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Antimicrobial activity of two novel antimicrobial peptides AA139 and SET-M33 against clinically and genotypically diverse *Klebsiella pneumoniae* isolates with differing antibiotic resistance profiles

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ABSTRACT

Colistin is an antimicrobial peptide (AMP) used as a drug of last resort, although plasmid-mediated colistin resistance (MCR) has been reported. AA139 and SET-M33 are novel AMPs currently in development for the treatment of multidrug-resistant (MDR) Gram-negative bacterial infections. As many AMPs have a similar mode of action to colistin, potentially leading to cross-resistance, the antimicrobial activity of AA139 and SET-M33 was investigated against a collection of 50 clinically and genotypically diverse *Klebsiella pneumoniae* isolates with differing antibiotic resistance profiles, including colistin-resistant strains. The collection was genotypically characterised and susceptibility to clinically relevant antibiotics was determined. Susceptibility to AA139 and SET-M33 did not differ among the collection despite differences in underlying mechanisms of resistance or susceptibility to colistin. For three colistin-susceptible and three colistin-resistant strains with distinct MDR profiles as well as an additional MCR-producing strain, the bactericidal activity of AA139, SET-M33 and colistin during 24 h of exposure was examined. Following 24 h of exposure to AA139, SET-M33 or colistin, the seven strains were tested for changes in susceptibility to the respective AMPs. AA139 and SET-M33 showed a concentration-dependent bactericidal effect irrespective of bacterial susceptibility to colistin. Exposure to low colistin concentrations resulted in the development of colistin resistance in colistin-susceptible strains, whereas susceptibility to AA139 and SET-M33 following exposure to the respective AMPs was maintained. The two novel AMPs remained effective against colistin-resistant strains and may be promising novel drugs for the treatment of clinically and genotypically diverse MDR *K. pneumoniae* infections, including infections associated with colistin-resistant bacteria.

1. Introduction

The spread of multidrug resistance has rendered a growing list of antibiotics ineffective in the treatment of antibiotic-resistant bacterial infections, whilst the past decades have seen a dearth in the discovery and development of new antibiotics [1]. This has caused concerns regarding an oncoming post-antibiotic era in which pandrug-resistant bacterial infections will be common in the clinical setting and there are little to no treatment options left available to clinicians [2]. Already this post-antibiotic era is being heralded by clinical reports of pandrug-resistant bacterial infections [3].

Due to the increase in antimicrobial-resistant infections, colistin has resurfaced as a drug of last resort in the clinic for the treatment of multidrug-resistant (MDR) bacterial infections [4]. This polypeptide antibiotic has been available for clinical use since 1959 but was largely abandoned due to issues with potential toxic side effects. The diminishing supply of effective antibiotics available for the treatment of MDR infections has caused a renewal in investigations on the potential of colistin in the clinical setting [5].

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Unfortunately, renewed use of colistin has led to the emergence and spread of colistin resistance [6]. The first report of a plasmid conferring mobilised colistin resistance (MCR) was published in 2015 [7]. Since then, different variants of plasmidic colistin resistance have been identified and isolated from patients across the world, rendering even this drug of last resort potentially ineffective [8].

Antimicrobial peptides (AMPs) are a family of naturally occurring antimicrobial compounds that were discovered in the first half of the 20th century, of which colistin is the most well-known example in the clinical setting [9]. In nature, AMPs are produced by all living organisms as a defensive mechanism against microorganisms, and thus far over 3000 different AMPs have been described in the Antimicrobial Peptide Database (http://aps.unmc.edu/AP/main.php) [10,11].

Most AMPs, including colistin, are cationic and share a broad-spectrum mechanism of antimicrobial killing that is non-specific but highly efficient [12]. This cationic mechanism works through disruption of the bacterial cytoplasmic membrane by interaction of the cationic peptide with anionic bacterial membrane lipids, leading to arrest of bacterial growth and cell death. Whilst most cationic AMPs have this membrane-disrupting mechanism of action in common, many have additional mechanisms of antimicrobial activity, including membrane protein targeting, intracellular activity and immunomodulation [13–15]. Studies have shown that AMPs differ in their potential for cross-resistance with colistin, which may be the result of differences between their additional mechanisms of antimicrobial activity [16–18]. Importantly, AMPs that do not share cross-resistance with colistin may remain a viable alternative in the treatment of colistin-resistant infections.

In the present study, the antimicrobial activity of two novel cationic AMPs (AA139 and SET-M33) was investigated using a collection of 50 clinically and genotypically diverse *Klebsiella pneumoniae* isolates with differing antibiotic resistance profiles. AA139 originates from arenicin-3, an AMP isolated from the marine lugworm *Arenicola marina* with a 21-residue amphipathic β-hairpin structure, and was developed from arenicin-3 based on decreases in plasma protein binding properties, cytotoxicity and haemolytic activity [19,20]. AA139 has shown potent in vitro antimicrobial activity against MDR Gram-negative bacteria and has shown promising in vivo results in a number of animal models of infectious diseases [20,21]. Studies into its mode of action have suggested a dual mode of action through direct binding of AA139 to membrane phospholipids followed by interruption of phospholipid transportation pathways, resulting in membrane dysregulation and subsequent bacterial cell death [19,22].

