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Evaluation of *in vitro* activity of fosfomycin, and synergy in combination, in Gram-negative bloodstream infection isolates in a UK teaching hospital

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Abstract

Introduction. Fosfomycin has retained activity against many multi-drug resistant (MDR) Gram-negatives, and may be useful against extended spectrum beta-lactamase (ESBL) producing and carbapenem-resistant Enterobacterales to improve clinical outcomes.

Hypothesis/Gap Statement. There are few data from the UK on the susceptibility of invasive Gram-negative isolates to fosfomycin, especially in the era of increasing use of oral fosfomycin for urinary tract infections (UTIs).

Aim. We evaluated fosfomycin susceptibility against 100 consecutive Gram-negative bloodstream isolates, both individually, and in combination with other mechanistically similar and differing antibiotics. The aim was to investigate the synergy between antibiotic combinations against several *E. coli, K. pneumoniae and P. aeruginosa* isolates with variable levels of resistance.

Methodology. Disc diffusion and MIC test strip methods applying revised EUCAST guidelines for Fosfomycin were used, followed by the MTS™ 'cross synergy' method for 'resistant' isolates as defined below: (a) Fosfomycin resistant by MIC test strip; (b) MDR isolates defined as being resistant to ≥3 classes of antibiotics (based on routine sensitivity testing; beta lactams were considered as a single class), and/or (c) AMP C or ESBL or carbapenemase producers (or carbapenem resistant). FIC Index (Fractional Inhibitory Concentration Index) calculations were used to interpret findings, whereby: FIC = (MICA combination A+B/ MIC agent A) + (MICB combination A+B/ MIC agent B). A result of ≤0.5 was taken to indicate 'synergy', >0.5 and ≤1.0 to indicate 'additive' effect, >1.0 and ≤4.0 to indicate 'indifference', and >4.0 to indicate 'antagonism'.

Results. We found that 95/100 isolates were susceptible to fosfomycin by MIC test strip, with 88/100 isolates susceptible to fosfomycin by disc, based on EUCAST guideline breakpoints. A total of 30/100 isolates (the more 'resistant' of the 100) were eligible for synergy testing according to our definitions (see *Methodology*), with the remaining 70 isolates not tested further. Seventeen out of 30 were MDR, 2/30 were AMP C producers and 9/30 were ESBL producers. Overall, 34/300 (11%) of all combination tests showed synergy and 161/300 (54%) were additive. Synergy was most commonly detected between fosfomycin and beta-lactam antibiotics, including piperacillin/tazobactam (10/30; 33%), ceftazidime/avibactam (10/30; 30%), and temocillin (8/30; 27%). An additive effect was most commonly detected with aztreonam (25/30; 83%) and meropenem (25/30; 83%), but 100% indifference was found with tigecycline (30/30). No antagonism was identified with any antibiotic combination.

Conclusion. Fosfomycin non-susceptibility by MIC test strip was unusual. Synergy was variable when combining fosfomycin with other antibiotics against the more 'resistant' isolates. Synergistic/additive effects were detected for beta-lactam/fosfomycin combinations in >80% of all such combinations, suggesting beta-lactams may be the preferred partner for fosfomycin. Agents with a discordant site of action were more likely to result in indifference. Antagonism was not detected.

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Keywords: synergy; additive; antibiotic resistance; fosfomycin.

Abbreviations: AMP-C, = AMP-C beta lactamase; CRE, = carbapenem-resistant Enterobacterales; DD, = disc diffusion; ESBL, = extended spectrum beta-lactamase; EUCAST, = European Committee on Antimicrobial Susceptibility Testing; FIC, = fractional inhibitory concentration index; MDR, = multi-drug resistant; MDRO, = multi-drug resistant organism; MIC, = minimum inhibitory concentration; UTI, = urinary tract infection; WHO, = World Health Organisation.

Six supplementary tables are available with the online version of this article.

INTRODUCTION

In an era of increasing antibiotic resistance, finding therapeutic options for multi-drug resistant organisms (MDRO), carbapenem-resistant *Enterobacterales* (CRE) and extended spectrum beta-lactamase (ESBL) producers represents a significant challenge [1]. With an associated poorer patient outcome [2], and increased economic cost in treating MDRO, the WHO (2020) declared antimicrobial resistance as 'one of the top 10 global public health threats facing humanity' [3].

