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# Oral fosfomycin activity against *Klebsiella pneumoniae* in a dynamic bladder infection *in vitro* model

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**Introduction:** The use of oral fosfomycin for urinary tract infections (UTIs) caused by non-*Escherichia coli* uropathogens is uncertain, including *Klebsiella pneumoniae*, the second most common uropathogen.

**Methods:** A multicompartment bladder infection *in vitro* model was used with standard media and synthetic human urine (SHU) to simulate urinary fosfomycin exposure after a single 3 g oral dose ($fAUC_{0-72}$ 16884 mg·h/L, $t_{1/2}$ 5.5 h) against 15 *K. pneumoniae* isolates including ATCC 13883 (MIC 2 to >1024 mg/L) with a constant media inflow (20 mL/h) and 4-hourly voiding of each bladder. The impact of the media (CAMHB + G6P versus SHU) on fosfomycin MIC measurements, drug-free growth kinetics and regrowth after fosfomycin administration was assessed. A low and high starting inoculum (5.5 versus 7.5 $\log_{10}$ cfu/mL) was assessed in the bladder infection model.

**Results:** Compared with CAMHB, isolates in SHU had a slower growth rate doubling time (37.7 versus 24.1 min) and reduced growth capacity (9.0 ± 0.3 versus 9.4 ± 0.3 $\log_{10}$ cfu/mL), which was further restricted with increased inflow rate (40 mL/h) and more frequent voids (2-hourly). Regrowth was commonly observed in both media with emergence of fosfomycin resistance promoted by a high starting inoculum in CAMHB (MIC rise to ≥1024 mg/L in 13/14 isolates). Resistance was rarely detected in SHU, even with a high starting inoculum (MIC rise to ≥1024 mg/L in 2/14 isolates).

**Conclusions:** Simulated in an *in vitro* UTI model, the regrowth of *K. pneumoniae* urinary isolates was inadequately suppressed following oral fosfomycin therapy. Efficacy was further reduced by a high starting inoculum.

## Introduction

Urinary tract infections (UTIs) are among the most commonly encountered infections and the second most common cause of sepsis.[1] There has been an 8-fold increase in antimicrobial resistance (AMR) among urinary pathogens over the past two decades.[2] Oral fosfomycin is commonly recommended as a first-line agent in treatment guidelines. In adult patients attending emergency departments between 2010 and 2016, fosfomycin susceptibility among all uropathogens was 87.8%, and higher for

*Escherichia coli* (97.5%).[3] High susceptibility rates are maintained among MDR strains.[4,5]

*Klebsiella pneumoniae* is the second most common uropathogen implicated in uncomplicated UTIs.[6] MDR strains of *K. pneumoniae* are challenging to treat and associated with increased mortality.[7,8] Oral fosfomycin can represent the only oral antibiotic option for some strains.[9,10] *K. pneumoniae* have a WT population for fosfomycin with MIC ≤64 mg/L. Yet, *in vitro* activity is limited by heteroresistance and multiple mechanisms of resistance, including a chromosomally encoded *fosA* gene,

defects in transporter genes, their regulators and target modifications.[11,12] CLSI restrict oral fosfomycin susceptibility for *E. coli* uropathogens (S ≤64 mg/L, R >128 mg/L).[13] EUCAST had considered oral fosfomycin susceptibility for all Enterobacterales; however, in 2021 they revised this to apply only to *E. coli*, and have lowered the breakpoint (S ≤8 mg/L, R >8 mg/L).[14] Problems with fosfomycin susceptibility testing, however, have been widely described.[15–19]

Accurate simulation of the urinary environment is critical when translating *in vitro* to *in vivo* pharmacodynamics (PD). The availability of nutritional factors is related to MIC measurements, with reduced nutrients resulting in both a slower growth rate and could promote higher kill rate.[20] Urine is nutritionally depleted and naturally antimicrobial, with hypertonicity, low pH, low oxygen content and high nitrites and urea. The measurement of fosfomycin activity, however, is promoted by the supplementation of laboratory media with glucose-6-phosphate (G6P), which is found in negligible concentrations in human urine.[21] Urodynamics is an additional important factor that can promote UTI clinical cure, with increased urine production and frequent bladder voiding. These are all important factors when performing pharmacokinetics (PK)/PD studies using a dynamic bladder infection *in vitro* model.

To assess fosfomycin activity against *K. pneumoniae* within the urinary environment, we have performed *in vitro* PD profiling against clinical urinary isolates. The experimental parameters investigated were selected to assess the impact of the media on MIC measurements, growth kinetics and fosfomycin activity in a urodynamic simulation of UTI treatment with a single 3 g dose of oral fosfomycin. To assess the impact of baseline fosfomycin heteroresistance on the emergence of resistance, low and high starting inocula were assessed, and genomic changes in the post-exposure regrowth explored.

## Methods

### Media and antibiotic

Cation-adjusted Mueller–Hinton II agar (MHA, Becton Dickinson, USA), CAMHB (Becton Dickinson) and synthetic human urine (SHU) were used. SHU components are presented in Table S1 (Table S1 is available as Supplementary data at *JAC* Online), originally described by Ipe *et al.*[22] Intravenous fosfomycin (Meiji Seika Pharma, Tokyo, Japan) was used. Where indicated, G6P (Sigma–Aldrich, USA) was added to CAMHB and SHU at a concentration of 25 mg/L, and to SHU at 0.2 mg/L to reflect the physiological urinary concentration.[21]

### K. pneumoniae isolates and susceptibility testing

Clinical, non-duplicate *K. pneumoniae* urinary isolates were obtained from a surveillance collection at a tertiary acute care hospital in metropolitan Melbourne, Australia (Table S2; Ethics approval 533/16). Isolates underwent fosfomycin susceptibility testing by agar dilution (AD) following standard methodology.[23] MIC ≤64 mg/L was used to distinguish WT from the non-WT strains. The differential effect of fosfomycin on 14 representative clinical isolates and ATCC 13883 was assessed (Table 1). These isolates underwent additional fosfomycin susceptibility testing by disc diffusion (FOT200 discs, Oxoid Ltd/Thermo Fisher Scientific, UK) and broth microdilution (BMD). Disc diffusion diameter >15 mm (ignoring inner colonies within inhibition zone) was used to distinguish WT from the non-WT strains.[24] The impact of the media on MIC measurements was quantified, with the

mean difference and SD calculated (see Supplementary Methods). High-level fosfomycin-resistant (HLR) subpopulations were assessed (see Supplementary Methods).

