

1 **Experiences in fosfomycin susceptibility testing and resistance mechanism**
2 **determination in *E coli* from urinary tract infections in the UK**

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18 Running title: UK fosfomycin resistance in *E. coli*

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22 **Abstract**

23 Purpose: As numbers of bacterial isolates resistant to first line antibiotics rise there
24 has been a revival in the use of older drugs including fosfomycin with novel
25 mechanisms of action. We aimed to investigate the prevalence and the genotypic
26 nature of fosfomycin resistance in *E. coli* from urinary tract infections (UTI) using the
27 various methods available in the clinical microbiology laboratory.

28 Methodology: 1000 culture positive urine samples were assessed for the presence of
29 *E. coli* and fosfomycin susceptibility was determined using the MAST Uri®system,
30 microbroth dilution, agar dilution and E-test strips.

31 Results/Key findings: Initial investigation using breakpoint susceptibility testing on the
32 MAST Uri®system, deemed 62 of 657 (9.5%) *E. coli* as fosfomycin resistant (MIC
33 ≥ 32 $\mu\text{g/ml}$) However, on further testing, a lower rate of 8 of the 62 (1.3%) were
34 robustly confirmed to be resistant using micro-broth dilution, agar dilution and E-test
35 strips These true resistant isolates belonged to diverse *E. coli* MLST types and each
36 had a unique set of chromosomal alterations in genes associated with fosfomycin
37 resistance. Fosfomycin resistant isolates were not multiply drug resistance and did
38 not carry plasmidic fosfomycin resistance genes. Therefore, the use of fosfomycin
39 may be unlikely to drive selection of a particular clone or movement of transferrable
40 resistance genes.

41 Conclusion: Fosfomycin remains a viable option for the treatment of *E. coli* in
42 uncomplicated UTIs, different susceptibility testing platforms can give very different
43 results regarding the prevalence of fosfomycin resistance with false positives a
44 potential problem that may unnecessarily limit use of this agent.

45 **Keywords:** Fosfomycin; Susceptibility testing; Antibiotic Resistance

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47 **1.1 Introduction**

48 Globally, increasing numbers of infections are caused by bacteria resistant to current
49 antibiotics.(1) As there is a lack of new antibiotics in development, the revival of older
50 drugs with distinct methods of action has been proposed as a short-term solution.(2)
51 One such drug is fosfomycin, a phosphonic-acid derivative cell wall inhibitor with a
52 novel mode of action and a broad spectrum of activity. (3) . In *Enterobacteriaceae*,
53 fosfomycin is taken up by mimicking the natural substrates of two nutrient transport
54 uptake systems GlpT and UhpT (inducible in the presence of glucose-6-
55 phosphate);(4) systems which require cyclic AMP (cAMP), cAMP-receptor protein
56 complexes and activator genes such as *uhpA*.(5-7) Once in the bacterial cytosol,
57 fosfomycin acts as a phosphoenolpyruvate analogue preventing the initial step of
58 cell wall synthesis, via inhibition of MurA.(4) leading to the prevention of
59 peptidoglycan biosynthesis and cell death.(8) As fosfomycin acts prior in the
60 biosynthesis pathway to other cell wall inhibitors β -lactams and glycopeptides it is not
61 inhibited by resistance determinants which act against these drugs such as extended
62 spectrum beta-lactamases (ESBLs).(9)

63 Historically the most commonly documented mechanism of fosfomycin resistance
64 has been impaired transport of fosfomycin into the cytoplasm, due to mutations in
65 structural or regulatory genes of the nutrient transport systems.(10); for example in
66 *E. coli*, insertions, deletions or mutations leading to amino-acid changes in *glpT*, *uhpT*
67 or *uhpA*. Alternatively, mutations in genes encoding adenylylase (*cyaA*) and
68 phosphotransferases (*ptsI*) are known to decrease intracellular levels of cyclic-AMP,
69 reducing the expression of *glpT* and *uhpT* and, consequently intracellular fosfomycin
70 levels.(11) Mutations in the gene encoding the drug target MurA, particularly those
71 that confer amino-acid changes in the active site and Cys115 residue have been
72 demonstrated to decrease the susceptibility of the organism by reducing its affinity
73 for fosfomycin.(12-14); however these are rare in nature and may impair bacterial
74 fitness.(10) Over-expression of *murA* has also been found both in mutants selected
75 *in-vitro* and in clinical isolates. It has been suggested this mechanism acts to saturate
76 fosfomycin molecules thereby allowing normal cellular function.(15, 16)

77 A final and, perhaps emerging mechanism of resistance is the acquisition of enzymes
78 that can inactivate fosfomycin by catalysing the opening of its oxirane ring.(17,
79 18(19))

80 Data from multiple studies has shown that exposure to fosfomycin *in-vitro* rapidly
81 selects resistant mutants, at a frequency of 10^{-7} - 10^{-8} .(20, 21) However, mutants
82 selected experimentally are typically physiologically impaired; with decreased growth
83 rates in culture media and urine when compared to wild-type strains.(20) It is also
84 thought that fosfomycin resistant isolates may have a reduced ability to adhere to
85 uroepithelial cells or catheters, and to have a higher sensitivity to polymorphonuclear
86 cells and serum complement killing.(22) Therefore, it has been speculated that
87 despite the rapid development of resistance *in-vitro*, significant biological fitness costs
88 prevent the establishment and propagation of resistant strains *in-vivo*. (2, 20)