SET-M33 is a synthetic tetrabranched peptide linked by a lysine core, providing high resistance to proteolytic degradation [23]. SET-M33 has likewise shown potent antimicrobial activity against Gram-negative bacteria and promising in vivo results in a number of animal models of infectious diseases [24]. Investigations into its mode of action have suggested that SET-M33 directly binds the bacterial lipopolysaccharide (LPS) and adopts an α-helix conformation in the membrane phospholipid bilayer, leading to membrane disruption resulting in bacterial cell death [25,26]. Further studies have indicated additional mechanisms that may contribute to the use of SET-M33 in treating infectious diseases, namely through immunomodulatory and anti-inflammatory activities [27], synergistic activity with other antibiotic families [28] and antibiofilm activity [25].

This study investigated the potential usefulness of AA139 and SET-M33 against clinically and genotypically diverse *K. pneumoniae* isolates with differing antibiotic resistance profiles, including colistin-resistant isolates.

2. Materials and methods

2.1. Bacterial isolates

A collection of 50 *K. pneumoniae* isolates was utilised in this study. The isolates were cultured from various clinical specimens, including blood, wound, mouth, throat, tracheal aspirate, rectum, catheter, urine and perineum. The collection contains isolates representing five different antibiotic resistance profiles, consisting of 10 isolates per profile: wild-type; extended-spectrum β-lactamase (ESBL)-producing; *K. pneumoniae* carbapenemase (KPC)-producing; OXA-48-like β-lactamase-producing; and New Delhi metallo-β-lactamase (NDM)-producing. Among the ESBL-producing isolates, there was one isolate positive for mobilised colistin resistance (MCR). The majority of clinical samples were collected between 2008 and 2015 from patients admitted to Erasmus MC University Medical Center (Rotterdam, the Netherlands). A KPC-producing isolate was obtained from a clinical sample from Greece and six NDM-producing isolates were obtained from clinical samples from Bangladesh.

2.2. Genotypic characterisation

PCR assays were used to verify the presence of the following resistance genes in the *K. pneumoniae* collection: CTX-M groups 1, 2, 8, 9 and 25 [29]; TEM [30]; SHV [31]; OXA-1-like [32]; OXA-48-like [33]; KPC [34]; NDM-1 [35]; and MCR-1 [7]. Multilocus sequence typing (MLST) was used to investigate genetic relatedness: partial DNA sequences of housekeeping genes were generated using a published high-throughput MLST (HiMLST) strategy that had been adapted for *K. pneumoniae* isolates [36]. The results were compared with the publicly available *K. pneumoniae* MLST profiles at http://bigsdb.pasteur.fr/. Pulsed-field gel electrophoresis (PFGE) was performed to further assess the genetic relationship between the isolates [37].

2.3. Antimicrobial agents

Ceftazidime hydrate, cefotaxime sodium salt, meropenem trihydrate, tigecycline and colistin sulfate salt were purchased from Sigma-Aldrich Chemie BV (Zwijndrecht, the Netherlands). AA139 in Ringer's acetate solution was obtained from Adenium Biotech ApS (Copenhagen, Denmark). Dry-frozen L-isomeric SET-M33-acetate [38] was obtained from Setlance srl (Siena, Italy).

2.4. Antimicrobial susceptibility testing

Minimum inhibitory concentrations (MICs) for the *K. pneumoniae* collection to clinically relevant antibiotics were determined using the broth microdilution method following European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines [39]. Two-fold antibiotic concentration ranges were used as follows: ceftazidime, 0.0625–512 mg/L; cefotaxime, 0.0625–512 mg/L; meropenem, 0.0156–128 mg/L; tigecycline, 0.0625–64 mg/L; AA139, 0.0625–64 mg/L; SET-M33, 0.0625–64 mg/L; and colistin, 0.0625–64 mg/L. Antimicrobial susceptibility of the collection was also determined using the VITEK®2 system and AST-N344 Gram-Negative Susceptibility Cards (bioMérieux Benelux BV, Zaltbommel, the Netherlands).

2.5. Selection of multidrug-resistant isolates

A panel of six *K. pneumoniae* isolates was selected from the collection for investigation of concentration- and time-dependent bactericidal activity and potential changes in susceptibility to

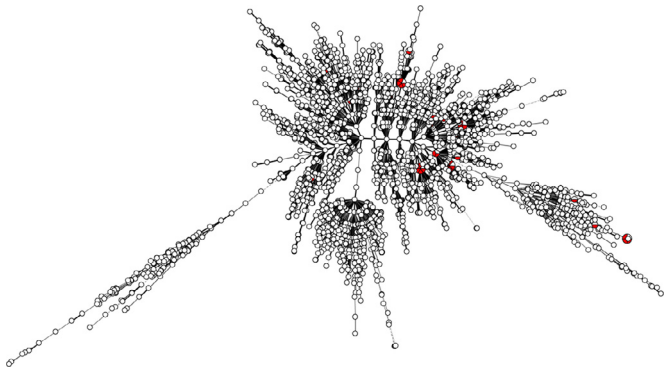

Fig. 1. Minimum spanning tree with logarithmic scaling of multilocus sequence typing (MLST) data of the collection of 50 *Klebsiella pneumoniae* isolates shown in comparison with a global *K. pneumoniae* collection of the Institut Pasteur (Paris, France). Red, Erasmus MC University Medical Center (Rotterdam, the Netherlands) collection; white, Institut Pasteur collection (http://bigsdb.pasteur.fr; accessed 20 June 2018).