### Bladder infection in vitro model

A modified multicompartment *in vitro* model that applies a continuous dilution system was used (see Supplementary Methods and Figure S1). Urinary excretion of fosfomycin after a single 3 g oral dose was simulated. Sixteen bladder compartments were run in parallel. Each bladder was inoculated with a *K. pneumoniae* isolate. Bladder filling and intermittent voiding mimicked normal human urodynamics. The main modification from the previous *in vitro* design[25] was an increase in media inflow to 20 mL/h into each bladder compartment (previously 3.75 mL/h). Bladder compartments underwent 4-hourly voiding that reduced the volume to 5 mL. The impact of the media (CAMHB with G6P versus SHU) and the starting inoculum (5.5 versus 7.5 $\log_{10}$ cfu/mL) on regrowth and emergence of resistance was assessed following fosfomycin administration.

### Fosfomycin PK

Observed urinary fosfomycin concentrations in healthy volunteers informed the *in vitro* simulation, with a peak concentration of 1000 mg/L after 6 h, urinary half-life 5.5 h and $fAUC_{0-72}$ 16884 mg·h/L.[26–28] Measurement of fosfomycin concentrations was performed by ultra-high-performance liquid chromatography–tandem MS (UHPLC–MS/MS) (see Supplementary Methods).[29] All 16 bladder compartments were sampled at peak concentration (6 h). Representative samples were collected from three bladder compartments at 4, 8, 24, 48 and 72 h. Measured concentrations were compared with target values using Bland–Altman plots and linear regression. Fosfomycin stability was confirmed, with <5% reduction in concentration after incubation at 37°C for 72 h.

### Quantification of bacterial growth

Drug-free bacterial growth under static incubation (37°C with vigorous shaking) and dynamic incubation (in the bladder infection model) were compared in both CAMHB (with G6P) and SHU. Under drug-free conditions, the bladder infection model was run at two speeds (20 mL/h, 4-hourly voids; 40 mL/h, 2-hourly voids) to assess the impact of simulated increased urodynamics. Drug-free growth rate (presented as generation time, $t_{gen}$) was obtained from linear regression of the growth curve over 2–4 h incubation, selected for when the growth was expected to be maximal. Growth capacity was the bacterial density after 24 h incubation. Comparison by one-way ANOVA.

Following fosfomycin administration in the bladder infection model, samples for bacterial density were collected at 4, 8, 24, 48 and 72 h. *K. pneumoniae* ATCC 13883 was run in duplicate as a biological replicate. Samples were collected directly from each bladder compartment. Bacterial cells were washed twice with saline and then underwent serial 10-fold dilutions in saline, of which 20 μL was plated from each dilution onto drug-free MHA (for total growth quantification) and on MHA with 512 mg/L fosfomycin and 25 mg/L G6P (for HLR quantification, performed every 24 h). Plates were incubated aerobically at 37°C for 16–20 h. Plates with fosfomycin were re-incubated for an additional 24 h. Limit of detection was 50 cfu/mL.

### Molecular assessment of fosfomycin resistance

Baseline (parent) isolates underwent WGS as reported previously.[30,31] A targeted exploration of key fosfomycin resistance genes and promoter regions was performed (*murA*, *fosA*, *uhpT*, *glpT*, *uhpA*, *uhpB*, *uhpC*, *cyaA*, *ptsI* and *glpR*) to identify sequence variations that distinguished non-WT isolates (fosfomycin MIC >64 mg/L) from those with lower MICs (see

**Table 1.** Baseline fosfomycin susceptibility and heteroresistance of the representative *K. pneumoniae* isolates

| Strain # | Standard susceptibility testing | | | | Heteroresistance | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | AD MIC (mg/L)[a] | | DD (inhibition zone, mm)[b] | | High inoculum AD (mg/L)[c] | High inoculum DD (mm)[d] | Disc elution (time, h)[e] | RSP proportion in CAMHB[f] | RSP proportion in SHU[f] |
| Clinical isolates | | | | | | | | | |
| INF014 | 2 | WT | 25 | WT | 64 | 22 | POS (72) | 1E-07 | – |
| INF321 | 2 | WT | 24 | WT | 64 | 22 | POS (48) | 1E-06 | – |
| INF215 | 4 | WT | 24 | WT | 64 | 17 | POS (48) | 1E-07 | – |
| INF174 | 4 | WT | 23 | WT | 128 | 19 | POS (48) | 4E-07 | – |
| INF044 | 8 | WT | 23 | WT | 256 | 19 | POS (48) | 2E-06 | – |
| INF344 | 8 | WT | 21 | WT | 256 | 17 | POS (48) | 4E-06 | 9E-07 |
| INF079 | 8 | WT | 20 | WT | 256 | 18 | POS (48) | 8E-07 | – |
| INF018 | 8 | WT | 21 | WT | 256 | 15 | POS (48) | 1E-06 | 4E-06 |
| INF171 | 16 | WT | 24 | WT | 256 | 13 | POS (48) | 3E-06 | 1E-06 |
| INF142 | 32 | WT | 22 | WT | 256 | 16 | POS (72) | 3E-06 | 1E-05 |
| INF223 | 32 | WT | 22 | WT | 128 | 14 | POS (24) | 5E-07 | – |
| INF161 | 32 | WT | 20 | WT | 256 | 16 | POS (48) | 1E-05 | 4E-06 |
| INF348[g] | 128 | non-WT | 11 | non-WT | 512 | no zone | POS (24) | 3E-05 | 8E-06 |
| INF249[g] | >1024 | non-WT | no zone | non-WT | >1024 | no zone | POS (24) | 1E+00 | 7E-01 |
| ATCC | | | | | | | | | |
| 13883 | 64 | WT | 21 | WT | 1024 | 20 | POS (48) | 1E-05 | 1E-05 |