89 In Japan, Spain, Germany, Austria, France, Brazil, North America and South Africa,
90 fosfomycin has been used extensively for >30 years(23). In these regions a soluble
91 salt form called fosfomycin-tromethamine (typically given as a single 3 g oral dose) is
92 widely used in the treatment of uncomplicated UTIs.(24) Until recently, fosfomycin-
93 trometamol was not distributed or commercially available in the UK; and any products
94 used were imported, and therefore unlicensed. Despite this, the NHS recorded a ten-
95 fold increase in fosfomycin-trometamol prescriptions from 100 to 1000 between 2012
96 and 2013; (25) and a further increase to 2,400 prescriptions in 2014. (25)

97 Renewed interest in fosfomycin has been for treatment of MDR organisms causing
98 UTIs where oral therapy choices may be limited. Considering these factors and the
99 possibility of introducing fosfomycin preparations into our formulary, our first aim was
100 to determine the proportion of organisms isolated from routine UTIs culture deemed
101 resistance to fosfomycin. In doing so the various methods of measuring susceptibility
102 to fosfomycin available to our clinical laboratory were assessed, and their relative
103 merits considered. The second aim was to investigate mechanisms of fosfomycin
104 resistance.

105 **1.2 Materials and Methods**

106 **1.2.1 Bacterial isolates**

107 Between July and August 2014, 2800 urine specimens received as part of standard
108 patient care (over 18 days in total) at Northampton General Hospital, a large 700 bed
109 tertiary hospital in the UK were collected. Subsequent analysis of isolates and
110 susceptibility testing followed the laboratory work-flow and methodologies used for
111 clinical investigation of specimens in this trust. Each was examined for signs of
112 infection using Iris IQSprint microscopy and those specimens meeting conventional
113 clinical criteria were cultured using the MAST Uri@system (n=1000) as per the
114 manufactures instructions. The susceptibility status of each cultured isolate to
115 fosfomycin was determined using a 96-well 'breakpoint' agar plate containing 32
116 µg/ml fosfomycin supplemented with 25 µg/l of glucose-6-phosphate (G6P) as
117 provided by MAST, and a presumptive species identification was carried out by
118 determining the colour of colonies growing on MAST CUTI chromogenic agar. A total
119 of 62 isolates putatively identified as fosfomycin resistant *E. coli* then had their
120 species confirmed using MALDI-TOF and were retained for further study. *E. coli* J53-
121 2 (NCTC 50167) was used as a fosfomycin susceptible control; *E. coli* NCTC 10418
122 was used as a quality control for susceptibility testing; and *E. coli* MG1655 (ATCC
123 700926) was used as a reference strain for genome comparisons.

124 **1.2.2 Antimicrobial susceptibility testing**

125 The minimum inhibitory concentrations (MIC) of an extended panel of antimicrobials
126 were determined using the BD PhoenixTM automated microbiology system with
127 antimicrobial susceptibility testing panel UNMIC-409 as per the manufacturer's
128 instructions. Fosfomycin MICs were further determined using fosfomycin E-tests®
129 (bioMérieux) and using the agar dilution method following the British Society of
130 Antimicrobial Chemotherapy (BSAC) guidelines.(26)

131 **1.2.3 Whole genome sequencing (WGS) and post sequencing analysis**

132 Isolates consistently considered resistant by all susceptibility testing methods were
133 genome sequenced by MicrobesNG using an Illumina MiSeq system. Velvet (Version
134 1.2.10)(27) was used for *de-novo* assembly of the genomes, and Prokka (Version
135 1.11)(28) used for annotation. Reads were also analysed using the 'nullarbor' pipeline
136 (v1.2) using a standard virtual machine on the MRC CLIMB framework. Pan genomes

137 were generated using 'roary' (v8.0), SNPs called with 'snippy' (v3.0) and antibiotic
138 resistance genes and mutations identified using 'ARIBA' (v2.8.1). Trees were
139 visualised with 'Phandango'. All packages used default parameters unless stated
140 otherwise. The Centre for Genomic Epidemiology
141 (<http://www.genomicepidemiology.org/>) provided software for interrogation of
142 genomes for multi-locus sequence type (MLST), *E. coli* serotype, plasmid replicons
143 and resistance associated genes (ResFinder); the Comprehensive Antibiotic
144 Resistance Database (CARD) was additionally used to seek resistance
145 determinants.(29)

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148 **1.3 Results**

149 **1.3.1 Fosfomycin resistance in UTI isolates using MAST urisystem**

150 From 1000 UTI culture positive isolates, 657 were confirmed as *E. coli* and 62 (9.5%)
151 were deemed fosfomycin resistant using breakpoint plates on the MAST Uri®system,
152 with growth on ≥80% of the culture well indicating an MIC >32 µg/ml.

