International Journal of Antimicrobial Agents 53 (2019) 74–79

Contents lists available at ScienceDirect

International Journal of Antimicrobial Agents

journal homepage: www.elsevier.com/locate/ijantimicag

Short Communication

Synergistic and bactericidal activities of mecillinam, amoxicillin and clavulanic acid combinations against extended-spectrum β-lactamase (ESBL)-producing *Escherichia coli* in 24-h time–kill experiments

Kari-Pekka Skarp, Ayda Shams, Hanna Montelin, Pernilla Lagerbäck, Thomas Tängdén*

Department of Medical Sciences, Section of Infectious Diseases, Uppsala University, SE 751 85 Uppsala, Sweden

ARTICLE INFO

Article history:
Received 9 May 2018
Accepted 9 September 2018

Editor: Jean-Marc Rolain

Keywords:
Amdinocillin
Mecillinam
Synergy
Extended-spectrum β-lactamase
ESBL
Urinary tract infection

ABSTRACT

This study aimed to evaluate the potential synergistic and bactericidal effects of mecillinam in combination with amoxicillin and clavulanic acid against extended-spectrum β-lactamase (ESBL)-producing *Escherichia coli*. Eight clinical *E. coli* isolates with varying susceptibility to mecillinam [minimum inhibitory concentrations (MICs) of 0.125 mg/L to >256 mg/L] and high-level resistance to amoxicillin (MICs > 256 mg/L) were used. Whole-genome sequencing was performed to determine the presence of β-lactamase genes and mutations in the *cysB* gene. The activities of single drugs and the combinations of two or three drugs were tested in 24-h time–kill experiments. Population analysis was performed for two strains before and after experiments. Only one strain had a mutation in the *cysB* gene resulting in an amino acid substitution. With the two-drug combinations, initial killing was observed both with mecillinam and amoxicillin when combined with clavulanic acid. Synergy was observed with mecillinam and clavulanic acid against one strain and with amoxicillin and clavulanic acid against three strains. However, following significant re-growth, a bactericidal effect was found only with amoxicillin and clavulanic acid against two strains. Pre-existing subpopulations with elevated mecillinam MICs were detected before experiments and were selected with mecillinam alone or in two-drug combinations. In contrast, the three-drug combination showed enhanced activity with synergy against six strains, a bactericidal effect against all eight strains, and suppression of resistance during 24-h antibiotic exposure. This combination may be of clinical interest in the treatment of urinary tract infections caused by ESBL-producing *E. coli*.

1. Introduction

The increasing prevalence of *Escherichia coli* producing extended-spectrum β-lactamases (ESBLs) has become a major challenge in the management of patients with urinary tract infections (UTIs) [1]. Therapeutic options are limited, especially for oral administration, owing to the frequent co-resistance to multiple β-lactam antibiotics, quinolones and trimethoprim in these strains [1]. Therefore, there is a need to re-evaluate old antibiotics that are still active against ESBL-producing *E. coli* and to learn more about how the efficacy of these drugs can be enhanced by optimal dosing or the use of combination therapy.

Mecillinam (also known as amdinocillin) is active against Enterobacteriaceae and has been used extensively for uncomplicated cystitis in Scandinavia since the 1980s. Susceptibility rates remain high (>90%) in clinical *E. coli* isolates [2], including ESBL-producing strains [3]. However, clinical data are sparse and indicate that the efficacy of mecillinam is uncertain when used for infections caused by ESBL-producing strains [4,5].

An inoculum effect, which can be reversed by adding a β-lactamase inhibitor, is usually observed in vitro with mecillinam and other β-lactam antibiotics against ESBL-producing bacteria [6]. In one study, a synergistic interaction with mecillinam in combination with clavulanic acid was found against 21 (44%) of 48 ESBL-producing *E. coli* strains in static time–kill experiments [3]. However, re-growth occurred in most experiments and a sustained synergistic effect after 24 h was found only for seven strains. Synergy against *E. coli* has also been reported in vitro and in vivo with mecillinam in combination with other β-lactam antibiotics, including amoxicillin and ampicillin [7]. The combination of amoxicillin and clavulanic acid is active against some ESBL-producing *E. coli* and has been used for cystitis with good clinical response in cases of susceptible strains with low minimum inhibitory concentrations (MICs) [8].