AD, agar dilution; DD, disc diffusion; RSP, resistant subpopulation; –, indicates resistant growth not detected after 24 h incubation.

[a]AD was performed in triplicate (median value reported). An MIC ≤64 mg/L was used to distinguish WT from the non-WT strains.

[b]DD was performed in triplicate (mean inhibition diameter reported). An inhibition diameter of >15 mm was used to distinguish WT from the non-WT strains. DD results applied the EUCAST reading guide that ignores any inner colonies within the inhibition zone.

[c]AD performed with $1 \times 10^6$ cfu/spot.

[d]DD performed with a lawn culture prepared directly using turbid overnight growth in CAMHB. Inhibition diameter ignores inner colonies within the inhibition zone.

[e]Disc elution screening test with six FOT200 discs (Oxoid Ltd/Thermo Fisher Scientific, UK) added to 2 mL CAMHB, inoculated with 100 μL of a $10^9$ cfu/mL bacterial suspension from an overnight culture in CAMHB, and assessed for turbidity (POS, positive) over 72 h incubation.

[f]RSP was determined after 24 h incubation in the bladder infection model in CAMHB with G6P, or SHU, with an inflow rate of 20 mL/h and 4-hourly voiding. The proportion was calculated from growth quantified on MHA with 512 mg/L fosfomycin (and 25 mg/L G6P) compared with total growth on drug-free MHA. All RSPs were confirmed to have a fosfomycin MIC of ≥1024 mg/L by standard AD methodology, except for INF174 that measured MIC 512 mg/L.

[g]Non-WT isolates (INF249 and INF348) both had a premature STOP codon in *uhpB*.

Supplementary Methods). Baseline HLR subpopulation and post-exposure regrowth strains were sequenced and compared with their respective parent strains. Sequence variations in fosfomycin resistance genes and promoter regions were assessed, as well as a broad exploration of single nucleotide variations (SNVs) across the chromosome (see Supplementary Methods). All sequence reads were deposited in the NCBI Sequence Read Archives (project accession PRJNA701073).

## Results

### *Baseline fosfomycin susceptibility*

Fifty *K. pneumoniae* clinical urinary isolates were selected for fosfomycin AD MIC testing. The fosfomycin MIC distribution was similar to EUCAST[32] and a collection published from the Netherlands[24] (Figure 1 and Table S2). Fourteen representative clinical isolates, and *K. pneumoniae* ATCC 13883, were selected for further testing based on their fosfomycin MIC (range 2 to >1024 mg/L). All isolates had disc diffusion diameters of ≤25 mm (Table 1). Isolates had a wide variety of STs, virulence and AMR genes (Table S3).

### *Baseline fosfomycin-resistant subpopulations*

All representative isolates had a detectable HLR subpopulation after overnight incubation in CAMHB with G6P. The HLR subpopulation was present in the WT strains at a proportion of $10^{-6}$–$10^{-7}$ (Table 1). When incubated in SHU, only 8 of 15 isolates had a detectable HLR subpopulation. Given that the total growth capacity in SHU was 1 $\log_{10}$ cfu/mL lower compared with incubation in CAMHB, the HLR subpopulation was likely below the limit of detection in some strains. Using a high-density inoculum with modified AD and disc diffusion testing demonstrated universal elevations in MIC measurements and reductions in inhibition zone diameters (Table 1). All isolates were positive by a modified disc elution fosfomycin heteroresistance screening test (see Supplementary Methods).

### *Impact of the media on fosfomycin MIC measurements*

Fosfomycin MIC measurements in different media are presented in Table S4. Compared with AD (MHA with 25 mg/L G6P), testing
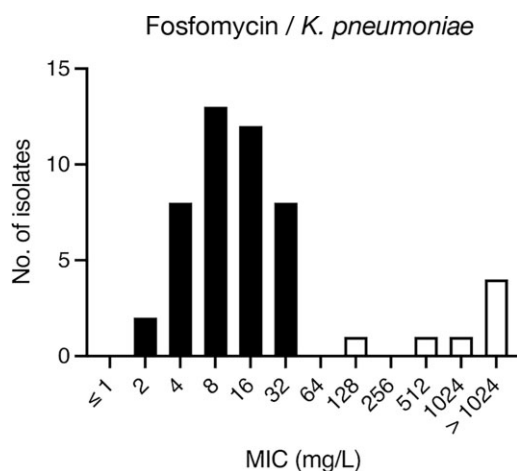
## Fosfomycin / *K. pneumoniae*



**Figure 1.** Fosfomycin MIC distribution among clinical *K. pneumoniae* urinary isolates (*n* = 50). MIC performed by agar dilution. Solid bars represent isolates with MICs within the WT range.