153 **1.3.2 Determination of fosfomycin minimum inhibitory concentrations**

154 **1.3.2.1 Fosfomycin MICs using BD Phoenix™**

155 Using an automated micro-broth dilution method (BD Phoenix™) 53/62 *E. coli*
156 isolates (85.5%) were found to have fosfomycin MICs of <16 µg/ml, three isolates
157 had an MIC of 32 µg/ml (4.8%) and six isolates had an MIC of 64 µg/ml (9.7%).
158 Therefore only six isolates showed concordance with data from the MAST
159 Uri®System, and were deemed resistant using BD interpretative software
160 (Epicentre™) with EUCAST breakpoints (>32 µg/ml).(30)

161 **1.3.2.2 Fosfomycin MICs using E-tests**

162 Due to the discrepancy between micro-broth dilution and breakpoint plate MIC
163 methods, E-tests were used as an alternative method for measuring fosfomycin MICs.
164 Two susceptible control strains, *E. coli* J53-2 and *E. coli* NCTC-10418 grew with
165 definitive zones of inhibition, revealing MICs of 0.25 µg/ml. Similarly, six selected
166 isolates deemed resistant using the MAST Uri®system but susceptible using the
167 micro-broth dilution (BD Phoenix™) were found to be sensitive to fosfomycin using
168 E-tests; each growing with a single defined zone of inhibition and MICs ranging from
169 0.19-0.75 µg/ml (Table 1).

170 All the isolates deemed resistant by both Mast Uri®system and BD Phoenix™ were
171 also categorised as resistant using E-test. Despite agreement of a resistance
172 interpretation between the three methods, there was little concordance between the
173 specific MICs determined by E-tests and the micro-broth dilution method (Table 1).

174 Of note was the difficulty in reading and interpreting E-tests. In each test a small
175 number of single colonies were observed within the clearance zone. As
176 recommended by others who have recorded the same phenomenon,(31) these
177 colonies were excluded from the E-test interpretation. Five isolates had a visible
178 'intermediate' zone of noticeably less dense growth, presenting two possible

179 interpretations. Due to the semi-confluent nature of the growth in these regions they
180 were not included in the zone of inhibition when reading the strips (Table 1).

181 *1.3.3.3 Investigation of fosfomycin MICs using modified agar dilution*

182 To further explore the differing growth phenotypes when using E-tests, a modified
183 agar dilution method was used whereby colonies were streaked on agar containing
184 different concentrations of fosfomycin and their growth observed. For the control
185 organisms and six Phoenix™/E-test determined fosfomycin susceptible organisms,
186 either no growth, or single colony/scanty growth was observed on agar containing a
187 low concentration of fosfomycin ($\leq 16 \mu\text{g/ml}$). Each of the nine resistant isolates
188 cultured on a low concentration of fosfomycin produced uniform colony morphologies;
189 when grown in the presence of higher concentrations of fosfomycin however each
190 produced a 'dual colony' growth phenotype.

191 **1.3.4 Characterisation of selected *E. coli* isolates**

192 WGS was used to characterise eight of the consistently fosfomycin resistant isolates
193 and two, randomly selected susceptible isolates. Fosfomycin resistance was present
194 in several different *E. coli* sequence types (6 different STs were seen in the 8 resistant
195 isolates, ST131 was the only ST seen more than once) indicating that resistance was
196 not distributed due to clonal expansion of one strain (Figure 1 and Table 2). The *E.*
197 *coli* sequence types found in this study include those previously reported as common
198 in UTI isolates in the UK; ST69, 73, 95 and 131.(32)

199 Each of the ten isolates were further characterised by investigating their antibiogram,
200 determined from their susceptibility profile to antimicrobials used in the treatment of
201 UTIs; and by interrogating WGS for genes and mutations known to confer
202 antimicrobial resistance (Table 2). Ampicillin resistance was detected in 8/10 isolates,
203 accompanied with the *in-silico* detection of *bla*_{TEM-1B}. Sulfamethoxazole resistance in
204 5/10 isolates corresponded with the detection of a *dfrA* gene and with either *sul1* or
205 *sul2*. Aminoglycoside resistance genes were identified in five of the isolates; of note
206 was a ST131 isolate possessing gentamicin resistance gene *aac(3)-IId* along with a
207 ciprofloxacin resistance conferring mutation in *gyrA*.

208 The *in-silico* analysis also showed the presence of many common
209 *Enterobacteriaceae* plasmid replicons including those of incompatibility group, IncF,
210 IncQ, IncX1, IncB/O/K/Z and plasmids from the group Col and Col156. Using CARD,
211 Resfinder and manual searches, no *fos*-like genes were detected in any of the strains,

212 suggesting an absence of known plasmid based transferrable fosfomycin resistance
213 genes in the resistant isolates.

214 **1.3.5 Amino-acid variation in proteins associated with fosfomycin resistance**

215 For each of the ten sequenced isolates, amino-acid changes or mutations in known
216 fosfomycin resistance genes *murA*, *glpT*, *uhpT*, *uhpA*, *ptsI* and *cyaA* were identified
217 from the WGS using *E. coli* MG1655 as a reference (Table 3). No *murA* changes
218 were identified in any of the fosfomycin resistant isolates, a single substitution of
219 Val389Ile was found in susceptible isolate, MU723432.

220 All sequenced isolates were found to have a Glu448Lys change in GlpT when
221 compared to MG1566. Fosfomycin resistant isolate MU721372 had an additional
222 three substitutions of Leu297Phe, Thr348Asn, Glu443Gln, however susceptible
223 isolate MU724857 also had a second GlpT change of Ala16Thr.