This study was carried out to determine the potential synergistic and bactericidal activity of the three-drug combination of mecillinam, amoxicillin and clavulanic acid. Eight clinical isolates

* Corresponding author. Tel.: +46 708 370 323.
E-mail address: thomas.tangden@medsci.uu.se (T. Tängdén).

https://doi.org/10.1016/j.ijantimicag.2018.09.011
0924-8579/

of ESBL-producing *E. coli* were exposed to the single drugs and to combinations in 24-h static time–kill experiments. The ability of the tested antibiotic regimens to suppress the emergence of mecillinam resistance was assessed by population analysis.

2. Materials and methods

2.1. Bacteria and media

Eight ESBL-producing *E. coli* clinical isolates were used in this study. All strains originated from urinary samples from patients treated for UTI in Uppsala County, Sweden. *Escherichia coli* resistant to cephalosporins were examined for ESBL production by the double-disk synergy test using ceftazidime, cefotaxime and cefepime with and without clavulanic acid according to European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines [9]. Bacteria were cultivated in cation-adjusted Mueller–Hinton II (MHII) broth and on MHII agar plates (Becton Dickinson, Oxford, UK).

2.2. Minimum inhibitory concentration determination and resistance genes

MICs were determined in triplicate by the gradient test method (Etest; bioMérieux, Lyon, France) in accordance with the manufacturer's instructions (mecillinam), or by broth microdilution in accordance with EUCAST guidelines (amoxicillin with and without clavulanic acid at 2 mg/L). The results were interpreted using EUCAST clinical breakpoints for uncomplicated UTI and systemic infections [10]. Whole-genome sequencing was performed using Illumina paired-end reads with a HiSeq sequencing approach (Illumina Inc., San Diego, CA). The fastq files were uploaded to ResFinder [11] to determine the presence of β-lactamase genes. The coding sequence of the *cysB* gene in the clinical isolates was compared with those of reference strains UTI89 (GenBank accession no. NC_007946) and MG1655 (GenBank accession no. NC_000913) using CLC Main Workbench 8.0 (QIAGEN, Hilden, Germany). Only sequence differences resulting in amino acid changes were considered potential resistance mutations.

2.3. Antibiotics

Mecillinam, amoxicillin and potassium clavulanate were purchased from Sigma-Aldrich Chemie GmbH (Schnelldorf, Germany). Stock solutions were prepared in sterile water. Mecillinam and amoxicillin were prepared freshly for each experiment, and stock solutions of clavulanic acid were stored at −80°C. In the initial time–kill experiments, mecillinam and amoxicillin were added at 32 mg/L and clavulanic acid at 4 mg/L. The three-drug combination was also tested using lower concentrations of mecillinam and amoxicillin corresponding to 1× and 2× mecillinam MIC against all strains except ARU752, which was highly resistant.

2.4. Time–kill experiments

An overnight culture from a single colony was diluted 1:100, was incubated for 1.5 h at 37°C to achieve exponential growth phase, was further diluted to a starting inoculum of ca. 5×10^6 CFU/mL and was incubated at 37°C with shaking during experiments. Samples were taken at 0, 2, 4, 6 and 24 h, were serially diluted in phosphate-buffered saline and were spread on plates. Colonies were counted following incubation overnight at 37°C. Drug carryover effects were controlled for by adding samples on a single spot on the plates and allowing them to diffuse briefly before spreading. If observed, the affected area was not counted. Results below the lower detection limit of 10 CFU/mL were counted as 1.0 $\log_{10}$ CFU/mL. All experiments were performed in duplicate and the mean values were used in the analysis. Synergy was defined as a $\geq$2 $\log_{10}$ CFU/mL decrease at 24 h with the combination compared with its most active compound [12]. The three-drug combination was compared with the most active single antibiotic as well as the most active two-drug combination. A bactericidal effect was defined as a $\geq$3 $\log_{10}$ reduction in CFU/mL at 24 h compared to the starting inoculum [12].

