using BD Phoenix for MIC measurement) was reported for 100% of *K. pneumoniae* and 98% of *E. coli* isolates. All patients received one or more doses 3g oral fosfomycin tromethamine (irrespective of sex/ complication status), at this United Kingdom-based medical center. Current guidelines state that all male UTI are complicated (cUTI), even simple cystitis, thus outside the uUTI approval for fosfomycin (61, 62). Previous studies suggest that cUTI are better treated with 2-3 doses of 3g fosfomycin over the course of 6 day (63), possibly explaining why women received a median of a single dose whereas men received a median of three doses. Based on the study's clinical and microbiological cure definitions, 22.2% (n/N= 2/9) of *K. pneumoniae* infections were cured (16).

Similarly, Neuner *et al.*, performed a retrospective chart review of the microbiological and clinical outcomes of UTI caused by multi-drug resistant (MDR) pathogens (11). This included carbapenem-resistant *K. pneumoniae* (n =13), and ESBL-positive *E. coli* (n = 5). Of the *K. pneumoniae* isolates 92% were fosfomycin susceptible and 80% of *E. coli* were fosfomycin susceptible per CLSI breakpoints using Etests. Microbiological cure was observed in just 46% of *K. pneumoniae* but 100% of *E. coli* infections.

A larger prospective study including carbapenem-resistant *K. pneumoniae* by van Duin *et al.*, included 19 hospitalized patients with a physician-diagnosed UTI treated with fosfomycin (17). Treatment success was seen in 10.5% (n = 2) of patients, which was defined as a negative urine culture following the index culture. Fosfomycin treatment failure was seen in 47.4% (n = 9) of patients; failure was defined as a patient having a recurrent carbapenem-resistant *K. pneumoniae*

seven days after the index culture. The remaining patients, 42.1% (n = 8), did not meet the definitions for treatment success or failure (17).

In hospital database review of UTI in kidney transplant recipients by Reid *et al.* 5 patients with infections caused by *K. pneumoniae* (carbapenemase producing) were treated with fosfomycin (18). All patients were 1-6 months post-transplant and received 3-7 doses of 3g oral fosfomycin. Bacterial clearance at 3 months post treatment was observed in only 1 patient, another experienced a persistent UTI, 2 patients had a recurrent UTI in the 3 months post-treatment, and one patient experienced both a persistent and recurrent UTI (18).

Seroy, *et al.*, performed a retrospective chart review and found 23 patients with *K. pneumoniae* (ESBL-positive, n = 8; carbapenem resistant, n = 13) caused UTIs that were treated with fosfomycin (14). Inclusion in this study required the isolated organism to be susceptible to fosfomycin per CLSI breakpoints using Etest. These patients received 1-14 doses of 3g oral fosfomycin. A persistent UTI was recorded for 8 patients (34.8%) with either ESBL-positive or carbapenem resistant infections. Recurrent UTIs were observed in 5 patients (21.7%) of which 4 were either ESBL-positive or carbapenem resistant. Resulting in an overall treatment failure rate of 56.5% (n/N = 13/23) patients within the study.

These studies concluded that *in vitro* susceptibility data for fosfomycin against *K. pneumoniae* was generally discordant with positive clinical outcomes. With treatment failure exceeding 47% in all studies despite the majority of the patients having susceptible *K. pneumoniae* infections

based on extrapolated breakpoints, evidence suggests that fosfomycin is best used to treat only *E. coli* (11, 16). Alternatively, some of the authors suggest that *K. pneumoniae* infections may be successfully treated using a different dosing regimen (14, 15).

Potential dosing regimen may include either prolonged treatment, a higher dose, or a combination of the two. Acute toxicity studies within animal models found that doses of up to 5g/kg were well tolerated in mice and rats (61). Adverse events of transient and mild watery stools were seen in rabbits and diarrhea with anorexia in dogs were associated with the increased dose of fosfomycin. The doses used in these toxicity studies represent 50-125 times greater doses than the 3g human dose (61). When studying the pharmacokinetics of multiple oral doses, Wenzler *et al.*, found both daily and every 48 hour dosing to be safe and tolerable with the only adverse events associated with fosfomycin being diarrhea reported by 58% of participants (64). Of the participants with noted diarrhea events, only one participant had *Clostridioides difficile*-associated diarrhea (64). Thus, it is safe to explore utilizing alternative dosing regimen for the oral treatment of *K. pneumoniae* uUTI.

2.4.2 Treatment of asymptomatic bacteriuria (ASB) with fosfomycin

In the study among pregnant patients from eight Indonesian health centers, 10.2% (n/N = 73/715) were positive for ASB (19). Of these patients *K. pneumoniae* was the second most common Gram-negative organism isolated behind *E. coli*. Based on *in vivo* susceptibility testing >80% of *K. pneumoniae* isolates and 100% of *E. coli* isolates were fosfomycin susceptible (19). Based on urinalysis results 76 patients were treated with a single 3g oral dose of fosfomycin-trometamol as

empiric treatment of the asymptomatic colonization per Infectious Diseases Society of America guidelines (19, 65). Risks associated with non-treatment of ASB in pregnant patients include preterm labor, low birth weight, preeclampsia, hypertension, maternal renal failure, and intrauterine fetal death (19, 65). This study reports 100% full-term and normal-birth weight babies and side effects of self-resolving diarrhea in two of the pregnant patients (19).

2.5 Observations of inner colonies and potential causes

The presence of colonies within the zone of inhibition (inner colonies) using DD and Etest have been noted among Enterobacterales (27–29). The frequency of their appearance ranges widely among the organisms tested. They have been noted as "frequent" (28) and were present in 19% (29) of *K. pneumoniae* when performing fosfomycin DD testing. Additional *E. coli* observations have noted inner colonies present in 0.8% (27), and 69% (29) of isolates, whose variability may be explained by both timing and geography isolate of collection. Transmission of resistance mechanisms among clinical isolates can take extended periods of time to reach new geographic locations or they may only persist in the geographic location in which they arise. It is because of this epidemiological records of antibiotic resistance are hyper-localized and hospital specific (66, 67).

E. coli and *K. pneumoniae* possess many of the same fosfomycin resistance mechanisms, however, *K. pneumoniae* possess more inherent fosfomycin resistance mechanisms (68). It is conventionally believed that some of these shared mechanisms, specifically the shared sugar phosphate transporters are the primary cause for the formation of IC and their resistance (27).

Though the inherently present *fosA* within some clinical *K. pneumoniae* must also be considered as a potential cause for the formation of increased resistance among an IC-subpopulation (28). However, the cause for the formation of these IC remains unknown and is only speculated to be related to known resistance genes within these Enterobacterales.

2.5.1 The role of sugar-phosphate transporters in the resistance of inner colonies

Mutations within the sugar-phosphate transporters UhpT and GlpT, which allow fosfomycin to enter the cell, are a suspected cause for the formation of fosfomycin resistant IC among otherwise susceptible Enterobacterales cultures. When *E. coli* IC strains were sequenced, four of the five had large deletions that included the *uhpT* gene that were not present in the IC-producer sequence (27). This gene is responsible for encoding the hexose-6-phosphate transporter—a sugar-phosphate antiporter— in Enterobacterales (69). The final *E. coli* IC strain had an intact *uhpT* but had a nonsense mutation in *uhpA* which is required for *in vivo* expression of *uhpT* (27, 70). UhpT is induced by its substrate—glucose-6-phosphate (G6P)—and is also partially responsible for fosfomycin uptake in Enterobacterales (70, 71). Presence of G6P in conjunction with a functional UhpT potentiates the activity of fosfomycin, requiring a lower concentration of fosfomycin to inhibit growth, thus a deletion or mutation in *uhpT* or another *uhp* would result in decreased susceptibility to fosfomycin (27, 70, 72). This type of mutation affects G6P utilization, and causes decreased bacterial fitness and is counter selected *in vivo* (27, 70). Counter selection is the process through which natural selection does not favor a particular mutation causing it to die out over several generations.

Similar to UhpT, GlpT (glycerol-3-P permease protein) is a cellular transporter that is responsible for fosfomycin uptake in Enterobacterales and induced by its substrate—glycerol-3-phosphate (69, 70, 73). Mutations in *glpT* are associated with increased fosfomycin resistance in *E. coli* clinical isolates as well as counter selection *in vivo* (70).

These mutations in *uhpT* and *glpT* are commonly seen in fosfomycin-resistant clinical *K. pneumoniae* isolates (74). Upstream mutations in *cyaA* that cause lower levels of cyclic-AMP (cAMP) are not seen *in vivo* as *uhpT* and *glpT* are positively regulated by cAMP (70, 75). Thus, having either a functional UhpT or GlpT transporter is necessary for *in vivo* growth (70).

2.5.2 Role of fosA *in resistant inner colonies*

Variations within *fosA* genes present and the expression levels of *fosA* may play a role in the formation of fosfomycin resistant IC. In a study where frequent inner colonies were noted among fosfomycin using DD testing against *K. pneumoniae,* all isolates were found to harbor *fosA* and all but one carried a *fosA6* allele (28). The *fosA* gene encodes fosfomycin resistant glutathione transferase, a metalloenzyme which inactivates fosfomycin by catalyzing the addition of glutathione (76, 77). Presence of chromosomal *fosA* has been seen among most clinical *K. pneumoniae* isolates across many studies, but not all *K. pneumoniae* possess this resistance mechanism. Various *fosA* alleles are found both chromosomally (*fosA5* and *fosA6*) in *K. pneumoniae* and on plasmids in many Enterobacterales (78–84). Nonetheless, even chromosomal *fosA5/fosA6* alleles are not found in all clinical *K. pneumoniae* isolates (46).

Plasmids carrying a *fosA* gene are often found in the presence of MDR-phenotypes, as these plasmids carry genes that encode resistance mechanism to other antibiotics (84, 85). Across Enterobacterales, all *fosA* alleles—aside from *fosA2*—have been found to be plasmid mediated (86). *FosA3* is a globally prevalent plasmid-carried allele and frequently transmitted by animals; it is extensively found in East Asia (86).

The presence of a *fosA* allele and fosfomycin heteroresistance are often found in the same isolates of *Klebsiella* spp. (87, 88). However, in cases where *K. pneumoniae* isolates screen positive for fosfomycin heteroresistance there is no evidence of sustained up-regulation of *fosA* (87).

2.5.3 Mutations in murA *among K. pneumoniae and inner colonies*

Fosfomycin targets UDP- N- acetylglucosamine enolpyruvate transferase (MurA), an enzyme responsible for initiation of peptidoglycan biosynthesis of the bacterial cell wall (68). Mutations in *murA* are associated with acquired fosfomycin resistance seen in *E. coli* (68, 89–94).

The occurrence of *murA* mutation-mediated fosfomycin resistance in clinical *K. pneumoniae* isolates is variable. In a study of carbapenem-resistant *K. pneumoniae* infections in Taiwan, no *murA* mutations were detected among fosfomycin-resistant strains (74). In opposition, another study found that 70% of fosfomycin non-susceptible isolates had a *murA* mutation (95). A third study found a mutant *murA* gene in a single (n/N = 1/48) fosfomycin non-susceptible *K. pnemoniae* (96). Thus, it is hypothesized that some *murA* mutations may still allow for a

functional MurA enzyme, meaning that other mechanisms are responsible for fosfomycin resistance among these *K. pneumoniae* isolates.

There have been no published instances of *murA* mutations occurring in IC produced by fosfomycin-susceptible Enterobacterales. However, the literature describing the presence of IC is limited to just a handful papers outside of our laboratory's work.

2.6 Conclusions

International studies using a wide variety of susceptibility testing methods and breakpoints have found the majority of *K. pneumoniae* clinical isolates to be susceptible to fosfomycin. This comes with limitations as many of these studies are extrapolating breakpoints that only apply to *E. coli*. Extrapolating fosfomycin susceptibility breakpoints for use in *K. pneumoniae* poorly predicts the clinical outcomes in UTI patients as compared to *E. coli* which these breakpoints were established for. Inner colonies are a frequent phenomenon among both species, however, with unexplained heteroresistance and more inherent resistance mechanisms in *K. pneumoniae*, the IC phenomena in *K. pneumoniae* must be investigated thoroughly to provide the best possible treatment for patients.

3. Chapter 3: Fosfomycin susceptibility of clinical *K. pneumoniae* isolates (97)

3.1 Introduction

Several fosfomycin studies have highlighted the observation of IC within the zone of inhibition during fosfomycin disk diffusion testing (27–29, 98). The presence of these inner colonies present during within Etest inner inhibition zones has been linked to (non-fosfomycin) antibiotic heteroresistant phenotypes in other organisms (99). The difference in recommendations for interpreting DD results between organizations can lead to isolates with the same minimal inhibitory concentration (MIC) having varying zone diameters, ultimately resulting in different susceptibility interpretations. In 2018, a CLSI ad hoc working group reviewed data surrounding these DD inner colonies. It was determined that the CLSI would not amend their methods because colonies within the zone were determined to be rare for *E. coli*, there was a lack of clinical outcomes data, and there were concerns that amending this method might imply that colonies could be ignored for other non-*E. coli* organisms where additional resistance genes (e.g., *fosA*) could be attributed to their occurrence (https://www.idsociety.org/idsa-newsletter/september-26-2018/clsi-updates-from-the-clsi-subcommittee-on-susceptibility-testing).

In addition to the two organizations having differing interpretations of IC they also have considerably different breakpoints with EUCAST having more conservative zone diameter measurements and lower MIC breakpoints, especially for the oral formulation of fosfomycin (24, 26). In 2020, EUCAST revised their oral fosfomycin breakpoints to reduce the MIC resistance threshold from >32 µg/mL down to >8 µg/mL, and these breakpoints were restricted for use only

in *E. coli* from their previous approval for all Enterobacterales (52). However, fosfomycin breakpoints have not been reviewed by CLSI again since the EUCAST change.

In light of these differing breakpoints and contradictory IC interpretive criteria published by CLSI and EUCAST that are being extrapolated for use in non-*E. coli* Enterobacterales, we sought to compare categorical agreement of DD and AD between the extrapolated breakpoints from CLSI and EUCAST oral breakpoints to the approved EUCAST IV breakpoints. Additionally, we sought to assess the implications of the IC interpretation on zone diameter readings among a convenience collection of 262 clinical *K. pneumoniae* isolates.

3.2 Methods

3.2.1 Bacterial isolates

This study included a convenience sample of 262 *K. pneumoniae* clinical isolates collected from five US locations from 2013-2016 (97, 100–102) and 2022-2023. Of these isolates 38 were obtained in Boston, MA; 36 from Philadelphia, PA; 6 from Houston, TX; 82 from Minneapolis, MN; and 100 from Richmond, VA. Henceforth, this collection of 262 *K. pneumoniae* isolates and any subsets of the collection will be referred to as the "clinical isolate collection" **(Table 6)**. The anatomical culture sites of the isolates included urine (n = 244), blood (n = 9), sputum (n = 5), wounds (n = 3), and bone (n = 1). Phenotypic susceptibility profiles varied and included 49 confirmed extended-spectrum beta-lactamase (ESBL) producing isolates (97, 100–102).

3.2.2 Susceptibility testing and disk diffusion interpretation

Fosfomycin susceptibility was determined in duplicate, on separate days, via AD and DD using both CLSI and EUCAST procedures (24, 52, 103). When performing disk diffusion, which both organizations state that the methods and breakpoints apply only to *E. coli*, two measurements were collected: (1) following the CLSI procedures to measure the innermost zone of inhibition when a minimum of one single discrete IC was present; and (2) following EUCAST procedures to measure the outermost zone of inhibition, ignoring the presence of any discrete inner colonies when present.

Test isolates were inoculated onto blood agar plates for overnight growth at 37°C. Single isolated colonies were used to inoculate sterile saline to a density of ~1.5 x 10^8 CFU/mL. For disk diffusion, a single, sterile swab was soaked in the inoculated saline for at least 30 seconds, rotated to remove excess liquid, and used to inoculate a dry Mueller-Hinton agar (MHA) plate. Following plate inoculation, commercially available fosfomycin disks (Becton and Dickinson, Franklin Lakes, NJ) containing 200 µg of fosfomycin and 50 µg of G6P were placed in the center of plates. For AD, the saline suspension was diluted to 2 x 10^6 CFU in Mueller-Hinton II broth (MHB) where 5 µL (10^4 CFU) of the MHB suspension was placed onto a series of MHA plates supplemented with fosfomycin plus 25 µg/mL of G6P. The concentrations of fosfomycin used for AD ranged from 1 to 256 µg/mL. *Enterococcus faecalis* ATCC 29212 was included as a control strain and was run in parallel with test isolates for each susceptibility test.

Due to the lack of established breakpoints for *K. pneumoniae* and oral fosfomycin, the oral fosfomycin MIC breakpoints and all DD breakpoints for *E. coli* using both CLSI and EUCAST criteria, and the EUCAST MIC breakpoints for IV fosfomycin were applied as written **(Table 5)**.

3.2.3 Correlation of susceptibility testing

Correlation between CLSI AD, CLSI DD, $EUCAST_{oral}$ AD, $EUCAST_{oral}$ DD and $EUCAST_{IV}$ DD results were calculated using $EUCAST_{IV}$ AD as the reference method. Categorical agreement was achieved when a test MIC value agreed with the interpretive criteria (susceptible / intermediate / resistant) results from the given reference method (24, 104). Error rates were calculated per the CLSI standards of minor error, major error, and very major error. A minor error was classified as when either a test method deemed an isolate to be intermediate while the reference method deemed the isolate to be resistant or susceptible, or when a test method deemed an isolate to be susceptible or resistant and the reference method deemed it to be intermediate. A major error occurred when the reference method determined the isolate to be susceptible and the test method deemed it to be resistant. Very major error occurred when the reference method deemed the isolate to be resistant and the test method deemed it to be susceptible (24, 104).

Scattergram comparisons depicting zone diameter measurements and MIC values were constructed using R desktop version and Rstudio (Rstudio, Boston, MA).

3.3 Results

3.3.1 Susceptibility testing

Using AD, the MIC range and $MIC_{50/90}$ remained the same between both organizations, with a range of 1 to >256 µg/mL and $MIC_{50/90}$ of 32/256 µg/mL. The resulting interpretive categories of the isolates varied based on the testing procedures and which organizations' breakpoints were applied or extrapolated for use (**Table 5**). When using AD, 58.0% (n = 152) of isolates were considered susceptible when applying EUCAST IV breakpoints, compared to 77.5% (n = 203) when extrapolating CLSI breakpoints, and 8.4% (n = 22) when extrapolating $EUCAST_{oral}$ breakpoints (**Table 7**).

When using DD per each organization's standard operating procedures (SOP) for interpreting the results of DD (per *E. coli* breakpoints), 58.4% (n = 153) were susceptible per CLSI, 50.4% (n = 132) per $EUCAST_{IV}$, while only 6.2% (n = 15) were susceptible per $EUCAST_{oral}$. A total of 230 (87.8%) isolates displayed discrete inner colonies during at least one replicate of DD testing, accounting for differences in zone diameter measurements based on SOP recommendations. Less than half (n = 117) of isolates had at least five discrete IC. The zones of inhibition for this isolate set ranged from 6 to 24 mm using CLSI, and 6 to 30 mm using EUCAST SOP (**Figure 1**). When utilizing the CLSI DD SOP, the median zone of inhibition was 17 mm (considered susceptible [S ≥ 16mm]). When utilizing the EUCAST DD SOP, the collection had a median zone of inhibition of 20 mm (considered resistant [R_{oral} < 24mm, R_{IV} < 21mm]).

Using the EUCAST procedure for DD, when inner colonies were present the zone of inhibition was 2 to 13 mm larger than CLSI measurements on the same plate, with a median difference of 4 mm. When inner colonies were present, zone diameter distributions followed visually distinct

patterns when comparing measurements (**Figure 1**). EUCAST data clustered near the median zone measurement (20.5 mm) with the majority of the isolates in this collection having a zone of inhibition around that peak (16 to 24 mm), with few isolates having a zone of inhibition outside of that range. However, the CLSI data had a more even distribution across zone measurements, not highly concentrated around the median zone diameter (16.5mm). Additionally, comparison of the zone diameters per each organization's SOP for individual MIC values shows a greater number of zone diameters measured using the CLSI SOP compared to EUCAST (**Figure 2**). EUCAST's SOP (panels A and B) resulted in more isolates with the same MIC sharing a zone diameter measurement as compared to CLSI (panel C) where there were a greater amount of isolates that did not share a zone diameter measurement with another isolate at that MIC.

3.3.2 Correlation of susceptibility testing methods

Categorical agreements calculated showed that the greatest agreement was with CLSI AD (72.5%; 190/262 isolates) where 8.0% (n/N = 21/262) of the isolates had minor error and 46.4% (n/N = 48/110) of the resistant isolates had very major error due to the one-dilution difference in susceptible breakpoints (**Table 8**). The poorest categorical agreement was with $EUCAST_{oral}$ DD (45.0%, 118/262 isolates), where, 92.8% (n/N = 141/151) of susceptible isolates having major errors, and 2.7% (n/N = 3/110) of resistant isolates having very major errors.

3.4 Discussion

Several recent fosfomycin studies have highlighted the observation of inner colonies within the zone of inhibition during fosfomycin disk diffusion testing (27–29, 98). However, conflicting

recommendations for interpretation of these colonies from CLSI and EUCAST can lead to varying zone diameters and susceptibility interpretations despite isolates having isolates identical MIC values.

This study sought to compare the susceptibility of a collection of *K. pneumoniae* isolates (n = 262) between CLSI and EUCAST procedures and breakpoints for both AD and DD testing. The results were widely distributed where 6.2–77.5% of the isolates were considered susceptible per the currently established CLSI and $EUCAST_{oral}$ breakpoints for *E. coli*, and the $EUCAST_{IV}$ breakpoints for all Enterobacterales. The CLSI AD breakpoints resulted in the most isolates being categorized as susceptible, and $EUCAST_{oral}$ DD resulted in the most isolates being categorized as resistant. This is a result of $EUCAST_{oral}$ lower susceptibility breakpoint MIC (≤8 μg/mL) compared to $EUCAST_{IV}$ (≤32 μg/mL) and CLSI (≤64 μg/mL) and the difference in zone diameter breakpoints. Additionally, nearly half of isolates displayed ≥5 inner colonies during DD testing (44.7%) resulting in remarkably different zone diameter measurements between CLSI and EUCAST before extrapolating their different interpretive categories. The practice of taking inner colonies into account when measuring the zone of inhibition resulted in greater inconsistencies in DD measurements for isolates with identical MIC values. Thus, the zone diameter measurements followed different distributions, indicating that the differing interpretation of IC, when present, affects the susceptibility categories.

When comparing CLSI AD and $EUCAST_{oral}$ AD to $EUCAST_{IV}$ AD there was greater agreement with CLSI (72.5%) than $EUCAST_{oral}$ (50.4%).This is strictly a reflection of the single two-fold

dilution difference between the reference $EUCAST_{IV}$ susceptible breakpoint and CLSI as compared to the two, two-fold dilution difference in the MIC breakpoints between the two EUCAST breakpoints. Additionally the greatest agreement between any DD methods and breakpoints as compared to the reference of $EUCAST_{IV}$ AD was $EUCAST_{IV}$ DD (61.5%), followed by CLSI (53.5%), and the worst being $EUCAST_{oral}$ DD (45.0%), albeit none of these are strong agreements and have large amounts of major (7.3-92.8%) and very major (2.7-46.4%) errors. The error rates exceeded CLSI's published acceptable error rates of <10% minor errors, 3% major errors, and 1.5% very major errors (104, 105). This can be attributed to both the difference in breakpoints and the difference in zone measurements from the two organizations for the 87.8% of isolates where inner colonies arose during DD testing.