AA139, SET-M33 and colistin. This panel of six isolates comprised one colistin-susceptible and one colistin-resistant strain from each of the three most extensively antibiotic-resistant profiles (KPC-producing, OXA-48-like-producing and NDM-producing). Selection of isolates was based on genotypic diversity and divergent susceptibilities to clinically relevant antibiotics, representing distinct groups of clinically relevant multidrug resistance. The MCR-producing isolate was also investigated using the same methods as the selected panel.

2.6. Concentration- and time-dependent bactericidal activity of antimicrobial peptides

Time–kill kinetic (TKK) assays were performed as described previously [40] in triplicate for each of the seven *K. pneumoniae* isolates. Four-fold increasing concentrations were used from 0.25–64 mg/L for all AMPs. At sampling time points, suspensions were centrifuged at 12 500 × *g* for 5 min to avoid antibiotic carry-over, were serially 10-fold diluted and were subcultured on Mueller–Hinton II agar plates. The plates were incubated at 37 °C for 20 h for CFU counting.

2.7. Change in susceptibility to antimicrobial peptides following exposure

The seven *K. pneumoniae* isolates exposed to AMPs for 24 h during the TKK assays were tested for changes in their susceptibility to the respective AMPs by MIC determination [39]. For colistin-resistant isolates, a concentration range of 0.5–512 mg/L colistin was used to be able to detect further increases in MIC.

3. Results

3.1. Characterisation of the Klebsiella pneumoniae collection

The clinical origins of the 50 isolates in the *K. pneumoniae* collection are shown in Supplementary Table S1. A variety of genetic lineages was observed within the collection when HiMLST data were compared with global *K. pneumoniae* MLST genotypes, although some isolates clustered together more than others (Fig. 1). PFGE data showed little overlap between the genetic profiles of the 50 isolates, with 40 clusters and singletons at 95% similarity (Supplementary Fig. S1).

PCR data showed that all 50 isolates were positive for the SHV resistance gene, as expected for *K. pneumoniae* (Supplementary Table S2). Wild-type isolates did not contain additional β-lactamase genes, except for one TEM-positive isolate. ESBL-producing isolates were all positive for CTX-M groups 1 or 9 and many were also positive for TEM (70%) or OXA-1 (40%). The *mcr* resistance gene was detected in one ESBL-producing isolate. The bla_{KPC}, $bla_{OXA\text{-}48\text{-}like}$ and bla_{NDM} resistance genes were restricted to their respective antibiotic resistance profiles, i.e. KPC-producing, OXA-48-like-producing and NDM-producing isolates. Other plasmid-mediated resistance genes were spread between the three multidrug resistance profiles with no obvious pattern.

3.2. Antimicrobial susceptibility of Klebsiella pneumoniae collection

The antimicrobial susceptibility of the *K. pneumoniae* collection against clinically relevant antibiotics (ceftazidime, cefotaxime, meropenem, tigecycline and colistin) as well as against two novel AMPs (AA139 and SET-M33) was compared based on antibiotic resistance profiles (Table 1).

Wild-type isolates were found to be phenotypically susceptible to all antibiotics tested, including the TEM-positive isolate. ESBL-producing and KPC-producing isolates were all resistant to ceftazidime and cefotaxime. ESBL-producing isolates remained susceptible to meropenem, whilst KPC-producing isolates were resistant to meropenem. OXA-48-like-producing isolates showed considerable variation in susceptibility to ceftazidime, cefotaxime and meropenem, with no obvious pattern. Moreover, 80% of the NDM-producing isolates were resistant to all antibiotics tested. Tigecycline-resistant and colistin-resistant isolates were found among all antibiotic resistance profiles, except among wild-type isolates. Antimicrobial susceptibility to AA139 and SET-M33 never exceeded a two-fold change, irrespective of the antibiotic resistance profile.

The *K. pneumoniae* collection was then divided into colistin-susceptible and colistin-resistant isolates based on colistin MICs, and the antimicrobial susceptibility of these two groupings was re-analysed (Table 2). The groupings showed differences in susceptibility to meropenem, colistin and tigecycline, with the colistin-susceptible isolates being susceptible and the colistin-resistant isolates being resistant to these antibiotics. The antimicrobial susceptibility to AA139 and SET-M33 did not exceed a two-fold change between the two groups.

VITEK® MICs for the *K. pneumoniae* collection based on antibiotic resistance profiles are shown in Supplementary Table S3. The VITEK® MICs matched the pattern of MICs found using the broth microdilution assay.