224 No amino-acid changes in the sequence of UhpT were identified in fosfomycin
225 susceptible isolates; however, 5/8 resistant isolates had changes in this protein. In
226 MU720214, both *uhpT* and *uhpA* were completely absent. Comparative analysis
227 against other *E. coli* genomes showed the presence of a phage integrase gene
228 adjacent to the *uhpT-uhpA* region within the assembled contig, suggestive of a
229 deletion event. Isolate MU720350 had two amino-acid changes at positions 31 and
230 39 predicted to confer premature stop codons leading to a truncated protein; four
231 strains had a Glu350Gln amino-acid substitution; and MU723240 had additional
232 substitutions of Tyr32Asn and Arg325Leu.

233 Only three isolates had changes in the *uhpA* gene, a deletion in MU720214, an
234 Arg46Cys substitution in susceptible isolate MU724857, and substitutions Arg14Gly
235 and Ala110Ser in fosfomycin resistant isolate MU721372 (Table 3).

236 When examining genes that affect levels of intracellular cAMP, all the isolates had
237 the substitution of Arg367Lys in PtsL and Asn142Ser in CyaA when compared to
238 MG1655; both changes are well represented in many *E. coli*. Two further substitutions
239 were identified in PtsL, Val25Ile in two of the resistant isolates (MU723051 and
240 MU723320) and Ala306Thr in one resistant (MU720214) and one susceptible *E. coli*
241 (MU724857). The amino-acid sequences of CyaA in each isolate fell broadly into two
242 groups, those with a single Asn142Ser change when compared to MG1655 (n=4),
243 and those with ≥ 3 additional amino-acid substitutions (Ser352Thr, Ala349Glu,
244 Ser356Lys, Gly359Glu and Ile514Val) (n=5 Table3) both containing susceptible and

245 resistant isolates. These amino-acid substitutions appeared to correlate more closely
246 with sequence type than with fosfomicin susceptibility status and were found
247 commonly in other *E. coli* strains.

248 1.4 Discussion

249 To investigate the extent of fosfomycin resistance in UTI isolates from routine clinical
250 specimens, different methods available to distinguish susceptible and non-
251 susceptible isolates using clinical laboratory protocols were explored. Use of
252 'breakpoint' plates on the MAST Uri®system for high throughput screening
253 determined the prevalence of resistance (MIC ≥ 32 $\mu\text{g/ml}$) in *E. coli* isolates as 12%;
254 a rate significantly higher than previously documented(33-35). However, on further
255 examination using automated micro-broth dilution, only nine of these isolates were
256 resistant (MIC ≥ 32 $\mu\text{g/ml}$). Furthermore, if CSLI guidelines had been applied none of
257 the isolates would be deemed resistant, as each had an MIC below the breakpoint
258 according to this scheme (S ≤ 64 , I=128 and R ≥ 256 $\mu\text{g/ml}$). (36, 37) Susceptibility
259 interpretations from the E-test method corroborated the findings from micro-broth
260 dilution, concordantly differentiating isolates deemed fosfomycin susceptible and
261 resistance. Therefore, both these methods agree that only 1.3% of *E. coli* within the
262 study should be regarded as fosfomycin resistant using current definitions; a
263 prevalence more in line with findings of previous studies both globally and within the
264 UK.(37, 38) The high prevalence of resistance recorded by the MAST Uri®system
265 reflects a large number of false positive results (53/62) given the interpretive criteria
266 followed. Whilst changes to fosfomycin susceptibility can occur relatively rapidly *in-*
267 *vitro* it is infeasible that a significant number of isolates initially identified as resistant
268 would have reverted to susceptibility in the time window of the laboratory
269 investigations. There may also however have been some false-susceptible results
270 given the methodologies we used

271 In the collection period, fosfomycin was not used in the trust or by community
272 pharmacists in this area, therefore patient exposure to the drug is likely to have been
273 low, and a 1.3% rate of resistance is likely to reflect spontaneous mutants which are
274 in the wider population of *E. coli*. Given the reports of fosfomycin resistance incurring
275 a significant fitness cost (10, 20) this level may be higher than expected given the
276 probable lack of direct selection in this population.

277 Lu *et al* (39) discussed the usefulness of disc-diffusion assays (39) in distinguishing
278 fosfomycin susceptible and resistant isolates despite reports of single colony
279 generation within the zone of inhibition.(31) A beneficial next step might be to directly
280 compare micro-broth dilution and E-test methods to disc-diffusion assays to establish
281 the most robust and practical method for determining fosfomycin susceptibilities

282 within a clinical laboratory setting and to assess the reproducibility each method for
283 those deemed susceptible and resistant. Interpretation of E-tests was obfuscated by
284 an intermediate zone of growth, resembling in appearance a 'small' colony phenotype
285 observed at higher concentrations of fosfomycin when isolates were streaked onto
286 plates. A similar 'dual colony' phenomenon in the presence of fosfomycin has been
287 described previously by Tsuruoka *et al.* who reported differences in growth and
288 carbohydrate uptake between colony types.(21) In the present study, these distinct
289 phenotypes were found to be transient and inconsistent, large and small colonies
290 going on after passage to produce daughter colonies of both phenotypes in the
291 presence of higher concentrations of fosfomycin (data not shown) further hindering
292 interpretation of susceptibility testing.