by BMD (CAMHB with 25 mg/L G6P) had a mean (± SD) difference of 1.1 ± 1.0 MIC dilutions. Testing without G6P supplementation increases the mean (± SD) difference in MIC measurements by 4.9 ± 2.0 dilutions (by AD in MHA) and by 4.0 ± 1.5 dilutions (by BMD in CAMHB [pH 7.3]), except isolate INF348 that maintained MIC within ≤1 2-fold dilution. All MIC values were ≥512 mg/L when tested in SHU (pH 5.6), with an insignificant change with the addition of a physiological amount of G6P (0.2 mg/L[21]). In contrast, when 25 mg/L G6P was added to SHU, all MIC measurements reduced, except for isolate INF348, which maintained an MIC of 1024 mg/L. In CAMHB, an acidic environment (pH 5.6) promoted a small increase in fosfomycin activity, reflected by a single MIC dilution reduction compared with pH 7.3

(mean difference: –0.7 ± 0.8 dilutions in CAMHB with G6P; –1.4 ± 0.6 dilutions in CAMHB without G6P). The impact of reducing the amount of nutrients in CAMHB was dependent on dilution with saline or water. Compared with full-strength CAMHB, MICs were maintained within ≤1 2-fold dilution with a 1:10 dilution with PBS (mean difference: –0.5 ± 1.0 dilutions in CAMHB with G6P; 0.5 ± 0.7 dilutions in CAMHB without G6P). In contrast, CAMHB (with and without G6P) diluted 1:10 with distilled water, MICs were greatly reduced (mean difference: –4.1 ± 1.7 dilutions in CAMHB with G6P; –4.0 ± 1.0 dilutions in CAMHB without G6P).

### Impact of the media and simulated urodynamics on bacterial growth

Bacterial growth under drug-free static conditions was slower in SHU compared with CAMHB ($t_{gen}$ 37.7 min in SHU versus 24.1 min in CAMHB [with G6P]) with a reduced growth capacity (9.0 ± 0.3 $\log_{10}$ cfu/mL in SHU versus 9.4 ± 0.3 $\log_{10}$ cfu/mL in CAMHB [with G6P], mean difference: –0.41 $\log_{10}$ cfu/mL, *P* = 0.0077) (Figure 2 and Table 2). When incubated in CAMHB under dynamic conditions in the bladder infection model, the change in bacterial density was most affected when the media inflow rate was increased to 40 mL/h with 2-hourly voids ($t_{gen}$: 73.4 min). Compared with static incubation, growth capacity in CAMHB was reduced during dynamic incubation (mean difference at 20 mL/h: –0.62 $\log_{10}$ cfu/mL, *P* = 0.0002; mean difference at 40 mL/h: –1.2 $\log_{10}$ cfu/mL, *P* < 0.0001). Dynamic incubation in SHU resulted in greater impacts in growth kinetics ($t_{gen}$ at 20 mL/h: 60.1 min; $t_{gen}$ at 40 mL/h: bacterial density failed to increase over the initial 6 h of incubation) (Figure 2 and Table 2). Compared with static incubation, growth capacity in SHU was further reduced during dynamic incubation (mean difference at 20 mL/h: –1.1 $\log_{10}$ cfu/mL, *P* ≤ 0.0001; mean difference at 40 mL/h: –2.2 $\log_{10}$ cfu/mL, *P* = 0.0001).

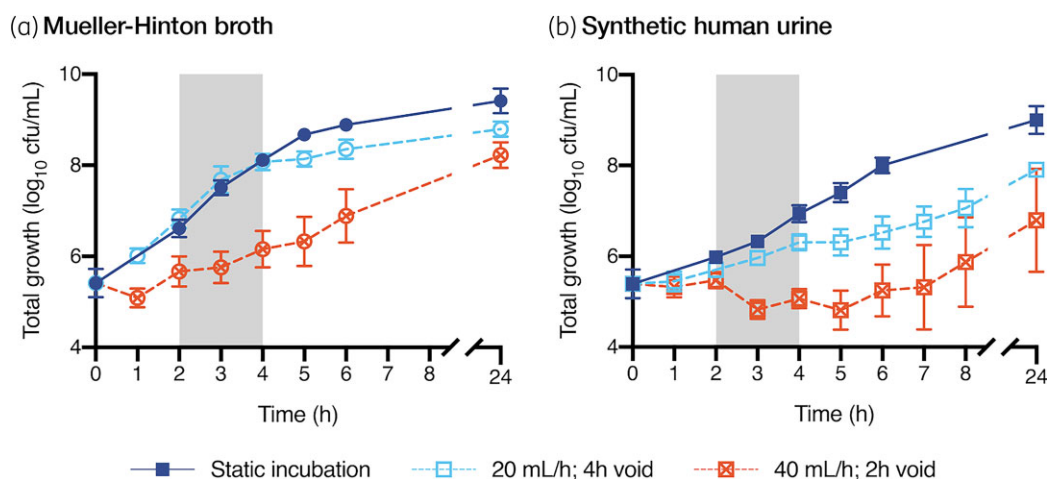(a) **Mueller-Hinton broth**  (b) **Synthetic human urine**



**Figure 2.** Drug-free growth under static and dynamic incubation conditions. Bacterial growth is presented as the average (± SD) of the 14 clinical isolates and the ATCC strain. Static incubation performed in 20 mL media, incubated at 36°C with vigorous shaking (200 rpm), without media inflow or outflow. Dynamic incubation performed in the bladder infection *in vitro* model under two different conditions: media inflow at 20 mL/h with 4-hourly voids compared with 40 mL/h with 2-hourly voids. The grey shaded area highlights the time over which the growth rate was assessed (2–4 h). This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