3.3. Concentration- and time-dependent bactericidal activity of antimicrobial peptides

The MIC results for the selected panel of three colistin-susceptible and three colistin-resistant *K. pneumoniae* isolates from the three most extensively antibiotic-resistant profiles (i.e. KPC-producing, OXA-48-like-producing and NDM-producing) against the AMPs tested are shown in Table 3. The bactericidal activity of the AMPs was investigated for this panel of six isolates using TKK assays (Fig. 2).

Regarding colistin-susceptible isolates, >99.9% of bacteria were killed after 2 h of exposure to ≥4 mg/L AA139, ≥4 mg/L SET-M33 or ≥0.25 mg/L colistin. Following initial bacterial killing, bacterial re-growth up to the level of non-exposed bacteria occurred after 24 h of exposure to ≤1 mg/L AA139, ≤4 mg/L SET-M33 or ≤4 mg/L colistin.

Regarding colistin-resistant isolates, >99.9% of bacteria were killed after 2 h of exposure to ≥16 mg/L AA139, ≥16 mg/L SET-M33 or ≥64 mg/L colistin. Following initial bacterial killing, bacterial re-growth up to the level of non-exposed bacteria occurred after

Table 1
Minimum inhibitory concentrations (MICs) of *Klebsiella pneumoniae* groups differing in antibiotic resistance profile.

Antibiotic resistance profile	No. of isolates	MIC (mg/L)[a]						
		Ceftazidime	Cefotaxime	Meropenem	Tigecycline	AA139	SET-M33	Colistin
Wild-type	10	0.5 (0.25 to 1)	0.09 (<0.06 to 1)	0.06 (0.03 to 0.5)	*2* (1 to *2*)	4 (4 to 4)	8 (8 to 16)	1 (0.5 to 2)
ESBL	10	**64** (*2* to **128**)	**512** (**128** to >**512**)	0.06 (0.03 to 0.13)	*2* (1 to **16**)	4 (4 to 4)	8 (4 to 16)	0.5 (0.5 to **16**)
KPC	10	**512** (**128** to >**512**)	**512** (**128** to >**512**)	**32** (**16** to **128**)	**4** (*2* to **8**)	4 (2 to 16)	16 (4 to 16)	1.5 (0.5 to **64**)
OXA-48-like	10	**256** (0.5 to **512**)	**512** (*2* to >**512**)	2 (1 to >**128**)	*2* (1 to **8**)	4 (4 to 8)	16 (8 to 32)	1 (0.5 to >**64**)
NDM	10	>**512** (>**512** to >**512**)	>**512** (**512** to >**512**)	**96** (**16** to >**128**)	**8** (*2* to **16**)	4 (2 to 8)	8 (8 to 16)	**3** (0.5 to **32**)

ESBL, extended-spectrum β-lactamase-producing; KPC, *K. pneumoniae* carbapenemase-producing; OXA-48-like, OXA-48-like β-lactamase-producing; NDM, New Delhi metallo-β-lactamase-producing.
[a] MIC assays were performed in triplicate for each isolate and the median and range are shown. MICs were interpreted as susceptible, intermediate (italic) or resistant (bold) according to European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines. Interpretation was not performed for the novel antimicrobial peptides AA139 and SET-M33.

Table 2
Minimum inhibitory concentrations (MICs) of *Klebsiella pneumoniae* groups differing in colistin (CST) susceptibility.

CST susceptibility	No. of isolates	MIC (mg/L)[a]						
		Ceftazidime	Cefotaxime	Meropenem	Tigecycline	AA139	SET-M33	CST
CST-susceptible	37	**128** (0.25 to >**512**)	**512** (<0.06 to >**512**)	0.5 (0.03 to **128**)	*2* (1 to **16**)	4 (2 to 8)	8 (4 to 16)	0.5 (0.5 to 2)
CST-resistant	13	>**512** (1 to >**512**)	**512** (*2* to >**512**)	**64** (0.13 to >**128**)	**4** (*2* to **16**)	4 (2 to 16)	16 (8 to 32)	**32** (**4** to >**64**)

[a] MIC assays were performed in triplicate for each isolate and the median and range are shown. MICs were interpreted as susceptible, intermediate (italic) or resistant (bold) according to European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines. Interpretation was not performed for the novel antimicrobial peptides AA139 and SET-M33.

Table 3
Minimum inhibitory concentrations (MICs) of selection of multidrug-resistant *Klebsiella pneumoniae* isolates.

Isolate selection	Isolate name	Antibiotic resistance profile	MIC (mg/L)[a]		
			AA139	SET-M33	Colistin
Colistin-susceptible	*K. pneumoniae* ESBL 1059	KPC	4	8	0.5
	K. pneumoniae R-DYK 4861	OXA-48-like	4	8	1
	K. pneumoniae B-DYK 9557	NDM	4	16	0.5
Colistin-resistant	*K. pneumoniae* R-DYK 3427	KPC	8	16	**64**
	K. pneumoniae R-DYK 7926	OXA-48-like	4	16	**32**
	K. pneumoniae ESBL 635	NDM	4	8	**16**
MCR-producing	*K. pneumoniae* R-DYK 11347	ESBL	4	8	**16**

KPC, *K. pneumoniae* carbapenemase-producing; OXA-48-like, OXA-48-like β-lactamase-producing; NDM, New Delhi metallo-β-lactamase-producing; ESBL, extended-spectrum β-lactamase-producing.
[a] MIC assays were performed in triplicate for each isolate and the median values are shown. MICs for colistin were interpreted as susceptible or resistant (bold) according to European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines. Interpretation was not performed for the novel antimicrobial peptides AA139 and SET-M33.