Mojica *et al.* recently conducted a similar analysis of *K. pneumoniae* isolates (n = 68) collected in Colombia; however, all were resistant to third-generation cephalosporins or carbapenems (29). They reported 90% of isolates to be susceptible per CLSI DD, 99% per CLSI AD, and 62% per EUCAST DD. This analysis was completed prior to EUCAST's AD breakpoint change (from 32 μg/mL to 8 μg/mL); therefore, a smaller amount of the 96% of isolates considered susceptible per EUCAST AD with the current breakpoint applied. Interestingly, the authors reported a much lower incidence (19%) of inner colonies whereas we saw them arise in 87.8% (44.7% of isolates having ≥5 IC) of our isolate collection (29). Similar to our results, Elliott *et al.* recently noted that inner colonies were "frequently present" for non-*E. coli* species during fosfomycin DD testing among a collection of *Klebsiella pneumoniae* carbapenemase (KPC)-producing isolates (28). All KPC-producing *K. pneumoniae* isolates (n = 24) carried *fosA* per whole-genome

sequencing. It is likely that most clinical *K. pneumoniae* isolates carry fosA and play a role in the formation of inner colonies. Even though our collection included isolates with susceptible phenotypes (n = 36), the $MIC_{50/90}$ values of 32/256 µg/mL were slightly higher than those reported (8/32 µg/mL) by Mojica *et al.* (29).

Furthermore, Abbott *et al.* reported a presence of *fosA* in all their *K. pneumoniae* isolates (n = 20) with 60% (n =12) of those screening positive for heteroresistance using a proposed disk elution method (87). Using an *in vitro* bladder infection model, the same authors found that baseline high-level heteroresistance in *K. pneumoniae* was associated with regrowth or treatment failure even when isolates had MICs that were lower than or equivalent to non-heteroresistant *E. coli* (106, 107). An evaluation of the correlation between heteroresistance and IC presence among our collection is currently being conducted.

Lucas *et al.* have also noted the presence of inner colonies in *E. coli* isolates during disk diffusion, but at a much lower incidence (8%) compared to the *K. pneumoniae* studies (27). This study was able to attribute some amount of the IC production to the presence of mutations in *uhpT,* which encodes the nutrient transport pump UhpT. Such mutations cause increased resistance to fosfomycin through reduced function in or loss of a functional UhpT pump which also reduced fosfomycin uptake. However, this increased resistance is believed to come at a fitness cost to the bacterial cells because they are less able or unable to utilize G6P as a nutrient with an altered UhpT pump; it is unknown how reliably these results can be applied to other non-*E. coli* Enterobacterales (27, 70).

One of the strengths of this study is the inclusion of both organizations' SOPs on the measurement of zone diameter as it results in different measurements when IC are present. The inclusion of an isolate set with diverse phenotypes collected from multiple geographic areas is also a strength of our work. However, this study is limited by its sample size as it is too small to perform an accurate epidemiological cutoff (ECOFF) value analysis or suggest appropriate breakpoints for *K. pneumoniae.* However, EUCAST's *MIC Distributions for Fosfomycin* database (retrieved 8/19/2022) displays the MIC distribution for 1396 *K. pneumoniae* isolates which follow a similar MIC distribution (ECOFF = 128 μg/mL) to the isolate collection in the current study (https://mic.eucast.org/search/).

In conclusion, our data suggest that CLSI and EUCAST frequently classify *K. pneumoniae* isolates with the same fosfomycin MIC into different interpretive categories based on the use of AD and DD, as well as the organizations' SOPs. Our data also show that the interpretation of IC greatly affects the resulting zone diameter and the interpretive category of the isolate, which supports both EUCAST and CLSI's current recommendations that DD is an unsupported method for non-*E. coli* organisms at the current breakpoints. The inconsistent categorization of isolates with identical MIC based on varying organizations' breakpoints and methods necessitates further studies to determine the clinical importance, most appropriate interpretation of IC present during fosfomycin DD testing, and hazards of extrapolating to non-*E. coli* Enterobacterales.

4. Chapter 4:Comparison of susceptibility among inner colony and clinical isolate collections susceptibility

4.1 Introduction

In 2018 a CLSI ad hoc working group (AHWG) was convened to evaluate the issue of IC during fosfomycin testing. After literature review—which focused on one paper (27) available at that time—the AHWG stated that IC were rare among the *E. coli* isolates for which these breakpoints apply (https://www.idsociety.org/idsa-newsletter/september-26-2018/clsi-updates-from-the-clsi-subcommittee-on-susceptibility-testing). These IC have been noted to occur at varying rates for different organisms ranging from 3.1-69% in *E. coli* and 19- 82.5% in *K. pneumoniae* (27, 29, 97). In real world settings, these breakpoints are extrapolated for use against other non-*E. coli* organisms which left the AHWG concerned that methods ignoring the IC would be extrapolated to organisms with more prevalent rates of IC production and additional resistance genes (e.g., *fosA*) (27–29, 87, 97, 106). Previous investigations have implicated *fosA* genes as a major source of fosfomycin resistance among *K. pneumoniae* due to the presence of both chromosomal and plasmid mediated variations in the *K. pneumoniae* population (28, 31–37).

In an effort to understand a possible contributor to the discordance between *in vitro* susceptibility testing results and *in vivo* clinical outcomes for fosfomycin treatment of *K. pneumoniae*, we sought to determine the frequency and susceptibility of IC as compared to their IC-producers among a geographically diverse collection of isolates (n = 262). We also aimed to investigate whether the heteroresistance status and presence of *fosA* among the clinical isolate collection contributed to the production of IC.

4.2 Methods

4.2.1 Bacterial isolates

This study included the same convenience sample of 262 *K. pneumoniae* clinical isolates outlined in section 3.2.1 **(Table 6)** (13, 97, 100, 101) , as well as the IC produced by these isolates during previous disk diffusion testing (n = 116) (97). As colony morphology was uniform for all IC, the closest IC to the disk was selected for subculturing and testing.

4.2.2 Susceptibility testing and interpretation

Fosfomycin susceptibility was determined in biological duplicate, on separate days, via BMD (24, 97). Isolates were tested on individual days in technical triplicate. The MIC for each test was read as the first concentration where there was no visible growth for all three technical replicates. The final MIC used for analysis was the modal MIC accounting for acceptable error of ±1 dilution.

Test isolates (clinical isolate collection: n = 262; IC collection n = 116) were inoculated onto blood agar plates for overnight growth at 37°C. Single isolated colonies were used to inoculate sterile saline to a density of ~1.5 x 10^8 CFU/mL. The saline suspension was diluted to 10^6 CFU in MHB where 50 µL of the MHB suspension was placed into three columns of a 96-well plate with each row containing a serial dilution of fosfomycin plus 25 µg/mL of G6P. Test concentrations of fosfomycin ranged from 2 to 256 µg/mL using 2-fold dilutions (24, 97). *Enterococcus faecalis* ATCC 29212 was included as a control strain and was run in parallel with

test isolates for each susceptibility test. All tests were performed according to recommendations in the CLSI M100 document (24).

Due to the lack of established oral fosfomycin breakpoints for *K. pneumoniae*, all analysis was extrapolated using oral breakpoints or directly applying IV breakpoints. CLSI and EUCAST *E. coli* oral breakpoints were extrapolated and pre-2024 $EUCAST_{IV}$ breakpoints were applied **(Table 5)**.

4.2.3 Heteroresistance screening

A modified disk elution screening test from Abbott *et al.* for heteroresistance was performed on a subset of the IC-producer and IC isolates to compare the frequency of heteroresistance populations between the susceptible isolates that produced ≥5 IC (n=44) and those that never produced IC (never producers; NP) (n=14) (87). Isolates were included in the screening if they were a susceptible isolate (per CLSI; ≤64 µg/mL) as either an IC-producer or NP. IC-producers needed to meet additional criteria of a resistant IC (per CLSI; ≥256 µg/mL), produced ≥5 IC, and a difference in IC and IC-producer MIC of at least three 2-fold dilutions **(Figure 3)**.

In the disk elution test, 6 commercially available fosfomycin disks (200µg fosfomycin, 50µg glucose-6-phosphate) (Becton and Dickinson, Franklin Lakes, NJ) were added to 1.9 mL of Mueller-Hinton broth in a test tube and allowed to elute for 90 minutes to approximate a fosfomycin concentration of ~420 µg/mL (68-70% of the total disk fosfomycin volume) (87).

Overnight broth cultures containing ~10^8 CFU/mL were used to inoculate both a disk-free control and the experimental tubes at 100µL. Tubes were incubated at 37 °C for 72 hours and assessed for turbidity visible to the unaided eye. An internal IC strain (B128D) with an MIC >1024 µg/mL was used as a positive growth control, *E. coli* ATCC 25922 as a negative growth control, and a bacteria-free negative control were run in parallel with test isolates.

Results underwent linear regression to determine the odds ratio for heteroresistance and being an IC-producer. Statistical data were processed using R version 4.3.2 and Rstudio Desktop version 2023.12.1 (Rstudio, Boston, MA). Packages used were the stats version 4.3.2 and base version 4.3.2.

4.2.4 Presence of fosA

Isolates that met the same inclusion criteria **(Figure 3)** as the heteroresistance screening were tested for the presence of *fosA* genes. DNA extractions were performed using the Dneasy mini kit (QIAGEN; Hilden, Germany). The presence of *fosA* was determined via PCR amplification using primers for *fosA* (F: ATCTGTGGGTCTGCCTGTCGT; R: ATGCCCGCATAGGGCTTCT) and *fosA3* (F:GCGTCAAGCCTGGCATTT; R:GCCGTCAGGGCTGAGAAA (23). The amplification product was run on gel electrophoresis for presence of an amplicon of ~271bp and ~282bp for *fosA* and *fosA3*, respectively (96). The presence of *fosA* and *fosA3* genes were also analyzed in relation to positive heteroresistance screenings via Fisher's Exact Test.

4.3 Results

4.3.1 Susceptibility testing and interpretation

The MIC values ranged from ≤2 to >256 µg/mL for the clinical isolate collection (n = 262) and 32 to >1024 µg/mL for IC (n = 116) **(Table 9)**. By extrapolating *E. coli* breakpoints, 50.8% (n = 133) of the clinical isolate collection were susceptible by CLSI breakpoints as well as 24.4% (n = 64) and 3.8% (n = 10) by $EUCAST_{IV}$ and $EUCAST_{oral}$ breakpoints, respectively. In addition, the same collection was resistant in 29.0% (n = 76), 75.6% (n = 198), and 96.2% (n = 251) of isolates per CLSI, $EUCAST_{IV}$, and $EUCAST_{oral}$ breakpoints, respectively. The $MIC_{50/90}$ for this collection was 64/256 µg/mL.

The IC collection was susceptible 3.4% (n =4) and 1.7% (n = 2) by CLSI and $EUCAST_{IV}$, respectively, with no susceptible isolates per $EUCAST_{oral}$ breakpoints. The same collection had 93.2% (n = 109) resistant isolates per CLSI, 98.3% (n = 114) per $EUCAST_{IV}$ and 100% (n = 116) per $EUCAST_{oral}$. The $MIC_{50/90}$ for this collection was >1024/>1024. IC isolates had an MIC that was up to seven 2-fold dilutions higher than their respective IC-producers and a modal increase of three dilutions **(Figure 4)**. A true increase in MIC was seen in 89.7% of isolates as two isolates had no increase in MIC and another group had an increase of a single 2-fold dilution (n = 10) which is acceptable error and considered the same MIC.

4.3.2 Heteroresistance screening

Of the susceptible collection of IC-producer strains, 95.3% (n/N = 41/43) screened positive for heteroresistance(**Table 10)**. The two isolates that had a negative screening were M063 (MIC [P/IC]: 32/1024 µg/mL) and V057 (MIC [P/IC]: 64/>1024 µg/mL). Among the NP isolates,

64.3% (n/N = 9/14) screened positive for heteroresistance. The NP isolates that screened negative had MIC of 8 µg/mL (B254, M041, M073), 32 µg/mL (M048), and 64 µg/mL (M055). The difference in rates of a positive heteroresistance screening was significant ($p < 0.01$). Additionally, IC-producers had 1.71 greater odds (95% confidence interval 1.24:2.34; $p < 0.01$) of screening heteroresistant as compared to NP isolates.

4.3.3 Presence of fosA/fosA3 genes

Of the NP isolates, 57.1% (n/N = 8/14) were *fosA* positive. Isolates M041 and M073 screened negative for the heteroresistant subpopulation and were *fosA* positive, while all other heteroresistance negative isolates were *fosA* negative. An additional three heteroresistance positive isolates (MIC: 64 µg/mL) were negative for *fosA*. There was no significant relationship between heteroresistance and *fosA* in NP ($p = 0.58$). None of the NP isolates contained a *fosA3* plasmid.

Of the IC-producer, 58.1% (n/N = 25/43) were *fosA* positive. Isolate V057 screened negative for the heteroresistant subpopulation and was *fosA* negative, in opposition to M063 that screened negative for heteroresistance but was *fosA* positive. An additional 15 isolates that screened positive for heteroresistance (MIC: 32-64 µg/mL) were negative for *fosA*. There was no significant relationship between heteroresistance and *fosA* in IC-producers ($p >0.9$). None of the IC-producers contained a *fosA3* plasmid.

Of the IC isolates, 39.5% (n/N = 17/43) were *fosA* positive. Some IC were positive for *fosA* despite the fact that they arose from *fosA* negative IC-producers (n = 4). Of the *fosA* negative IC isolates 46.2% (n/N = 12/26) came from *fosA* positive IC-producers. There was no significant relationship between the presence of *fosA* in the IC and heteroresistance of the IC-producer from which these IC arose (p >0.9). None of the IC isolates contained a *fosA3* plasmid.

4.4 Discussion

In addition to the occurrence of more frequent IC, the presence of inherent *fosA* genotypes and noted heteroresistance are cited reasons why oral fosfomycin may be considered a poor treatment option for *K. pneumoniae* UTI (28, 87). This study sought to compare the frequency and susceptibility of the IC arising from fosfomycin susceptibility testing as compared to their IC-producers. This was further expanded to explore the rates of heteroresistance among IC-producers and NP, as well as the presence of *fosA* among all three phenotypes (NP, IC-producers, and IC).

While the interpretation of the susceptibility testing differs by the breakpoints extrapolated or applied, the MIC range of the clinical isolate collection (n = 262) was lower (≤2 to >256 µg/mL) as compared to the IC collection (n = 116, 32 to >1024 µg/mL). The modal increase of three 2-fold dilutions from IC-producer to IC was also reflected in the $MIC_{50/90}$ of 64/256 µg/mL and >1024/>1024 µg/mL for the clinical isolate and IC collections, respectively.

Despite different phenotypes, both the IC-producers and NP had isolates that screened positive for heteroresistance using the modified screening test. IC-producers had greater odds (1.71; 95% CI 1.24:2.34; $p < 0.01$) of having a positive heteroresistance screening as compared to the NP. Heteroresistance appeared to be unrelated to *fosA* among the clinical isolate collection, as there was no significant relationship between heteroresistance and presence of *fosA* in IC- producers (($p > 0.9$) or NP ($p = 0.58$) per a Fisher's exact test. Additionally, 46.2% (n/N =12/26) of the IC that arose from *fosA* positive IC-producers lost or did not have an intact chromosomal *fosA* gene. The *fosA3* gene, which is most commonly seen on plasmids, was not present in any of the isolates.

Mojica *et al.* conducted BMD and DD testing on an isolate collection including *K. pneumoniae* isolates (n = 68) (29). Of their collection, 76 and 85% of isolates were susceptible per $EUCAST_{IV}$ and CLSI breakpoints, respectively. The authors reported lower $MIC_{50/90}$ values (8/32 µg/mL) compared to the current study (64/256 µg/mL). In addition to the lower MIC, the authors noted a lower incidence of IC (19%) as compared to the current study (44.3%). Elliott *et al.* also noted that IC were frequently present for non-*E. coli* species during fosfomycin DD testing among a collection of *Klebsiella pneumoniae* carbapenemase (KPC)-producing isolates (28). All KPC-producing isolates (n = 24) in their collection carried *fosA* per whole-genome sequencing- notably higher than the 50.9% (n/N = 29/57) within the clinical isolate collection carrying a chromosomal *fosA* gene in the current study.

Additionally, Abbott *et al.* reported the presence of *fosA* in their *K. pneumoniae* isolates (n = 20), which is more in agreement with the findings of Elliott *et al.* than the current study (106). The IC-production phenotype was unknown for these isolates. Using the disk elution method modified for the current study, 60% (n/N = 12/20) of the Abbott's isolates screened positive for heteroresistant population with a high-level resistant subpopulation. The authors were unable to attribute the heteroresistance to an upregulation in *fosA* with quantitative PCR methods. In another study using an *in vitro* bladder infection model, it was shown that a baseline heteroresistance in *K. pneumoniae* was associated with regrowth and treatment failure despite having an MIC lower than or equivalent to non-heteroresistant *E. coli* (87, 107).

One of the strengths of the current study is addressing the problem from the three phenotype approach, looking at NP, IC-producers, and the IC themselves. Previous studies have looked at *fosA* presence in IC-producers but did not address the other two phenotypes. When heteroresistance was studied, it was done without the context of IC-production. This study is limited by the inclusion of only *K. pneumoniae* and the exclusion of *E. coli*; however, the regrowth with a susceptible MIC phenomenon is not seen as commonly in *E. coli* (107). Additionally, due to the volume of IC present among the 116 IC-producers we only subcultured and tested the closest IC to the disk, this means we may not have captured variations in the presence of *fosA* genes among the IC. The current study is limited in the geographic locations of isolate collection being only from United States locations and may only be representative of trends in North America. The lack of global diversity in isolates highlighted by the lack of *fosA3* within the collection, a *fosA* allele common across East Asia (86).

The production of ≥5 IC among clinical *K. pneumoniae* isolates is common and, in most cases, results in a subpopulation with increased MIC. The production of these IC results in greater odds of producing an heteroresistant population, however, there is still a notable amount of NP that also had a positive heteroresistance screening. The presence of chromosomal *fosA* appears to be unrelated to both IC production and a positive heteroresistance screening. The production of IC during fosfomycin disk diffusion is a predictor of heteroresistant populations, which is associated with poor clinical outcomes. Thus, the frequent presence of *K. pneumoniae* IC were associated with heteroresistance and should prompt avoidance of fosfomycin treatment when IC are present during DD testing.

5. Chapter 5: *In vitro* fitness and whole genome sequence analysis of fosfomycin susceptible and resistant isolates

5.1 Introduction

In disk diffusion (DD), one of the most frequently used susceptibility testing methods performed for fosfomycin, the presence of colonies growing within the zone of inhibition have been noted (27–29, 97). Enterobacterales utilize two main nutrient transport systems (*uhpT* and *glpT*) which are also responsible for fosfomycin uptake (76). UhpT is induced by glucose-6-phosphate (G6P), the supplementation of which is standard in commercially available disks (76). These IC have increased minimal inhibitory concentrations (MIC) as compared to the isolates from which they arise. However, it is conventionally hypothesized that the IC have reduced fitness compared to the respective IC-producer as well due to mutations within *uhpT*, due to the inability to utilize its substrate, G6P (27). Furthermore, mutations in *glpT* are associated with increased fosfomycin resistance in *E. coli* clinical isolates (70). These type of mutation, those disrupting the proper transcription and translation of *uhpT* and *glpT*, affect sugar phosphate utilization (27, 70, 76). The loss of ability to utilize G6P and G3P has been associated with decreased bacterial fitness and believed to cause counter selection—where loss of fitness mutants are unable to thrive in an environment with isolates without these mutations causing the mutants to die and non-mutants to thrive—*in vivo* (27, 70). It was further described that there was a reduced fitness in fosfomycin resistant *E. coli* as compared to susceptible isolates (70).

Taking into account the discordance between fosfomycin susceptibility testing results and clinical outcomes, where *K. pneumoniae* isolates with susceptible MIC (based on extrapolated *E.*

coli breakpoints) result in frequent treatment failure and regrowth (11, 14–18), and the frequency of IC, we aimed to determine the *in vitro* fitness of three phenotypes: IC never producers (NP), IC-producers, and IC. Additionally, this study aimed to investigate whether any genetic differences between IC and their IC-producer strains may be related to their occurrence during DD testing.

5.2 Methods

5.2.1 Bacterial isolates

This study included a convenience sample of 23 *K. pneumoniae* urine isolates collected from four US locations during 2013-2016 (n = 5) and 2022-2023 (n = 9) (13, 97, 100, 101). Susceptible isolates NP (n = 5), susceptible IC-producers (n = 9) and their corresponding resistant IC (isolates given the "D" designation [n = 9]) were included. Of these, one (M001) was confirmed to be an ESBL-producing isolate. IC-producer/IC pairs were selected from the 44 IC-producers that met inclusion criteria in section 4.3.2, and narrowed down to the IC producers with the largest number of dilutions separating their MIC from the respective IC. The 5 NP were those with the lowest MIC from each geographic collection location with NP.

5.2.2 Time growth experiments

Each isolate was grown in three media types: full-strength cation-adjusted Mueller-Hinton broth (CaMHB), ¼-strength CaMHB, and filtered sterilized pooled human urine (Lee BioSolutions, Maryland Heights, Missouri, USA) for 24 hours at 37°C, with samples taken at standard time intervals. Overnight cultures were utilized to inoculate 15mL flasks of media at ~10^5 CFU/mL. At hours 0, 1, 2, 3, 4, 6, 8, and 24, a 2.5mL sample of culture was taken for two technical

replicates, diluted in 10-fold serial dilutions and plated onto Mueller-Hinton agar (MHA). Plate counts were utilized to determine the CFU/mL present at the time of the measurement. Experiments were conducted in biological duplicate on separate days for each media type.

A growth curve of the mean value of four plate counts for each timepoint per media type was made for each isolate to visualize the different phases of bacterial growth. Graphs were normalized to a true starting concentration of 10^5 CFU/mL **(Figure 5, 6)**.

5.2.3 Generation time

Using the growth curve and generation time equation **(Figure 7)**, the average generation time during the logarithmic phase of growth was calculated for each isolate per media type. Student's T-tests were performed for each media type comparing individual IC-producer and IC, as well as the averages across IC-producers and IC, and IC-producers and NP.