Multi-drug resistant Gram-negative bacterial infections have increased in recent years [4], with resistance in *Klebsiella pneumoniae* to last resort treatments (e.g. carbapenems) now more than 50% in some countries [3]. Resistance in *E. coli* is also extensive, with fluoroquinolone antibiotics potentially ineffective in over 50% of patients in many parts of the world [5]. The prevalence of ESBL producing *E. coli* has also increased in both community and healthcare settings between 2014 and 2020 [6].

Although several new antibiotics have been approved or are under development, the availability of efficacious antimicrobials in treating highly resistant Gram-negatives remains limited [7]. It is therefore important to explore alternative treatment regimens, such as combinations of antibiotics and associated synergy thereof, to establish the most active/reliable antibiotic therapies.

In clinical practice, invasive infections due to MDRO are often treated with combination therapy, particularly in patients with life, limb, or sight-threatening infections [8]. This is often prescribed according to the susceptibility profile of the isolate following laboratory testing and clinicians' discretion, but there remains limited laboratory and clinical evidence to support the efficacy of this approach [9].

Brochado *et al*. (2018) [10] profiled approximately 3000 drug combinations against three Gram-negative pathogens (*Escherichia coli*, *Salmonella enterica* serovar Typhimurium, and *Pseudomonas aeruginosa*), all of which belong to the highest risk group for development of antibiotic resistance [11]. Whilst many of the tested antibiotic combinations revealed an antagonistic effect (typically seen when partner drugs targeted different cellular processes), synergy was often found when using antibiotic combinations that targeted similar cellular processes simultaneously. It is possible that the use of such an approach, alongside the use of effective new agents, may therefore lead to improved clinical outcomes in patients infected with MDRO pathogens.

Fosfomycin

There is emerging laboratory evidence that fosfomycin may be particularly suited for use against ESBL producers and CRE [12], and may be a good choice of antimicrobial to use in combination with other antibiotics to improve clinical outcomes [13]. Fosfomycin is a broad-spectrum cell wall acting antibiotic, and inhibits the formation of the peptidoglycan precursor UDP N-acetylmuramic acid (UDP-MurNAc) [13]. It is widely used orally in the treatment of uncomplicated urinary tract infections (UTIs) [12], but also in the intravenous treatment of more severe infections. It has retained activity against many problematic community and nosocomial bacteria, including MDROs [14].

With relatively few published data from the UK on the susceptibility of invasive Gram-negative isolates to fosfomycin, especially in the era of increasing use of oral fosfomycin for the treatment of UTIs in the community (based on Public Health England guidance),[15] we evaluated fosfomycin susceptibility against 100 consecutive Gram-negative bloodstream infection isolates, both individually, and in combination with other mechanistically similar and differing antibiotics .

METHODS

The study was performed in the National Health Service (NHS) microbiology laboratory of a 1200-bedded teaching hospital that has all major medical and surgical sub-specialties other than transplantation. A total of one hundred (100) consecutive clinical isolates of either *E. coli*, *K. pneumoniae* or *P. aeruginosa* from positive blood cultures were tested (isolated 10/06/18 - 31/07/18). This was an arbitrary number chosen to reflect local bloodstream infection isolates that commonly pose resistance challenges clinically with proportions reflecting the respective local incidences of studied bacteria in consecutive blood culture isolates. Other Enterobacterales were not tested. The identification of bacteria, and antimicrobial susceptibility-testing, other than for fosfomycin alone and in combination with other antibiotic partners, was according to standard NHS microbiology laboratory methods [15]. Three reference (control) strains with known antimicrobial susceptibilities, including to fosfomycin alone and in combination, were also used: *E. coli* ATCC 25922, *E. coli* NCTC 13846 (colistin combination testing only) and *P. aeruginosa* ATCC 27853.

Susceptibility to Fosfomycin alone *in vitro*

Fosfomycin susceptibility was evaluated in the 100 isolates according to European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines (for *E. coli*), using the EUCAST DD (disc diffusion) method [16] and by MIC test strip (MTS™) [17]. In the absence of EUCAST guidelines at the time of testing, *K. pneumoniae* and *P. aeruginosa* isolates were tested using the same approach as for *E. coli*. See Table S1 (available in the online version of this article) for Fosfomycin MIC/DD data.