293 *In-silico* MLST and whole genome comparison of the fosfomycin resistant *E. coli*
294 showed that the isolates were of diverse sequence-types, and that resistance and
295 plasmid profiles differed in each isolate. Therefore, resistance had not disseminated
296 in this population due to expansion of one clone. Examination of the mechanisms of
297 resistance found no evidence for mobile elements being involved in fosfomycin
298 resistance, the absence of any plasmid located *fos* genes suggests that resistance in
299 these *E. coli* were due to chromosomal mutations. When examining sequences of
300 genes known to contribute to fosfomycin resistance, no two isolates had the same
301 set of substitutions or mutations. As in other studies, changes in GlpT and
302 UhpT/UhpA transport systems responsible for uptake of fosfomycin were the most
303 commonly identified; with 6/8 resistant organisms possessing amino-acid changes or
304 deletions within these systems that were absent in the susceptible strains. This
305 included the complete deletion of the *uhpT/uhpA* region; location of a premature stop
306 codon predicted to lead to a truncated UhpT protein; and the commonly reported
307 UhpT substitution Glu350Gln;(14, 40) all speculated to result in reduced uptake of
308 fosfomycin. Substitutions in GlpT were less common in this study than other recent
309 reports, only a single isolate (MU721372) accumulating many changes in this region.
310 Of note is the Glu448Lys substitution, identified previously in other fosfomycin
311 resistant isolates.(14) This change was identified in all the sequenced isolates when
312 compared to MG1655, including those deemed susceptible, but was not found during
313 a search of an extended panel of sequenced *E. coli* submitted to Genbank. This
314 suggests that either this substitution does not confer resistance to fosfomycin,
315 contradicting speculation by others;(14) or that it acts to reduce susceptibility,
316 perhaps below our defined breakpoints in the absence of other changes within the

317 protein. It may be that low-level changes to susceptibility account for why some
318 isolates were deemed resistant using screening with the MAST Uri®system, whilst
319 remaining sensitive using other testing methods.

320 Only a single substitution (Val389Ile) was identified in MurA within the sequence of
321 one of the susceptible isolates. Although the modification has been reported by others
322 in fosfomycin resistant isolates,(40) its location outside the active site of this enzyme
323 means its role in resistance is ambiguous. The role of changes in CyaA and PtsI
324 proteins in this study is less clear. The amino-acid sequence of CyaA appeared to
325 divide into two groups both with substitutions which can be found in other fosfomycin
326 susceptible *E. coli*. This suggests that these changes may be unrelated to fosfomycin
327 susceptibility but may correspond to the *E. coli* phylogeny.

328 While many of the substitutions identified in this study have previously been linked to
329 fosfomycin resistance by others, our detection of amino acid changes in both
330 susceptible and non-susceptible strains raises doubts regarding their contribution to
331 fosfomycin resistance. Mutations within the transport systems could be further
332 investigated by growing these organisms on minimal media with or without glucose-
333 6-phosphate or glycerol-3-phosphate to elucidate their functional status.

334 The use of fosfomycin for treatment of UTIs and other infections is likely to increase.
335 In this study, the prevalence of fosfomycin resistance in *E. coli* isolated from UTIs
336 was found to be relatively low and resistant isolates were divergent. The identification
337 of chromosomal based changes in genes associated with fosfomycin susceptibility,
338 and the absence of *fos* genes on conjugative plasmids indicates that resistance in
339 these isolates was not transferrable, and that co-location with other resistance genes
340 did not appear to lead to co-selection. Therefore, in this setting fosfomycin remains a
341 useful agent in the treatment of UTIs, equipping us with an extra option for hard to
342 treat UTIs and providing an alternative to drugs such as carbapenems which may
343 drive selection of resistant organisms further. Current methods to identify fosfomycin
344 resistant *E. coli* isolates in urine can give very different results, there is a need for
345 more consistency to accurately define real rates of resistance which is important in
346 monitoring any evolution of resistance as fosfomycin use is likely to increase.

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355 **Conflicts of interest**

356 None to declare

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361 **References**

- 362 1. **Livermore DM.** 2009. Has the era of untreatable infections arrived? Journal
363 of Antimicrobial Chemotherapy **64**:29-36.
- 364 2. **Falagas ME, Grammatikos AP, Michalopoulos A.** 2008. Potential of old-
365 generation antibiotics to address current need for new antibiotics. Expert Rev
366 Anti Infect Ther **6**:593-600.
- 367 3. **Michalopoulos AS, Livaditis IG, Gougoutas V.** 2011. The revival of
368 fosfomycin. Int J Infect Dis **15**:E732-E739.
- 369 4. **Kahan FM, Kahan JS, Cassidy PJ, Kropp H.** 1974. Mechanism of action of
370 fosfomycin (phosphonomycin). Ann N Y Acad Sci **235**:364-386.
- 371 5. **Alper MD, Ames BN.** 1978. Transport of antibiotics and metabolite analogs
372 by systems under cyclic-amp control - positive selection of Salmonella-
373 typhimurium-cya and crp mutants. Journal of Bacteriology **133**:149-157.
- 374 6. **Larson TJ, Ye SH, Weissenborn DL, Hoffmann HJ, Schweizer H.** 1987.
375 Purification and characterization of the repressor for the sn-glycerol 3-
376 phosphate regulon of escherichia-coli-k12. J Biol Chem **262**:15869-15874.
- 377 7. **Sonna LA, Ambudkar SV, Maloney PC.** 1988. THE MECHANISM OF
378 GLUCOSE-6-PHOSPHATE TRANSPORT BY ESCHERICHIA-COLI. J Biol
379 Chem **263**:6625-6630.
- 380 8. **Brown ED, Vivas EI, Walsh CT, Kolter R.** 1995. MurA (MurZ), the enzyme
381 that catalyzes the first committed step in peptidoglycan biosynthesis, is
382 essential in *Escherichia coli*. Journal of Bacteriology **177**:4194-4197.
- 383 9. **Falagas ME, Kastoris AC, Kapaskelis AM, Karageorgopoulos DE.** 2010.
384 Fosfomycin for the treatment of multidrug-resistant, including extended-
385 spectrum β -lactamase producing, Enterobacteriaceae infections: a systematic
386 review. The Lancet Infectious Diseases **10**:43-50.