**Table 2.** Drug-free growth under static and dynamic incubation settings

| | | Time period | | |
|---|---|---|---|---|
| | | Initial growth (2–4 h) | | Maximum growth (24 h) |
| Media | Incubation conditions[a,b] | Δ in bacterial density ($\log_{10}$ cfu/mL) | Average $t_{gen}$ (min) | Total bacterial density ($\log_{10}$ cfu/mL) |
| CAMHB + G6P | Static | 1.5 ($\pm$0.2) | 24.1 | 9.4 ($\pm$0.3) |
| CAMHB + G6P | 20 mL/h; 4 h void | 1.3 ($\pm$0.2) | 28.9 | 8.8 ($\pm$0.2) |
| CAMHB + G6P | 40 mL/h; 2 h void | 0.5 ($\pm$0.3) | 73.4 | 8.2 ($\pm$0.3) |
| SHU | Static | 1.0 ($\pm$0.1) | 37.7 | 9.0 ($\pm$0.3) |
| SHU | 20 mL/h; 4 h void | 0.6 ($\pm$0.2) | 60.1 | 7.9 ($\pm$0.1) |
| SHU | 40 mL/h; 2 h void | −0.4 ($\pm$0.2) | NA | 6.8 ($\pm$1.1) |

NA, not applicable (due to a reduction in bacterial density that was observed over the initial 2–4 h time period).

Bacterial growth is presented as the average ($\pm$ SD) of the 14 clinical isolates and the ATCC strain. The $t_{gen}$ (min) is calculated from the average change in bacterial density over a 2 h time period (2–4 h).

[a]Static incubation in 20 mL media, incubated at 36°C with vigorous shaking (200 rpm), without any media inflow or outflow.

[b]Dynamic incubation in the bladder infection *in vitro* model with constant media inflow and intermittent voiding.

### Response to fosfomycin exposure in the bladder infection model

*In vitro* fosfomycin concentrations from the bladder compartments confirmed the targeted simulated fosfomycin exposure after a single 3 g oral dose (Figure S2). Despite humanized fosfomycin exposure, post-exposure regrowth was commonly observed across all representative *K. pneumoniae* isolates, regardless of baseline fosfomycin MIC (Table 3). HLR clinical isolate, INF249 (baseline MIC ≥1024 mg/L), regrew maximally in all dynamic experiments, with the quantification of the resistant subpopulation equal to the total growth at all sampling timepoints.

#### CAMHB with G6P

For the remaining 14 isolates (excluding the HLR strain INF249), following a high starting inoculum (7.5 $\pm$ 0.1 $\log_{10}$ cfu/mL), 13 isolates had maximal regrowth at 72 h with a rise in fosfomycin MIC to ≥1024 mg/L. Only isolate INF321 (MIC 2 mg/L) had undetected regrowth (Figure 3a). A resistant subpopulation was detected in the starting inoculum in half of the isolates (INF018, INF171, INF142, INF223, INF161, INF348, ATCC 13883 [both replicates]) at a density between 2.0 and 3.4 $\log_{10}$ cfu/mL. In contrast, following a low starting inoculum (5.4 $\pm$ 0.2 $\log_{10}$ cfu/mL), three isolates had undetected regrowth (INF321, INF215 and ATCC 13883 [one of two replicates]) (Figure 3b), only two isolates had a rise in MIC to ≥1024 mg/L (INF142 and ATCC 13883 [single replicate]) and the remaining isolates regrew without a significant change in fosfomycin MIC. Resistant subpopulations were below the limit of detection in the low starting inoculum (except INF249). There was a trend in isolates with baseline MICs ≤16 mg/L to have sub-maximal regrowth at 72 h (≤7.0 $\log_{10}$ cfu/mL) without a detectable resistant subpopulation (Table 3).

#### SHU

Regardless of the starting inoculum, following fosfomycin exposure, all isolates regrew to a maximal growth capacity, but with limited post-exposure amplification of the resistant subpopulation (Figure 3c and d). Resistant subpopulations were detected in the high and low starting inoculums in the same isolates as the CAMHB experiments. Following a high starting inoculum (7.5 $\pm$ 0.1 $\log_{10}$ cfu/mL), only two isolates had a rise in MIC to ≥1024 mg/L (INF215 and INF348), and two isolates had smaller rises in MIC (INF014 [MIC rise from 2 to 512 mg/L] and INF161 [MIC rise from 32 to 256 mg/L]). With a low starting inoculum (5.3 $\pm$ 0.1 $\log_{10}$ cfu/mL), only one isolate had a post-exposure rise in MIC ≥1024 mg/L (INF215) (Table 3).

### Molecular mechanisms of fosfomycin resistance in baseline clinical isolates

Comparative WGS on the 50 *K. pneumoniae* clinical isolates demonstrated 5 of 7 non-WT strains had a premature STOP codon in *uhpB* (*n*=4 ST29, *n*=1 ST34). The other 2 isolates had unique mutations: INF011 (ST1393) L14S in *glpT* and T63A in *uhpC*; INF319 (ST461) L100M in *uhpC* (Table S2). Of the 15 isolates tested in the bladder infection model, two genomes failed quality control (INF018-postexp-SHU and INF321-HLR-MHBG6P). When compared with their respective parent strains, the HLR subpopulation and post-exposure regrowth derivates had a median seven chromosomal SNVs (range 0–26; Table S5). Non-synonymous mutations were identified in 36 distinct genes, the majority of which (*n*=28, 78%) were mutated in only a single genome. Putative deletions were commonly detected in a wide variety of genes, the most common identified in a sodium/solute symporter family protein and was observed in 8/15 isolates (INF018, INF044, INF079, INF161, INF215, INF223, INF321, INF344) (Table S6).