2.5. Population analysis

Population analysis was performed in duplicate for ARU653 and ARU652 to describe the presence of resistant populations before and after experiments. Samples were taken at 0 h and 24 h and were plated on agar containing mecillinam at concentrations of 0–16 mg/L. Viable counts were performed as described above.

3. Results

3.1. Antimicrobial susceptibility and resistance genes

MICs, antimicrobial susceptibilities and β-lactamase genes of the eight isolates are shown in Table 1. Six strains were susceptible to mecillinam (MICs of 0.125–4 mg/L) and two were resistant (MICs of 16 mg/L and >256 mg/L). All strains were highly resistant to amoxicillin alone and six strains were susceptible (uncomplicated UTI) to amoxicillin when combined with clavulanic acid (MICs of 4–32 mg/L). Genes encoding CTX-M-type ESBLs were detected in all isolates, most commonly $bla_{\text{CTX-M-15}}$, and up to three

Table 1
Minimum inhibitory concentrations (MICs), classification of antibiotic susceptibility according to European Committee on Antimicrobial Susceptibility Testing (EUCAST) definitions, and β-lactam resistance genes. Clavulanic acid was added at a fixed concentration of 2 mg/L. The susceptibility breakpoint for mecillinam (8 mg/L) applies only for uncomplicated urinary tract infections (UTIs). Susceptibilities for amoxicillin/clavulanic acid are presented using the clinical breakpoints for uncomplicated UTI and systemic infections (32 mg/L and 8 mg/L, respectively).

Strain	MIC (mg/L) [susceptibility]			β-lactamase gene(s)
	MEC	AMX	AMX + CLA[a]	
ARU752	>256 [R]	>256 [R]	>32 [R/R]	$bla_{\text{TEM-1D}}$, $bla_{\text{CTX-M-1}}$, $bla_{\text{OXA-1}}$
ARU646	16 [R]	>256 [R]	>32 [R/R]	$bla_{\text{TEM-1B}}$, $bla_{\text{CTX-M-15}}$
ARU652	4 [S]	>256 [R]	32 [S/R]	$bla_{\text{CTX-M-15}}$, $bla_{\text{OXA-1}}$
ARU654	2 [S]	>256 [R]	16 [S/R]	$bla_{\text{TEM-1B}}$, $bla_{\text{CTX-M-15}}$
ARU660	1 [S]	>256 [R]	8 [S/S]	$bla_{\text{TEM-1B}}$, $bla_{\text{CTX-M-15}}$
ARU651	0.5 [S]	>256 [R]	32 [S/R]	$bla_{\text{CTX-M-15}}$, $bla_{\text{OXA-1}}$
ARU653	0.25 [S]	>256 [R]	4 [S/S]	$bla_{\text{CTX-M-3}}$
ARU648	0.125 [S]	>256 [R]	4 [S/S]	$bla_{\text{CTX-M-14}}$

MEC, mecillinam; AMX, amoxicillin; CLA, clavulanic acid; S, susceptible; R, resistant.
[a] MIC of AMX in the presence of 2 mg/L CLA.

β-lactamase genes were detected in the same strain (ARU752). A single mutation in the *cysB* gene resulting in an amino acid substitution (L256P) was found in ARU652, whereas the other strains were wild-type for *cysB*.

3.2. Time–kill experiments

Bacterial reductions of 0.4–3.0 $\log_{10}$ CFU/mL were observed after 2–6 h with mecillinam alone (Fig. 1). However, substantial re-growth occurred in all experiments and after 24 h no bactericidal effect was observed despite using concentrations of up to 256 × MIC. Negligible activity was noted with amoxicillin or clavulanic acid alone.