- 387 10. **Castañeda-García A, Blázquez J, Rodríguez-Rojas A.** 2013. Molecular
388 Mechanisms and Clinical Impact of Acquired and Intrinsic Fosfomycin
389 Resistance, vol 2, p 217-236. *Antibiotics*.
- 390 11. **Cordaro JC, Melton T, Stratis JP, Atagun M, Gladding C, Hartman PE,**
391 **Roseman S.** 1976. Fosfomycin resistance - selection method for internal and
392 extended deletions of phosphoenolpyruvate - sugar phosphotransferase
393 genes of salmonella-typhimurium. *Journal of Bacteriology* **128**:785-793.
- 394 12. **Kim DH, Lees WJ, Kempell KE, Lane WS, Duncan K, Walsh CT.** 1996.
395 Characterization of a Cys115 to Asp substitution in the Escherichia coli cell
396 wall biosynthetic enzyme UDP-GlcNAc enolpyruvyl transferase (MurA) that
397 confers resistance to inactivation by the antibiotic fosfomycin. *Biochemistry*
398 **35**:4923-4928.
- 399 13. **Venkates.Ps, Wu HC.** 1972. Isolation and characterization of a
400 phosphonomycin resistant mutant of *Escherichia coli* K-12. *Journal of*
401 *Bacteriology* **110**:935-&.
- 402 14. **Takahata S, Ida T, Hiraishi T, Sakakibara S, Maebashi K, Terada S,**
403 **Muratani T, Matsumoto T, Nakahama C, Tomono K.** 2010. Molecular
404 mechanisms of fosfomycin resistance in clinical isolates of Escherichia coli.
405 *International Journal of Antimicrobial Agents* **35**:333-337.
- 406 15. **Horii T, Kimura T, Sato K, Shibayama K, Ohta M.** 1999. Emergence of
407 fosfomycin-resistant isolates of shiga-like toxin-producing Escherichia coli
408 O26. *Antimicrobial Agents and Chemotherapy* **43**:789-793.
- 409 16. **Couce A, Briales A, Rodriguez-Rojas A, Costas C, Pascual A, Blazquez**
410 **J.** 2012. Genomewide Overexpression Screen for Fosfomycin Resistance in
411 Escherichia coli: MurA Confers Clinical Resistance at Low Fitness Cost.
412 *Antimicrobial Agents and Chemotherapy* **56**:2767-2769.

- 413 17. **Mendes AC, Rodrigues C, Pires J, Amorim J, Ramos MH, Novais A, Peixe**
414 **L.** 2016. Importation of Fosfomycin Resistance fosA3 Gene to Europe. *Emerg*
415 *Infect Dis* **22**:346-348.
- 416 18. **Rigsby RE, Fillgrove KL, Beihoffer LA, Armstrong RN.** 2005. Fosfomycin
417 resistance proteins: A nexus of glutathione transferases and epoxide
418 hydrolases in a metalloenzyme superfamily, p 367-379. *In* Sies H, Packer L
419 (ed), *Gluthione Transferases and Gamma-Glutamyl Transpeptidases*, vol 401.
420 Elsevier Academic Press Inc, San Diego.
- 421 19. **Fillgrove KL, Pakhomova S, Newcomer ME, Armstrong RN.** 2003.
422 Mechanistic diversity of fosfomycin resistance in pathogenic microorganisms.
423 *Journal of the American Chemical Society* **125**:15730-15731.
- 424 20. **Nilsson AI, Berg OG, Aspevall O, Kahlmeter G, Andersson DI.** 2003.
425 Biological costs and mechanisms of fosfomycin resistance in *Escherichia coli*.
426 *Antimicrobial Agents and Chemotherapy* **47**:2850-2858.
- 427 21. **Tsuruoka T, Yamada Y.** 1975. Characterization of spontaneous fosfomycin
428 (phosphonomycin) resistant cells of *Escherichia coli* B *in-vitro*. *J Antibiot*
429 (Tokyo) **28**:906-911.
- 430 22. **Schito GC.** 2003. Why fosfomycin trometamol as first line therapy for
431 uncomplicated UTI? *International journal of antimicrobial agents* **22 Suppl**
432 **2**:79-83.
- 433 23. **Popovic M, Steinort D, Pillai S, Joukhadar C.** 2010. Fosfomycin: an old, new
434 friend? *European Journal of Clinical Microbiology & Infectious Diseases*
435 **29**:127-142.
- 436 24. **Patel SS, Balfour JA, Bryson HM.** 1997. Fosfomycin tromethamine - A
437 review of its antibacterial activity, pharmacokinetic properties and therapeutic