*Fosfomycin gene mutations in the baseline HLR subpopulation*

Isolates INF249 (MIC >1024 mg/L) and INF348 (MIC 128 mg/L) had the same truncation in *uhpB* in all derivative strains that was detected in the parent strain (Table S5). In CAMHB with G6P, only three HLR subpopulation strains (INF142, INF171,

**Table 3.** Post-exposure outcome (change in bacterial density at 72 h from starting inoculum) following single dose fosfomycin, comparison of high and low starting inoculum and incubation in different media

| Strain # | Media: CAMHB + G6P | | | | Media: SHU | | | |
|---|---|---|---|---|---|---|---|---|
| | Low inoculum[a] | | High inoculum[b] | | Low inoculum[c] | | High inoculum[d] | |
| | TG (MIC) | RSP (Proportion) | TG (MIC) | RSP (Proportion) | TG (MIC) | RSP (Proportion) | TG (MIC) | RSP (Proportion) |
| Clinical isolates | | | | | | | | |
| INF014 | −3.2 (2) | – | 1.6 (>1024) | 7.1 (5E-01) | 2.8 (2) | – | 0.5 (512) | 3.2 (7E-04) |
| INF321 | – | – | – | – | 3.0 (8) | – | 0.2 (8) | – |
| INF215 | 1.8 (4) | – | 1.4 (>1024) | 7.1 (8E-01) | 3.1 (>1024) | 4.2 (3E-03) | 0.8 (>1024) | 5.2 (7E-02) |
| INF174 | – | – | 2.1 (>1024) | 6.9 (6E-01) | 2.6 (8) | 0.3 (1E-06) | 0.3 (16) | 1.9 (9E-05) |
| INF044 | −0.4 (8) | – | 1.4 (>1024) | 7.0 (2E-01) | 2.9 (16) | 1.3 (5E-05) | 0.8 (16) | 1.2 (5E-06) |
| INF344 | −1.5 (8) | – | 1.8 (>1024) | 6.8 (8E-01) | 3.1 (8) | 2.2 (3E-05) | 1.2 (16) | 2.3 (3E-05) |
| INF079 | 1.7 (16) | – | 1.5 (>1024) | 6.7 (5E-03) | 2.7 (32) | 0.5 (1E-06) | 0.6 (16) | 4.0 (5E-03) |
| INF018 | 0.8 (16) | – | 1.6 (>1024) | 6.8 (5E-02) | 3.0 (32) | 1.0 (5E-06) | 0.5 (16) | 2.5 (1E-03) |
| INF171 | 3.1 (16) | 2.4 (8E-05) | 1.4 (1024) | 4.6 (5E-03) | 3.1 (8) | 1.9 (2E-05) | 0.8 (32) | 0.9 (2E-05) |
| INF142 | 3.1 (>1024) | 6.2 (2E-01) | 1.3 (>1024) | 5.5 (5E-02) | 2.4 (32) | 1.7 (5E-05) | −0.3 (32) | – |
| INF223 | 3.3 (32) | 0.3 (3E-07) | 1.3 (>1024) | 5.4 (3E-01) | 2.8 (32) | – | −0.1 (32) | 0.2 (2E-05) |
| INF161 | 3.2 (32) | 1.3 (3E-06) | 1.3 (>1024) | 6.2 (5E-01) | 2.7 (32) | 1.3 (9E-06) | 0.9 (256) | −0.1 (2E-05) |
| INF348 | 3.5 (256) | 4.2 (1E-03) | 1.5 (>1024) | 6.0 (1) | 2.8 (256) | 2.9 (3E-04) | 0.5 (>1024) | 2.9 (1) |
| INF249 | 3.0 (>1024) | 3.1 (8E-01) | 1.1 (>1024) | 1.2 (1) | 3.1 (>1024) | 2.9 (8E-01) | 1.4 (>1024) | 1.5 (1) |
| ATCC[e] | | | | | | | | |
| 13883 | 3.7 (>1024) | 6.6 (3E-01) | 1.5 (>1024) | 6.2 (1) | 2.5 (128) | 2.7 (4E-04) | 1.0 (64) | 2.6 (1E-02) |
| | – | – | 1.7 (>1024) | 5.6 (3E-01) | 2.5 (64) | 4.1 (1E-02) | 0.8 (128) | 1.8 (4E-03) |

TG, total growth; RSP, resistant subpopulation; –, indicates no growth detected at 72 h, either TG or RSP growth.

TG, change in $\log_{10}$ cfu/mL at 72 h of the TG quantified on drug-free MHA, and the MIC determined by AD; RSP, change in $\log_{10}$ cfu/mL at 72 h of the RSP growth quantified on MHA with 512 mg/L fosfomycin (and 25 mg/L G6P), and the proportion that the RSP growth makes up of the total population. The RSP growth was confirmed by AD to have an MIC $\geq$1024 mg/L.

The bladder infection model was run with an inflow rate of 20 mL/h with 4-hourly intermittent voiding.

All fosfomycin MICs were performed by AD.

[a]Average starting inoculum 5.4 ($\pm$0.2) $\log_{10}$ cfu/mL.

[b]Average starting inoculum 7.5 ($\pm$0.1) $\log_{10}$ cfu/mL.

[c]Average starting inoculum 5.3 ($\pm$0.1) $\log_{10}$ cfu/mL.

[d]Average starting inoculum 7.5 ($\pm$0.1) $\log_{10}$ cfu/mL.

[e]ATCC strain tested in duplicate.

INF174) had new genomic changes compared with their parent strain. These included mutations in *uhpC*, *uhpT* and *ptsI*, respectively (Table S5). When incubated in SHU, four HLR subpopulation strains (INF018, INF161, INF171, INF348) had new genomic changes compared with their parent strain. Only one strain (INF171) had a mutation in the same gene (*uhpT*) identified after incubation in CAMHB with G6P. The other identified strains (INF018, INF161, INF348) had mutations in *uhpT*, *ptsI* and *glpT*, respectively (Table S5).