The two-drug combination of mecillinam and amoxicillin at 32 mg/L resulted in 1.1–3.5 $\log_{10}$ CFU/mL reductions after 2–6 h but was not synergistic or bactericidal (Fig. 1). Mecillinam and clavulanic acid showed 0.3–4.1 $\log_{10}$ CFU/mL reductions at 2–6 h and synergistic activity against one strain (ARU660). All other strains had 24-h bacterial concentrations higher than the starting inoculum with this combination. Amoxicillin and clavulanic acid was the most active double combination, exhibiting synergistic and bactericidal effects against three and two strains, respectively.

The three-drug combination of mecillinam and amoxicillin at 32 mg/L and clavulanic acid at 4 mg/L resulted in a 3.7–5.6 $\log_{10}$ CFU/mL decrease in bacterial concentrations after 2–6 h (Fig. 1). Synergy was achieved against six strains. For ARU648 and ARU653 this combination was not synergistic compared with the two-drug combination of amoxicillin and clavulanic acid, which was also highly active. A bactericidal effect was found against all tested strains and re-growth was observed only with ARU652 (3.2 $\log_{10}$ CFU/mL at 24 h). The activity was reduced when using the lower concentrations of mecillinam and amoxicillin but a bactericidal effect was still noted for three strains (ARU646, ARU654 and ARU660) (Supplementary Fig. S1). As the single drugs and two-drug combinations were not tested at these concentrations, synergy could not be assessed.

3.3. Population analysis

The population analysis with ARU653 and ARU652 showed growth on plates containing mecillinam at concentrations up to 8 mg/L and 16 mg/L, respectively, corresponding to 32 × and 4 × MIC for these strains (Fig. 2). This was observed also at 0 h and after 24 h without antibiotic exposure (positive control) indicating the presence of pre-existing resistant subpopulations. Selection of resistant populations was pronounced after exposure to mecillinam alone and in two-drug combinations with amoxicillin or clavulanic acid. With ARU653, bacterial growth at 24 h was similar (<1 $\log_{10}$ CFU/mL difference) on plates containing mecillinam and on non-antibiotic containing plates. The same pattern was observed with ARU652 in one of the replicates, but the proportions of bacteria growing on antibiotic-containing plates were similar to the positive control in the other replicate. Single and double combination regimens not containing mecillinam resulted in similar proportions of bacteria growing on antibiotic-containing plates as the positive control. Suppression of resistance was found with the triple combination against both strains and with the combination of amoxicillin and clavulanic acid against ARU653.

4. Discussion

In this study, the effects of amoxicillin, mecillinam and clavulanic acid combinations against ESBL-producing *E. coli* were investigated. The three-drug combination demonstrated synergy against six strains and a bactericidal effect against all eight strains. With this combination, synergistic and bactericidal effects were also observed against strains that were highly resistant to the single antibiotics. The three-drug combination was recently shown to lower the MICs of *E. coli* resistant to mecillinam owing to high-level expression of TEM-1 [13]. A plausible mechanistic explanation for the observed interaction is that mecillinam and amoxicillin act simultaneously on different target molecules [penicillin-binding protein 2 (PBP2) [14] and PBP1a [15], respectively] of the same pathway resulting in enforced inhibition of cell wall synthesis and maintenance, whilst clavulanic acid counteracts the enzymatic degradation of these antibiotics. With lower antibiotic concentrations corresponding to 1 × and 2 × mecillinam MIC, a bactericidal effect was still detected against three strains.

Several possible explanations exist for the re-growth observed during experiments also at very high antibiotic concentrations, such as an inoculum effect, antibiotic degradation, and selection of pre-existing or emerging resistant subpopulations. Mutations resulting in inactivation of the *cysB* gene, involved in the cysteine biosynthesis pathway, are frequently found in clinical isolates of mecillinam-resistant *E. coli* [16]. In the current study, a single non-silent mutation in the *cysB* gene was found in one of the strains but the function of the resulting amino acid substitution has not been described. Based on the population analysis, we conclude that the observed re-growth was at least in part caused by pre-existing subpopulations with elevated mecillinam MICs. Selection of resistant populations occurred with mecillinam alone and in combinations with one of the other drugs but not with the three-drug combination.