5.2.4 Competition time growth curves/rates

Isolate pairs consisting of an IC-producer and its respective IC were co-inoculated in flasks containing full-strength CaMHB for 72 hours to test the fitness and growth rates when isolates were combined. Overnight cultures were utilized to inoculate 15mL flasks of media at 10^5 CFU/mL, where equal parts of the inoculum came from the IC-producers and IC. Samples containing 2.5 mL were taken at hours 0, 1, 2, 3, 4, 6, and 8 in technical duplicate. At hours 12, 24, 36, 48, and 60, 10µL of culture (culture A) was used to inoculate a 10ml flask of fresh MHB (culture B) to extend the logarithmic growth phase. Samples of 2.5mL were taken from both

cultures A and B at 12-hour increments as well as a final 2.5mL sample taken at hour 72, each in technical duplicate. Selective plating was utilized to differentiate between the growth from the IC-producer and IC. Each replicate was plated on standard MHA and on fosfomycin-supplemented MHA (containing 25 µg/mL G6P) at a concentration between the MIC of the IC-producers and IC at appropriate dilutions and incubated overnight. Both isolate types grew on the standard MHA, but only the IC grew on the fosfomycin-supplemented plates. The difference between the plate counts was used to determine the IC-producers' CFU/mL.

5.2.5 Whole genome sequence analysis

DNA extractions were performed on all isolates using the Dneasy mini prep using the Gram-negative extraction protocol (QIAGEN; Hilden, Germany) and the genomic DNA was sequenced by SeqCoast Genomics (Portsmouth, NH). Sequencing was performed using short read Illumina sequencing performed on NextSeq2000. The sequence data were assembled and annotated using the *Comprehensive Genome Analysis* tool from the Bacterial and Viral Bioinformatic Resource Center (BV-BRC) (108). The annotated genomes were uploaded to Geneious Prime (Dotmatics; Boston, MA) for further analysis.

Single gene sequence comparisons were completed for six genes associated with fosfomycin resistance **(Table 11)** (27, 28, 37, 68, 70, 73, 76, 78–84, 89–92, 94, 109–111). The copy numbers of genes of interest were assessed through Geneious Prime annotation search tools. The search function allowed for both the copy number and location of these genes to be assessed on the annotated genomes. Once located the *extract region* and *reverse complement*, when needed, tools

were used to isolate the singular gene sequence for comparison. Theses single sequences were aligned and compared for IC-producer/IC pairs using the *pairwise align* function. The alignment data provided percentage of shared sequence identity in both DNA and amino acid sequences as well as locations of any variations.

Genome wide analysis was conducted on Geneious Prime by mapping the IC contigs to the respective IC-producer sequence. The *annotate and predict* functions were used to find all single nucleotide polymorphisms (SNP) and larger variations. Variations were narrowed to those that caused amino acid changes within coding regions. The genes with variations between IC-producers and IC were compared across the nine pairs to identify the most common mutations from IC-producers to IC.

5.3. Results

5.3.1. Time growth experiments

The growth curves for full-strength and ¼-strength MHB followed approximately the same curve for each isolate when comparing to only itself while the urine curves were noticeably lower **(Figures 5, 6)**. Growth curves between individual IC-producers and IC pairs also followed approximately the same shape when looking at each media type **(Figures 5, 6)**.

5.3.2. Generation Time

Generation times among the isolate pairs ranged from 21.6-31.2 minutes in full-strength MHB, 23.4-32.4 minutes in ¼-strength MHB, and 21.0-34.8 minutes in urine **(Table 12)**. Differences in

generation time for IC-producer and IC pairs in all media conditions ranged from <0.6-4.2 minutes. Generation times were not significantly different ($\alpha = 0.5$) in any media type, with p-values ranging from 0.06-0.98 **(Table 13)**.

Generation times between IC-producers and IC as a collection were not significantly different in any media type. Inner colonies had shorter average generation times (difference of <0.6 minutes) in full- and (difference of 0.6 minutes) ¼-strength MHB, while IC-producers had shorter average generation times (difference of 1.2 minutes) in urine **(Table 14)**.

Between IC-producers and NP, as a collection, generation times did not yield clear evidence of a difference in fitness. NP had a significantly shorter average generation time of 3.0 minutes ($p = 0.03$) in full-strength MHB. There was no significant difference in generation times in ¼-strength MHB and urine (difference of 0.6 minutes), with IC-producers having a slightly shorter generation time among the urine cultures (3.6 minutes, $p = 0.06$) **(Table 14)**.

5.3.3. Competition growth curves/rates

The competition growth curves demonstrated a near steady logarithmic phase for both IC-producers and IC for most isolates **(Figure 8)**. The exception to this was isolates V044D and V001D, which began to experience death phase between hours 24-36 and 60-72, respectively, whereas their IC-producers (V044 and V001) did not **(Figure 8A and 7F)**.

Generation times among the IC-producers within the competition experiments ranged from 49.8-51.6 minutes and 47.4-70.2 minutes among the IC **(Table 12)**. There were no significant differences in the generation times between the IC-producers and their IC in competition (Table 3). However, the generation times for both the IC-producers and the IC doubled (22.2 – 31.2 minutes to 47.4 – 70.2 minutes) when in competition as compared to solo inoculation in full-strength MHB.

5.3.1. Whole genome sequence analysis

Most IC-producers shared 100% sequence identity with their IC among the six resistance genes analyzed. Of the IC that had mutations compared to their IC-producers, many of the mutations were silent single nucleotide polymorphisms (SNP).

The relevant mutations were within the sugar phosphate transporters (*glpT* and *uhpT*). One isolate pair (B131/B131D) was missing *glpT,* another pair (M001/M001D) had *glpT* gene split into two locations, similar to the split of *glpT* in B209D **(Table 15)**. As all bacterial genes are contiguous, a separation or split among the sequence is considered a variation/mutation. Three IC (B131D, M063D, and M001D) had different mutations in *uhpT.* One of the NP (B254) did not contain a copy of *fosA* and an IC isolate (V044D) had a mutation in *fosA.* Two NP (B254 and B275) had duplicate copies of *murA*, and one IC (M063D) had a mutation in the same gene. A single IC had a mutation in *pykF* as compared to its IC-producer.

All IC isolates had variations causing translational changes within an uncharacterized Major Facilitator Superfamily (MFS)-type transporter. No other variant gene or variants among a gene family were seen across all IC. Many of the isolates had mutations in multiple MFS-type transporters that were shared with other IC isolates. Aside from B131D, all IC isolates had a variant in at least one of three uncharacterized MFS-type transporters. Five isolates (B209D, M009D, M063D, V044D, and V094D) had mutations in a 1218bp MFS-type gene. Four isolates (M001D, V001D, V044D, and V094D) had mutations in a 1428bp MFS-type gene. The third MFS-type gene (1371bp) had three isolates with mutations (B209D, P550D, and V001D).

5.4. Discussion

The occurrence of fosfomycin-resistant IC among a collection of susceptible isolates of *K. pneumoniae* was common (n/N = 43/133; 32%), IC-producers and IC had reduced *in vitro* fitness compared to NP due to sugar-phosphate transporter mutations (17, 20). This study sought to explore the relationship between these IC and *in vitro* fitness, as well as potential genetic causes for the production of these IC among clinical *K. pneumoniae* isolates.

Among this collection, no significant *in vitro* fitness loss was observed from IC-producers to IC isolates in full-strength MHB, ¼-strength MHB, or pooled human urine. A small but significant ($p = 0.03$) difference in generation time was observed between the collections of IC-producers and NP isolates, where the NP had a shorter mean doubling time (3.0 minutes). This indicates that in most media types the phenotypic presentation (NP, IC-producer, or IC) had no effect on growth rate. When grown in competition, both IC-producers and IC had increased generation

times where observed generation time was nearly twice as long for single culture time growths. This implies that the competition for resources within the same culture had a greater effect on generation time. Additionally, the occurrence of two IC isolates that underwent the death phase as early as 24-36 hours post initial inoculation point to a difference in stability of the mutations among the IC isolates.

The potential genetic causes for the IC were not consistent across all isolate pairs, 56% (n = 5) of the isolate pairs had near identical copies of *uhpT* and *glpT* between the IC-producers and IC with unchanged amino acid sequences. The remaining pairs (n = 4) had variations changing the amino acid sequences in one or both of these transporters. Furthermore, three of the isolate pairs had no variations in the translational sequence of any of the six examined genes examined. New genes of interest were established as variations across MFS-type transporters were identified among all IC.

These data are contradictory to the findings in previous studies which exclusively studied *E. coli* IC. Lucas *et al.* sequenced five *E. coli* IC-producer/IC pairs when studying the presence of IC among fosfomycin DD testing (27). The study found deletions in *uhpT* in four IC that were not present in the IC-producer. This type of mutation affects G6P utilization, and has caused decreased bacterial fitness and is counter selected *in vivo* (27, 70). While the current study saw mutations within *uhpT* among three isolate pairs there was no loss of fitness observed in any of three media types for solo incubation or when grown in competition.

Furthermore, Nilsson *et al.*, found fosfomycin resistance was associated with mutations in *uhpT* and *glpT,* or the associated genes in *E. coli* (70). These isolates had mutations in the sugar-phosphate transporters which resulted in increased generation time in both the presence and absence of fosfomycin compared to isolates without these variants. In the current study, variations within *glpT* from IC-producer to IC were seen in a single isolate pair (B209/D) with no loss of *in vitro* fitness.

Abbott *et al.*, utilized an *in vitro* bladder infection model to study the treatment of *K. pneumoniae* UTI with fosfomycin (106). This study simulated the regular emptying (every two hours) of the bladder during both the infection and treatment portions of the model, far more frequently than the re-inoculation in the current study. In another study, Abbott *et al.*, examined heteroresistance of these isolates by exposing isolates to high-dose fosfomycin in MHB for 72 hours to visualize which isolates were able to grow despite the fosfomycin presence (87). They determined that a high level resistant heteroresistant subpopulation (similar to that seen with the IC) was associated with regrowth and treatment failure despite having an MIC lower than or equivalent to non-heteroresistant *E. coli* (87, 106).

Identification of mutations in MFS-type transporters as a mechanism for fosfomycin resistance in *K. pneumoniae* is novel to our knowledge; however, Sharma et. al, showed that a transporter in this same family is responsible for fosfomycin efflux in *Acinetobacter baumannii* (112). Other members of the MFS family exist as multidrug efflux pumps for many organisms including *K. pneumoniae* (113, 114). This includes *kpnGH*, an MFS-transporter responsible for the efflux of

drugs from several antibiotic classes among *K. pneumoniae* (115). The relationship between the fosfomycin-resistant IC and these common variations in the uncharacterized MFS-type transporter needs further study to determine if they are a factor in the production of these *K. pneumoniae* IC.

The strengths of this study are highlighted by the inclusion and comparison of the three IC-production phenotypes. By studying both IC/IC-producer pairs as well as NP isolates, the current study was able to observe and compare the *in vitro* fitness of three different types of isolates.

Among this collection of *K. pneumoniae* clinical isolates, there was no significant loss of *in vitro* fitness from the IC isolates. While the genetic cause of the IC remains unknown, some of the same genes where variations had previously been noted in *E. coli* studies were seen in this collection without the associated fitness loss seen in those studies. With the additional knowledge that heteroresistant *K. pneumoniae* are associated with regrowth and treatment failure in modeled systems, *K. pneumoniae* IC should be treated as clinically relevant pending *in vivo* testing as they have near equal fitness to their IC-producer.

6. Chapter 6: *In vivo* fitness of IC and IC-producers

6.1. Introduction

Discrete IC occurring within the zone of inhibition during fosfomycin disk diffusion (DD) testing have been documented among many organisms including *Klebsiella pneumoniae* (27–29, 97). These IC often have elevated fosfomycin minimal inhibitory concentration (MIC) values when compared to the IC-producers from which they arise. This is particularly problematic due to the discordance in guidelines for reading DD results as discussed in Chapter 3. The European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines state to measure the entire zone of inhibition, despite the presence of IC, whereas the Clinical and Laboratory Standards Institute (CLSI) guidelines state to measure the IC-free zone. Despite both organizations' clear statements that these breakpoints and guidelines apply only to *Escherichia coli*, they are frequently extrapolated to non-*E. coli* Enterobacterales (32, 33, 35–38, 41, 43, 45–49, 51, 56).

Previous studies that have documented fosfomycin non-susceptible IC have noted genetic differences between the IC-producers and IC (27). Some of the most common mutations and deletions between the IC-producers to IC cultures occur in sugar phosphate transporters, UhpT and GlpT. Both are cellular transporters responsible for fosfomycin uptake in Enterobacterales and are induced by their substrates—glucose-6-phosphate (G6P) and glycerol-3-phosphate (G3P) for UhpT and GlpT, respectively (69, 70, 73). Thus, disruptions in the expression of these genes affect the utilization of their substrates, can cause decreased bacterial fitness, leading to the conventional belief that only the IC-producer isolate is able to grow *in vivo*

(27, 70). However, most of these fitness studies occurred *in vitro* and limited work using a mouse model has occurred only recently among *E. coli* (110).

As Enterobacterales species, both *K. pneumoniae* and *E. coli* possess many of the same fosfomycin resistance mechanisms, however, *K. pneumoniae* have more inherent fosfomycin resistance mechanisms (68, 116). FosA is a metalloenzyme which inactivates fosfomycin by catalyzing the addition of glutathione. Unlike in *E. coli*, this resistance gene can be carried both chromosomally and via plasmids in *K. pneumoniae* (76, 77). Several studies have reported chromosomally carried *fosA* alleles among most *K. pneumoniae* isolates in their collections (28, 78–84).

We therefore sought to determine the urinary tract colonizing abilities of IC-producer/IC pairs, with known genotypes (*fosA*, *uhpT*, and *glpT*), in competition using a validated ascending urinary tract infection (UTI) mouse model.

6.2. Methods

6.2.1. Bacterial isolates

A subset of four isolate pairs, from 5.2.1, with varying fosfomycin MIC values and varied geographic collection sites was used for the ascending mouse UTI model work. Four susceptible IC-producer isolates and their corresponding resistant IC (isolates given the "D" designation) were included. Isolates were chosen for the large differences in MIC values between IC-producer and IC. B131/D and B209/D were collected from Boston, Massachusetts, in 2013 (100). B131/D

are both known to be missing *glpT*, and B131D has mutations within *uhpT*. B209D is known to have mutations in glpT not seen in B209. Isolates collected from Boston were known to come from patients with symptomatic infections; however, these data were not available for other geographic cites of collection (117). M001/D was collected in 2022 from Minneapolis, Minnesota, and confirmed to be an ESBL-producing isolate. M001/D contained a non-continuous copy of *glpT* and M001D has a mutation within *uhpT*. P550/D was collected in 2016 from Philadelphia, Pennsylvania (101). P550D contains no mutations among *fosA*, *uhpT*, *glpT*, *murA*, *pykA*, and *pykF* compared to P550.

6.2.2. Ascending urinary tract infection mouse model

An established ascending UTI mouse model, validated in *E. coli*, was used to determine the colonizing abilities of the IC-producers and IC in competition (118–121). All procedures involving animals were reviewed and approved by the Institutional Animal Care and Use Committee of the University of Minnesota (IACUC protocol number: 2102-38878A). The total duration of the model was five days from culture preparation to results, with inoculation occurring on day zero. Four isolate pairs were used to inoculate a total of 144 mice (35 to 39 mice per isolate pair).

On day -1, a single colony of overnight culture of the IC-producers and IC were used to inoculate separate flasks containing 250 mL of cation-adjusted Muller Hinton broth (MHB). Following overnight incubation at 37° C in a water shaker bath, the contents of the flasks were centrifuged to produce a pellet with a concentration of ~10^{11} colony forming units (CFU)/mL. This

concentration was estimated by serial dilution to ~10^8 CFU/mL followed by spectrophotometry and comparison to an established optical density (OD)/concentration curve. Concentration was confirmed via further serial dilution (~10^1 and 10^2 CFU/mL) and plating 200µL to count on day one. On day zero, the ~10^{11} CFU/mL inoculum of each strain (IC-producer and IC pair) was mixed and used to inoculate sedated mice via urinary catheter at a volume of 1µL/g mouse weight. Mice had water removed at least 15 minutes prior to being sedated via isoflurane. The mixed inoculum was delivered via pump (1070 Micron; Foredom Electric Company; Bethel, Connecticut) at a rate of 0.5µL/second, to reduce the likelihood of injury to the bladders of sedated mice. Water was returned to mice 30 minutes after the last mouse in the cage was inoculated.

On day two, mice were humanely sacrificed via carbon dioxide, and their bladders and kidneys were aseptically harvested and placed into separate sterile collection vials. After determining organ weight, each vial was homogenized in 2mL of sterile phosphate buffered saline (PBS). Homogenate was plated onto both drug-free and fosfomycin-containing Mueller-Hinton agar (MHA) plates. The drug-supplemented plates contained 25 µg/mL G6P and fosfomycin at a concentration at least two dilutions higher than the MIC of the IC-producer and at least two dilutions lower than the MIC of the IC in order to inhibit growth of the IC-producer isolate. The difference between the plate counts was used as the IC-producer count. This selective plating process was used to calculate bacterial burden and average CFU/mg of tissue for each experiment.

6.2.3. Whole genome sequence analysis of urinary tract associated virulence factors

DNA extractions of the isolates grown outside of the mouse model were performed as outlined in section 5.2.5. Single gene sequence comparisons were completed for virulence factor genes associated with bladder colonization (*fimF, fimG, fimH,* and *papC*) (49, 122–124). The *fimH* gene, a type 1 fimbrial adhesion factor regulated by both *fimF* and *fimG*. The *papC* gene, a homotypic cell adhesion factor.

6.3. Results

6.3.1. Ascending urinary tract infection mouse model

Of the mice inoculated (n = 144), 15% (n = 22) died prior to sacrifice **(Figure 9)**. This was near equally spread among the isolate pairs, where 14% of the inoculated mice (n/N = 5/35) died from each B131/D, M001/D, and B209/D, and 17% of inoculated mice (n/N = 7/39) died from the P550/D pair. Cause of death was not able to be assessed in the mice because they were either removed from cages by animal care staff or the postmortem interval was too long to harvest the tissues. The harvested mice had colonization in 53-80% in bladder tissue **(Table 16)**. Of the mice with bladder colonization, 53-81% had progression to kidney colonization in either one or both kidneys.

Colonization density was much greater among bladder tissue as compared to kidney tissues for both IC-producers and IC **(Table 17)**. Due to the wide variability in colonization density, most isolate pairs had no significant difference in density between IC-producer and IC. However, in

bladder tissue, isolate P550 had significantly higher ($p < 0.01$) colonization density than its IC (P550D).

The proportion of the bacterial burden attributable to each isolate type varied based on organ tissue and isolate pair **(Figure 10)**. On average the IC were a greater proportion of the bacterial burden in the kidney tissue for B131/D, bladder tissue for M001/D, and both bladder and kidney tissue for B209/D. The higher proportion of burden of B131D was significantly greater ($p = 0.046$) than B131. A significantly higher proportion of the bacterial burden was attributed to the IC-producer in kidney tissue for M001/D ($p = 0.016$) and both bladder ($p = 0.042$) and kidney tissue ($p = 0.036$) among P550/D.

6.3.2. Whole genome sequence analysis of urinary tract associated virulence factors

All isolate pairs contained all four virulence genes: *fimF, fimG, fimH,* and *papC* **(Table 18)**. None of the isolate pairs contained any variations with all isolate pairs sharing 100% sequence identity between corresponding IC-producers and IC.

6.4. Discussion

Conventional belief suggests that some of the most common genetic variations between fosfomycin IC-producers and their IC cause a decrease in bacterial fitness and would be selected against *in vivo* (27, 70). However, all previous studies have been conducted utilizing *E. coli* which do not contain the same inherent *fosA* genes and have less frequent heteroresistant cultures

(87, 107). This study sought to determine the urinary tract colonizing abilities of IC-producer/IC pairs in competition.

Of the mice that survived to harvest, 53-80% of mice had bladder colonization based on the isolate pairs. Kidney colonization occurred in 53-81% of the mice with bladder colonization. Colonization density was highly variable in both bladder and kidney tissue, with a much greater density observed in bladder tissue. The proportion of bacterial burden attributed to the IC was greater in B131/D in kidney tissue ($p = 0.046$) M001/D in bladder tissue and B209/D in both bladder and kidney tissue. Thus, IC have the ability to colonize the urinary tract at near equal rates as their IC-producer and in the case of 131D in kidney tissue at a significantly higher proportion.

All isolates contained copies of virulence factor genes associated with bladder colonization (*fimF, fimG, fimH,* and *papC*). Each isolate pair had 100% shared sequence identity between the IC-producer and IC for all four genes. This indicates that for these *fimH* and *papC* encoded adhesion factors there are no mutagenic caused differences in the ability of these isolates to adhere to the urinary tract via FimH or PapC. Thus, the differences in abilities to adhere to bladder tissue among isolate pairs is unrelated to these genes and is caused by an unrelated loss or gain of fitness within the IC.

Bermudez *et al.*, used a similar bladder colonization model with *E. coli* IC with known genetic mutations within *uhpBA, pykA,* and *pykF* (110) *UhpA* and *uhpB* are regulatory genes for uhpT

and necessary for its expression *in vivo*. Mice underwent transurethral inoculation of a single isolate, either a wild type or mutagenic *E. coli* strain. At 24 hours post inoculation there were comparable organ titers between the wild type and *pykA* mutants, *pykF* mutants, and *pykA/pykF* double mutants. At 16 hours post inoculation they noted comparable bacterial titers in both bladder and kidney tissue for wild type and *uhpBA* mutants (110). In contrast, the current study inoculated isolates in competition, which improved upon the understanding of the fitness of these isolate types when competing for the same limited space and nutrients.

The competition aspect of this study, inoculating isolates via a mixed culture, is one of its greatest strengths. *In vitro*, separate isolate inocula did not produce significantly different generation times in any tested medium; however, when in mixed culture, both isolates had extended generation times, and some IC isolates underwent a death phase. While the ascending mouse UTI model is translational and does not exactly replicate a human host, the competition aspect of the model does mimic the same mixed culture conditions within a human since clinical isolates collected from patients were used. However, this work is limited by the small number of isolates tested.

Among this collection, IC had varying abilities to colonize the urinary tract, though on average they had some presence in both tissue types across the four isolate pairs. Furthermore, they were able to be the primary bacterial burden in half the tissues with a significantly greater proportion of the burden in kidney tissue coming from B131D. This work reveals that genetic variations within *uhpT* and *glpT* did not alter the *in vivo* fitness of *K. pneumoniae* when colonizing the

mouse urinary tract emphasizing that fosfomycin IC should be considered clinically relevant, and not ignored during DD testing.

7. Chapter 7: Reflections and future directions

7.1. Clinical relevance of fosfomycin resistant *K. pneumoniae* inner colonies

The work described herein had provided valuable insight into the clinical relevance of fosfomycin resistant *K. pneumoniae* IC. The occurrence of IC among fosfomycin disk diffusion testing against *K. pneumoniae* was common, arising from 87.8% (n/N = 230/262) of the clinical isolate collection. When approached through the inclusion criteria of ≥5 IC, 44.7% (n/N = 116/262) of the collection was included for further analysis. A smaller subsection of these clinically collected isolates had a susceptible MIC when extrapolating *E. coli* breakpoints (n = 49). Most of the IC that arose from a susceptible *K. pneumoniae* clinical isolate were resistant to fosfomycin (n/N = 44/49). These IC isolates had a modal increase in MIC of three, 2-fold dilutions from their IC-producer. Furthermore, the production of these IC was associated with significantly greater odds of a positive heteroresistance screening (odds ratio: 1.68; 95% CI: 1.21-2.32; $p < 0.01$).

