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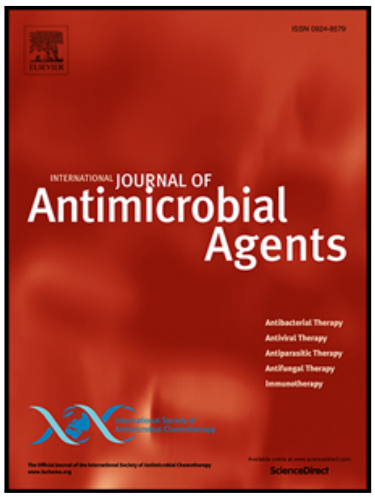

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Highlights:

- Susceptibility to AA139 and SET-M33 did not deviate among clinically relevant *K. pneumoniae*
- AA139 and SET-M33 showed a bactericidal effect irrespective of colistin susceptibility
- Exposure to low concentrations of colistin resulted in development of colistin resistance
- Susceptibility to AA139 or SET-M33 was maintained after exposure to respective antimicrobials

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 plasmidal resistance (MCR) has been reported. AA139 and SET-M33 are novel AMPs currently in development for the treatment of multidrug-resistant Gram-negative infections. As many AMPs have a similar mode of action as colistin, which can potentially lead 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 characterized and susceptibility to clinically relevant antibiotics was determined. Susceptibility to AA139 and SET-M33 did not deviate among the collection, despite differences in underlying mechanisms of resistance or susceptibility to colistin. For 3 colistin-susceptible and 3 colistin-resistant strains with distinct multidrug-resistant profiles, and an additional MCR-producing strain, the bactericidal activity of AA139, SET-M33, and colistin during 24 hours exposure was examined. After the 24 hours exposure to AA139, SET-M33, or colistin, the 7 strains were tested for changes in susceptibility towards the respective AMPs. AA139 and SET-M33 showed a concentration-dependent bactericidal effect irrespective of the susceptibility of the bacteria to colistin. Exposure to low concentrations of colistin resulted in the development of colistin resistance in colistin-susceptible strains, whereas susceptibility to AA139 and SET-M33 after 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 multidrug-resistant *K. pneumoniae* infections, including infections associated with colistin-resistant bacteria.

Keywords: antimicrobial peptides; colistin; antimicrobial activity; antimicrobial resistance;

Gram-negative bacteria; Klebsiella pneumoniae

1. Introduction

The spread of multidrug-resistance has rendered a growing list of antibiotics ineffective in the treatment of antibiotic-resistant bacterial infections, while the past decades have seen a dearth in the discovery and development of new antibiotics[1]. This has caused concerns about an oncoming post-antibiotic era, in which pan-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 pan-resistant bacterial infections[3].

Due to the increase of antibiotic-resistant infections, colistin has resurfaced as a drug of last resort in the clinic for the treatment of multidrug-resistant 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 multidrug-resistant infections has caused a renewal in investigations on the potential of colistin in the clinical setting[5].

Unfortunately, the renewed use of colistin has led to the emergence and spread of colistin resistance[6]. The first report of a plasmid conferring mobilized colistin resistance (MCR) was published in 2015[7]. Since then, different variants of plasmidal 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 towards micro-organisms, 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 which 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, which leads to the arrest of bacterial growth and cell death. While 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 hemolytic activity[19, 20]. AA139 has shown potent *in vitro* antimicrobial activity against multidrug-resistant Gram-negative bacteria and has shown promising *in vivo* results in a number of animal models of infectious disease[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 resulting in bacterial cell death[19, 22].

SET-M33 is a synthetic tetra-branched 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 disease[24]. Investigations into its mode of action have suggested that SET-M33 directly binds the bacterial 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 which may contribute to the use of SET-M33 in treating infectious diseases, namely through immunomodulatory and anti-inflammatory activity[27], synergistic activity with other antibiotic families[28], and anti-biofilm activity[25].