24 h of exposure to ≤4 mg/L AA139, ≤4 mg/L SET-M33 or ≤16 mg/L colistin.

3.4. Change in susceptibility to antimicrobial peptides following exposure

After 24 h of exposure to AA139, SET-M33 or colistin in TKK assays, susceptibility to the respective antibiotic of exposure was determined for the three colistin-susceptible and three colistin-resistant *K. pneumoniae* isolates (Table 4).

Regarding colistin-susceptible isolates, susceptibility to AA139 remained unchanged except after exposure to AA139 at its MIC, which led to a 4-fold MIC increase. Susceptibility to SET-M33 showed some minor changes but never exceeded a 2-fold increase in MIC after exposure to SET-M33. Exposure to colistin led to the development of colistin resistance even at low concentrations of colistin.

Regarding colistin-resistant isolates, susceptibility to AA139 remained unchanged except after exposure to AA139 at its MIC, which led to a 4-fold MIC increase. Susceptibility to SET-M33 remained unchanged except after exposure of SET-M33 at its MIC, which led to a 2-fold increase in MIC. Exposure to high concentrations of colistin led to a further increase in the MICs for colistin.

Table 4
Change in susceptibility to antimicrobial peptides (AMPs) following 24 h of exposure to the respective AMP of *Klebsiella pneumoniae* isolates.

AMP concentration (mg/L)	MIC (mg/L)[a]					
	CST-susceptible isolates			CST-resistant isolates		
	AA139	SET-M33	CST	AA139	SET-M33	CST
0	4	8	1	4	16	**64**
0.25	4	8	**16**	4	16	**32**
1	4	8	**32**	4	16	**32**
4	16	16	**32**	16	16	**64**
16	nd	nd	nd	nd	32	**128**
64	nd	nd	nd	nd	nd	**256**

MIC, minimum inhibitory concentration; CST, colistin; nd, not determined (no regrowth in original time–kill kinetic assay).
[a] MIC assays were performed in triplicate for each isolate; the median MICs of three isolates pooled together are shown. MICs for CST were interpreted as susceptible or resistant (bold) according to European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines. Interpretation was not performed for the novel AMPs AA139 and SET-M33.

3.5. Antimicrobial activity of antimicrobial peptides against an MCR-producing isolate

The MICs for an MCR-producing *K. pneumoniae* isolate towards AMPs can be found in Table 3. The TKK results for the MCR-producing isolate are shown in Fig. 3. Bacterial killing at ≥99.9%