Upon analysis of genomic variations between IC-producers/IC, there was not a consistently mutated gene across the isolate pairs. Common mutations occurred among the sugar phosphate transporters where three IC had variations in *uhpT* not present in their respective IC-producers. Two of those isolate pairs had either shared absence or splitting of *glpT* with their respective IC-producer. An additional IC isolate contained unique variations compared to the respective IC-producer. Further variations were noted among single IC isolates within *fosA*, *murA*, and *pykA*.

Among the subset of isolates which underwent *in vitro* fitness testing, the generation times of isolates ranged from 21.6-31.2 minutes in full-strength MHB, 23.4-32.4 minutes in ¼-strength MHB, and 21.0-34.8 minutes in urine. With generation time differences in all media types not exceeding 4.2 minutes, there was no significant difference in the *in vitro* fitness of the IC compared to the IC-producers in monoculture. However, when comparing the NP isolate collection to the IC-producer isolate collection, the NP isolates had a significantly shorter average generation time of 3.0 minutes ($p = 0.03$) as monoculture in full-strength MHB. This demonstrates a lack of fitness change among IC isolates in monoculture as compared to IC-producers.

When generation times were assessed among a competition culture of IC-producers/IC, they were approximately double those of the monoculture generation times for both IC-producers and IC. Thus, when both isolate types were competing for nutrients, their growth was slowed. In addition, two of the nine IC among the isolate pairs experienced a death phase that was not seen among their respective IC-producers within the 72-hour culture period. This indicated that some of the hypothesized mutagenic causes for the IC production are more stable than others.

Among the isolates that underwent *in vivo* fitness testing via the ascending mouse UTI model, proportional bacterial burden varied between isolate pairs. On average, three isolate pairs (B131/D, M001/D, and B209/D) demonstrated higher average bacterial burden among IC in either bladder tissue, kidney tissue or both. Of which, the IC burden was significantly higher ($p = 0.046$) among B131D in kidney tissue. Isolate pair M001/D had a significantly greater ($p =$

0.016) IC-producer burden in the kidney tissue. Furthermore, P550/D had a significantly higher proportion of the burden attributable to the IC-producer in both the bladder ($p = 0.042$) and kidney tissues ($p = 0.036$). Thus, the IC isolates had the ability to colonize the urinary tract at near equal rates to their IC-producers and in the case of B131D, caused a significantly greater proportion of the colonization in some (kidney) tissues.

The works of this dissertation lead to the conclusion that fosfomycin-resistant *K. pneumoniae* IC should likely be considered clinically relevant. Due to their near equal generation times *in vitro* and colonizing abilities *in vivo*, their occurrence should not be ignored. These IC are likely to colonize the human urinary tract with fosfomycin-resistant organisms, and as Abbott *et al.*, has proved, these heteroresistant populations are associated with failed treatment and regrowth using a dynamic bladder model (87, 106). While not an approved susceptibility testing method by either CLSI or EUCAST, fosfomycin DD testing may be beneficial in determining clinical outcomes of fosfomycin treatment. The association between IC and heteroresistance could be critical in determining the likelihood of microbiological cure versus treatment failure and regrowth.

7.2. Future laboratory research

7.2.1. Fosfomycin dose dependent expression via RT qPCR

To understand how the presence of fosfomycin alters the expression of various resistance mechanisms, I propose a fosfomycin dose-dependent expression study utilizing reverse transcription quantitative polymerase chain reaction (RT qPCR).

Isolates will be exposed to two concentrations of fosfomycin, chosen to simulate the concentration of a susceptible MIC (8 µg/mL) (24, 26)and the average concentration seen clinically in the first 24 hours post fosfomycin dosage (350 µg/mL) (64, 125, 126). This will be accomplished by using pre-warmed (37° C) flasks of 15mL of MHB supplemented with 25 µg/mL of G6P (127) and either 0 µg/mL, 8 µg/mL, or 350 µg/mL of fosfomycin inoculated with 200µL of overnight broth culture—each containing ~10^8 CFU/mL. Flasks would be incubated for 2 hours in a 37° C shaker bath. Using optical density and an appropriate *K. pnemoniae* growth curve to determine the concentration of the culture 7.5x10^8 cells would be stabilized for RNA extraction using RNAprotect (QIAGEN; Hilden, Germany) (112, 128).

RNA extractions would be performed using the rNeasy mini kit (QIAGEN; Hilden, Germany). Template RNA will be converted to cDNA using iScript cDNA synthesis kit (Bio-Rad; Hercules, CA, USA). Reverse transcription quantitative polymerase chain reaction (RT qPCR) would be performed on QuantStudio 5 (Thermo Fisher Scientific; Waltham, MA, USA) using SYBR green chemistry.

Genes of interest would include *uhpT, glpT, murA,* and *fosA* to determine if fosfomycin exposure alters the expression of these resistance mechanisms. An additional reference gene (*recA*) would be recommended for use in analysis and normalization. While *K. pneumoniae* does not have an established reference gene for RT qPCR, Gomes *et al.*, determined that *recA* meets all the requirements for a reference gene (129).

7.2.2. Characterizing causal relationships between genetic variations and fosfomycin resistance

Following the genetic findings of this work it would be critical to determine any causative relationship between these variations and fosfomycin resistance. I would propose an approach similar to how Srinivasan *et al.* were able to characterize a novel multidrug efflux pump within *K. pneumoniae* (115).

This would be accomplished in part by creating primers and transforming them into an *E. coli* reference strain. Though the chromosomal genes would remain intact it would allow for the understanding of whether the additive nature of these gene products resulted in fosfomycin resistance in the cases of *fosA* and uncharacterized MFS variants identified in Chapter 5.3.4.

In several of the variations seen, the presence of a functional gene product would nullify any effects seen from an additional plasmid mediated copy. Variations within *uhpT, glpT, murA,* and *pykF* need to be transformed into a susceptible *K. pneumoniae* isolate with disrupted chromosomal copy(s) using plasmid mediated variant as a replacement. Because the MFS variants are currently uncharacterized it will also be necessary to complete this step for those genes.

These transformed isolates will then be subject to AD along with the unaltered isolates as reference to determine if an increase in MIC is observed due to the variation(s) in each of the genes of interest.

7.3. Future clinical research

To determine the relationship between fosfomycin IC and treatment failure, I propose a retrospective analysis of patients given fosfomycin to treat their *K. pneumoniae* UTI along with collection of their causative isolate. This would involve obtaining both patient records regarding the UTI treated with fosfomycin per prescriber decision. For ethical purposes, antibiotic choice should not be influenced by a study (thus a retrospective analysis is necessary) as there is evidence that fosfomycin may not be equivalent to other available oral antibiotics for UTI caused by *E. coli*, and should be reserved for difficult cases where other IV or PO options may not be available (130).

Study design would require the inclusion of any patients who received fosfomycin for the treatment of a *K. pneumoniae* UTI with an available culture for duplicate DD testing within a research laboratory setting to determine IC-production status. Patients should be excluded should they have a history of UTI within the last six months or any antibiotic use within 30-days prior to culture. Other variables to collect would include classification of UTI as either a cUTI or uUTI, urinary catheter use, and complicating factors such as a uretal stent, neurogenic bladder, and polycystic kidney disease should be collected as well. Patient records would then be assessed 30 days after initial culture to determine if the patient returned for UTI symptoms. If a patient had a positive urine culture for *K. pneumoniae* or returned with UTI symptoms within those 30 days, the patient case would be considered a microbiological and clinical failure. Should a patient not return for UTI based follow-up within 30 days, they would be considered to have treatment

success. Patients could be lost to follow up if they returned to a different institution for UTI symptoms within the 30 days post initial culture, and this limitation would need to be considered in the analysis of these results.

Due to the limited use of fosfomycin against *K. pneumoniae* UTI, it may be beneficial to approach this as a multicenter study utilizing collaborators across the United States. By assessing multiple geographic areas, the patient/isolate collection would be larger and likely more representative of the types of *K. pneumoniae* UTI seen in the United States.

Table 1. Collection and susceptibility testing practices for fosfomycin against *Klebsiella* spp. in international studies.

Number of isolates	Isolated organism	Geographic location	Source of Isolate	Testing Method	Breakpoints used	Author
n = 250	*Klebsiella* spp.	Poland	[a]Urine, wounds, blood, lower respiratory, intra-abdominal infection, cerebrospinal fluid, and other clinical materials	Agar dilution	$EUCAST_{IV}$	Kowalska-Krochmal, *et al.*, 2022 (53)
n = 30	*K. pneumoniae*	Germany, United States	--	Agar dilution	$EUCAST_{IV}$	Goer, *et al.*, 2022 (54)
n = 68	*K. pneumoniae*	United States	--	Agar dilution, disk diffusion, ETEST	CLSI	Endimaiani, et.al., 2010 (31)
n = 155	*Klebsiella* spp.	Belgium, United Kingdom, Italy, Spain, Russia	Urine	Agar dilution, disk diffusion	$EUCAST_{oral}$	Tutone, *et al.*, 2022 (55)
n = 1350	*K. pneumoniae* (n = 1252), *K. oxytoca* (n = 98)	South Africa	Urine	Disk diffusion	CLSI	Mothibi, *et al.*, 2020 (32)
n = 28	*K. pneumoniae*	Iran	Urine	Disk diffusion	CLSI	Yekani, *et al.*, 2018 (33)
n = 46	*K. pneumoniae*	India	Urine	Agar dilution, disk diffusion	CLSI	Behera, *et al.*, 2018 (34)
n = 232	*K. pneumoniae*	Lebanon	Urine, body screen,	Disk	CLSI	Araj, et, al.,

			wound/pus/abscess, blood, respiratory, and other fluids	diffusion		2018 (35)
n = 643	*K. pneumoniae*	Czech Republic	Urine	Disk diffusion	EUCAST[b]	Fajfr *et al.*, 2017 (56)
n = 98	*Klebsiella* spp.	Turkey	Skin/ soft tissues, sputum, blood, genitourinary, sterile body fluids	Disk diffusion	CLSI	Demir and Buyukguclu, 2017 (36)
n = 56	*Klebsiella* spp.	Pakistan	Urine	Disk diffusion	CLSI	Khan, *et al.*, 2014 (37)
n = 305	*Klebsiella* spp.	Israel	Urine	Disk diffusion, Vitek 2	CLSI	Peretz, *et al.*, 2019 (38)
n = 50	*K. pneumoniae*	Germany	Urine, lower airway, wounds	Agar dilution	EUCAST[b]	Kaase, *et al.*, 2014 (57)
n = 9	*K. pneumoniae*	China	Urine	Broth microdilution	CLSI	Qiao, et.al., 2013 (39)
n = 19	*K. pneumoniae*	United States	Urine	ETEST	CLSI	Alexander, *et al.*, 2012 (40)
n = 100	*K. pneumoniae*	Taiwan	--	Agar dilution	EUCAST[b]	Lu, *et al.*, 2011 (59)
n = 66	*K. pneumoniae*	Taiwan	Urine	Disk diffusion	CLSI	Liu, *et al.*, 2010 (41)
n = 138	*K. pneumoniae*	Spain	Urine	Agar dilution	CLSI	de Cueto, *et al.*, 2006 (42)
n = 82	*K. pneumoniae*	Mexico	Urine	Disk diffusion	CLSI	Miranda-Novales, *et al.*, 2022 (43)
n = 988	*K. pneumoniae*	Italy	Urine, respiratory tract, blood,	BD Phoenix	EUCAST	Della Rocca, *et al.*, 2022(58)

			medical devices, wounds			
n = 38	*K. pneumoniae*	India	Urine	Agar dilution, disk diffusion, ETEST	CLSI, EUCAST	Bir, *et al.*, 2022 (44)
n = 32	*K. pneumoniae*	Turkey	Wound, urine, blood, and rectal	Agar dilution	EUCAST	Aydemir, *et al.*, 2022 (60)
n = 37	*K. pneumoniae*	Paksitan	Urine	Disk diffusion	CLSI	Iqbal, *et al.*, 2021 (45)
n = 100	*K. pneumoniae*	Iran	--	Disk diffusion	CLSI	Kashefieh, *et al.*, 2021 (46)
n = 472	*K. pneumoniae* (n = 381), *K. oxytoca* (n = 91)	Brazil	Urine	Disk diffusion	CLSI	Lima, *et al.*, 2021 (47)
n = 52	*K. pneumoniae*	Iran	Wound, urine, blood, tracheal aspirate	Disk diffusion	CLSI	Sharahi, *et al.*, 2021 (48)
n = 200	*K. pneumoniae*	Pakistan	Urine, blood, pus, catheter tip, body fluid, bile, nasal/tracheal swab, sputum, tissue	Disk diffusion	CLSI	Imtiaz, *et al.*, 2021 (49)
n = 139	*K. pneumoniae*	Spain	Urine, blood, bile, abdominal	Agar dilution	CLSI	Ballestero-Tellez, *et al.*, 2017 (50)
n = 1617	*K. pneumoniae*	Pakistan	Urine	Disk diffusion	CLSI	Abdullah, *et al.*, 2013 (51)

[a] Source of culture was not separated by isolated organism

[b] Analyzed data prior to EUCAST changing oral fosfomycin breakpoints in 2020

Table 2. Studies that extrapolated CLSI *E. coli* breakpoints for used in fosfomycin susceptibility testing against *Klebsiella* spp.

Number of Isolates	Isolated Organism	Testing Method	Susceptible n (%)	Resistant n (%)	$MIC_{50/90}$ (µg/mL) $IZ_{50/90}$ (mm)	MIC range (µg/mL) IZ range (mm)	Author
n = 68	*K. pneumoniae*	Agar dilution (CLSI)	63 (92.6)	3 (4.4)	16/64 µg/mL	4 - 256 µg/mL	Endimaiani, et.al., 2010 (31)
		Disk diffusion (CLSI)	43 (63.2)	2 (2.9)	≥16/≥14mm	≤10 - ≥20mm	
		ETEST (CLSI)	40 (58.8)	10 (14.7)	64/≥512 µg/mL	16 - ≥512 µg/mL	
n = 1350	*K. pneumoniae* (n = 1252), *K. oxytoca* (n = 98)	Disk diffusion (CLSI)	(94.0)	(6.0)	--	--	Mothibi, *et al.*, 2020 (32)
n = 28	*K. pneumoniae*	Disk diffusion (CLSI)	--	1 (3.6)	--	--	Yekani, *et al.*, 2018 (33)
n = 46	*K. pneumoniae*	Agar Dilution (CLSI)	42 (91.3)	2 (4.3)	16/64 µg/mL	1 - >512 µg/mL	Behera, *et al.*, 2018 (34)
n = 232	*K. pneumoniae*	Disk diffusion (CLSI)	(66-73)[a]	(27-34)[a]	--	--	Araj, et, al., 2018 (35)
n = 98[c]	*Klebsiella* spp.	Disk diffusion (CLSI)	--	35 (35.7)	--	--	Demir and Buyukguclu, 2017 (36)
n = 56	*Klebsiella* spp.	Disk diffusion (CLSI)	36 (64.0)	--	--	--	Khan, *et al.*, 2014 (37)

n = 305	*Klebsiella* spp.	Disk diffusion (CLSI)	--	192 (63.1)[d]	--	--	Peretz, *et al.*, 2019 (38)
n = 9	*K. pneumoniae*	Broth microdilution (CLSI)	9 (100)	0 (0.0)	1/32 µg/mL	--	Qiao, et.al., 2013 (39)
n = 19	*K. pneumoniae*	ETEST (CLSI)	--	--	16/205 µg/mL[e]	--	Alexander, *et al.*, 2012 (40)
n = 100	*K. pneumoniae*	Agar dilution (EUCAST)	85 (85.0)	15 (15.0)	8/64 µg/mL	1 - >512 µg/mL	Lu, *et al.*, 2011 (59)
n = 66	*K. pneumoniae*	Disk diffusion (CLSI)	38 (57.6)	--	--	--	Liu, *et al.*, 2010 (41)
n = 138	*K. pneumoniae*	Agar dilution (CLSI)	128 (92.8)	4 (2.9)	16/64 µg/mL	1 - 512 µg/mL	de Cueto, *et al.*, 2006 (42)
n = 82	*K. pneumoniae*	Disk diffusion (CLSI)	--	20 (24.4)	--	--	Miranda-Novales, *et al.*, 2022 (43)
n = 38	*K. pneumoniae*	Agar dilution (CLSI)	35 (92.1)	3 (7.9)	8/64 µg/mL	8 - 512 µg/mL	Bir, *et al.*, 2022 (44)
		Disk diffusion (CLSI)	32 (84.2)	6 (15.8)	--	--	
		ETEST	33 (86.8)	3 (7.9)	8/128 µg/mL	0.125 – 1024	

		(CLSI)				µg/mL	
n = 37	*K. pneumoniae*	Disk diffusion (CLSI)	28 (75.7)	9 (24.3)[d]	--	--	Iqbal, *et al.*, 2021 (45)
n = 100	*K. pneumoniae*	Disk diffusion (CLSI)	--	11 (11.0)	--	--	Kashefieh, *et al.*, 2021 (46)
n = 472	*K. pneumoniae* (n = 381), *K. oxytoca* (n = 91)	Disk diffusion (CLSI)	409 (86.7)	63 (13.3)	--	--	Lima, *et al.*, 2021 (47)
n = 52	*K. pneumoniae*	Disk diffusion (CLSI)	36 (69.2)	14 (26.9)	--	--	Sharahi, *et al.*, 2021 (48)
n = 200	*K. pneumoniae*	Disk diffusion (CLSI)	--	43 (22)	--	--	Imtiaz, *et al.*, 2021 (49)
n = 139	*K. pneumoniae*	Agar dilution (CLSI)	90 (64.7)	49 (35.3)	--	--	Ballestero-Tellez, *et al.*, 2017 (50)
n = 1617	*K. pneumoniae*	Disk diffusion (CLSI)	1253 (77.5)	173 (10.7)	--	--	Abdullah, *et al.*, 2013 (51)

[a] Susceptibility was tested in comparison to isolates susceptibility to ertapenem, imipenem, and meropenem

[b] Analyzed data prior to EUCAST changing oral fosfomycin breakpoints in 2020

[c] 79 of these isolates are ESBL-positive

[d] Denoted as non-susceptible, includes both resistant and intermediate isolates

[e] $MIC_{50/90}$ represents the entire isolate collection of *K. pneumoniae* (n = 19) and *Citrobacter freundii* (n = 1)

Table 3. Studies that applied or extrapolated EUCAST breakpoints for used in fosfomycin susceptibility testing against *Klebsiella* spp.

Number of Isolates	Isolated Organism	Testing Method	Susceptible n (%)	Resistant n (%)	$MIC_{50/90}$ (µg/mL) $IZ_{50/90}$ (mm)	MIC range (µg/mL) IZ range (mm)	Author
n = 250	*Klebsiella* spp.	Agar dilution[a]	165 (66.0)	85 (34.0)	32/512 µg/mL	0.5 - ≥512 µg/mL	Kowalska-Krochmal, *et al.*, 2022 (53)
n = 30	*K. pneumoniae*	Agar dilution[a]	20 (66.7)	10 (33.3)	--	--	Goer, *et al.*, 2022 (54)
n = 155	*Klebsiella* spp.	Agar dilution[b]	42 (27.1)	113 (72.9)	--	--	Tutone, *et al.*, 2022 (55)
		Disk diffusion[b]	3 (1.9)	151 (97.4)	--	--	
n = 643	*K. pneumoniae*	Disk diffusion[c]	517 (80.4)	64 (10.0)	--	--	Fajfr *et al.*, 2017 (56)
n = 50	*K. pneumoniae*	Agar dilution [c]	34 (68.0)	16 (32.0)	16/256 µg/mL	0.5 - >1024 µg/mL	Kaase, *et al.*, 2014 (57)
n = 100	*K. pneumoniae*	Agar dilution [c]	85 (85.0)	15 (15.0)	8/64 µg/mL	1 - >512 µg/mL	Lu, *et al.*, 2011 (59)
n = 988	*K. pneumoniae*	BD Phoenix [c]	744 (75.4)	244 (24.6)	--	--	Della Rocca, *et al.*, 2022 (58)
n = 38	*K. pneumoniae*	Agar dilution[c]	33 (86.9)	5 (13.1)	8/64 µg/mL	8 - 512	Bir, *et al.*, 2022

						µg/mL	(44)
		Disk diffusion[c]	19 (50)	19 (50)	--	--	
		ETEST[c]	34 (89.5)	4 (10.5)	8/128 µg/mL	0.125 – 1024 µg/mL	
n = 32	*K. pneumoniae*	Agar dilution[a]	29 (90.6)	3 (9.4)	--	--	Aydemir, *et al.*, 2022 (60)

[a] Analysis performed after EUCAST changed fosfomycin breakpoints in 2020, IV breakpoints applied

[b] Analysis performed after EUCAST changed fosfomycin breakpoints in 2020, oral breakpoints extrapolated

[c] Analysis performed prior to EUCAST changing oral fosfomycin breakpoints in 2020

Table 4. Clinical outcomes of fosfomycin monotherapy against susceptible *Klebsiella* spp. UTI compared to *E. coli*.

Citation, year of publication	Location	Study type	Fosfomycin dosing regimen	Number of patients	Fosfomycin susceptibility	Study outcomes	
						Clinical cure	Microbiological cure
Tumturk *et al.*, 2019	Turkey	Retrospective chart review	Single 3g oral dose	n = 12 *(Klebsiella* spp.)	100% susceptible per CLSI breakpoints	70.8% (n/N = 34/48) Clinical response rate was only reported as an entire collection (including *Enterobacter* spp. [n =4])	50% (n/N = 6/12)
				n = 32 (*E. coli*)	100% susceptible per CLSI breakpoints		79% (n/N = 25/32)
Matthews *et al.*,	United Kingdom	Retrospective cohort	n = 53 received one dose n = 8 received two doses n =14 received three or more doses	n = 9 *(K. pneumoniae*)	100% susceptible per EUCAST[a] breakpoints	22.2% (n/N = 2/9)	22.2% (n/N = 2/9)
				n = 52 (*E. coli*)	100% susceptible per EUCAST[a] breakpoints	76.9% (n/N = 40/52)	36.5% (n/N = 19/52)

Neuner *et al.*,	Clevland, Ohio, United States	Retrospective chart review	Average of 2.9 fosfomycin doses	n = 13 *(K. pneumoniae*)	92% susceptible per CLSI breakpoints	NA	46% (n/N = 6/13) *K. pneumoniae*
				n = 5 (*E. coli*)	80% susceptible per CLSI breakpoints	NA	100% (n/N = 5/5) *E. coli*
van Duin *et al.*,	United States	Prospective	NA	n = 15 *(K. pneumoniae*)	Testing not reported	NA	13.3% (n/N = 2/15)
Reid *et al.*,	United States	Retrospective review of kidney transplant patients	3-7 doses of 3g oral fosfomycin	n = 5 *(K. pneumoniae*)	Testing not reported	NA	20.0% (n/N = 1/5)

Seroy *et al.*,	United States	Retrospective chart review	1-14 doses of 3g oral fosfomycin	n = 23 *(K. pneumoniae*)	88.9% (n/N = 8/9) of isolates reported were susceptible per CLSI	43.5% (n/N = 10/23) without persistent or recurrent UTI	NA

[a] Analysis performed prior to EUCAST changing oral fosfomycin breakpoints in 2020; NA: not available

Table 5. CLSI and EUCAST breakpoints for oral and intravenous (IV) fosfomycin formulations.