The finding that the combination of mecillinam and clavulanic acid was superior to mecillinam alone is in line with the results of the study by Lampri et al. where enhanced initial killing was found in almost one-half of the tested ESBL-producing *E. coli*. However, also in this study re-growth was common and a 24-h synergistic effect was observed only against 15% of the strains [3]. The combination of amoxicillin and clavulanic acid has been used for cystitis caused by ESBL-producing *E. coli* with an overall clinical cure rate of 84%, and significantly higher in cases of MICs ≤ 8 mg/L than with higher MICs (93% vs. 56%) in one study [8]. Still, clinical data are limited and our results suggest that the addition of mecillinam to this combination can enhance its antibacterial activity by synergistic interaction.

In clinical practice, mecillinam is administered orally as the prodrug pivmecillinam, and clavulanic acid is available in fixed combinations with amoxicillin. The antibiotic concentrations used in this study are normally exceeded in urine for most of the dosing interval. For mecillinam, peak (C_{max}) urine concentrations of 176–1324 mg/L have been detected and the estimated half-life is 1.79 h [17]. Persistent high concentrations in urine have also been observed with amoxicillin (131 mg/L after 8–12 h) and clavulanic acid (8 mg/L after 4–8 h) [18]. However, blood concentrations are significantly lower. The C_{max} of mecillinam has been reported to be 3.5 mg/L after administration of 200 mg pivmecillinam [19] and, although recommended for pyelonephritis in Denmark and Norway, clinical evidence for this indication is insufficient. With amoxicillin/clavulanic acid 500/125 mg, maximum blood concentrations of ca. 8 mg/L and 4 mg/L, respectively, have been reported [20]. Thus, the drug concentrations used in the current study are clearly relevant for lower UTI but are usually not achieved in patient blood. However, both mecillinam and amoxicillin are well tolerated and a higher than normal dosage may be considered.

This study has several limitations. Only eight isolates were tested in the experiments and more studies are needed to validate our findings. The absence of an immune response, which probably has an important role in preventing the re-growth of resistant bacteria, is an inherent limitation of all in vitro infection models. There may also be differences in fitness of mutants in rich growth medium such as MHII broth, which was used in these experi-