In this manuscript, the authors investigate 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 utilized in this study. The isolates were cultured from various clinical specimens: 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: Wildtype, extended-spectrum β-lactamase (ESBL)-producing, *K. pneumoniae* carbapenemase (KPC)-producing, OXA β-lactamase 48-like (OXA-48-like)-producing, and New Delhi metallo-β-lactamase (NDM)-producing. Among the ESBL-producing isolates was one isolate positive for mobilized colistin resistance (MCR). The majority of the clinical samples had been collected between 2008 and 2015 from patients admitted at the Erasmus MC University Medical Center Rotterdam (Erasmus MC), the Netherlands. A KPC-producing isolate was obtained from a clinical sample from Greece, and 6 NDM-producing isolates were obtained from clinical samples from Bangladesh.

2.2. Genotypic characterization

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, 25[29]; TEM[30]; SHV[31]; OXA-1-like[32], OXA-48-like[33]; KPC[34]; NDM-1[35]; and MCR-1[7]. 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 to the publicly available *K. pneumoniae* MLST profiles at

http://bigsdb.pasteur.fr/. PFGE was performed to further assess the genetic relation 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

The MICs of the *K. pneumoniae* collection to clinically relevant antibiotics were determined using the broth microdilution method following EUCAST guidelines[39]. Two-fold antibiotic concentration ranges were used; 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), 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 6 *K. pneumoniae* isolates was selected from the collection for the investigation of concentration- and time-dependent bactericidal activity and potential changes in susceptibility

to AA139, SET-M33 and colistin. This panel of 6 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, 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 investigated using the same methods as the selected panel.

2.6. Concentration- and time-dependent bactericidal activity of AMPs

Time-kill kinetics (TKK) assays were performed as described previously[40] in triplicate for each of the 7 *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 minutes to avoid antibiotic carry-over, serially 10-fold diluted, and sub-cultured on Mueller-Hinton II Agar plates. The plates were incubated at 37°C during 20 hours for cfu counting.

2.7. Change in susceptibility towards AMPs after exposure

The 7 *K. pneumoniae* isolates exposed to AMPs for 24 hours during the TKK assays were tested for changes in their susceptibility towards 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. Characterization of K. pneumoniae collection

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

PCR data showed that all 50 isolates were positive for the SHV resistance gene as expected for *K. pneumoniae* (Supplementary Table 2S). Wildtype 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. KPC, OXA-48-like, and NDM resistance genes were restricted to their respective antibiotic resistance profiles, i.e. KPC-producing, OXA-48-like-producing, and NDM-producing isolates. The other plasmid-mediated resistance genes were spread between these 3 multidrug-resistance profiles in no obvious pattern.

3.2. Antimicrobial susceptibility of K. pneumoniae collection

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

Wildtype 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, while KPC-producing isolates were resistant to meropenem. OXA-48-like-producing isolates showed considerable variation in susceptibility towards ceftazidime, cefotaxime, and meropenem, with no obvious pattern. 80% of NDM-producing isolates were resistant to all antibiotics tested. Tigecycline-resistant and colistin-resistant isolates were found among all antibiotic resistance profiles, except among Wildtype isolates. Antimicrobial susceptibility towards 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 MIC values, and the antimicrobial susceptibility of these two groupings was reanalyzed (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 3S. The VITEK® MICs matched the pattern of MICs found using the broth microdilution assay.

3.3. Concentration- and time-dependent bactericidal activity of AMPs

The MIC results for the selected panel of 3 colistin-susceptible and 3 colistin-resistant *K. pneumoniae* isolates from the three most extensively antibiotic-resistant profiles (KPC-producing, OXA-48-like-producing, NDM-producing) against the AMPs tested are shown in Table 3. The bactericidal activity of the AMPs was investigated for this panel of 6 isolates using TKK assays (Figure 2).