Mueller Hinton II agar plates were used, with inoculum by sterile swab of a suspension in saline to 0.5 McFarland, and incubation at 35±2 °C ambient for 24 h. Fosfomycin 200 µg discs (with 50 µg glucose-6-phosphate) and Fosfomycin MTS™ were placed onto

separate inoculated agar plates simultaneously. When reading the fosfomycin 200 µg inhibition zones, isolated colonies within the inhibition zone were ignored, and only the outer zone edge read, according to recommendations [16]. The following zone diameter breakpoints were applied: Fosfomycin susceptible (S)≥24 mm; resistant (R)<24 mm. MIC breakpoints: Fosfomycin S≤32 mg l^{-1}; R>32 mg l^{-1}. As EUCAST breakpoints were not available for *K. pneumoniae* or *P. aeruginosa* vs. Fosfomycin in the 2018 (for both) or 2021 (for *P. aeruginosa*) guidelines, the above breakpoint values were also used for initial susceptibility interpretation for these species as a means of testing for Fosfomycin resistance [16, 18].

The MICs of the reference strains were determined in triplicate for all methods, before testing of clinical specimens. To audit and quality assure the accuracy of our results, the first ten clinical isolates, and thereafter every one in five isolates, were tested in duplicate, with results recorded in parallel and analysed for agreement. When discordant results were ascertained a third test was performed for clarification according to a priori discordancy rules (See Table S2).

Susceptibility testing to fosfomycin based antibiotic combinations *in vitro*

Isolates fulfilling one or more of the following criteria were selected for combination testing:

(a) Fosfomycin resistance by DD +/or MTS (based on the methods described above)
(b) AMP C or ESBL or carbapenemase producing, or carbapenem resistant
(c) Any MDRO, as defined by the isolate being resistant to three or more different classes of antibiotics (all beta-lactams were considered as one class) according to the prior standard NHS sensitivity testing

The antibiotics tested (in combination with fosfomycin) were: Meropenem, Gentamicin, Ciprofloxacin, Tigecycline (not versus *Pseudomonas*), Colistin, Ceftazidime/avibactam, Ceftolozane/tazobactam, Aztreonam, Temocillin (not versus *Pseudomonas*), Piperacillin/tazobactam, and Tobramycin (for *Pseudomonas* only).

Synergy testing of Fosfomycin in combination with each of the above antibiotics was performed for each eligible isolate to determine a combination MIC, using the MTS™ 'cross' synergy method (described below) [19]. Combination MICs were then compared with MICs for each of the antibiotics when used alone, and used to calculate the Fractional Inhibitory Concentration (FIC) as determined below:

$$FIC = (MIC\ 'A'_{\text{combination 'A'+'B'}} / MIC_{\text{agent 'A'}}) + (MIC\ 'B'_{\text{combination 'A'+'B'}} / MIC_{\text{agent 'B'}})$$

Interpretation: FIC≤0.5 (synergy); >0.5 and ≤1.0 (additive); >1 and ≤4.0 (indifference); >4.0 (antagonism) [19].

MTS™ 'cross' synergy method: [19]

An MTS of fosfomycin was placed with tweezers on to the MTS synergy applicator platform, such that the MIC value for fosfomycin (tested alone) was positioned at the base intersection (see Fig. 1 below). An MTS of antibiotic 'B' (the partner antibiotic) was then carefully placed on to the MTS synergy applicator platform, such that the MIC value of antibiotic 'B' was positioned at the base insertion at a 90° angle to the fosfomycin MTS in a 'cross' formation. The MTS synergy delivery tool was used to move the appropriately positioned MTS 'cross' onto an inoculated agar plate. The MTS synergy delivery tool was then removed, ensuring MTS strips were pushed onto the agar surface with tweezers as required. Plates were then incubated for 24 h. All synergy tests were photographed for future third-party reference/re-interpretation as needed.

Fig. 1. MTS™ synergy applicator platform plus MTS antibiotic strips [19].