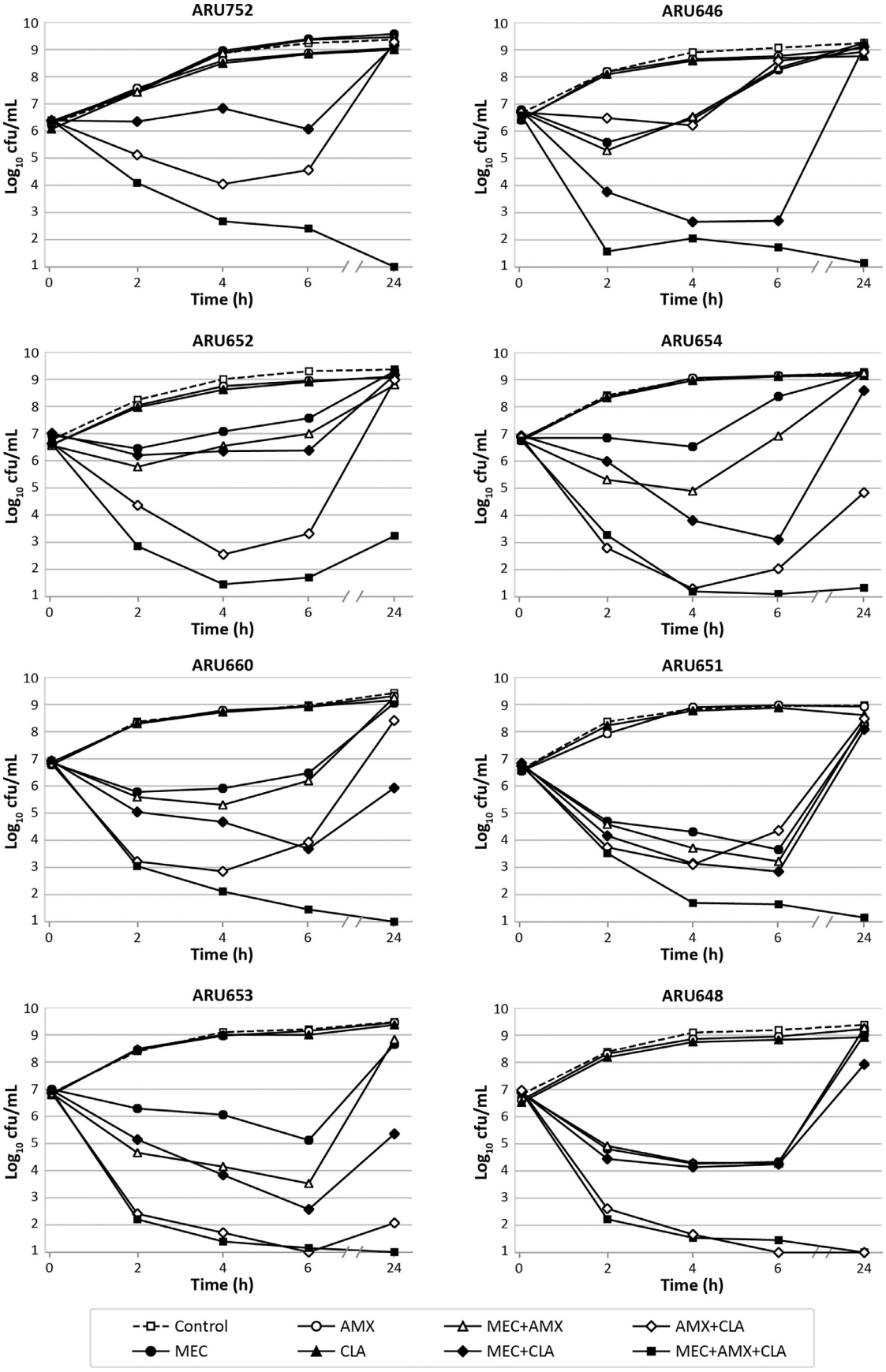

Fig. 1. Time–kill experiments for eight clinical *Escherichia coli* isolates with concentrations of mecillinam (MEC) and amoxicillin (AMX) at 32 mg/L and clavulanic acid (CLA) at 4 mg/L.