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

Regarding colistin-resistant isolates, more than 99.9% of bacteria were killed after 2 hours of exposure to ≥16 mg/L AA139, ≥16 mg/L SET-M33, or ≥64 mg/L colistin. After initial bacterial killing, bacterial re-growth up to the level of non-exposed bacteria occurred after 24 hours exposure to ≤4 mg/L AA139, ≤4 mg/L SET-M33, or ≤16 mg/L colistin.

3.4. Change in susceptibility towards AMPs after exposure

After 24 hours of exposure to AA139, SET-M33 or colistin in TKK assays, the susceptibility to the respective antibiotic of exposure was determined for the 3 colistin-susceptible and 3 colistin-resistant *K. pneumoniae* isolates (Table 4).

Regarding colistin-susceptible isolates, susceptibility towards AA139 remained unchanged except after exposure to AA139 at its MIC, which led to a 4-fold MIC increase. Susceptibility towards 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 towards AA139 remained unchanged except after exposure to AA139 at its MIC, which led to a 4-fold MIC increase. Susceptibility towards 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 MIC values for colistin.

3.5. AMP antimicrobial activity towards an MCR-producing isolate

MIC results of 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 Figure 3. Bacterial killing at ≥99.9% was observed after 2 hours of exposure to ≥4 mg/L AA139, ≥4 mg/L SET-M33, or ≥64 mg/L colistin. After initial bacterial killing, bacterial re-growth up to the level of non-exposed bacteria occurred after 24 hours exposure to ≤1 mg/L AA139, ≤4 mg/L SET-M33, or ≤4 mg/L colistin.

As shown in Table 5, after 24 hours 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.

4. Discussion

AA139 and SET-M33 are two promising novel AMPs currently in development as potential antibiotics for the treatment of Gram-negative multidrug-resistant infections. To examine the clinical relevance of AA139 and SET-M33, a collection of 50 *K. pneumoniae* isolates from clinical samples was established representing 5 distinct antibiotic resistance profiles: Wildtype, 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). The antimicrobial susceptibility against clinically relevant antibiotics and the presence of plasmid-mediated antibiotic resistance genes was determined for all 50 *K. pneumoniae* isolates.

The susceptibility to AMPs AA139 and SET-M33 of all 50 isolates remained in the same order of magnitude regardless of 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 to 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 multidrug-resistant 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 hours of exposure. Despite these differences in initial bactericidal activity, regrowth of the colistin-susceptible bacteria after 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 when compared to colistin-susceptible isolates, with a 99.9% cfu/mL reduction and no regrowth 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 to 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* when compared to 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 for both AA139 and SET-M33.

After 24 hours 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 hours exposure to low concentrations of colistin, up to a 64-fold increase in MIC. Conversely, changes in susceptibility of colistin-susceptible isolates towards AA139 or SET-M33 after 24 hours were only observed after exposure to the respective AMPs at their own MIC, but never exceeded a 4-fold increase in MIC. In isolates which were already colistin-resistant, a further decrease in susceptibility to colistin up to 4-fold decrease was observed after 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 after exposure to

SET-M33. This finding matches our earlier study[26] where we showed that colistin-resistance was easily selected in *K. pneumoniae* and *P. aeruginosa* isolates after 24 hours exposure to colistin, while 24 hours 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 to colistin[24].

Finally, antimicrobial activity and susceptibility changes were also examined for an MCR-producing isolate in order to investigate a difference 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 to colistin-susceptible isolates, and no significant changes in susceptibility were observed after 24 hours exposure. This suggests that plasmid-mediated colistin-resistance does not confer cross-resistance towards 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 to colistin. Future studies will be needed to show that this is the same in other Gram-negative bacterial species. In spite of 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 values of AA139 and SET-M33 are substantially higher than the MIC values of colistin when compared in weight per volume, and that colistin showed bactericidal activity within the first 6 hours 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 to colistin, and when the MIC values are calculated based on their respective molarities, the MIC values 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 which provide 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. aerigunosa*[23], a lethal sepsis model caused by *E. coli*[38], and three neutropenic models of sepsis, lung infection, and skin infection caused by *P. aerigunosa*[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 which 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 multidrug-resistant *K. pneumoniae* infections, even when the bacteria have developed colistin resistance following previous treatment with colistin.