	MIC (µg/mL)			DD[a] (mm)		
	Susceptible	Intermediate	Resistant	Susceptible	Intermediate	Resistant
CLSI[b]	≤ 64	128	≥ 256	≥ 16	13-15	≤ 12
$EUCAST_{IV}$[c]	≤ 32	NA	> 32	≥ 21	NA	<21
$EUCAST_{oral}$[d]	≤ 8	NA	> 8	≥ 24	NA	<24

[a]only uncomplicated UTI caused by *E. coli*

[b]CLSI M100, 33rd edition applies only to *E. coli* from the urinary tract and *E. faecalis*

[c]EUCAST IV 2023 breakpoints for use against all Enterobacterales

[d]EUCAST oral breakpoints only uncomplicated UTI caused by *E. coli*

CLSI: Clinical and Laboratory Standards Institute; EUCAST: European Committee on Antimicrobial Susceptibility Testing

Table 6. Clinical isolates collection stratified by geographic location.

Collection location	Number of isolates	Anatomical site of culture	Collection period	Proportion of IC-producers	Number of IC collected	Citations
Boston, MA	n = 38	Urine: n = 38	2013-2014	73.7%	n = 28	Hirsch *et al.*, 2015 (100) Chen *et al.*, 2017 (102) Hirsch *et al.*, 2020 (101) Bixby *et al.*, 2023 (97)
Philadelphia, PA	n = 36	Urine: n = 25 Sputum: n = 5 Blood: n = 3 Wound: n = 3 Bone: n = 1	2016	33.3%	n = 12	Chen *et al.*, 2017 (102) Hirsch *et al.*, 2020 (101) Bixby *et al.*, 2023 (97)
Houston, TX	n = 6	Blood: n = 6	2008-2015	33.3%	n = 2	Chen *et al.*, 2017 (102) Bixby *et al.*, 2023 (97)
Minneapolis, MN	n = 82	Urine: n = 82	2022-2023	23.2%	n = 19	
Richmond, VA	n = 100	Urine: n = 100	2022	56.0%	n = 56	

Table 7. Comparison of susceptibility for agar dilution and disk diffusion using both CLSI and EUCAST breakpoints.

	Susceptible (S)	Intermediate (I)	Resistant (R)	MIC/Zone Range	$MIC_{50/90}$
EUCAST oral breakpoints (n = 262)					
Agar dilution n (%)	22 (8.4)	NA	240 (91.6)	1 to >256 µg/mL	32/256 µg/mL
Disk diffusion n (%)	15 (6.2)	NA	247 (93.8)	6 to 30 mm	20/18 mm
EUCAST intravenous breakpoints (n = 262)					
Agar dilution[a] n (%)	152 (58.0)	NA	110 (42.0)	1 to >256 µg/mL	32/256 µg/mL
Disk diffusion n (%)	132 (35.0)	NA	130 (65.0)	6 to 30 mm	20/18 mm
CLSI breakpoints (n = 262)					
Agar dilution n (%)	203 (77.5)	21 (8.0)	38 (14.5)	1 to >256 µg/mL	32/256 µg/mL
Disk diffusion n (%)	153 (58.4)	54 (20.6)	55 (21.0)	6 to 24 mm	17/10 mm

[a] Approved breakpoint for all Enterobacterales, including *K. pneumoniae.*

CLSI: Clinical and Laboratory Standards Institute; EUCAST: European Committee on Antimicrobial Susceptibility Testing

Table 8. Agreement between CLSI and EUCAST susceptibility testing results as compared to $EUCAST_{IV}$ agar dilution (AD) as the reference method.

Method	Categorical agreement	Minor error	Major error	Very major error
$EUCAST_{IV}$ DD	161/262 (61.5)	NA	61/152 (40.1)	40/110 (36.4)
$EUCAST_{oral}$ AD	132/262 (50.4)	NA	130/152 (85.5)	0/110 (0.0)
$EUCAST_{oral}$ DD	118/262 (45.0)	NA	141/152 (92.8)	3/110 (2.7)
CLSI AD	190/262 (72.5)	21/262 (8.0)	0/152 (0)	51/110 (46.4)
CLSI DD	141/262 (53.8)	54/262 (20.6)	19/152 (7.3)	48/110 (43.6)

CLSI: Clinical and Laboratory Standards Institute; EUCAST: European Committee on Antimicrobial Susceptibility Testing; DD: disk diffusion

Table 9. Interpretation of MIC fosfomycin susceptibility testing results for clinical and inner colonies.

	Susceptible n (%)	Intermediate n (%)	Resistant n (%)	MIC Range (µg/mL)	$MIC_{50/90}$ (µg/mL)
Clinical isolate collection (n = 262)					
CLSI	133 (50.8)	53 (20.2)	76 (29.0)	≤2 to >256	64/256
$EUCAST_{IV}$	64 (24.4)	NA	198 (75.6)		
$EUCAST_{oral}$	10 (3.8)	NA	252 (96.2)		
Inner colony isolates (n = 116)					
CLSI	4 (3.4)	3 (2.6)	109 (93.9)	32 to >1024	>1024/>1024
$EUCAST_{IV}$	2 (1.7)	NA	114 (98.3)		
$EUCAST_{oral}$	0 (0.0)	NA	116 (100.0)		

CLSI: Clinical and Laboratory Standards Institute; EUCAST: European Committee on Antimicrobial Susceptibility Testing

Table 10. Heteroresistance screening and the presence of *fosA* for the clinical (n = 57) isolate collection, which was comprised of the inner colony (IC)-producers (n = 43) and never-producers (n = 14), along with the IC (n = 43) isolate collections.

	IC-producer (Y/N)	MIC (µg/mL)		Heteroresistance screening	*fosA*		*fosA*3	
		Clinical isolate	Inner colony		Clinical isolates	Inner colony	Clinical isolates	Inner colony
V044	Y	8	512	+	+	+	-	-
B131	Y	8	1024	+	+	+	-	-
B035	Y	16	1024	+	+	-	-	-
V046	Y	16	1024	+	-	-	-	-
M009	Y	16	>1024	+	+	+	-	-
B069	Y	32	256	+	+	-	-	-
M049	Y	32	256	+	-	+	-	-
B068	Y	32	1024	+	+	-	-	-
M063	Y	32	1024	-	+	+	-	-
V025	Y	32	1024	+	-	-	-	-
B120	Y	32	>1024	+	-	-	-	-
M001	Y	32	>1024	+	+	+	-	-
V001	Y	32	>1024	+	+	+	-	-
V033	Y	32	>1024	+	+	+	-	-

V094	Y	32	>1024	+	+	+	-	-
B019	Y	64	512	+	+	-	-	-
B090	Y	64	512	+	+	-	-	-
M021	Y	64	512	+	-	-	-	-
V065	Y	64	512	+	+	+	-	-
V083	Y	64	512	+	-	+	-	-
B093	Y	64	1024	+	+	-	-	-
B096	Y	64	1024	+	+	+	-	-
B121	Y	64	1024	+	-	-	-	-
B137	Y	64	1024	+	-	-	-	-
P550	Y	64	1024	+	+	+	-	-
V085	Y	64	1024	+	-	-	-	-
B132	Y	64	>1024	+	-	-	-	-
B209	Y	64	>1024	+	+	+	-	-
B233	Y	64	>1024	+	-	-	-	-
M054	Y	64	>1024	+	-	+	-	-
M058	Y	64	>1024	+	+	-	-	-
P570	Y	64	>1024	+	+	-	-	-

P615	Y	64	>1024	+	+	+	-	-
V008	Y	64	>1024	+	+	-	-	-
V014	Y	64	>1024	+	-	-	-	-
V018	Y	64	>1024	+	+	-	-	-
V027	Y	64	>1024	+	-	-	-	-
V037	Y	64	>1024	+	-	-	-	-
V055	Y	64	>1024	+	-	-	-	-
V057	Y	64	>1024	-	-	+	-	-
V058	Y	64	>1024	+	+	-	-	-
V076	Y	64	>1024	+	-	-	-	-
V096	Y	64	>1024	+	+	-	-	-
B254	N	8	NA	-	-	NA	-	NA
B275	N	8	NA	+	+	NA	-	NA
M041	N	8	NA	-	+	NA	-	NA
M073	N	8	NA	-	+	NA	-	NA
V078	N	16	NA	+	+	NA	-	NA
M048	N	32	NA	-	-	NA	-	NA
P653	N	32	NA	+	+	NA	-	NA

M010	N	64	NA	+	-	NA	-	NA
M011	N	64	NA	+	-	NA	-	NA
M055	N	64	NA	-	-	NA	-	NA
P628	N	64	NA	+	+	NA	-	NA
P531	N	64	NA	+	-	NA	-	NA
P547	N	64	NA	+	+	NA	-	NA
V016	N	64	NA	+	+	NA	-	NA

N: no; NA: not applicable; Y: yes

Table 11. Common resistance genes analyzed among whole genome sequence analysis.

	Function	Cause for fosfomycin resistance
fosA	Fosfomycin deactivating enzyme	Renders fosfomycin inactive once in the bacterial cell
uhpT	Sugar-phosphate (G6P) antiporter	Transporter of fosfomycin into bacterial cell, loss of function mutations prevents fosfomycin from reaching the cite of action
glpT	Sugar-phosphate (G3P) transporter	
murA	Enzymatic catalyst for cell wall peptidoglycan synthesis	Fosfomycin cite of action, certain mutations prevent from fosfomycin binding
pykA, *pykF*	Pyruvate kinase isozyme	Essential in the glycolysis portion of cell wall formation, mutations causing a reduction in activity of pyruvate kinases have been associated with fosfomycin resistance.

Table 12. Average generation times for isolates in full-strength Mueller-Hinton broth (MHB), ¼-strength MHB, filter sterilized urine, and in competition (full-strength MHB).

Isolate	Isolate type	MIC (μg/mL)	Generation Time (minutes)			
			Full-strength MHB	¼-strength MHB	Urine	Comp. MHB
V044	P	8	27.6 ± 1.8	30.6 ± 0.6	28.8 ± 1.2	53.4 ± 3.0
V044D	IC	512	26.4 ± 1.2	25.8 ± 3.6	32.4 ± 5.4	70.2 ± 7.2
B131	P	8	28.8 ± 2.4	25.8 ± 1.2	29.4 ± 2.4	51.6 ± 3.0
B131D	IC	1024	31.2 ± 1.2	27.6 ± 3.6	27.0 ± 3.0	56.0 ± 1.8
M009	P	16	28.2 ± 6.0	23.4 ± 2.4	23.4 ± 1.2	56.0 ± 1.8
M009D	IC	>1024	24.0 ± 0.6	23.4 ± 1.2	24.0 ± 1.2	53.4 ± 3.6
M063	P	32	27.0 ± 1.8	25.2 ± 1.2	24.6 ± 0.6	51.6 ± 2.4
M063D	IC	1024	24.6 ± 3.6	27.6 ± 3.6	25.8 ± 1.8	49.8 ± 3.0
M001	P	32	22.2 ± 4.2	25.2 ± 4.2	24.6 ± 0.6	49.8 ± 2.4
M001D	IC	>1024	22.2 ± 5.4	24.6 ± 3.0	25.8 ± 1.2	52.2 ± 4.2
V001	P	32	28.8 ± 0.6	30.0 ± 1.8	31.2 ± 0.6	49.8 ± 4.2
V001D	IC	>1024	31.2 ± 1.2	32.4 ± 2.4	34.8 ± 3.0	47.4 ± 5.4
V094	P	32	24.0 ± 4.2	27.0 ± 2.4	26.4 ± 1.2	49.2 ± 3.6
V094D	IC	>1024	24.6 ± 1.8	25.8 ± 2.4	26.4 ± 0.6	47.4 ± 3.6
P550	P	64	27.6 ± 1.2	26.4 ± 2.4	24.6 ± 1.8	51.6 ± 2.4
P550D	IC	1024	30.0 ± 3.0	27.0 ± 3.0	25.8 ± 1.2	50.4 ± 3.0
B209	P	64	28.8 ± 4.2	28.2 ± 2.4	26.0 ± 0.6	50.4 ± 5.4
B209D	IC	>1024	26.4 ± 4.8	27.6 ± 1.8	22.8 ± 2.4	50.4 ± 1.2
B254	NP	8	27.0 ± 4.2	28.8 ± 2.4	29.4 ± 3.6	--
B275	NP	8	24.6 ± 1.8	26.4 ± 1.2	26.4 ± 1.2	--
M041	NP	8	21.6 ± 2.4	24.6 ± 0.6	25.2 ± 1.8	--
M073	NP	8	22.8 ± 3.0	25.2 ± 0.6	25.2 ± 1.2	--
V078	NP	16	23.4 ± <0.6	26.4 ± 1.2	29.4 ± 2.4	--

Table 13. Difference in average generation times for individual IC-producers and IC pairs in full-strength Mueller-Hinton broth (MHB), ¼-strength MHB, filter sterilized urine, and in competition (Full-strength MHB).

Isolate	Media type	Difference in generation time (minutes)	p-value
V044/V044D	Full-strength MHB	1.2[a]	0.36
	¼-strength MHB	3.6	0.08
	Urine	3.6	0.32
	Competition	16.8	0.21
B131/B131D	Full-strength MHB	2.4	0.18
	¼-strength MHB	1.8	0.25
	Urine	2.4[a]	0.30
	Competition	3.6[a]	0.87
M009/M009D	Full-strength MHB	4.2[a]	0.26
	¼-strength MHB	<0.6	3.64
	Urine	3.6	0.16
	Competition	2.4	0.74
M063/M063D	Full-strength MHB	2.4[a]	0.38
	¼-strength MHB	2.4	0.16
	Urine	1.2	0.11
	Competition	1.8[a]	0.41
M001/M001D	Full-strength MHB	<0.6	0.98
	¼-strength MHB	1.2[a]	0.32
	Urine	1.2	0.36
	Competition	2.4	0.44
V001/V001D	Full-strength MHB	2.4	0.06
	¼-strength MHB	2.4	0.27
	Urine	3.6	0.07

	Competition	2.4[a]	3.69
V094/V094D	Full-strength MHB	3.6	0.72
	¼-strength MHB	1.2[a]	0.40
	Urine	<0.6[a]	0.51
	Competition	1.8[a]	0.72
B209/B209D	Full-strength MHB	2.4[a]	0.54
	¼-strength MHB	3.6[a]	0.84
	Urine	1.8	0.34
	Competition	<0.6	0.96
P550/P550D	Full-strength MHB	2.4	0.25
	¼-strength MHB	3.6	0.73
	Urine	1.2	0.38
	Competition	1.2[a]	3.67

[a] IC had the shorter generation time

Table 14. Difference in average generation time among never producer (NP), IC-producer, and IC collections.

Isolate	Media type	Difference in generation time (minutes)	p-value
Producers/IC	Full-strength MHB	<0.6[a]	0.30
	¼-strength MHB	<0.6[a]	0.98
	Urine	1.2	0.32
	Competition	3.6	0.48
NP/Producers	Full-strength MHB	3.0	0.03
	¼-strength MHB	0.6	0.40
	Urine	3.6[b]	0.06

[a] IC had the shorter generation time

[b] IC-producers had the shorter generation time

IC: inner colony; IC-producers: inner colony producer

Table 15. Genetic variations within common fosfomycin resistance genes among isolates.

	fosA	*glpT*	*uhpT*	*murA*	*pykA*	*pykF*
V044	+	+	+	+	+	+
V044D	+[a]	+	+	+	+	+
B131	+	-	+	+	+	+
B131D	+	-	+[b]	+	+	+
M009	+	+	+	+	+	+
M009D	+	+	+	+	+	+
M063	+	+	+	+	+	+
M063D	+	+	+[c]	+[d]	+	+
M001	+	±	+	+	+	+
M001D	+	±	+[e]	+	+	+
V001	+	+	+	+	+	+
V001D	+	+	+	+	+	+[f]
V094	+	+	+	+	+	+
V094D	+	+	+	+	+	+
B209	+	+	+	+	+	+
B209D	+	±[g]	+	+	+	+
P550	+	+	+	+	+	+
P550D	+	+	+	+	+	+
B254	-	+	+	+[h]	+	+
B275	+	+	+	+[h]	+	+
M041	+	+	+	+	+	+
M073	+	+	+	+	+	+
V078	+	+	+	+	+	+

Many isolates had silent mutations from IC-producer to IC that are not noted as the amino acid chain was conserved

± gene is split into two locations and is likely non-functional, but functionality is unknown

[a] Non-conserved missense mutation at 52

[b] Many mutations including a frame shift at 1320 that lead to a premature stop codon

[c] Conserved missense mutation at 1300

[d] Non-conserved missense mutation at 617 and conserved missense mutation at 629

[e] Insertion of 9 new bp at 517

[f] Conserved missense mutation at 397

[g] Missing 3 bases at 265

[h] Two copies of this gene are present

Table 16. Rates of tissue colonization among mouse bladder and kidneys. “Mice w/ bladder colonization” was calculated as the number of the mice whose organs were harvested that had bacterial growth in bladder tissue. “Mice w/ kidney colonized” was calculated as the number of mice with bladder colonization that progressed to bacterial growth in one or both kidneys. “Total kidneys colonized” was calculated as the number of kidneys with bacterial growth among the mice with bladder colonization as colonization in either one or both kidneys was not consistent.

Isolate pair	**Mice w/ bladder colonized**		**Mice w/ kidney colonized**		**Total kidneys colonized**	
	Total	**n (%)**	**Total**	**n (%)**	**Total**	**n (%)**
B131/D	30	24 (80)	24	16 (67)	48	25 (52)
M001/D	30	21 (70)	21	17 (81)	42	30 (71)
P550/D	30	17 (53)	17	9 (53)	34	16 (47)
B209/D	32	16 (53)	16	10 (63)	32	10 (31)

Table 17. Average tissue concentration of bacterial cells stratified by isolate pair and tissue type. Bacterial concentration was normalized to the measured weight of the tissue prior to homogenization. The difference in concentrations of IC-producers and IC were compared using Fisher's exact test to determine if there was a significant difference in concentration for each tissue type.

	Bladder (CFU/mg tissue)			**Kidney (CFU/mg tissue)**		
	IC-producer	IC	p-value	IC-producer	IC	p-value
B131/D	73.95 ± 144.3	84.8 ± 227.0	0.15	9.1 ± 31.7	8.5 ± 16.7	0.20
M001/D	93.4 ± 172.8	252.7 ± 516.5	0.09	3.03 ± 4.4	6.7 ± 8.8	0.14
P550/D	74.4 ± 110.0	18.07 ± 31.3	<0.01	32.5 ± 75.2	4.6 ± 8.6	0.06
B209/D	13.1 ± 23.0	14.5 ± 30.6	0.11	0.8 ± 0.8	1.0 ± 1.4	0.07

Table 18. Presence of common bladder colonization virulence factors.

	fimF	*fimG*	*fimH*	*papC*
B131	+	+	+	+
B131D	+	+	+	+
M001	+	+	+	+
M001D	+	+	+	+
P550	+	+	+	+
P550D	+	+	+	+
B209	+	+	+	+
B209D	+	+	+	+

Figure 1. Histogram of measured fosfomycin disk diffusion zone of inhibition diameters using both EUCAST and CLSI guidelines for inner colony (IC) interpretation. EUCAST measurements were the entire diameter of the zone of inhibition, irrespective of presence of IC presence. CLSI measurements were the IC free zone of inhibition.

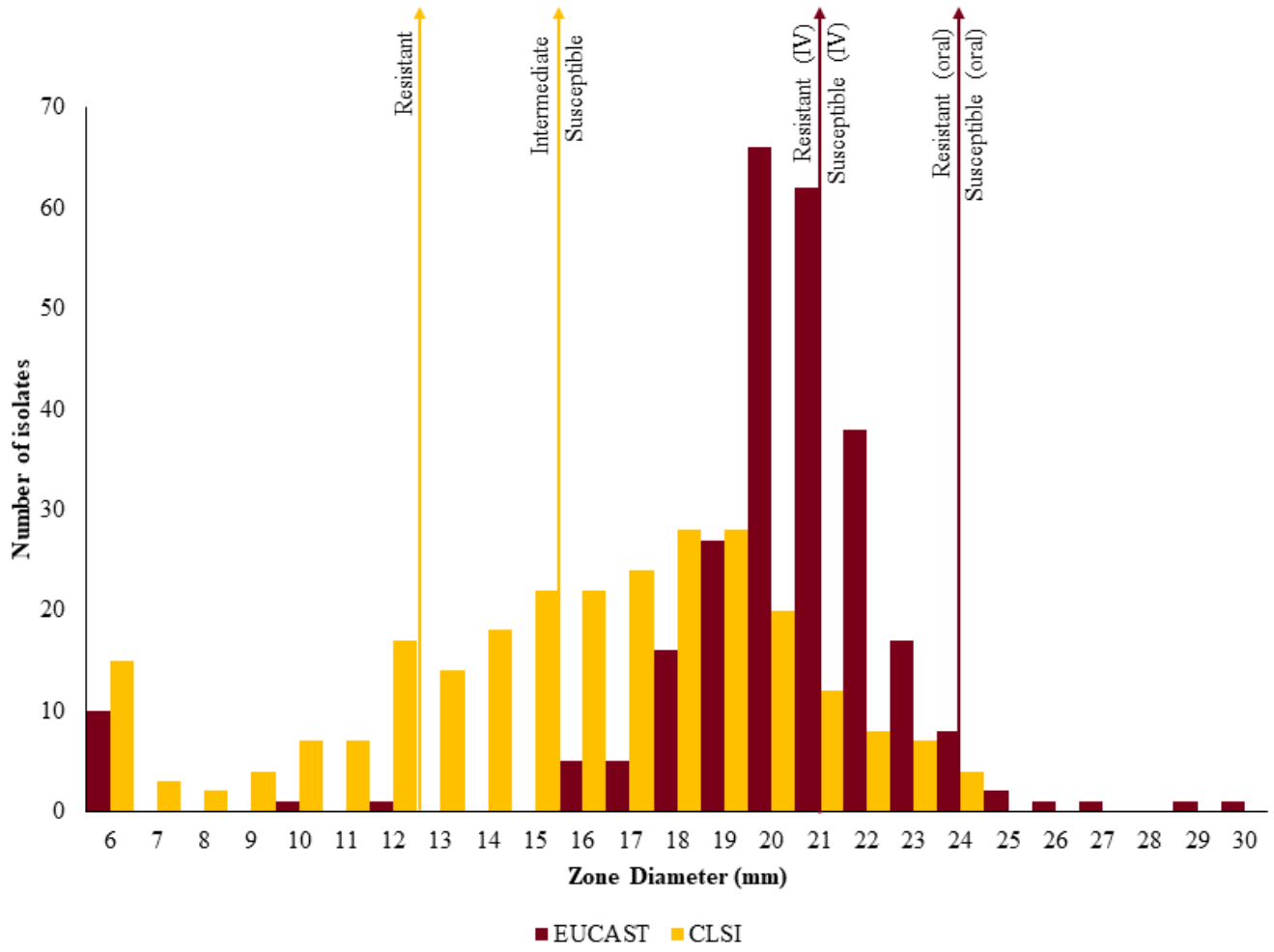


Figure 2. Scattergram comparison of agar dilution MIC measurements and disk diffusion zone diameter measurements for $EUCAST_{IV}$ (A) $EUCAST_{oral}$ (B) and CLSI (C) breakpoints.