Table 1. NHS susceptibility testing data for 30 'resistant' isolates

	% Antibiotic Susceptibility (by standard NHS methods)										
Antibiotic class* →	**1**	**1**	**1**	**2**	**3**	**4**	**1**	**5**	**1**	**1**	**1**
Isolate (*n*=30) / 'Resistant' criteria ↓	**Amoxicillin**	**Co-amoxiclav**	**Piperacillin/ tazobactam**	**Co-trimoxazole**	**Gentamicin**	**Ciprofloxacin**	**Meropenem**	**Chloramphenicol**	**Cefotaxime**	**Aztreonam**	**Ertapenem**
E. coli (*N*=21)	0% (0/21)	14% (3/21)	86% (18/21)	33% (7/21)	48% (10/21)	62% (13/21)	100% (21/21)	86% (18/21)	57% (12/21)	52% (11/21)	100% (12/12)
K. pneumoniae (*N*=8)	0% (0/8)	50% (4/8)	75% (6/8)	75% (6/8)	100% (8/8)	62.5% (5/8)	100% (8/8)	75% (6/8)	100% (8/8)	100% (8/8)	100% (4/4)
P. aeruginosa (*N*=1)	–	–	100% (1/1)	–	100% (1/1)	100% (1/1)	100% (1/1)	–	–	–	–
ESBL producer (*N*=9)	0% (0/9)	22% (2/9)	100% (9/9)	33% (3/9)	56% (5/9)	33% (3/9)	100% (9/9)	100% (9/9)	0% (0/9)	0% (0/9)	100% (6/6)
AMP C producer (*N*=2)	0% (0/2)	0% (0/2)	50% (1/2)	100% (2/2)	100% (2/2)	100% (2/2)	100% (2/2)	100% (2/2)	100% (2/2)	50% (1/2)	100% (1/1)
MDRO (*N*=17)	0% (0/17)	12% (2/17)	88% (15/17)	18% (3/17)	35% (6/17)	53% (9/17)	100% (17/17)	71% (12/17)	64% (11/17)	64% (11/17)	100% (9/9)

*Where: 1=Penicillin/Cephalosporin/Carbapenem/Monobactam, 2=Sulphonamide, 3=Aminoglycoside, 4=Fluoroquinolone, 5=Chloramphenicol

Table 2. Resistance mechanisms in 30 'resistant' isolates (nearest %)

	Isolates (n)	AMP C+only	ESBL+only	MDRO only	MDRO and ESBL+	Fosfo R (disc) only	Fosfo R (disc) and ESBL+	Fosfo R (disc) and MDRO
E. coli	21	10% (2/21)	10% (2/21)	38% (8/21)	29% (6/21)	5% (1/21)	5% (1/21)	5% (1/21)
K. pneumoniae†*	8	0% (0/8)	0% (0/8)	0% (0/8)	0% (0/8)	75% (6/8)	0% (0/8)	25% (2/8)
P. aeruginosa	1	0% (0/1)	0% (0/1)	0% (0/1)	0% (0/1)	100%* (1/1)	0% (0/1)	0% (0/1)
Totals	30	7% (2/30)	7% (2/30)	27% (8/30)	20% (6/30)	27% (8/30)	3% (1/30)	10% (3/30)

*EUCAST breakpoints not available for *P. aeruginosa* vs. Fosfomycin in 2018 or 2021 revised guidelines. Breakpoint values for *E. coli* used for interpretation.
†EUCAST breakpoints not available for *K. pneumoniae* at the time of testing, but has been included within the 2021 revised guidelines for interpretation [18].

RESULTS

Susceptibility to fosfomycin alone *in vitro*

Most isolates were *E. coli* (*N*=83), followed by *K. pneumoniae* (13) and *P. aeruginosa* (four). Almost all (95/100) isolates were susceptible to fosfomycin by MIC test strip. We found 88/100 isolates were susceptible to fosfomycin by disc method, based on 2018 EUCAST guidelines [16]. Fosfomycin MIC and DD data is presented in Table S1 (note variable breakpoints by method). Since completion of the study, EUCAST 2022 guidelines are now available which show limited changes in interpretation of our results (see Table S3).

Of the four isolates resistant to fosfomycin by MIC test strip, one was a MDRO, one was an ESBL producer, and the remaining two isolates were neither AMP C/ESBL producers nor defined as a MDRO.