*Fosfomycin gene mutations in the regrowth population after fosfomycin exposure*

Isolates INF249 (MIC >1024 mg/L) and INF348 (MIC 128 mg/L) maintained the same truncation in *uhpB* detected in the parent strains. In CAMHB with G6P, only four strains (INF079, INF142, INF171, INF348) had new mutations compared with their parent strain, despite 13 strains demonstrating a rise in fosfomycin MIC to $\geq$1024 mg/L. These include mutations in *glpT* in three strains,

and INF142 had an insertion sequence upstream of *fosA*. These mutations were not identified in the baseline HLR subpopulation of these strains. When exposed to fosfomycin in SHU, only two strains (INF079, INF171) had new mutations in the fosfomycin resistance genes, including mutations in *glpT* and *uhpT*, respectively. Neither of these strains demonstrated a post-exposure rise in fosfomycin MIC. INF079 did not have mutations detected in a baseline HLR subpopulation. INF171 had a mutation detected in the same gene (*uhpT*) in the baseline HLR subpopulation; however, the mutation in the post-exposure regrowth was a different deletion and present in 50% of the population (Table S5).

## Discussion

The regrowth of *K. pneumoniae* urinary isolates appears to be inadequately suppressed following urinary fosfomycin exposure after a simulated single 3 g oral dose in an *in vitro* bladder infection model. Efficacy was reduced with a high starting inoculum
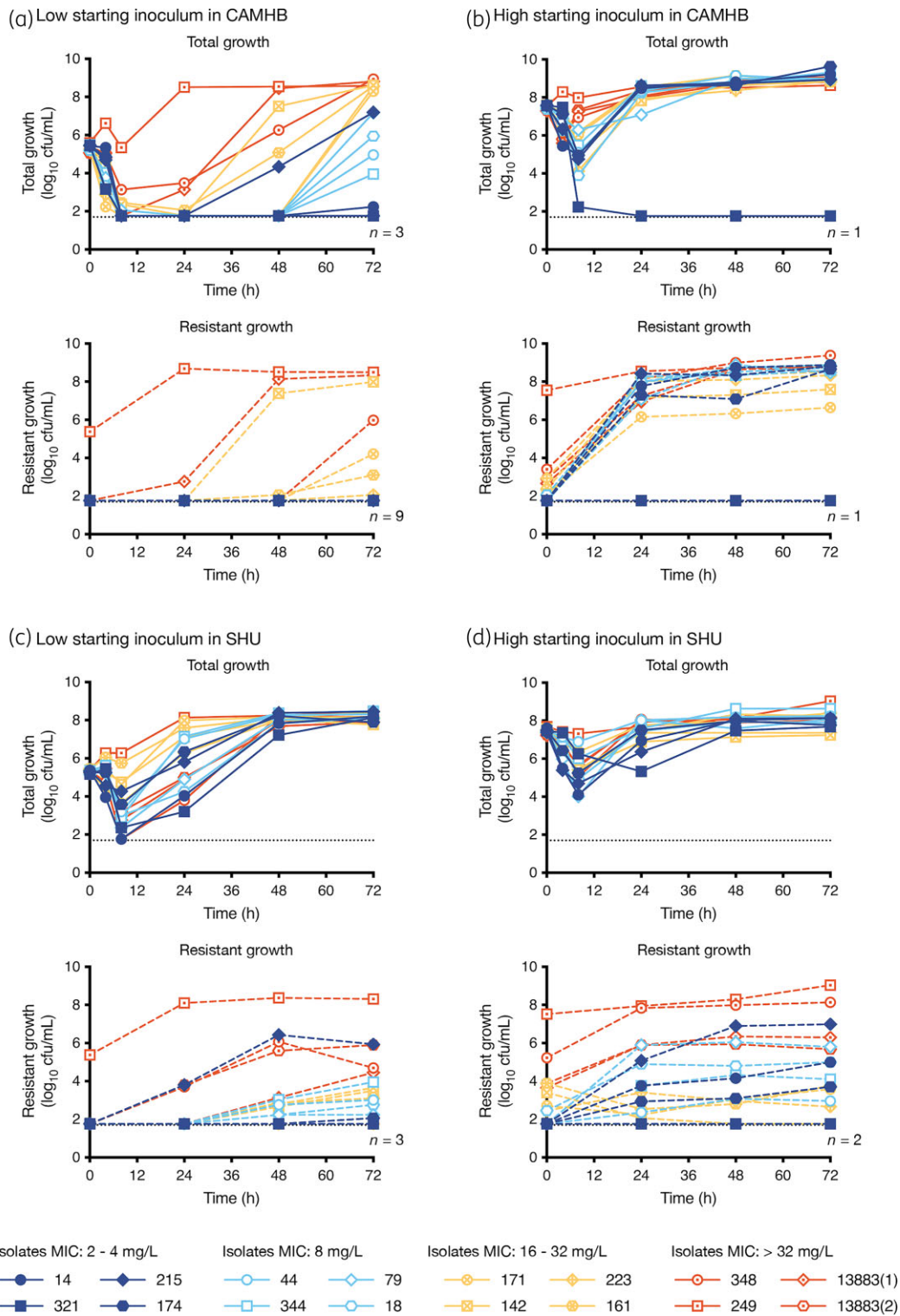
**Figure 3.** Growth response after a simulated single 3 g dose of oral fosfomycin. Each experiment is represented by two graphs illustrating the total growth (quantitative growth on drug-free MHA) and the fosfomycin-resistant growth (quantitative growth on MHA supplemented with 512 mg/L fosfomycin and 25 mg/L G6P). (a) Testing in CAMHB with 25 mg/L G6P with a low starting inoculum. (b) Testing in CAMHB with 25 mg/L G6P with a high starting inoculum. (c) Testing in SHU with a low starting inoculum. (d) Testing in SHU with a high starting inoculum. Solid lines represent the total growth. Dashed lines represent fosfomycin resistant growth. The 15 isolates are grouped by their baseline fosfomycin MIC measurement (2–4, 8, 16–32 and >32 mg/L). ATCC 13883 was run in duplicate. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

and in nutrient-depleted media simulating the biomatrix of urine. A low starting inoculum enabled the most fosfomycin activity. Although WGS testing identified mutations in derivate strains across the chromosome and in known fosfomycin resistance genes, many strains had elevated fosfomycin MIC measurements without an identified causative genomic change.