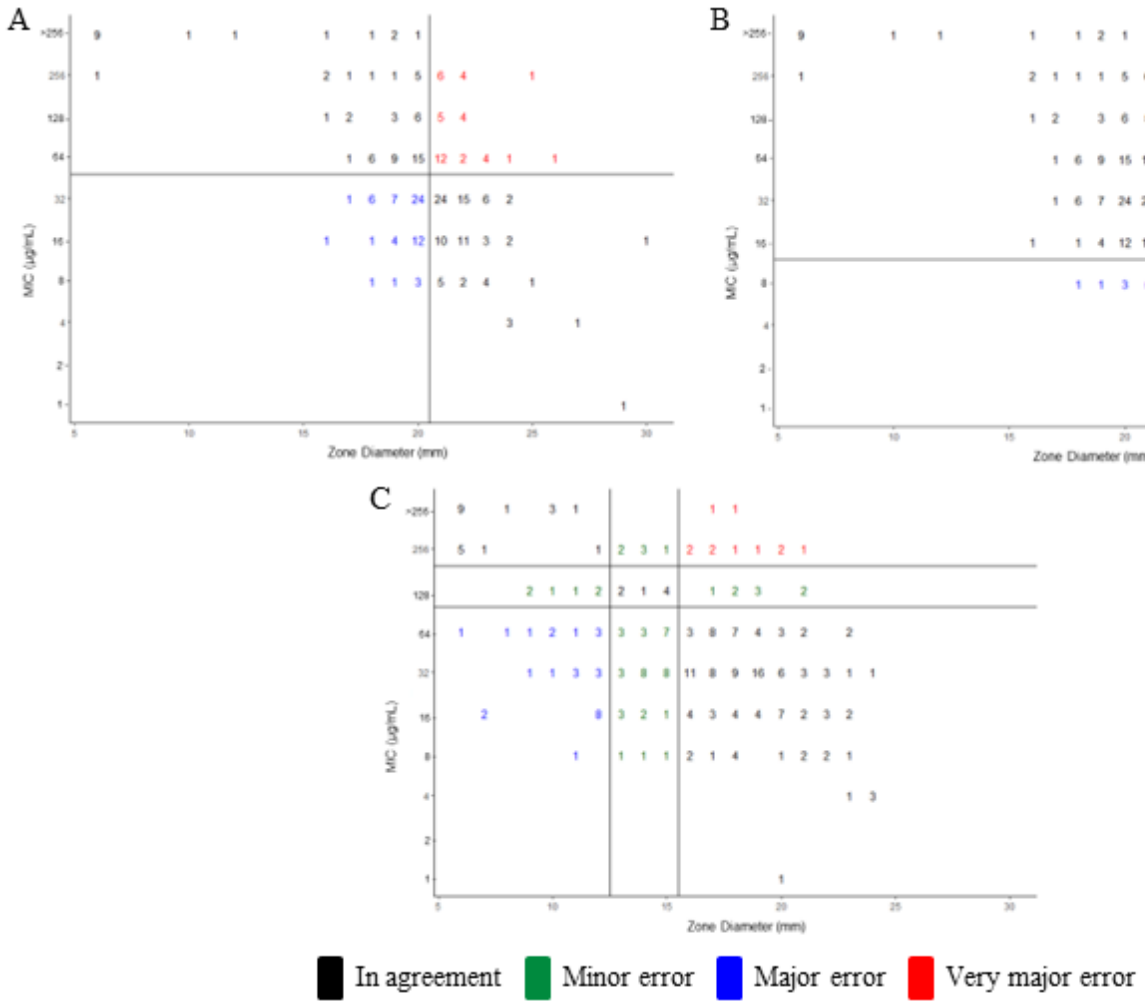


Figure 3. Flowchart of inclusion criteria for the heteroresistance screening for the clinical isolate collection (n = 262).

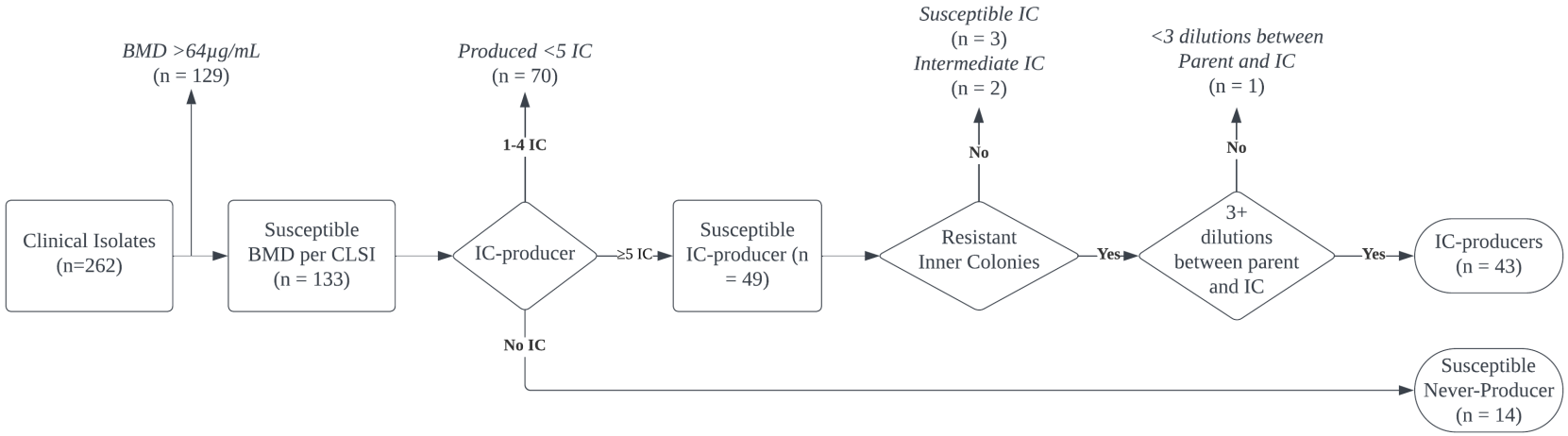


Figure 4. Number of 2-fold dilution increase in MIC value of inner colony isolates arising from each IC-producer isolate.

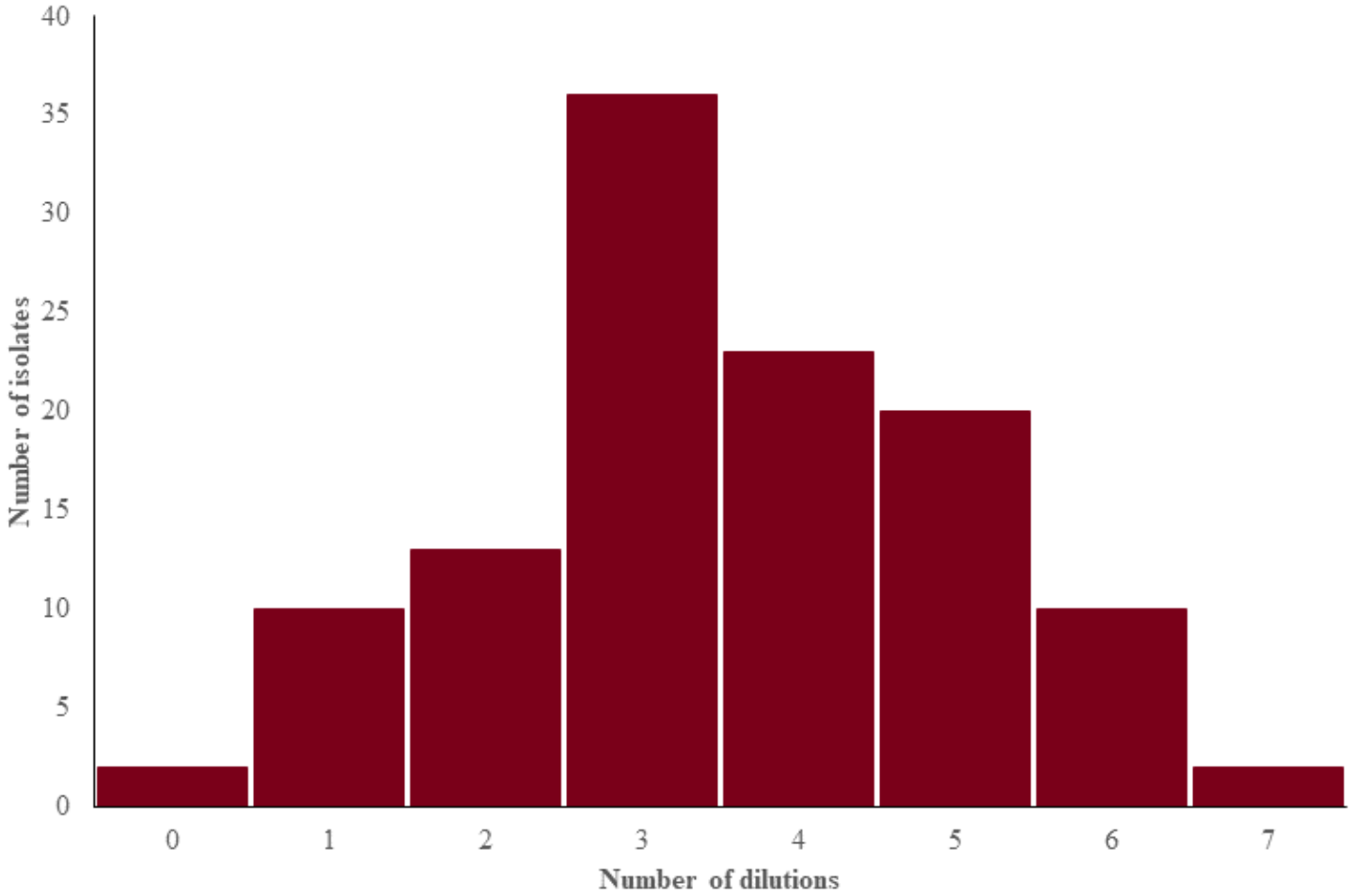


Figure 5. Growth curves for IC-producer/IC pairs (n = 9) in full-strength Mueller-Hinton broth (MHB), ¼-strength MHB, and filter sterilized urine.

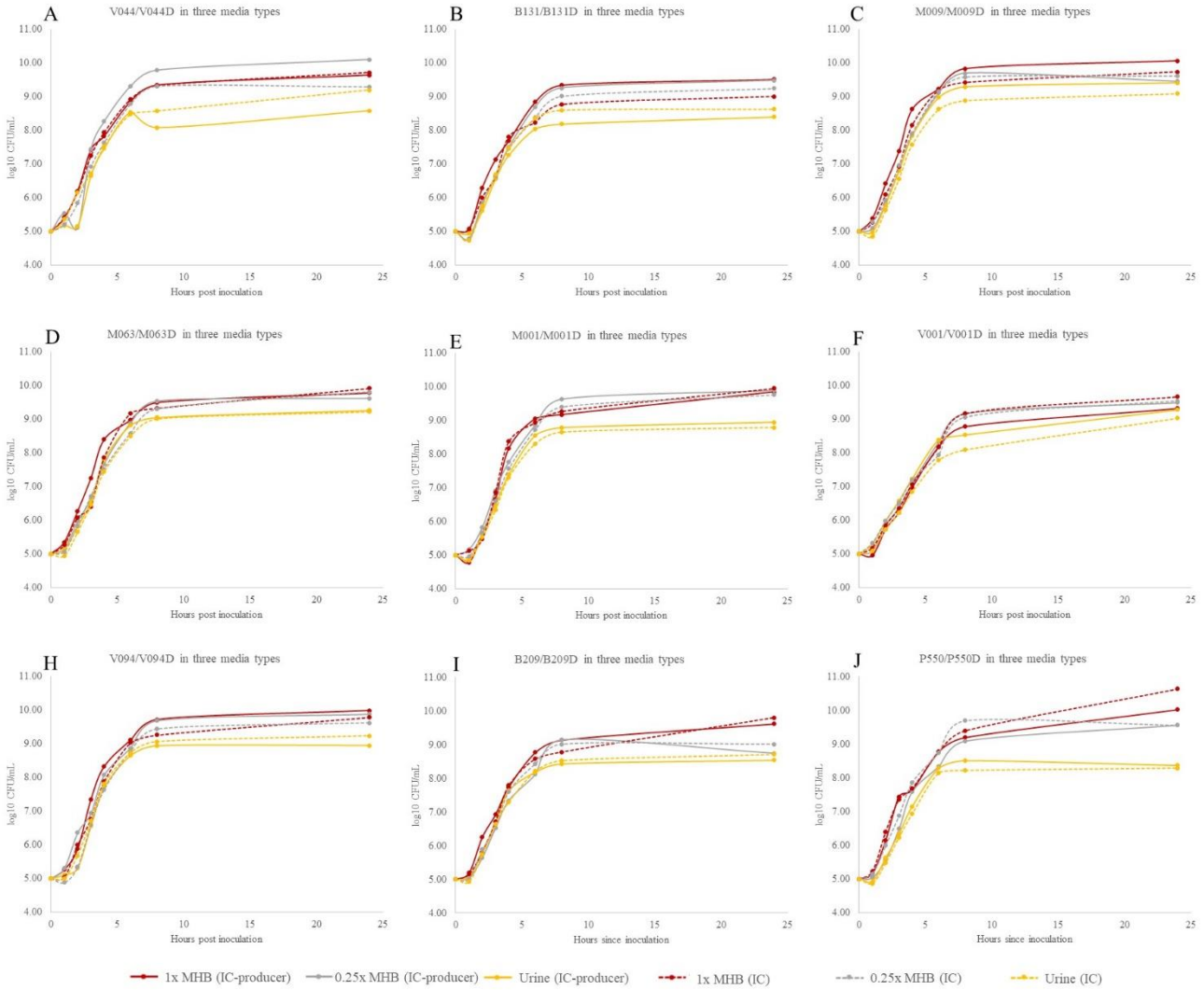


Figure 6. Growth curve for inner colony never producer isolates (n = 5) in full-strength Mueller-Hinton broth (MHB), ¼-strength MHB, and filter sterilized urine.

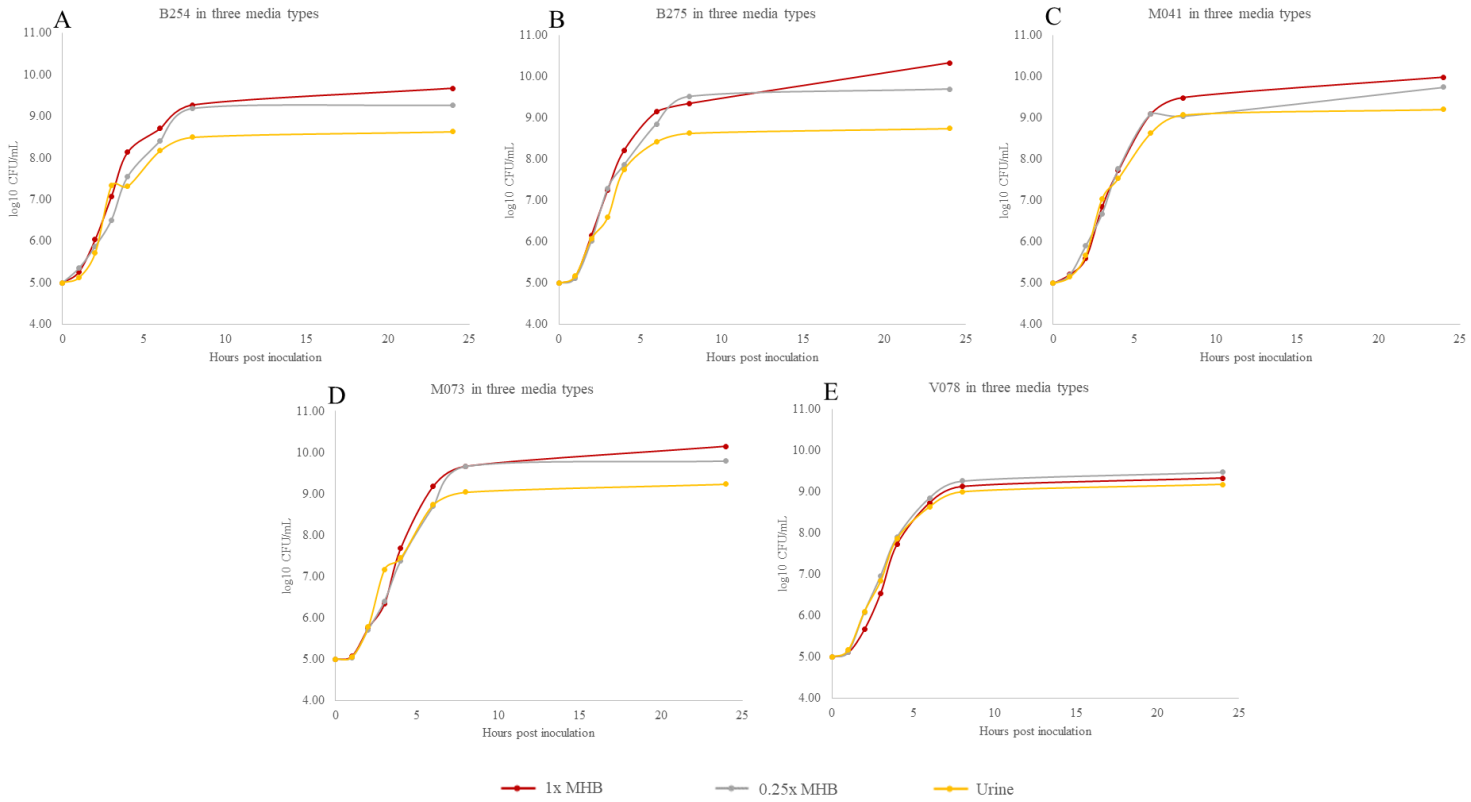


Figure 7. Generation time equation used to calculate the doubling time of cultures during the logarithmic growth phase.

$$G = \frac{t_1 - t_0}{\left(\log_{10} \mathrm{CFU}_{t_1} - \log_{10} \mathrm{CFU}_{t_0}\right)/\log_{10} 2}$$

Figure 8. Competition growth curves for IC-producer/IC pairs in full-strength Muller-Hinton broth over 72 hours. Flasks were inoculated at hour zero with both IC and IC-producer cultures at $5x10^4$ CFU/mL each. Every 12 hours broth was replaced by adding 10 µL of the grown flask into 10 mL of fresh broth.

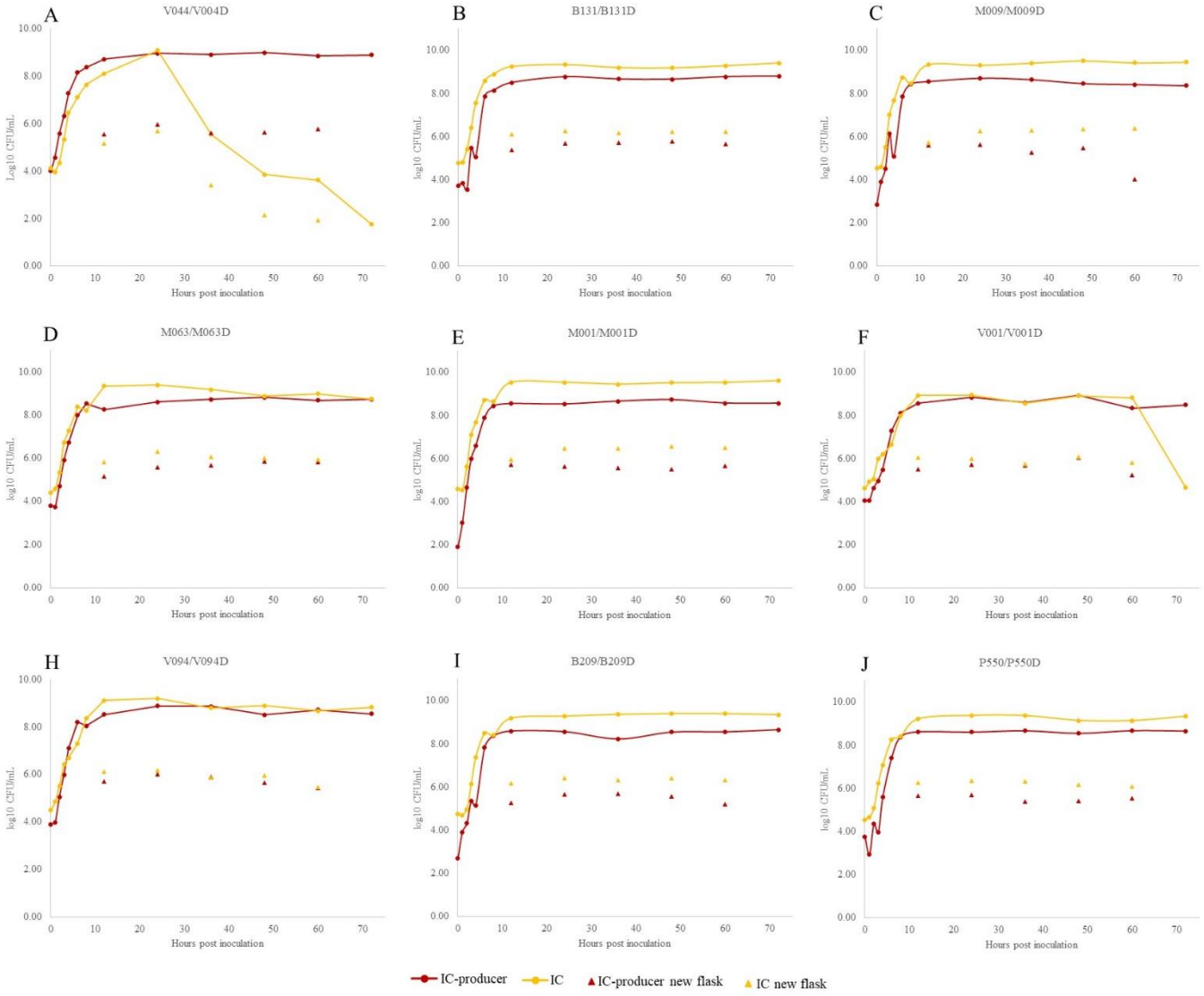


Figure 9. Flowchart of mouse inoculation, survival, and bacterial colonization for the four isolate pairs used in the ascending UTI mouse model.