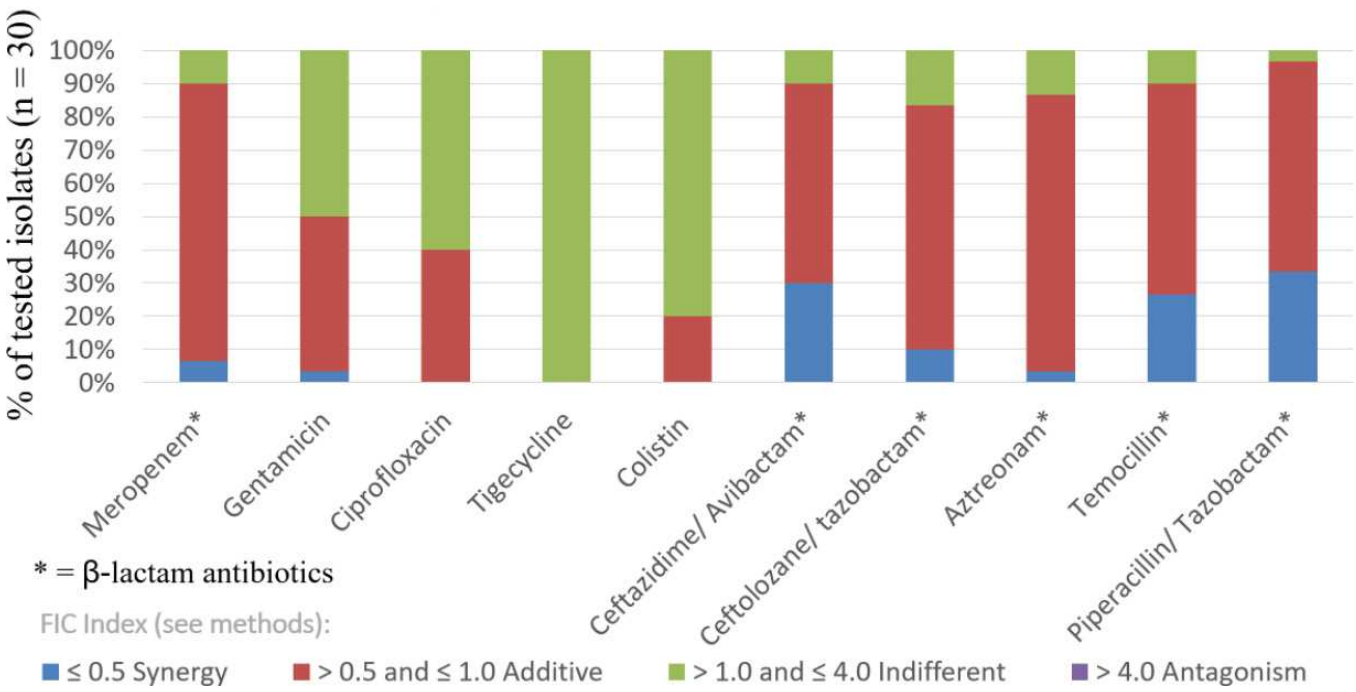

Fig. 2. Evaluation of *in vitro* Fosfomycin in combination with other antibiotics by FIC Index in *E. coli*, *K. pneumoniae*, and *P. aeruginosa* isolates from 30 hospitalised patients.