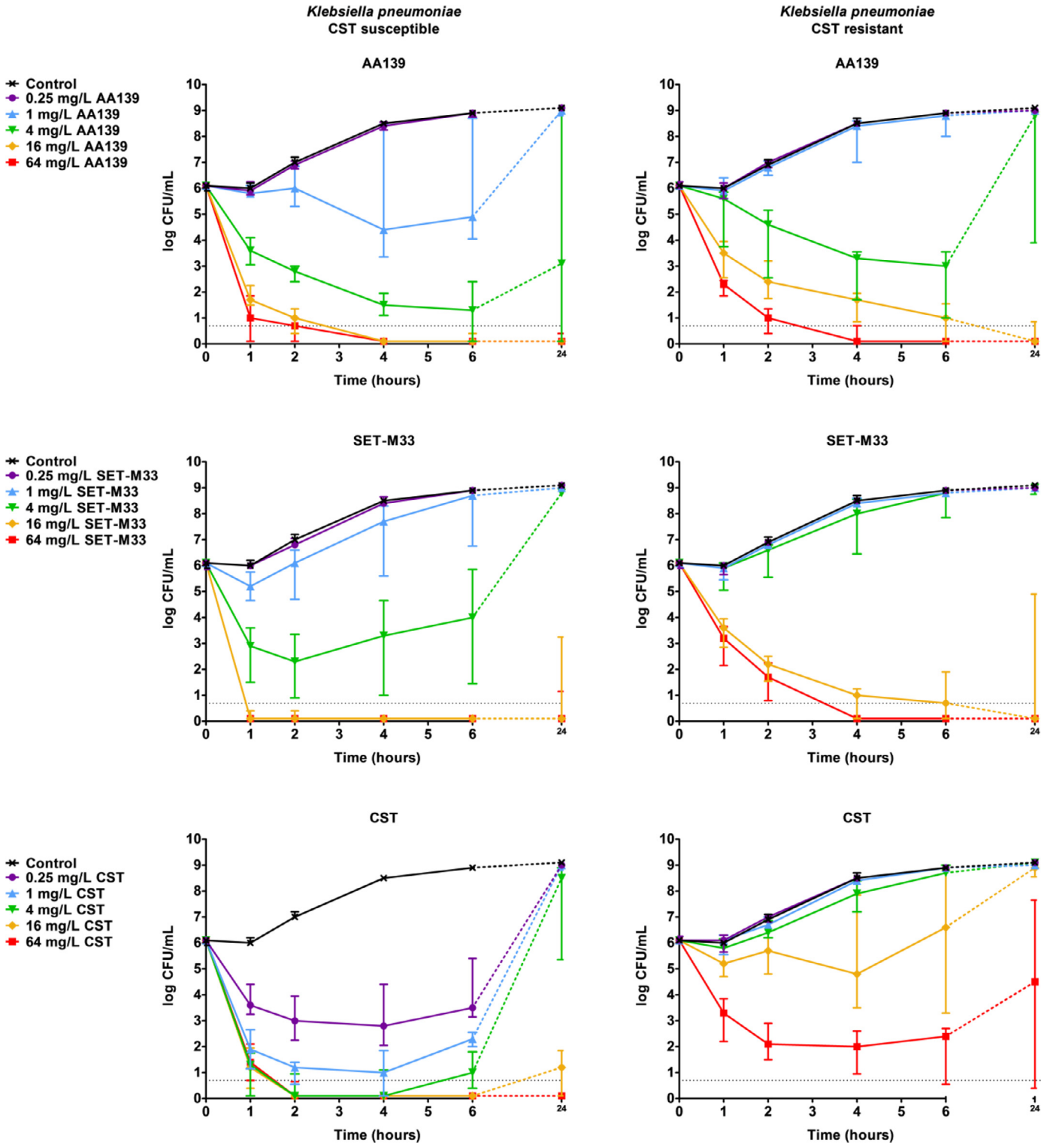

Fig. 2. Concentration- and time-dependent bactericidal activity of AA139, SET-M33 and colistin (CST) against three CST-susceptible and three CST-resistant *Klebsiella pneumoniae* isolates producing KPC, OXA-48-like and NDM. Shown here are the median and interquartile range for three CST-susceptible or three CST-resistant *K. pneumoniae*; experiments were performed in triplicate for all six isolates. The dashed grey line indicates the lower limit of quantification (log 0.7 CFU/mL).

was observed after 2 h of exposure to ≥4 mg/L AA139, ≥4 mg/L SET-M33 or ≥64 mg/L colistin. Following initial bacterial killing, bacterial re-growth up to the level of non-exposed bacteria occurred after 24 h of exposure to ≤1 mg/L AA139, ≤4 mg/L SET-M33 or ≤4 mg/L colistin.

As shown in Table 5, after 24 h of exposure to AA139, SET-M33 or colistin in the TKK assay, the change in MIC of SET-M33 for the MCR-producing isolate never exceeded a 2-fold increase and there was no change in the susceptibility of the isolate to colistin or AA139.