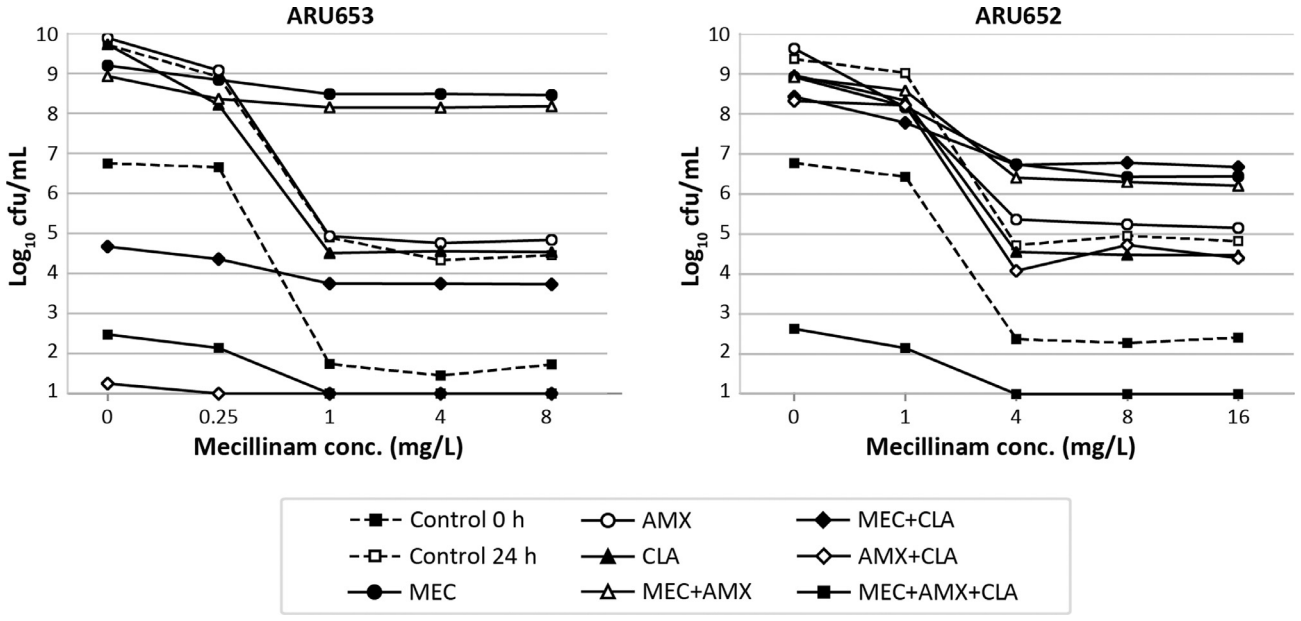

Fig. 2. Population analysis with *Escherichia coli* strains ARU653 and ARU652 performed at 0 h and at 24 h in experiments without addition of antibiotics (control) or after exposure to the single drugs or drug combinations. Samples were spread on agar containing mecillinam at concentrations of 0–16 mg/L. MEC, mecillinam; AMX, amoxicillin; CLA, clavulanic acid.

ments, compared with the in vivo situation where mutants may be more challenged [16]. Furthermore, we did not assess antibiotic concentrations during experiments or the genetic characteristics of surviving resistant populations. However, the main purpose was to evaluate potential synergistic and bactericidal effects with the three-drug combination, which was clearly demonstrated.

In conclusion, the three-drug combination of mecillinam, amoxicillin and clavulanic acid showed enhanced bacterial killing against ESBL-producing *E. coli* compared with the single antibiotics and two-drug combinations and was able to suppress the emergence of resistance during 24 h. The efficacy of mecillinam and amoxicillin/clavulanic acid is uncertain also when the isolated pathogen is deemed susceptible. Thus, concurrent administration of all three drugs could be considered to improve the clinical outcome in patients with lower UTI. More research is required to establish pharmacokinetic/pharmacodynamic targets and the potential clinical use of this combination also for systemic infections. If sufficient activity is found, perhaps with the requirement of higher dosing, this combination could be valuable in the management of patients with pyelonephritis.

Acknowledgments

The authors wish to thank Anna Hallgren for providing the clinical isolates, and Pikkei Wistrand-Yuen for technical assistance.

Funding

This study was supported by the AFA Insurance Foundation, the Family Olinder-Nielsen Foundation and the SSAC Foundation.

Competing interests

None declared.

Ethical approval

Not required.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ijantimicag.2018.09.011.