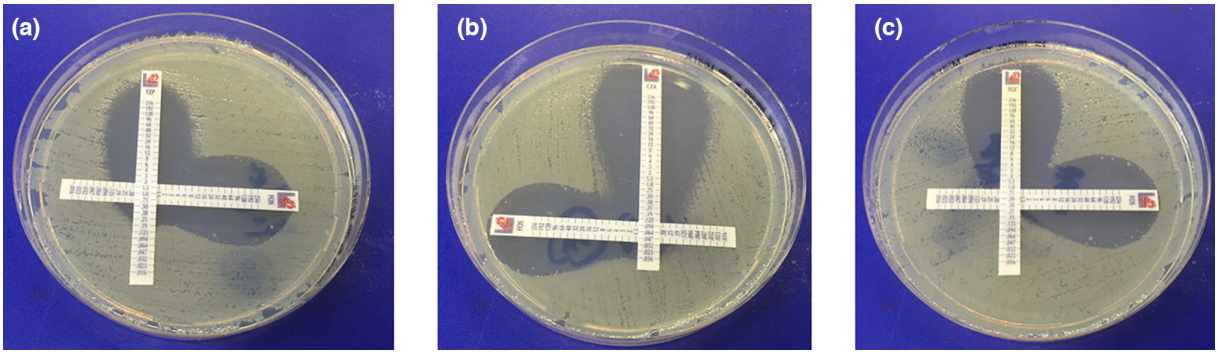

Fig. 3. MIC test strips in combination showing an example of synergy (a), additive effect (b) and indifference (c).

Table 3. *In vitro* combination activity according to antibiotic class

	Fractional Inhibitory Concentration Index (FIC)			
Type of antibiotic	**≤0.5 (Synergy)**	**>0.5 and ≤1.0 (Additive)**	**>1.0 and ≤4.0 (Indifference)**	**>4.0 (Antagonism)**
Beta-lactams (*n*=30; combinations=180)	18% (33/180)	71% (127/180)	11% (20/180)	0% (0/180)
Non-beta-lactams (*n*=30; combinations=119)	0.83% (1/119)	27% (32/119)	72.27% (86/119)	0% (0/119)

Susceptibility testing to fosfomycin based antibiotic combinations *in vitro*

Thirty isolates (30%) were eligible for synergy testing, according to our definitions (see Methods). The remaining 70 isolates were not tested further. The NHS susceptibility testing data for these isolates is shown in Table 1. Of the 30 identified isolates, 21 were *E. coli*, eight were *K. pneumoniae*, and one was *P. aeruginosa*, with 17 being of MDRO status, two AMP C producers and nine ESBL producers; see Table 2 for further information. See also S5-S6 for antibiotic MICs (when combined with fosfomycin by MTS™ 'cross' synergy method).

The 30 isolates and 10 fosfomycin partner antibiotics generated 299 isolate-combination antibiotic tests with 32/299 (11%) showing synergy and 160/299 (54%) an additive effect overall. Synergy was most detected between fosfomycin and beta-lactam antibiotics; piperacillin/tazobactam (10/30; 33%), ceftazidime/avibactam (10/30; 30%), and temocillin (8/30; 27%). An additive effect was most detected with aztreonam (26/30; 87%) and meropenem (25/30; 83%). A rate of 100% indifference was found when fosfomycin was combined with tigecycline (30/30). No antagonism was identified with any antibiotic combination; see Fig. 2, Table S4. Fig. 3 shows an example of synergy, additive effect and indifference.

When stratified according to antibiotic class (beta-lactam versus non-beta-lactam), 18% of beta-lactam combinations showed synergy with 71% an additive effect versus only 0.83% synergy and 27% an additive effect in non-beta-lactam combinations; see Table 3. When results were stratified by isolate, they were comparable; see Table 4.

Table 4. *In vitro* combination activity by organism

	Fractional Inhibitory Concentration Index (FIC)			
Organism (*N*=30)	**≤0.5 (Synergy)**	**>0.5 and ≤1.0 (Additive)**	**>1.0 and ≤4.0 (Indifference)**	**>4.0 (Antagonism)**
E. coli (*N*=21; combinations=210)	12% (25/210)	53% (112/210)	35% (73/210)	0% (0/210)
K. pneumoniae (*N*=8; combinations=80)	8.75% (7/80)	52.5% (42/80)	38.75% (31/80)	0% (0/80)
P. aeruginosa (*N*=1; combinations=9)	11% (1/9)	67% (6/9)	22% (2/9)	0% (0/9)