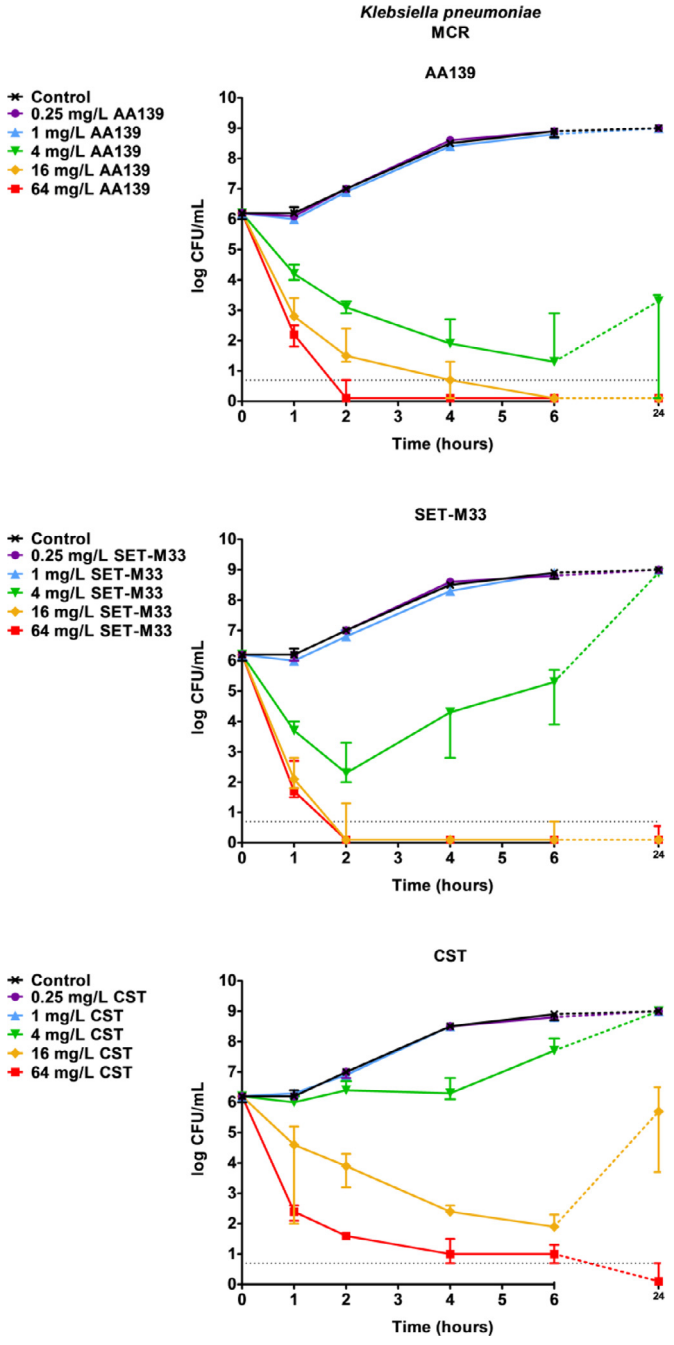

Fig. 3. Concentration- and time-dependent bactericidal activity of AA139, SET-M33 and colistin (CST) against an MCR-producing *Klebsiella pneumoniae* extended-spectrum β-lactamase-producing isolate. Shown here are the median and range from triplicate experiments. The dashed grey line indicates the lower limit of quantification (log 0.7 CFU/mL).

Table 5
Change in susceptibility to antimicrobial peptides (AMPs) after 24 h of exposure to the respective AMP of an MCR-producing *Klebsiella pneumoniae* isolate.

AMP concentration (mg/L)	MIC (mg/L)[a]		
	AA139	SET-M33	Colistin
0	4	8	**16**
0.25	2	8	**16**
1	2	16	**16**
4	nd	16	**16**
16	nd	nd	nd
64	nd	nd	nd

nd, not determined (no re-growth in the original time–kill kinetic assay).

[a] MIC assays were performed in triplicate and the median values are shown. MICs for colistin were interpreted as susceptible or resistant (bold) according to European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines. Interpretation was not performed for the novel AMPs AA139 and SET-M33.

4. Discussion

AA139 and SET-M33 are two promising novel AMPs currently in development as potential antibiotics for the treatment of MDR Gram-negative bacterial infections. To examine the clinical relevance of AA139 and SET-M33, a collection of 50 *K. pneumoniae* isolates from clinical samples was established representing five distinct antibiotic resistance profiles: wild-type; ESBL-producing; KPC-producing; OXA-48-like-producing; and NDM-producing. These isolates were clinically diverse and genotypically distinct at the global (MLST) and individual level (PFGE). Antimicrobial susceptibility against clinically relevant antibiotics as well as the presence of plasmid-mediated antimicrobial resistance genes was determined for all 50 *K. pneumoniae* isolates.

Susceptibility to the AMPs AA139 and SET-M33 of all 50 isolates remained in the same order of magnitude regardless of the antibiotic resistance profile or susceptibility of the isolates to colistin. This implies that the underlying mechanism(s) of resistance in these isolates did not affect the susceptibility of the strains to AA139 and SET-M33. Given that the growing emergence and spread of colistin resistance in the past few years [6,7] has been coupled with the potential of colistin cross-resistance [16–18], it is necessary to test whether novel AMPs such as AA139 and SET-M33 possess antimicrobial activity against colistin-resistant isolates. These results suggest that AA139 and SET-M33 have potential in the treatment of MDR infections, including those associated with colistin-resistant isolates.

In TKK assays using colistin-susceptible isolates, AA139, SET-M33 and colistin showed concentration-dependent bactericidal activity, with colistin showing activity at lower concentrations than AA139 or SET-M33 within the first 6 h of exposure. Despite these differences in initial bactericidal activity, re-growth of the colistin-susceptible bacteria following 24 h of exposure to levels similar to non-colistin-exposed bacteria was observed at similar concentrations for AA139, SET-M33 and colistin. The activity of colistin was reduced against colistin-resistant isolates compared with colistin-susceptible isolates, with a 99.9% CFU/mL reduction and no re-growth of colistin-resistant bacteria only being observed at the highest colistin concentration used (64 mg/L). The novel AMPs showed a milder decrease in bactericidal activity compared with colistin, with a 99.9% CFU/mL reduction and no re-growth of colistin-resistant bacteria observed at concentrations of >16 mg/L for AA139 and SET-M33. This reinforces our earlier study on SET-M33 [26] which found that the antimicrobial activity of SET-M33 was similarly mildly diminished in colistin-resistant mutants of *K. pneumoniae* and *Pseudomonas aeruginosa* compared with the decrease in antimicrobial activity of colistin. Here we demonstrated that this is the case for a selection of clinically and genotypically diverse *K. pneumoniae* isolates both for AA139 and SET-M33.