The evidence supporting fosfomycin efficacy for UTIs caused by non-*E. coli* urinary isolates is limited, including complicated UTIs and the use of multiple doses of fosfomycin. Clinical studies have reported higher persistence and recurrence rates in *K. pneumoniae* isolates treated with oral fosfomycin, and a divergence between *in vitro* susceptibility (92%) and microbiological cure (46%).[10,33] Fosfomycin heteroresistance and treatment selection of a pre-existing resistant subpopulation have been implicated in limited efficacy.[16,34,35] Our study, however, demonstrated different genomic changes in the baseline HLR subpopulation compared with the post-exposure derivative strains, when aligned with the parent strain.

The mechanisms of fosfomycin resistance are multiple in both gene and gene function. It is difficult to link the fosfomycin phenotype to a specific genotype. FosA, an enzyme that catalyses the $Mn^{2+}$-and $K^+$-dependent glutathione-mediated degradation of fosfomycin, is an intrinsic gene conserved among all *K. pneumoniae* strains.[12] This contrasts with *E. coli* strains with an acquired plasmid-borne *fosA3* gene, which affords an increase in the fosfomycin MIC to $\geq 1024$ mg/L.[36,37] Among the 50 baseline isolates, we identified 5 of 7 of the non-WT strain with a premature STOP codon in *uhpB*. UhpB encodes the kinase of the two-component response regulator (UhpA) required for *uhpT* expression. UhpT is a fosfomycin–G6P symporter. Down-regulation, or complete lack of expression, reduces the import of fosfomycin into the bacterial cell. This is supported by the fosfomycin susceptibility results observed in our isolate INF348, where G6P supplementation failed to enhance fosfomycin activity in the setting of having a premature STOP codon in *uhpB*. While we did not identify any experimentally derived isolates with acquired mutations in *uhpB*, we did identify five with disruptions in *uhpT* (four with elevated MICs) and one with a disruption in *uhpC* (with elevated MIC). Additionally, there were five derived isolates with acquired mutations in *glpT* (four with elevated MICs), which encodes for a second fosfomycin symporter. One additional isolate carried an insertion sequence insertion upstream of *fosA*, which may result in increased expression of this gene. In agreement with Ortiz-Padilla *et al*.,[38] we speculate that an elevation in fosfomycin MIC >64 mg/L is due to the combination of impaired fosfomycin symporters (UhpT and GlpT) with the chromosomally mediated FosA production. However, most of our experimentally derived isolates, including 30 with elevated fosfomycin MICs, did not acquire mutations in *uhp*, *glpT* or *fosA* or promoter regions, indicating that additional, yet unknown, mechanisms play a role in fosfomycin resistance. Our analyses also identified many distinct acquired SNVs in these isolates; however, these were distributed across different regions of the chromosome (Table S5), and we were unable to identify specific candidate resistance-causing mutations.

The site of infection is a vital aspect when translating *in vitro* simulations to human infections. To simulate the bladder environment, a synthetic urine alternative, SHU, was tested in parallel with standard laboratory media. We found that although regrowth was common in both media, there was reduced emergence of resistance in SHU. This was also supported by the observation that all isolates had fosfomycin MIC measurements $\geq 512$ mg/L when performed by BMD in SHU (Table S4). The reduced nutrients and pH 5.6 of SHU, compared with CAMHB, had a much less of an impact on MIC measurements compared with the presence or absence of G6P. This exemplifies how the environment impacts upon antibiotic activity and PD outcome, particularly important when considering the urinary environment.[39] Similarly, Hughes and Andersson[40] describe different bacterial evolutionary trajectories to antibiotic resistance, reporting that strong antibiotic selection pressure results in higher resistance and reduced fitness, whereas weak antibiotic selection selects for less resistance but mutants with greater fitness.

A major modification to the *in vitro* model compared with previous testing was a >5-fold increase in the media flow rate into each bladder compartment, thereby providing an improved dynamic simulation of human urodynamics. In this study, applying a 20 mL/h inflow rate resulted in faster and larger volume increases in the bladder compartments, before each timed, high-speed void. The simulation of urodynamics and large volume shifts is vital for informing UTI PD. This dynamic incubation also serves as an inherent test of uropathogen fitness, where successful strains must maintain an adequate growth rate to sustain a viable regrowth population.

The limitations of our *in vitro* model include the lack of an immune system and bladder tissue architecture. Furthermore, we demonstrated an occasion where the ATCC strain had discrepant results with its biological replicate, likely due to random differences between the two replicates in relation to the resistant subpopulation proportion included in the starting inocula. This is further reflective of the multiple experimental variables at play. However, testing multiple different strains with a broad spectrum of baseline susceptibility ensures no single result greatly impacts upon the final conclusions.

In this study, fosfomycin failed to eradicate *K. pneumoniae* uropathogens, regardless of baseline susceptibility. This suggests caution when considering the use of fosfomycin as monotherapy for the treatment of UTIs due to *K. pneumoniae*. These data support the recent change in the EUCAST oral fosfomycin breakpoints to restrict susceptibility testing to *E. coli*. The increased activity of fosfomycin observed with a low starting bacterial inoculum may support the use of fosfomycin if administered early in infection, along with increased fluid intake, frequent voiding and a functioning immune system. The role of combination antibiotic therapy, and the development of a FosA inhibitor, may facilitate a more certain role for fosfomycin against infections caused by *K. pneumoniae*.

## Transparency declarations

## Supplementary data
Methods, Tables S1 to S6 and Figures S1 and S2 are available as Supplementary data at *JAC* Online.

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