Table 5. *In vitro* combination activity by 'resistance' mechanism

	Fractional Inhibitory Concentration Index (FIC)			
'Resistance' mechanism	**≤0.5 (Synergy)**	**>0.5 and ≤1.0 (Additive)**	**>1.0 and ≤4.0 (Indifference)**	**>4.0 (Antagonism)**
Fosfomycin resistant [by MTS] only (*N*=2, combinations=20)	10% (2/20)	20% (4/20)	70% (14/20)	0% (0/20)
Fosfomycin resistant [by MTS]+/-ESBL+/- AMP C+/-MDRO) (*N*=4; combinations=40)	10% (4/40)	25% (10/40)	65% (26/40)	0% (0/40)
Fosfomycin resistant [by disc method] (*N*=12; combinations=119)	9.2% (11/119)	51.3% (61/119)	39.5% (47/119)	0% (0/119)
ESBL+ (*N*=9; combinations=90)	19% (17/90)	58% (52/90)	23% (21/90)	0% (0/90)
AMP C+ (*N*=2; combinations=20)	0% (0/20)	70% (14/20)	30% (6/20)	0% (0/20)
MDRO (*N*=17; combinations=170)	10.5% (18/170)	56% (95/170)	33.5% (57/170)	0% (0/170)
All 'resistant' isolates (*n*=30; combinations=299)	11.4% (34/299)	53.1% (159/299)	35.5% (106/299)	0% (0/299)

Synergy or an additive effect (mostly) was detected in 60% of tests versus fosfomycin resistant isolates (by disc method), 35% versus fosfomycin resistant isolates (by MTS), 70% versus AMP-C producers, 67% versus MDRO isolates and 77% versus ESBL producers compared to 65% in all isolate-fosfomycin-partner combinations tested; see Table 5.

Synergy or an additive effect was detected in 67.5% of isolates when susceptible to both antibiotics used in combination testing. This was 61% when isolates were susceptible to only one of the two antibiotics, and 67% when resistant to both antibiotics used; see Table 6.

DISCUSSION

Fosfomycin non-susceptibility was unusual, with 95% susceptibility by MTS and 88% susceptibility by disc method. This high rate is reassuring, and in keeping with other studies. Hareendranath *et al.* (2021) demonstrated 98% susceptibility to fosfomycin in 375 *in vitro E. coli* isolates in India, of which 150 were MDR [20]. Low resistance to fosfomycin in uropathogens was also seen by Batra *et al.* (2021), who reviewed 7295 isolates cultured from patients in India with uncomplicated UTI over 4 years [21]. This suggests fosfomycin continues to represent a good choice of antimicrobial for treating resistant uropathogens causing uncomplicated UTIs but may also be a useful combination partner for the less commonly occurring severe and invasive resistant Gram-negative infections when combination therapy is felt to be appropriate.

Synergy was variable when combining fosfomycin with other antibiotics. Synergistic or additive effects were detected for beta-lactam/fosfomycin combinations in a high proportion of isolates; >80% for all, suggesting that such combinations may be preferable when considering fosfomycin combination therapy. Beta-lactam antibiotics target the bacterial cell wall penicillin binding proteins (PBPs), whilst fosfomycin inhibits the peptidoglycan precursor UDP N-acetylmuramic acid (UDP-MurNAc) involved in peptidoglycan biosynethesis [22]. There is therefore a suggestion that targeting similar cellular processes simultaneously, at

Table 6. *In vitro* combination activity by isolate resistance to antibiotics tested in each combination

		Fractional Inhibitory Concentration Index (FIC)			
Isolate level of resistance to each combination of antibiotics	**n**	**≤0.5 (Synergy)**	**>0.5 and ≤1.0 (Additive)**	**>1.0 and ≤4.0 (Indifference)**	**>4.0 (Antagonism)**
BOTH Susceptible	151	13.2% (20/151)	54.3 % (82/151)	32.5% (49/151)	0% (0/151)
1 Susceptible	142	7.7% (11/142)	53.5% (76/142)	38.7% (55/142)	0% (0/142)
BOTH Resistant	6	33.3% (2/6)	33.3% (2/6)	33.3% (2/6)	0% (0/6)

least *in vitro*, provides a greater chance of synergistic/additive effects against Gram-negative isolates. This is an outcome mirrored by the study by Brochado *et al.* (2018) (including fosfomycin combinations) [10], as well as a study by Ojdana *et al.* (2019) who showed ceftazidime-avibactam used in combination with fosfomycin reduced MICs to less than the susceptibility breakpoint among 19 tested carbapenemase-producing *K. pneumoniae in vitro*, using a similar cross synergy method [23]. Agents used alongside fosfomycin with a different target of antibiotic action (i.e. the non-beta-lactams), however, were more likely to result in indifference, with antagonism not detected in our study.