Following 24 h of antibiotic exposure in the TKK assays, the isolates were tested for changes in susceptibility that could suggest adaptation to a resistant phenotype. Colistin-susceptible isolates became colistin-resistant after 24 h of exposure to low concentrations of colistin (up to a 64-fold increase in MIC). Conversely, changes in susceptibility of colistin-susceptible isolates to AA139 or SET-M33 after 24 h were only observed after exposure to the respective AMPs at their own MIC but never exceeded a 4-fold

increase in MIC. In isolates that were already colistin-resistant, a further decrease in susceptibility to colistin up to 4-fold decrease was observed following exposure to colistin. Conversely, susceptibility of colistin-resistant isolates to AA139 remained unchanged except for a 4-fold increase in MIC after exposure to AA139 at its own MIC, and susceptibility to SET-M33 never decreased more than 2-fold following exposure to SET-M33. This finding matches our earlier study [26] where it was shown that colistin resistance was easily selected in *K. pneumoniae* and *P. aeruginosa* isolates after 24 h of exposure to colistin, whilst 24 h of exposure to SET-M33 did not lead to significant changes in susceptibility to SET-M33 in these isolates. Another study likewise suggested that SET-M33 has a lower propensity for resistance selection compared with colistin [24].

Finally, antimicrobial activity and susceptibility changes were also examined for an MCR-producing isolate to investigate differences between a plasmid-mediated colistin-resistant strain and a chromosomal colistin-resistant strain. The bactericidal activity of AA139 and SET-M33 against this strain was similar compared with colistin-susceptible isolates and no significant changes in susceptibility were observed after 24 h of exposure. This suggests that plasmid-mediated colistin resistance does not confer cross-resistance to AA139 or SET-M33 and that treatment with either of these AMPs will not readily select AMP-resistant mutants.

The findings of this study suggest that there is only limited cross-resistance between the two novel AMPs and colistin for clinical *K. pneumoniae* isolates and that the two novel AMPs have a lower propensity to select for resistant mutants compared with colistin. Future studies will be needed to show that this is the same in other Gram-negative bacterial species. Despite the shared cationic mechanism of action between these compounds [12], cross-resistance may be limited due to differences between their additional mechanisms of antimicrobial activity, which studies have suggested for AA139 [19,22], SET-M33 [24,26,27,41] and colistin [15]. These different additional mechanisms of antimicrobial activity may also explain the apparent difference between the two novel AMPs and colistin in their propensity to select for resistant mutants. AA139 and SET-M33 may be of interest for future studies on the mechanisms of antimicrobial activity and resistance for AMPs.

An interesting point for discussion with respect to AMPs is that the MICs of AA139 and SET-M33 are substantially higher than the MICs of colistin when compared in weight per volume and that colistin showed bactericidal activity within the first 6 h of exposure at lower concentrations than AA139 or SET-M33 when compared in weight per volume. However, it should be noted that the molecular weights of AA139 and SET-M33 are substantially higher compared with colistin, and when the MICs are calculated based on their respective molarities the MICs for AA139 and SET-M33 are very similar to those of colistin [23]. Ultimately, the usefulness of any given antibiotic for the clinic depends on its therapeutic window of effect, and the MIC is only an indicator of the minimum effective dose. Other pre-clinical investigations providing further insight into the therapeutic window of effect have been performed for both of the novel AMPs.

The in vivo efficacy of AA139 has been investigated in mice disease models of a peritonitis/bacteraemia neutropenic *Escherichia coli* infection, a neutropenic thigh *E. coli* infection as well as a urinary tract *E. coli* infection [42–44]. These studies demonstrated in vivo efficacy of AA139 against Gram-negative bacteria at similar concentrations as the effective concentrations found in the present study, with low toxicity and safety issues reported [21].

SET-M33 demonstrated in vitro haemolytic activity [26] and ex vivo cytotoxic activity [38] only at concentrations well above the effective concentrations found in the current study. In vivo toxicity studies on SET-M33 in mice showed no to mild toxic signs of acute toxicity, depending on the route of administration, at the effective concentrations found in the present study [24,38]. The in vivo efficacy of SET-M33 has been tested in various mouse models of disease; lethal intraperitoneal infection models caused by *E. coli* or *P. aeruginosa* [23], a lethal sepsis model caused by *E. coli* [38] and three neutropenic models of sepsis, lung infection and skin infection caused by *P. aeruginosa* [24]. Notably, when colistin was administered at similar concentrations in terms of weight per volume as SET-M33, it resulted in far more severe toxic side effects, including death, whereas no toxic side effects were reported for SET-M33 [24].

An important conclusion is that in comparative studies with high-molecular-weight antibiotics and low-molecular-weight antibiotics, it may be more insightful to express antibiotic concentrations in terms of molarity rather than weight per volume.

In conclusion, AA139 and SET-M33 are two novel AMPs currently in development for clinical application that showed consistent antimicrobial activity towards clinically and genotypically diverse *K. pneumoniae* isolates with differing antibiotic resistance profiles. Both AA139 and SET-M33 remained effective against colistin-resistant isolates, including an isolate carrying plasmid-mediated colistin resistance. Exposure of *K. pneumoniae* isolates to the two novel AMPs did not lead to significant change in susceptibility. These findings suggest that AA139 and SET-M33 are promising novel antibiotics for the treatment of MDR *K. pneumoniae* infections even when the bacteria have developed colistin resistance following previous treatment with colistin.

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Competing interests

This study was performed in collaboration with Adenium Biotech ApS (Copenhagen, Denmark) and Setlance srl (Siena, Italy), who are, respectively, the patent holders of AA139 and SET-M33.

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.2019.05.019.

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