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Declarations

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Competing Interests: This study was performed in collaboration with Adenium Biotech ApS and Setlance srl, 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:

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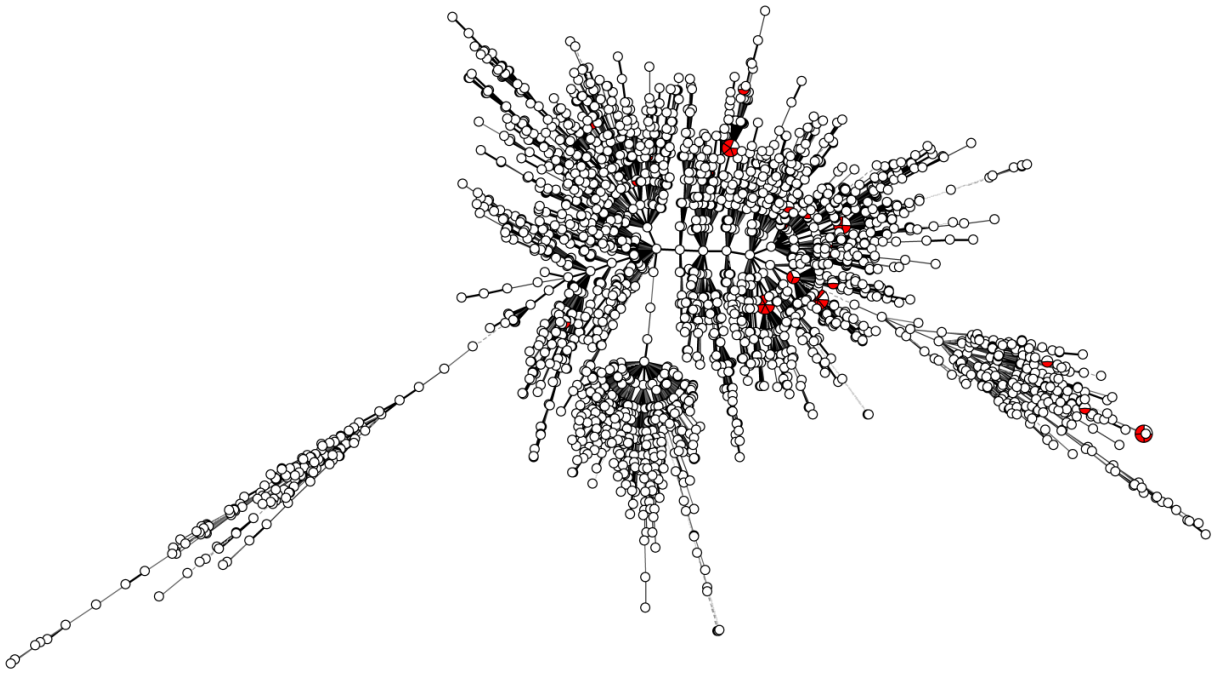

Figure 1. Minimum spanning tree with logarithmic scaling of MLST data of the collection of 50 *K. pneumoniae* isolates shown in comparison to a global *K. pneumoniae* collection of the Pasteur Institute, Paris, France. Red; Erasmus MC collection. White; Pasteur collection available at http://bigsdb.pasteur.fr accessed at 20 June 2018.