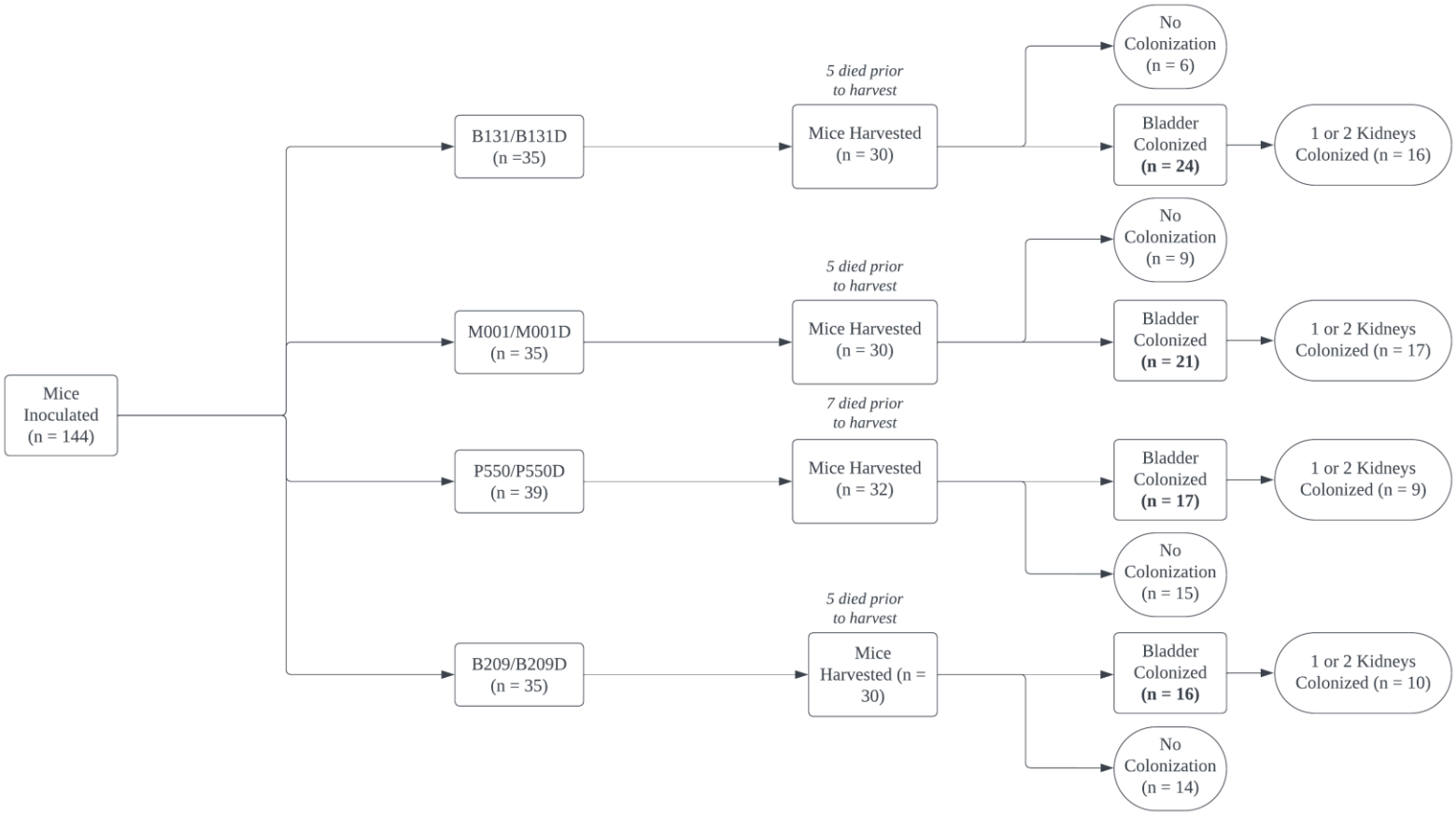
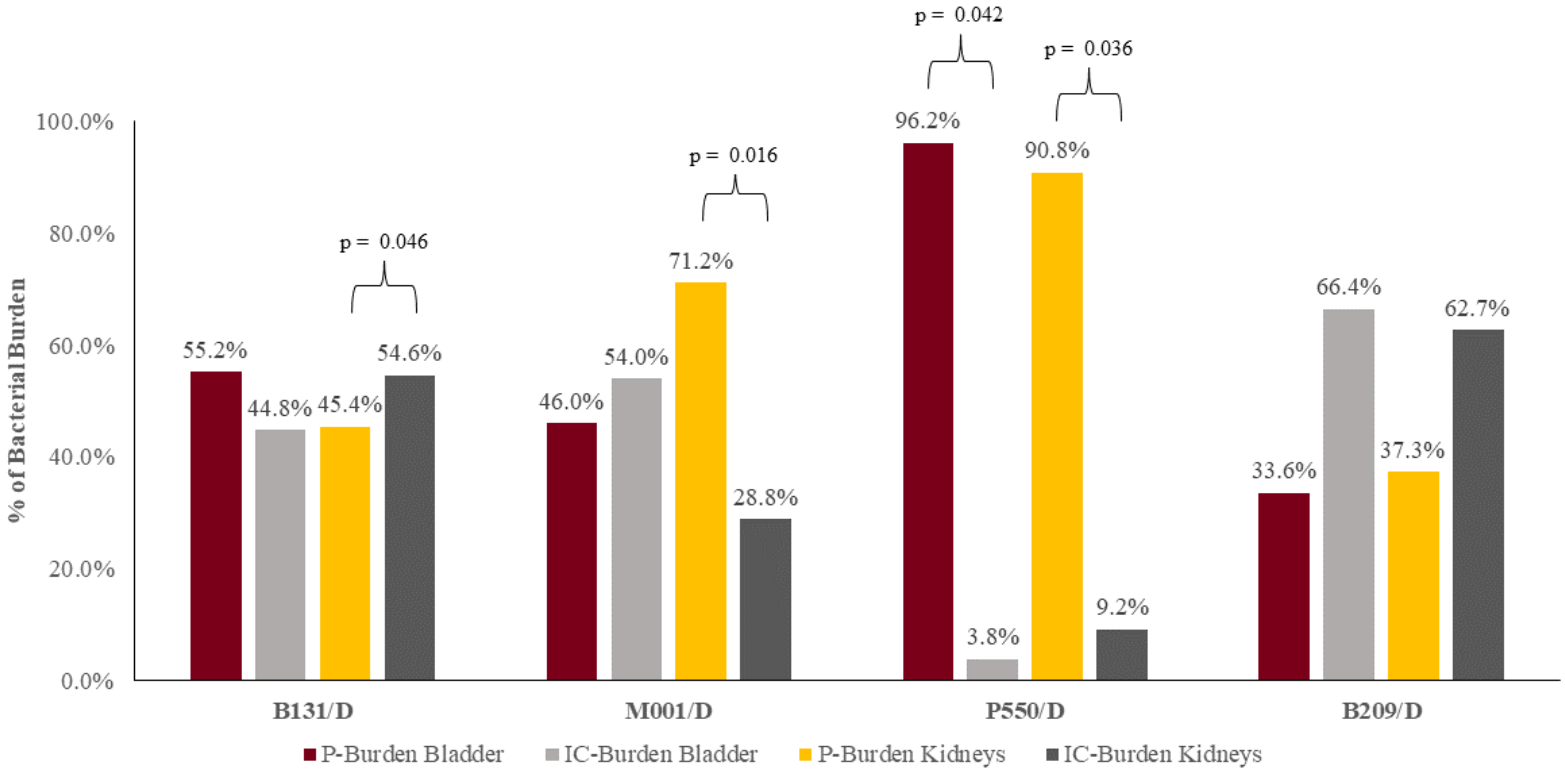


Figure 10. Proportion of bacterial burden attributable to IC-producers and corresponding IC. Bacterial burden was calculated for only organs with colonization where the proportion of the burden attributable to the IC-producer (P) was compared to the proportion of the burden attributable the IC in both bladder and kidney tissue.



References

1. Magill SS, O'Leary E, Ray SM, Kainer MA, Evans C, Bamberg WM, Johnston H, Janelle SJ, Oyewumi T, Lynfield R, Rainbow J, Warnke L, Nadle J, Thompson DL, Sharmin S, Pierce R, Zhang AY, Ocampo V, Maloney M, Greissman S, Wilson LE, Dumyati G, Edwards JR, Emerging Infections Program Hospital Prevalence Survey Team. 2021. Antimicrobial use in US hospitals: comparison of results from emerging infections program prevalence surveys, 2015 and 2011. Clin Infect Dis Off Publ Infect Dis Soc Am 72:1784–1792.

2. Gupta K, Hooton TM, Naber KG, Wullt B, Colgan R, Miller LG, Moran GJ, Nicolle LE, Raz R, Schaeffer AJ, Soper DE. 2011. International clinical practice guidelines for the treatment of acute uncomplicated cystitis and pyelonephritis in women: a 2010 update by the Infectious Diseases Society of America and the European Society for Microbiology and Infectious Diseases. Clin Infect Dis 52:e103–e120.

3. Hendlin D, Stapley EO, Jackson M, Wallick H, Miller AK, Wolf FJ, Miller TW, Chaiet L, Kahan FM, Foltz EL, Woodruff HB, Mata JM, Hernandez S, Mochales S. 1969. Phosphonomycin, a New Antibiotic Produced by Strains of Streptomyces | Science. Science 166:122–123.

4. Falagas ME, Vouloumanou EK, Samonis G, Vardakas KZ. 2016. Fosfomycin. Clin Microbiol Rev 29:321–347.

5. Falagas ME, Kastoris AC, Karageorgopoulos DE, Rafailidis PI. 2009. Fosfomycin for the treatment of infections caused by multidrug-resistant non-fermenting Gram-negative bacilli: a systematic review of microbiological, animal and clinical studies. 2. Int J Antimicrob Agents 34:111–120.

6. Castañeda-García A, Rodríguez-Rojas A, Guelfo JR, Blázquez J. 2009. The Glycerol-3-Phosphate Permease GlpT Is the Only Fosfomycin Transporter in Pseudomonas aeruginosa | Journal of Bacteriology. 22. J Bacteriol 191:6968–6974.

7. Reeves DS. 1994. Fosfomycin trometamol. 6. J Antimicrob Chemother 34:853–858.

8. Zhanel GG, Zhanel MA, Karlowsky JA. 2018. Intravenous Fosfomycin: An Assessment of Its Potential for Use in the Treatment of Systemic Infections in Canada. Can J Infect Dis Med Microbiol 2018:e8912039.

9. Edimiani A, Patel G, Hujer K, Swaminathan M, Perez F, Rice LB, Jacobs MR, Robert A Bonomo. 2010. In Vitro Activity of Fosfomycin against blaKPC-Containing Klebsiella pneumoniae Isolates, Including Those Nonsusceptible to Tigecycline and/or Colistin. 1. Antimicrob Agents Chemother 54:526–529.

10. Karlowsky JA, Denisuik AJ, Lagacé-Wiens PRS, Adam HJ, Baxter MR, Hoban DJ, Zhanel GG. 2014. In Vitro activity of fosfomycin against Escherichia coli isolated from patients

with urinary tract infections in Canada as part of the CANWARD surveillance study. Antimicrob Agents Chemother 58:1252–1256.

11. Neuner EA, Sekeres J, Hall GS, van Duin D. 2012. Experience with fosfomycin for treatment of urinary tract infections due to multidrug-resistant organisms. 11. Antimicrob Agents Chemother 56:5744–5748.

12. Babiker A, Clarke L, Doi Y, Shields RK. 2019. Fosfomycin for treatment of multidrug-resistant pathogens causing urinary tract infection: A real-world perspective and review of the literature. Diagn Microbiol Infect Dis 95:114856.

13. Giancola SE, Mahoney MV, Hogan MD, Raux BR, McCoy C, Hirsch EB. 2017. Assessment of fosfomycin for complicated or multidrug-resistant urinary tract infections: patient characteristics and outcomes. 2. Chemotherapy 62:100–104.

14. Seroy JT, Grim SA, Reid GE, Wellington T, Clark NM. 2016. Treatment of MDR urinary tract infections with oral fosfomycin: a retrospective analysis. J Antimicrob Chemother 71:2563–2568.

15. Tumturk A, Tonyali S, Tezer Tekce AY, Isikay L, Cime H. 2019. Fosfomycin in the treatment of extended spectrum beta-lactamase-producing Enterobacteriaceae- related urinary tract infections. J Infect Dev Ctries 13:73–76.

16. Matthews PC, Barrett LK, Warren S, Stoesser N, Snelling M, Scarborough M, Jones N. 2016. Oral fosfomycin for treatment of urinary tract infection: a retrospective cohort study. BMC Infect Dis 16:556.

17. van Duin D, Cober E, Richter SS, Perez F, Kalayjian RC, Salata RA, Evans S, Fowler VG Jr, Kaye KS, Bonomo RA. 2015. Impact of therapy and strain type on outcomes in urinary tract infections caused by carbapenem-resistant *Klebsiella pneumoniae*. J Antimicrob Chemother 70:1203–1211.

18. Reid GE, Grim SA, Layden JE, Akkina S, Tang I, Campara M, Clark NM. 2013. The use of fosfomycin to treat urinary tract infections in kidney transplant recipients. Transplantation 96:e12.

19. Rosana Y, Ocviyanti D, Halim M, Harlinda FY, Amran R, Akbar W, Billy M, Akhmad SRP. 2020. Urinary tract infections among Indonesian pregnant women and its susceptibility pattern. Infect Dis Obstet Gynecol 2020:9681632.

20. Michalopoulos AS, Livaditis IG, Gougoutas V. 2011. The revival of fosfomycin. Int J Infect Dis 15:e732–e739.

21. Falagas ME, Maraki S, Karageorgopoulos DE, Kastoris AC, Kapaskelis A, Samonis G. 2010. Antimicrobial susceptibility of Gram-positive non-urinary isolates to fosfomycin. Int J Antimicrob Agents 35:497–499.

22. Falagas ME, Maraki S, Karageorgopoulos DE, Kastoris AC, Mavromanolakis E, Samonis G. 2010. Antimicrobial susceptibility of multidrug-resistant (MDR) and extensively drug-resistant (XDR) Enterobacteriaceae isolates to fosfomycin. Int J Antimicrob Agents 35:240–243.

23. Maraki S, Samonis G, Rafailidis PI, Vouloumanou EK, Mavromanolakis E, Falagas ME. 2009. Susceptibility of Urinary Tract Bacteria to Fosfomycin. Antimicrob Agents Chemother 53:4508–4510.

24. Clinical and Laboratory Standards Institute. 2023. Performance standards for antimicrobial susceptibility testing, 33rd ed. CLSI supplement M100. Clinical and Laboratory Standards Institute, Wayne, PA.

25. 2021. The European Committee on Antimicrobial Susceptibility Testing. EUCAST disk diffusion method for antimicrobial susceptibility testing Version 10.0, 2022.

26. The European Committee on Antimicrobial Susceptibility Testing. Breakpoint tables for interpretation of MICs and zone diameters. Version 12.0, 2022. http://www.eucast.org.

27. Lucas AE, Ito R, Mustapha MM, McElheny CL, Mettus RT, Bowler SL, Kantz SF, Pacey MP, Pasculle AW, Cooper VS, Doi Y. 2017. Frequency and mechanisms of spontaneous fosfomycin nonsusceptibility observed upon disk diffusion testing of *Escherichia coli*. 1. J Clin Microbiol 56:e01368-17.

28. Elliott ZS, Barry KE, Cox HL, Stoesser N, Carroll J, Vegesana K, Kotay S, Sheppard AE, Wailan A, Crook DW, Parikh H, Mathers AJ. 2019. The role of *fosA* in challenges with fosfomycin susceptibility testing of multispecies *Klebsiella pneumoniae* carbapenemase-producing clinical isolates. 10. J Clin Microbiol 57:e00634-19.

29. Mojica MF, De La Cadena E, Hernández-Gómez C, Correa A, Appel TM, Pallares CJ, Villegas MV. 2020. Performance of disk diffusion and broth microdilution for fosfomycin susceptibility testing of multidrug-resistant clinical isolates of Enterobacterales and *Pseudomonas aeruginosa*. J Glob Antimicrob Resist 21:391–395.

30. Fuchs PC, Barry AL, Brown SD. 1997. Susceptibility testing quality control studies with fosfomycin tromethamine. Eur J Clin Microbiol Infect Dis Off Publ Eur Soc Clin Microbiol 16:538–540.

31. Endimiani A, Patel G, Hujer K, Swaminathan M, Perez F, Rice LB, Jacobs MR, Robert A Bonomo. 2010. *In vitro* activity of fosfomycin against blaKPC-containing *Klebsiella pneumoniae* isolates, including those nonsusceptible to tigecycline and/or colistin. 1. Antimicrob Agents Chemother 54:526–529.

32. Mothibi LM, Bosman NN, Nana T. 2020. Fosfomycin susceptibility of uropathogens at Charlotte Maxeke Johannesburg Academic Hospital. South Afr J Infect Dis 35:173.

33. Yekani M, Baghi HB, Sefidan FY, Azargun R, Memar MY, Ghotaslou R. 2018. The rates of quinolone, trimethoprim/sulfamethoxazole and aminoglycoside resistance among Enterobacteriaceae isolated from urinary tract infections in Azerbaijan, Iran. GMS Hyg Infect Control 13:Doc07.

34. Behera B, Mohanty S, Sahu S, Praharaj AK. 2018. *In vitro* activity of fosfomycin against multidrug-resistant urinary and nonurinary gram-negative isolates. Indian J Crit Care Med Peer-Rev Off Publ Indian Soc Crit Care Med 22:533–536.

35. Araj GF, Avedissian AZ, Itani LY, Obeid JA. 2018. Antimicrobial agents active against carbapenem-resistant *Escherichia coli* and *Klebsiella pneumoniae* isolates in Lebanon. J Infect Dev Ctries 12:164–170.

36. Demir T, Buyukguclu T. 2017. Fosfomycin: in vitro efficacy against multidrug-resistant isolates beyond urinary isolates. J Glob Antimicrob Resist 8:164–168.

37. Khan IU, Mirza IA, Ikram A, Ali S, Hussain A, Ghafoor T. 2014. In vitro activity of fosfomycin tromethamine against extended spectrum beta-lactamase producing urinary tract bacteria. J Coll Physicians Surg--Pak JCPSP 24:914–917.

38. Peretz A, Naamneh B, Tkhawkho L, Nitzan O. 2019. High Rates of Fosfomycin Resistance in Gram-Negative Urinary Isolates from Israel. Microb Drug Resist Larchmt N 25:408–412.

39. Qiao L-D, Chen S, Yang Y, Zhang K, Zheng B, Guo H-F, Yang B, Niu Y-J, Wang Y, Shi B-K, Yang W-M, Zhao X-K, Gao X-F, Chen M, Tian Y. 2013. Characteristics of urinary tract infection pathogens and their in vitro susceptibility to antimicrobial agents in China: data from a multicenter study. BMJ Open 3:e004152.

40. Alexander BT, Marschall J, Tibbetts RJ, Neuner EA, Dunne WM, Ritchie DJ. 2012. Treatment and clinical outcomes of urinary tract infections caused by KPC-producing Enterobacteriaceae in a retrospective cohort. Clin Ther 34:1314–1323.

41. Liu H-Y, Lin H-C, Lin Y-C, Yu S-H, Wu W-H, Lee Y-J. 2011. Antimicrobial susceptibilities of urinary extended-spectrum beta-lactamase-producing *Escherichia coli* and *Klebsiella pneumoniae* to fosfomycin and nitrofurantoin in a teaching hospital in Taiwan. J Microbiol Immunol Infect Wei Mian Yu Gan Ran Za Zhi 44:364–368.

42. de Cueto M, López L, Hernández JR, Morillo C, Pascual A. 2006. In vitro activity of fosfomycin against extended-spectrum-beta-lactamase-producing *Escherichia coli* and *Klebsiella pneumoniae*: comparison of susceptibility testing procedures. Antimicrob Agents Chemother 50:368–370.

43. Miranda-Novales G, Flores-Moreno K, López-Vidal Y, Ponce de León-Rosales S, Pucra Network Members null. 2022. Limited therapeutic options in Mexico for the treatment of urinary tract infections. Antibiot Basel Switz 11:1656.

44. Bir R, Mohapatra S, Kumar A, Arif N, Tyagi S, Ak AP, Gautam H, Sood S, Das BK, Kapil A. 2022. Genomic analysis of fosfomycin resistance in multi-drug resistant uropathogens and comparison of in-vitro susceptibility methods uropathogens. Iran J Microbiol 14:636–644.

45. Iqbal Z, Mumtaz MZ, Malik A. 2021. Extensive drug-resistance in strains of *Escherichia coli* and *Klebsiella pneumoniae* isolated from paediatric urinary tract infections. J Taibah Univ Med Sci 16:565–574.

46. Kashefieh M, Hosainzadegan H, Baghbanijavid S, Ghotaslou R. 2021. The molecular epidemiology of resistance to antibiotics among *Klebsiella pneumoniae* isolates in Azerbaijan, Iran. J Trop Med 2021:9195184.

47. Lima RC, Faria CA, Carraro-Eduardo JC, Morales PS, Fonseca ABM. 2021. Evaluation of sensitivity profiles to fosfomycin in bacterial urine samples from outpatients. Eur J Obstet Gynecol Reprod Biol 262:184–187.

48. Sharahi JY, Hashemi A, Ardebili A, Davoudabadi S. 2021. Molecular characteristics of antibiotic-resistant *Escherichia coli* and *Klebsiella pneumoniae* strains isolated from hospitalized patients in Tehran, Iran. Ann Clin Microbiol Antimicrob 20:32.

49. Imtiaz W, Syed Z, Rafaque Z, Andrews SC, Dasti JI. 2021. Analysis of antibiotic resistance and virulence traits (genetic and phenotypic) in *Klebsiella pneumoniae* clinical isolates from Pakistan: identification of significant levels of carbapenem and colistin resistance. Infect Drug Resist 14:227–236.

50. Ballestero-Téllez M, Docobo-Pérez F, Rodríguez-Martínez JM, Conejo MC, Ramos-Guelfo MS, Blázquez J, Rodríguez-Baño J, Pascual A. 2017. Role of inoculum and mutant frequency on fosfomycin MIC discrepancies by agar dilution and broth microdilution methods in Enterobacteriaceae. Clin Microbiol Infect Off Publ Eur Soc Clin Microbiol Infect Dis 23:325–331.

51. Abdullah FE, Mushtaq A, Irshad M, Rauf H, Afzal N, Rasheed A. 2013. Current efficacy of antibiotics against *Klebsiella* isolates from urine samples - a multi-centric experience in Karachi. Pak J Pharm Sci 26:11–15.

52. The European Committee on Antimicrobial Susceptibility Testing. Rationale for the EUCAST clinical breakpoints. Version 2.0, 2020.

53. Kowalska-Krochmal B, Mączyńska B, Rurańska-Smutnicka D, Secewicz A, Krochmal G, Bartelak M, Górzyńska A, Laufer K, Woronowicz K, Łubniewska J, Łappo J, Czwartos M, Dudek-Wicher R. 2022. Assessment of the susceptibility of clinical Gram-negative and Gram-positive bacterial strains to fosfomycin and significance of this antibiotic in infection treatment. Pathogens 11:1441.