- 438 efficacy as a single-dose oral treatment for acute uncomplicated lower urinary
439 tract infections. *Drugs* **53**:637-656.
- 440 25. **Centre HHaSci**. 2014. Prescription cost analysis England 2012/2013, Health
441 and Social Care information centre. V1.0 (2013/2014).
- 442 26. **Andrews JM**. 2002. Determination of minimum inhibitory concentrations (vol
443 48, S1, pg 5, 2001). *Journal of Antimicrobial Chemotherapy* **49**:1049A-1049A.
- 444 27. **Zerbino DR**. 2010. Using the Velvet de novo assembler for short-read
445 sequencing technologies. *Current protocols in bioinformatics / editorial board*,
446 Andreas D Baxevanis [et al] **Chapter 11**:Unit 11.15.
- 447 28. **Seemann T**. 2014. Prokka: rapid prokaryotic genome annotation.
448 *Bioinformatics* **30**:2068-2069.
- 449 29. **McArthur AG, Waglechner N, Nizam F, Yan A, Azad MA, Baylay AJ,**
450 **Bhullar K, Canova MJ, De Pascale G, Ejim L, Kalan L, King AM, Koteva**
451 **K, Morar M, Mulvey MR, O'Brien JS, Pawlowski AC, Piddock LJV,**
452 **Spanogiannopoulos P, Sutherland AD, Tang I, Taylor PL, Thaker M, Wang**
453 **WL, Yan M, Yu T, Wright GD**. 2013. The Comprehensive Antibiotic
454 Resistance Database. *Antimicrobial Agents and Chemotherapy* **57**:3348-
455 3357.
- 456 30. **(EUCAST) TEcoast**. 2016. Breakpoint tables for the interpretation of MICs
457 and zone diameters. Version 6.0; 2016. <http://www.eucast.org>.
- 458 31. **Pasteran F, Lucero C, Rapoport M, Guerriero L, Barreiro I, Albornoz E,**
459 **Veliz O, Corso A**. 2012. Tigecycline and intravenous fosfomycin zone
460 breakpoints equivalent to the EUCAST MIC criteria for Enterobacteriaceae.
461 *Journal of Infection in Developing Countries* **6**:452-456.
- 462 32. **Doumith M, Day M, Ciesielczuk H, Hope R, Underwood A, Reynolds R,**
463 **Wain J, Livermore DM, Woodford N**. 2015. Rapid Identification of Major

- 464 Escherichia coli Sequence Types Causing Urinary Tract and Bloodstream
465 Infections. J Clin Microbiol **53**:160-166.
- 466 33. **Kahlmeter G.** 2003. An international survey of the antimicrobial susceptibility
467 of pathogens from uncomplicated urinary tract infections: the ECO.SENS
468 Project. Journal of Antimicrobial Chemotherapy **51**:69-76.
- 469 34. **Falagas ME, Maraki S, Karageorgopoulos DE, Kastoris AC,**
470 **Mavromanolakis E, Samonis G.** 2010. Antimicrobial susceptibility of
471 multidrug-resistant (MDR) and extensively drug-resistant (XDR)
472 Enterobacteriaceae isolates to fosfomycin. International Journal of
473 Antimicrobial Agents **35**:240-243.
- 474 35. **Maraki S, Samonis G, Rafailidis PI, Vouloumanou EK, Mavromanolakis E,**
475 **Falagas ME.** 2009. Susceptibility of Urinary Tract Bacteria to Fosfomycin.
476 Antimicrobial Agents and Chemotherapy **53**:4508-4510.
- 477 36. **Anonymous.** Clinical and Laboratory Standards Institute (2013) M100-S23
478 Performance Standards for Antimicrobial susceptibility testing; twenty-third
479 information supplement; January 2013;
480 http://reflab.yums.ac.ir/uploads/clsi_m100-s23-2013.pdf.
- 481 37. **Chin TL, MacGowan AP, Bowker KE, Elder F, Beck CR, McNulty C.** 2015.
482 Prevalence of antibiotic resistance in Escherichia coli isolated from urine
483 samples routinely referred by general practitioners in a large urban centre in
484 south-west England. Journal of Antimicrobial Chemotherapy **70**:2167-2169.
- 485 38. **Chislett RJ, White G, Hills T, Turner DPJ.** 2010. Fosfomycin susceptibility
486 among extended-spectrum-beta-lactamase-producing Escherichia coli in
487 Nottingham, UK. Journal of Antimicrobial Chemotherapy **65**:1076-1077.
- 488 39. **Lu CL, Liu CY, Huang YT, Liao CH, Teng LJ, Turnidge JD, Hsueh PR.**
489 2011. Antimicrobial Susceptibilities of Commonly Encountered Bacterial

490 Isolates to Fosfomycin Determined by Agar Dilution and Disk Diffusion
491 Methods. Antimicrobial Agents and Chemotherapy **55**:4295-4301.
492 40. **Li Y, Zheng B, Zhu SN, Xue F, Liu J.** 2015. Antimicrobial Susceptibility and
493 Molecular Mechanisms of Fosfomycin Resistance in Clinical Escherichia coli
494 Isolates in Mainland China. Plos One **10**:7.
495

496 **Table 1: Fosfomycin minimum inhibitory concentrations and growth characteristics**