References

[1] Adler A, Katz DE, Marchaim D. The continuing plague of extended-spectrum β-lactamase-producing Enterobacteriaceae infections. Infect Dis Clin North Am 2016;30:347–75.
[2] Kahlmeter G, Poulsen HO. Antimicrobial susceptibility of *Escherichia coli* from community-acquired urinary tract infections in Europe: the ECO•SENS study revisited. Int J Antimicrob Agents 2012;39:45–51.
[3] Lampri N, Galani I, Poulakou G, Katsarolis I, Petrikkos G, Giamarellou H, et al. Mecillinam/clavulanate combination: a possible option for the treatment of community-acquired uncomplicated urinary tract infections caused by extended-spectrum β-lactamase-producing *Escherichia coli*. J Antimicrob Chemother 2012;67:2424–8.
[4] Jansåker F, Frimodt-Møller N, Sjögren I, Dahl Knudsen J. Clinical and bacteriological effects of pivmecillinam for ESBL-producing *Escherichia coli* or *Klebsiella pneumoniae* in urinary tract infections. J Antimicrob Chemother 2014;69:769–72.
[5] Søraas A, Sundsfjord A, Jørgensen SB, Liestøl K, Jenum PA. High rate of per oral mecillinam treatment failure in community-acquired urinary tract infections caused by ESBL-producing *Escherichia coli*. PLoS One 2014;9:e85889.
[6] Thomas K, Weinbren MJ, Warner M, Woodford N, Livermore D. Activity of mecillinam against ESBL producers in vitro. J Antimicrob Chemother 2006;57:367–8.
[7] Grunberg E, Cleeland R, Beskid G, DeLorenzo WF. In vivo synergy between 6 β-amidinopenicillanic acid derivatives and other antibiotics. Antimicrob Agents Chemother 1976;9:589–94.
[8] Rodríguez-Baño J, Alcalá JC, Cisneros JM, Grill F, Oliver A, Horcajada JP, et al. Community infections caused by extended-spectrum β-lactamase-producing *Escherichia coli*. Arch Intern Med 2008;168:1897–902.
[9] European Committee on Antimicrobial Susceptibility Testing (EUCAST). *EUCAST guidelines for detection of resistance mechanisms and specific resistances of clinical and/or epidemiological importance. Version 1.0, 2013*. http://www.eucast.org [accessed 15 August 2018].
[10] European Committee on Antimicrobial Susceptibility Testing (EUCAST). *Breakpoint tables for interpretation of MICs and zone diameters. Version 8.1, 2018*. http://www.eucast.org [accessed 15 August 2018].
[11] Zankari E, Hasman H, Cosentino S, Vestergaard M, Rasmussen S, Lund O, et al. Identification of acquired antimicrobial resistance genes. J Antimicrob Chemother 2012;67:2640–4.
[12] Clinical and Laboratory Standards Institute (CLSI). *Methods for determining bactericidal activity of antimicrobial agents; approved guideline*. CLSI document M26-A. Wayne, PA: 1999.

[13] Birgy A, Delecourt M, Geslain G, Desselas E, Caseris M, Magnan M, et al. A combination of mecillinam and amoxicillin/clavulanate can restore susceptibility of high-level TEM-1-producing *Escherichia coli* to mecillinam. J Antimicrob Chemother 2017;72:1911–14.
[14] Spratt BG. The mechanism of action of mecillinam. J Antimicrob Chemother 1977;3(Suppl B):13–19.
[15] Gerrits MM, Godoy AP, Kuipers EJ, Ribeiro ML, Stoof J, Mendonça S, et al. Multiple mutations in or adjacent to the conserved penicillin-binding protein motifs of the penicillin-binding protein 1A confer amoxicillin resistance to *Helicobacter pylori*. Helicobacter 2006;11:181–7.
[16] Thulin E, Sundqvist M, Andersson DI. Amdinocillin (mecillinam) resistance mutations in clinical isolates and laboratory-selected mutants of *Escherichia coli*. Antimicrob Agents Chemother 2015;59:1718–27.
[17] Kerrn MB, Frimodt-Møller N, Espersen F. Urinary concentrations and urine ex-vivo effect of mecillinam and sulphamethizole. Clin Microbiol Infect 2004;10:54–61.
[18] Ponte C, Gracia M, Giménez M-J, Aguilar L, Maín CM, Carpintero Y, et al. Urinary concentrations and bactericidal activity against amoxicillin-nonsusceptible strains of *Escherichia coli* with single-dose, oral, sustained-release amoxicillin/clavulanic acid: a phase I, open-label, noncomparative clinical trial in healthy volunteers. Clin Ther 2005;27:1043–9.
[19] Bryskier A. Penicillins. In: Bryskier A, editor. Antimicrobial agents: antibacterials and antifungals. Washington, DC: American Society of Microbiology; 2005. p. 113–62.
[20] Adam D, de Visser I, Koeppe P. Pharmacokinetics of amoxicillin and clavulanic acid administered alone and in combination. Antimicrob Agents Chemother 1982;22:353–7.