54. Goer A, Blanchard LS, Van Belkum A, Loftus KJ, Armstrong TP, Gatermann SG, Shortridge D, Olson BJ, Meece JK, Fritsche TR, Pompilio M, Halimi D, Franceschi C. Multicenter evaluation of the novel ETEST fosfomycin for antimicrobial susceptibility testing of Enterobacterales, *Enterococcus faecalis*, and *Staphylococcus* species. J Clin Microbiol 60:e00021-22.

55. Tutone M, Bjerklund Johansen TE, Cai T, Mushtaq S, Livermore DM. 2022. Susceptibility and resistance to fosfomycin and other antimicrobial agents among pathogens causing lower urinary tract infections: findings of the SURF study. Int J Antimicrob Agents 59:106574.

56. Fajfr M, Louda M, Paterová P, Ryšková L, Pacovský J, Košina J, Žemličková H, Broďák M. 2017. The susceptibility to fosfomycin of Gram-negative bacteria isolates from urinary tract infection in the Czech Republic: data from a unicentric study. BMC Urol 17:33.

57. Kaase M, Szabados F, Anders A, Gatermann SG. 2014. Fosfomycin susceptibility in carbapenem-resistant Enterobacteriaceae from Germany. J Clin Microbiol 52:1893–1897.

58. Della Rocca MT, Foglia F, Crudele V, Greco G, De Filippis A, Franci G, Finamore E, Galdiero M. 2022. Antimicrobial resistance changing trends of *Klebsiella pneumoniae* isolated over the last 5 years. New Microbiol 45:338–343.

59. Lu C-L, Liu C-Y, Huang Y-T, Liao C-H, Teng L-J, Turnidge JD, Hsueh P-R. 2011. Antimicrobial susceptibilities of commonly encountered bacterial isolates to fosfomycin determined by agar dilution and disk diffusion methods. Antimicrob Agents Chemother 55:4295–4301.

60. Aydemir Ö, Şahin EÖ, Ayhancı T, Ormanoğlu G, Aydemir Y, Köroğlu M, Altındiş M. 2022. Investigation of in-vitro efficacy of intravenous fosfomycin in extensively drug-resistant *Klebsiella pneumoniae* isolates and effect of glucose 6-phosphate on sensitivity results. Int J Antimicrob Agents 59:106489.

61. Monurol (fosfomycin tromethamine)[package insert]. Madison, NJ: Allergan USA. Rev. May 2018.

62. Nicolle L. 2005. Complicated urinary tract infection in adults. Can J Infect Dis Med Microbiol 16:349–360.

63. Derington CG, Benavides N, Delate T, Fish DN. 2020. Multiple-dose oral fosfomycin for treatment of complicated urinary tract infections in the outpatient setting. 2. Open Forum Infect Dis 7.

64. Wenzler E, Bleasdale SC, Sikka M, Bunnell KL, Finnemeyer M, Rosenkranz SL, Danzinger LH, Rodvold KA. 2018. Phase I study to evaluate the pharmacokinetics, safety, and tolerability of two dosing regimens of oral fosfomycin tromethamine in healthy adult participants. 8. Antimicrob Agents Chemother 62.

65. Nicolle LE, Gupta K, Bradley SF, Colgan R, DeMuri GP, Drekonja D, Eckert LO, Geerlings SE, Köves B, Hooton TM, Juthani-Mehta M, Knight SL, Saint S, Schaeffer AJ, Trautner B, Wullt B, Siemieniuk R. 2019. Clinical practice guideline for the management of asymptomatic bacteriuria: 2019 update by the Infectious Diseases Society of America. Clin Infect Dis Off Publ Infect Dis Soc Am 68:e83–e110.

66. Joshi S. 2010. Hospital antibiogram: a necessity. Indian J Med Microbiol 28:277–280.

67. Truong WR, Hidayat L, Bolaris MA, Nguyen L, Yamaki J. 2021. The antibiogram: key considerations for its development and utilization. JAC-Antimicrob Resist 3:dlab060.

68. Falagas ME, Athanasaki F, Voulgaris GL, Triarides NA, Vardakas KZ. 2019. Resistance to fosfomycin: mechanisms, frequency and clinical consequences. 1. Int J Antimicrob Agents 53:22–28.

69. Kahan FM, Kahan JS, Cassidy PJ, Kropp H. 1974. The mechanism of action of fosfomycin (phosphonomycin). 0. Ann N Y Acad Sci 235:364–386.

70. Nilsson AI, Berg OG, Aspevall O, Kahlmeter G, Andersson DI. 2003. Biological costs and mechanisms of fosfomycin resistance in *Escherichia coli*. 9. Antimicrob Agents Chemother 47:2850–2858.

71. Castañeda-García A, Rodríguez-Rojas A, Guelfo JR, Blázquez J. 2009. The glycerol-3-phosphate permease GlpT is the only fosfomycin transporter in *Pseudomonas aeruginosa*. 22. J Bacteriol 191:6968–6974.

72. Grimm H. 1979. In vitro investigations with fosfomycin on mueller-hinton agar with and without glucose-6-phosphate. 5. Infection 7:256–259.

73. Elvin CM, Hardy CM, Rosenberg H. 1985. Pi exchange mediated by the GlpT-dependent sn-glycerol-3-phosphate transport system in *Escherichia coli*. J Bacteriol 161:1054–1058.

74. Wang Y-P, Chen Y-H, Hung I-C, Chu P-H, Chang Y-H, Lin Y-T, Yang H-C, Wang J-T. 2022. Transporter genes and *fosA* associated with fosfomycin resistance in carbapenem-resistant *Klebsiella pneumoniae*. Front Microbiol 13.

75. Alper MD, Ames BN. 1978. Transport of antibiotics and metabolite analogs by systems under cyclic AMP control: positive selection of *Salmonella typhimurium* cya and crp mutants. J Bacteriol 133:149–157.

76. Castañeda-García A, Blázquez J, Rodríguez-Rojas A. 2013. Molecular mechanisms and clinical impact of acquired and intrinsic fosfomycin resistance. 2. Antibiotics 2:217–236.

77. Ito R, Tomich AD, McElheny CL, Mettus RT, Sluis-Cremer N, Doi Y. 2017. Inhibition of fosfomycin resistance protein FosA by phosphonoformate (foscarnet) in multidrug-resistant Gram-negative pathogens. Antimicrob Agents Chemother 61:e01424-17.

78. Wachino J, Yamane K, Suzuki S, Kimura K, Arakawa Y. 2010. Prevalence of fosfomycin resistance among CTX-M-producing *Escherichia coli* clinical isolates in Japan and identification of novel plasmid-mediated fosfomycin-modifying enzymes. Antimicrob Agents Chemother 54:3061–3064.

79. Ho P-L, Chan J, Lo W-U, Lai EL, Cheung Y-Y, Lau TCK, Chow K-H. 2013. Prevalence and molecular epidemiology of plasmid-mediated fosfomycin resistance genes among blood and urinary *Escherichia coli* isolates. J Med Microbiol 62:1707–1713.

80. Jiang Y, Shen P, Wei Z, Liu L, He F, Shi K, Wang Y, Wang H, Yu Y. 2015. Dissemination of a clone carrying a *fosA3*-harbouring plasmid mediates high fosfomycin resistance rate of KPC-producing *Klebsiella pneumoniae* in China. Int J Antimicrob Agents 45:66–70.

81. Ma Y, Xu X, Guo Q, Wang P, Wang W, Wang M. 2015. Characterization of *fosA5*, a new plasmid-mediated fosfomycin resistance gene in *Escherichia coli*. Lett Appl Microbiol 60:259–264.

82. Guo Q, Tomich AD, McElheny CL, Cooper VS, Stoesser N, Wang M, Sluis-Cremer N, Doi Y. 2016. Glutathione-S-transferase FosA6 of *Klebsiella pneumoniae* origin conferring fosfomycin resistance in ESBL-producing *Escherichia coli*. J Antimicrob Chemother 71:2460–2465.

83. Zurfluh K, Treier A, Schmitt K, Stephan R. 2020. Mobile fosfomycin resistance genes in Enterobacteriaceae—An increasing threat. MicrobiologyOpen 9:e1135.

84. Zhou Y, Ai W, Cao Y, Guo Y, Wu X, Wang B, Rao L, Xu Y, Zhao H, Wang X, Yu F. 2021. The Co-occurrence of NDM-5, MCR-1, and FosA3-encoding plasmids contributed to the generation of extensively drug-resistant *Klebsiella pneumoniae*. Front Microbiol 12:811263.

85. Loras C, González-Prieto A, Pérez-Vázquez M, Bautista V, Ávila A, Campoy PS, Oteo-Iglesias J, Alós J-I. 2021. Prevalence, detection and characterisation of fosfomycin-resistant *Escherichia coli* strains carrying fosA genes in Community of Madrid, Spain. J Glob Antimicrob Resist 25:137–141.

86. Yang T-Y, Lu P-L, Tseng S-P. 2019. Update on fosfomycin-modified genes in Enterobacteriaceae. J Microbiol Immunol Infect 52:9–21.

87. Abbott IJ, Dekker J, van Gorp E, Wijma RA, Raaphorst MN, Klaassen CHW, Meletiadis J, Mouton JW, Peleg AY. 2020. Impact of bacterial species and baseline resistance on fosfomycin efficacy in urinary tract infections. 4. J Antimicrob Chemother 75:988–996.

88. Lim T-P, Teo JQ-M, Goh AW-L, Tan S-X, Koh T-H, Lee WH-L, Cai Y, Tan T-T, Kwa AL-H. 2020. *In vitro* pharmacodynamics of fosfomycin against carbapenem-resistant *Enterobacter cloacae* and *Klebsiella aerogenes*. Antimicrob Agents Chemother 64:e00536-20.

89. Horii T, Kimura T, Sato K, Shibayama K, Ohta M. 1999. Emergence of fosfomycin-resistant isolates of shiga-like toxin-producing *Escherichia coli* O26. Antimicrob Agents Chemother 43:789–793.

90. Marchese A, Gualco L, Debbia EA, Schito GC, Schito AM. 2003. In vitro activity of fosfomycin against Gram-negative urinary pathogens and the biological cost of fosfomycin resistance. Int J Antimicrob Agents 22:53–59.

91. Scortti M, Lacharme-Lora L, Wagner M, Chico-Calero I, Losito P, Vázquez-Boland JA. 2006. Coexpression of virulence and fosfomycin susceptibility in Listeria: molecular basis of an antimicrobial in vitro–in vivo paradox. 5. Nat Med 12:515–517.

92. Takahata S, Ida T, Hiraishi T, Sakakibara S, Maebashi K, Terada S, Muratani T, Matsumoto T, Nakahama C, Tomono K. 2010. Molecular mechanisms of fosfomycin resistance in clinical isolates of *Escherichia coli*. Int J Antimicrob Agents 35:333–337.

93. Tsutomu Tsuruoka, Aiko Miyata, Yujiro Yamada. 1978. Two kinds of mutants defective in multiple carbohydrate utilization isolated from in vitro fosfomycin-resistant strains of <i> Escherichia coli<i/> k-12. J Antibiot (Tokyo) 31.

94. Arca P, Reguera G, Hardisson C. 1997. Plasmid-encoded fosfomycin resistance in bacteria isolated from the urinary tract in a multicentre survey. J Antimicrob Chemother 40:393–399.

95. Lu P-L, Hsieh Y-J, Lin J-E, Huang J-W, Yang T-Y, Lin L, Tseng S-P. 2016. Characterisation of fosfomycin resistance mechanisms and molecular epidemiology in extended-spectrum β-lactamase-producing *Klebsiella pneumoniae* isolates. Int J Antimicrob Agents 48:564–568.

96. Liu P, Chen S, Wu Z-Y, Qi M, Li X-Y, Liu C-X. 2020. Mechanisms of fosfomycin resistance in clinical isolates of carbapenem-resistant *Klebsiella pneumoniae*. J Glob Antimicrob Resist 22:238–243.

97. Bixby ML, Salay JM, Krueger AR, Mathers AJ, Hirsch EB. 2023. Fosfomycin disk diffusion testing among *Klebsiella pneumoniae* results in frequent inner colonies and categorical disagreement based on conflicting breakpoint organization recommendations. Microbiol Spectr 11:e0336322.

98. Smith EC, Brigman HV, Anderson JC, Emery CL, Bias TE, Bergen PJ, Landersdorfer CB, Elizabeth B Hirsch. 2020. Performance of four fosfomycin susceptibility testing methods against an international collection of clinical *Pseudomonas aeruginosa* isolates. 10. J Clin Microbiol 58.

99. Rodríguez-Villodres Á, Ortiz de la Rosa JM, Álvarez-Marín R, Pachón J, Aznar J, Lepe JA, Smani Y. 2017. Heteroresistance to piperacillin-tazobactam in clinical isolates of *Escherichia coli* sequence type 131. Antimicrob Agents Chemother 62:e01923-17.

100. Hirsch EB, Raux BR, Zucchi PC, Kim Y, McCoy C, Kirby JE, Wright SB, Eliopoulos GM. 2015. Activity of fosfomycin and comparison of several susceptibility testing methods against contemporary urine isolates. 6. Int J Antimicrob Agents 46:642–647.

101. Hirsch EB, Brigman HV, Zucchi PC, Chen A, Anderson JC, Eliopoulos GM, Cheung N, Gilbertsen A, Hunter RC, Emery CL, Bias TE. 2020. Ceftolozane-tazobactam and ceftazidime-avibactam activity against β-lactam-resistant *Pseudomonas aeruginosa* and extended-spectrum β-lactamase-producing Enterobacterales clinical isolates from U.S. medical centres. J Glob Antimicrob Resist 22:689–694.

102. Chen A, Smith KP, Whitfield BA, Zucchi PC, Lasco TM, Bias TE, Kirby JE, Hirsch EB. 2017. Activity of minocycline against *Klebsiella pneumoniae* carbapenemase (KPC)-producing Enterobacteriaceae clinical isolates, with comparison to doxycycline and tigecycline. Diagn Microbiol Infect Dis 88:365–367.

103. 2021. EUCAST: Disk diffusion methodology. https://www.eucast.org/ast_of_bacteria/disk_diffusion_methodology/. Retrieved 1 July 2021.

104. Humphries RM, Ambler J, Mitchell SL, Castanheira M, Dingle T, Hindler JA, Koeth L, Sei K, CLSI Methods Development and Standardization Working Group of the Subcommittee on Antimicrobial Susceptibility Testing. 2018. CLSI methods development and standardization working group best practices for evaluation of antimicrobial susceptibility tests. J Clin Microbiol 56:e01934-17.

105. Clinical and Laboratory Standards Institute. 2023. Development of in vitro susceptibility testing criteria and quality control parameters. 6th ed. CLSI guidelines M23. Clinical and Laboratory Standards Institute, Wayne, PA.

106. Abbott IJ, Mouton JW, Peleg AY, Meletiadis J. 2021. Pharmacokinetic/pharmacodynamic analysis of oral fosfomycin against Enterobacterales, *Pseudomonas aeruginosa* and *Enterococcus* spp. in an in vitro bladder infection model: impact on clinical breakpoints. J Antimicrob Chemother 76:3201–3211.

107. Abbott IJ, van Gorp E, Wijma RA, Meletiadis J, Roberts JA, Mouton JW, Peleg AY. 2020. Oral fosfomycin efficacy with variable urinary exposures following single and multiple doses against Enterobacterales: the importance of heteroresistance for growth outcome. Antimicrob Agents Chemother 64:e01982-19.

108. Olson RD, Assaf R, Brettin T, Conrad N, Cucinell C, Davis JJ, Dempsey DM, Dickerman A, Dietrich EM, Kenyon RW, Kuscuoglu M, Lefkowitz EJ, Lu J, Machi D, Macken C, Mao C, Niewiadomska A, Nguyen M, Olsen GJ, Overbeek JC, Parrello B, Parrello V, Porter JS, Pusch GD, Shukla M, Singh I, Stewart L, Tan G, Thomas C, VanOeffelen M, Vonstein V, Wallace ZS, Warren AS, Wattam AR, Xia F, Yoo H, Zhang Y, Zmasek CM, Scheuermann RH, Stevens RL. 2023. Introducing the Bacterial and Viral Bioinformatics Resource Center

(BV-BRC): a resource combining PATRIC, IRD and ViPR. Nucleic Acids Res 51:D678–D689.

109. Ito R, Mustapha MM, Tomich AD, Callaghan JD, McElheny CL, Mettus RT, Shanks RMQ, Sluis-Cremer N, Doi Y. 2017. Widespread fosfomycin resistance in Gram-negative bacteria attributable to the chromosomal *fosA* gene. 4. mBio 8.

110. Bermudez TA, Brannon JR, Dudipala N, Reasoner S, Morales G, Wiebe M, Cecala M, DaCosta M, Beebout C, Amir O, Hadjifrangiskou M. 2023. Raising the alarm: fosfomycin resistance associated with non-susceptible inner colonies imparts no fitness cost to the primary bacterial uropathogen. Antimicrob Agents Chemother 68:e0080323.

111. Taguchi A, Nakashima R, Nishino K. 2023. Functional and structural characterization of *Streptococcus pneumoniae* pyruvate kinase involved in fosfomycin resistance. J Biol Chem 299:104892.

112. Sharma A, Sharma R, Bhattacharyya T, Bhando T, Pathania R. 2017. Fosfomycin resistance in *Acinetobacter baumannii* is mediated by efflux through a major facilitator superfamily (MFS) transporter—AbaF. J Antimicrob Chemother 72:68–74.

113. Pasqua M, Bonaccorsi di Patti MC, Fanelli G, Utsumi R, Eguchi Y, Trirocco R, Prosseda G, Grossi M, Colonna B. 2021. Host-bacterial pathogen communication: the Wily role of the multidrug efflux pumps of the MFS family. Front Mol Biosci 8:723274.

114. Campos AC da C, Andrade NL, Couto N, Mutters NT, de Vos M, Rosa AC de P, Damasco PV, Lo Ten Foe JR, Friedrich AW, Chlebowicz-Flissikowska MA, Rossen JWA. 2020. Characterization of fosfomycin heteroresistance among multidrug-resistant *Escherichia coli* isolates from hospitalized patients in Rio de Janeiro, Brazil. J Glob Antimicrob Resist 22:584–593.

115. Srinivasan VB, Singh BB, Priyadarshi N, Chauhan NK, Rajamohan G. 2014. Role of novel multidrug efflux pump involved in drug resistance in *Klebsiella pneumoniae*. PloS One 9:e96288.

116. Zheng D, Bergen PJ, Landersdorfer CB, Hirsch EB. 2022. Differences in fosfomycin resistance mechanisms between *Pseudomonas aeruginosa* and Enterobacterales. Antimicrob Agents Chemother 66:e0144621.

117. Bixby ML, Raux BR, Bhalla A, McCoy C, Hirsch EB. 2022. Opportunities for antibiotic stewardship in emergency department or hospitalized patients with asymptomatic bacteriuria: identifying risk factors for antibiotic treatment. Antimicrob Steward Healthc Epidemiol 2:e16.

118. Johnson JR, Brown JJ. 1996. Defining inoculation conditions for the mouse model of ascending urinary tract infection that avoid immediate vesicoureteral reflux yet produce renal and bladder infection. 3. J Infect Dis 173:746–749.

119. Johnson JR, Jelacic S, Schoening LM, Clabots C, Shaikh N, Mobley HLT, Tarr PI. 2005. The IrgA homologue adhesin Iha is an *Escherichia coli* virulence factor in murine urinary tract infection. 2. Infect Immun 73:965–971.

120. Johnson JR, Magistro G, Clabots C, Porter S, Manges A, Thuras P, Schubert S. 2018. Contribution of yersiniabactin to the virulence of an *Escherichia coli* sequence type 69 ("clonal group A") cystitis isolate in murine models of urinary tract infection and sepsis. Microb Pathog 120:128–131.

121. Johnson JR, Russo TA, Drawz SM, Clabots C, Olson R, Kuskowski MA, Rosen H. 2013. OxyR contributes to the virulence of a clonal group a *Escherichia coli* strain (O17:K+:H18) in animal models of urinary tract infection, subcutaneous infection, and systemic sepsis. Microb Pathog 64:1–5.

122. Tarchouna M, Ferjani A, Ben-Selma W, Boukadida J. 2013. Distribution of uropathogenic virulence genes in *Escherichia coli* isolated from patients with urinary tract infection. Int J Infect Dis 17:e450–e453.

123. Caneiras C, Lito L, Mayoralas-Alises S, Díaz-Lobato S, Melo-Cristino J, Duarte A. 2019. Virulence and resistance determinants of *Klebsiella pneumoniae* isolated from a Portuguese tertiary university hospital centre over a 31-year period. 6. Enfermedades Infecc Microbiol Clin Engl Ed 37:387–393.

124. Rezatofighi SE, Mirzarazi M, Salehi M. 2021. Virulence genes and phylogenetic groups of uropathogenic *Escherichia coli* isolates from patients with urinary tract infection and uninfected control subjects: a case-control study. BMC Infect Dis 21:361.

125. Zhanel GG, Walkty AJ, Karlowsky JA. 2016. Fosfomycin: A First-Line Oral Therapy for Acute Uncomplicated Cystitis. Can J Infect Dis Med Microbiol 2016:e2082693.

126. Wijma RA, Koch BCP, Gelder T van, Mouton JW. 2018. High interindividual variability in urinary fosfomycin concentrations in healthy female volunteers. Clin Microbiol Infect 24:528–532.

127. Cattoir V, Pourbaix A, Magnan M, Chau F, de Lastours V, Felden B, Fantin B, Guérin F. 2020. Novel Chromosomal Mutations Responsible for Fosfomycin Resistance in *Escherichia coli*. Front Microbiol 11:575031.

128. Martin LW, Robson CL, Watts AM, Gray AR, Wainwright CE, Bell SC, Ramsay KA, Kidd TJ, Reid DW, Brockway B, Lamont IL. 2018. Expression of *Pseudomonas aeruginosa* Antibiotic Resistance Genes Varies Greatly during Infections in Cystic Fibrosis Patients. Antimicrob Agents Chemother 62:e01789-18.

129. Gomes AÉI, Stuchi LP, Siqueira NMG, Henrique JB, Vicentini R, Ribeiro ML, Darrieux M, Ferraz LFC. 2018. Selection and validation of reference genes for gene expression studies

in *Klebsiella pneumoniae* using reverse transcription quantitative real-time PCR. 1. Sci Rep 8:9001.

130. Sojo-Dorado J, López-Hernández I, Rosso-Fernandez C, Morales IM, Palacios-Baena ZR, Hernández-Torres A, Merino de Lucas E, Escolà-Vergé L, Bereciartua E, García-Vázquez E, Pintado V, Boix-Palop L, Natera-Kindelán C, Sorlí L, Borrell N, Giner-Oncina L, Amador-Prous C, Shaw E, Jover-Saenz A, Molina J, Martínez-Alvarez RM, Dueñas CJ, Calvo-Montes J, Silva JT, Cárdenes MA, Lecuona M, Pomar V, Valiente de Santis L, Yagüe-Guirao G, Lobo-Acosta MA, Merino-Bohórquez V, Pascual A, Rodríguez-Baño J, REIPI-GEIRAS-FOREST group. 2022. Effectiveness of fosfomycin for the treatment of multidrug-resistant *Escherichia coli* bacteremic urinary tract infections: a randomized clinical trial. JAMA Netw Open 5:e2137277.