Isolate	Fosfomycin MIC ($\mu\text{g/ml}$)		
	MastUri	BD Phoenix	E-test
MU721372	≥ 32	64	512 (24)
MU723051	≥ 32	64	384
MU715908	≥ 32	64	384 (98)
MU720214	≥ 32	64	384 (48)
MU723320	≥ 32	64	256
MU723292	≥ 32	64	192
MU720350	≥ 32	32	256
MU723240	≥ 32	32	256 (12)
MU720142	≥ 32	32	96 (4)
MU723432	≥ 32	<16	0.38
MU724857	≥ 32	<16	0.75
MU719876	≥ 32	<16	0.25
MU724367	≥ 32	<16	0.19
MU725806	≥ 32	<16	0.5
MU725463	≥ 32	<16	0.25
NCTC 10418	<16	<16	0.25
J53-2			0.25

497

498 MIC values in brackets represent interpretations of the E-test which include 'intermediate' growth within the zone of inhibition

499

500 **Table 2: Genotypic characterisation of selected *E. coli* isolates**

Isolate	Fosfomycin MIC (Phoenix)	Serotype	ST	Antibiogram (Phoenix)	Resfinder/ CARD: Presence of resistance genes	Plasmid replicons
MU721372	64 µg/ml	O17/O77:H18	69	Fos, Amp, Trim	<i>bla</i> _{TEM-1B} , <i>sul2</i> , <i>dfrA17</i> , <i>aph(6)Ib</i> , <i>aph(3')Ib</i> ,	IncFII, IncFIB, Col156, IncQ1
MU723051	64 µg/ml	O16:H5	131	Fos, Amp, Cefurox, Gent, Cipro	<i>bla</i> _{TEM-1B} , <i>aac(3)-IId</i> , <i>gyrA</i>	IncFII, IncFIB, IncFIA
MU715908	64 µg/ml	O111:H21	40	Fos	-	-
MU720214	64 µg/ml	O6:H1	73	Fos, Amp, Trim	<i>bla</i> _{TEM-1B} , <i>sul1</i> , <i>dfrA5</i>	IncFIB, Col156
MU723320	64 µg/ml	O16:H5	131	Fos, Amp	<i>bla</i> _{TEM-1B}	IncFII, IncFIB, Col156
MU720350	32 µg/ml	O75:H5	550	Fos	-	IncFII, IncFIB, IncX1, Col156, Col
MU723240	32 µg/ml	-:H4	131	Fos, Amp, Trim	<i>bla</i> _{TEM-1B} , <i>sul1</i> , <i>dfrA17</i> , <i>aadA5</i> ,	IncFII, IncFIA
MU720142	32 µg/ml	O6:H31	127	Fos, Amp, Trim	<i>bla</i> _{TEM-1B} , <i>sul2</i> , <i>dfrA14</i> , <i>aph(3')Ib</i> , <i>aph(6)Ib</i>	IncFII, IncFIB, IncB/O/Z/K, Col156
MU723432	<16 µg/ml	O83:H33	567	Amp, Trim	<i>bla</i> _{TEM-1B} , <i>sul2</i> , <i>dfrA8</i> , <i>dfrA14</i> , <i>strB</i> , <i>aph(3')Ib</i> , <i>aph(6)Ib</i> ,	IncFII, IncFIB, IncFII(pCoo)
MU724857	<16 µg/ml	O25:H4	95	Amp, Coamox, PipTaz	<i>bla</i> _{TEM-1B}	IncFII, ColpVC, IncFIB, IncB/O/Z/K

501 Fos, fosfomycin; Amp, ampicillin; Trim, trimethoprim; Coamox, coamoxiclav; Cefurox, cefuroxime; Cipro, ciprofloxacin; Gent, gentamicin; PipTaz, Tazocin

502

Table 3: Fosfomycin-associated mutations found in resistant *E. coli* isolates

Isolate	Fos MIC (Phoenix)	Amino-acid substitutions or sequence variations					
		MurA	GlpT	UhpT	UhpA	PstI	CyaA
MU721372	64 µg/ml	None	Leu297Phe Thr348Asn Glu443Gln	None	Arg14Gly Ala110Ser	None	Ser352Thr Ala349Glu Ser356Lys Gly359Glu
MU723051	64 µg/ml	None	None	Glu350Gln	None	Val25Ile	None
MU715908	64 µg/ml	None	None	None	None	None	None
MU720214	64 µg/ml	None	None	No peptide	No peptide	Ala306Thr	Ala349Glu Ser356Lys Gly359Glu Ile514Val
MU723320	64 µg/ml	None	None	Glu350Gln	None	Val25Ile	None
MU720350	32 µg/ml	None	None	Glu350Gln (Nonsense: premature stop codon at 31 and 39)	None	None	Ala349Glu Ser356Lys Gly359Glu
MU723240	32 µg/ml	None	None	Tyr32Asn Arg325Leu Glu350Gln	None	None	None
MU720142	32 µg/ml	None	None	None	None	None	Ala349Glu Ser356Lys Gly359Glu Ile514Val
MU723432	<16 µg/ml	Val389Ile	None	None	None	None	Ala349Glu Ser356Lys Gly359Glu Ile514Val
MU724857	<16 µg/ml	None	Ala16Thr	None	Arg46Cys	Ala306Thr	Ala349Glu Ser356Lys Gly359Glu Ile514Val
Present in all strains vs MG1655		None	Glu448Lys	None	None	Arg367Lys	Asn142Ser

503

504 **Figure Legend**

505

506

507 **Figure 1.** Phylogenetic reconstruction of population structure of the Fosfomycin
508 resistant *E. coli* isolates produced by Roary. (R) and (S) indicate resistant and sensitive
509 isolates respectively. ST121 strain EC958 was used as a reference.

510