In this study, the combination of colistin plus fosfomycin did not result in synergy (20% additive, 80% indifference). This contrasts with a recent meta-analysis that suggested a 'moderate' rate of synergy based on eight pharmacokinetic/pharmacodynamic studies and two using time-kill methods in carbapenem resistant *K. pneumoniae* isolates [24]. This may reflect the different methodologies, and that our sample was mostly *E. coli*, but it is also unclear as to whether synergy is a phenomenon that is more likely to be identified in highly resistant isolates. In this study, synergy or an additive effect was more common in ESBL producers (19% synergy, 58% additive) than in the four isolates resistant to fosfomycin by MTS (10% synergy, 25% additive), of which two did not have MDRO status and were not AMP C or ESBL producers. When synergistic and additive effects were considered together, the number of antibiotics the isolate was susceptible to did not appear to make a difference (61–67.5%), although the number in the resistant/resistant group were small.

Studies of combination therapy to date have predominantly focused on high resistance organisms causing severe infections with the aim of improving *in vitro* and *in vivo* antibacterial activity and clinical outcomes. Most antimicrobials are still used for lower acuity, less resistant infections, however, so key outcomes in this cohort of patients, such as bacterial regrowth, clinical recrudescence, and the (further) development of resistance, also needs to be investigated to see if combination therapy has advantages or simply causes more adverse effects and/or resistance.

Limitations

Whilst these results appear promising, it is necessary to maintain caution before applying such findings clinically. The sample size was relatively small (N=100; N=30 for combination testing) and from a single centre in the UK so the wider generalisability is debatable, and readers should consider our results in the context of their own local microbial epidemiology and clinical and laboratory practices. A review of several antimicrobial synergy testing methods highlighted results are often inconsistent between the various combination-testing methods, and that synergy is 'neither universal nor predictable', ranging from antagonism to >80% synergy across several studies [7, 9]. There is therefore a lack of clear consensus on which pragmatic methods are appropriate for synergy evaluation in clinical and research practice, and how they compare to gold standard methods [9]. There can also be discordancy between susceptibility results and clinical outcomes, as can be seen in the treatment of chronic *P. aeruginosa* infections in cystic fibrosis [25].

In addition to the MTS™ cross synergy testing method used in this study [19], Khan *et al.* (2021) reviewed several other *in vitro* susceptibility testing methodologies, focussing on ten *Enterobacterales* and six *Pseudomonas aeruginosa* isolates (including carbapenem resistant and metallo-beta-lactamase producers) vs. aztreonam (ATM) and ceftazidime-avibactam (CZA) combinations. This included two disc-based methods (disc stacking and broth disc elution), and two MIC-based methods (gradient strip stacking, and gradient strip crossing- as used in our study), with the modified broth micro-dilution method used as the reference 'gold standard'. A reproducibility analysis compared modified micro-dilution with the above methods and demonstrated agreement of between 94 and 100%, with the MTS™ cross synergy method and broth disc elution found to be the most accurate and precise methods for ATM-CZA combination testing [26]. This is important as such methods may be more feasible in low-income settings where the burden of resistant infections is often higher.

As well as the need for further *in vitro* work, it will be important to test, versus monotherapy, the most promising identified combination antimicrobial therapies in randomised clinical trials. This will be important, given that other factors, such as the patient immune system, drug-drug interactions, and adverse effects, may impact clinical outcomes [9]. The challenges of performing such trials, especially in high severity, high resistant infections however, are considerable.

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J.S., Orchid ID: 0000-0001-5543-6555. Role: conceptualisation; methodology; validation; data curation; formal analysis; investigation; writing- original draft and review and editing, visualisation, project administration. D.M.: writing- review and editing. M.Vd.W.: conceptualisation; methodology; writing- review and editing D.W.: conceptualisation; methodology; writing- review and editing; supervision P.B.: conceptualisation; methodology T.S.:

conceptualisation, methodology, resources G.B.: conceptualisation; methodology; validation; formal analysis; writing- original draft and review and editing; supervision; project administration; funding.

Conflicts of interest
The authors declare that there are no conflicts of interest.

Ethical statement
All authors consented to publication.

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