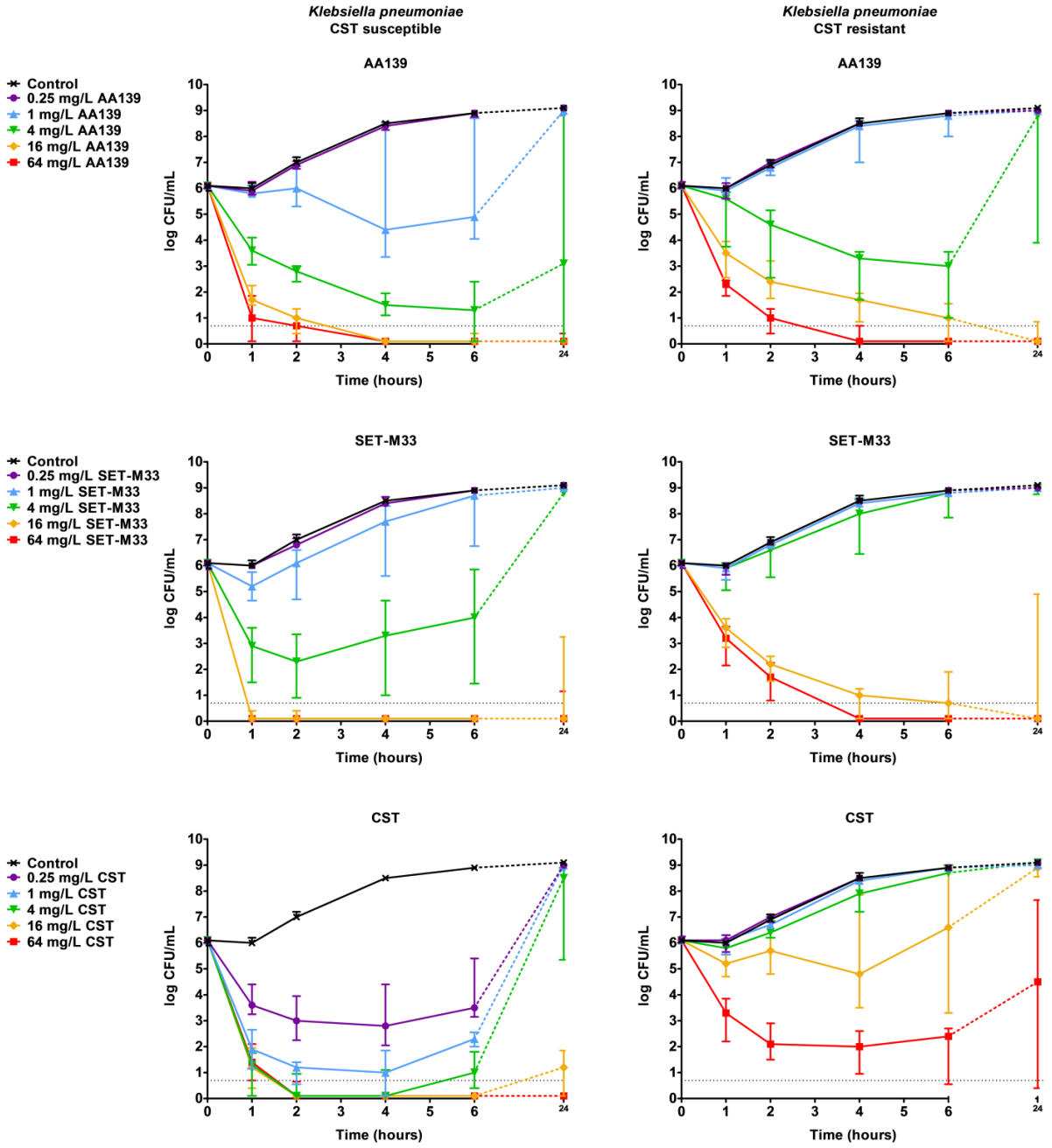

Figure 2. Concentration- and time-dependent bactericidal activity of AA139, SET-M33 and colistin (CST) against 3 colistin-susceptible and 3 colistin-resistant *K. pneumoniae* KPC, OXA-48-like and NDM isolates. Shown here are the median and interquartile range for 3 colistin-susceptible or 3 colistin-resistant *K. pneumoniae*; experiments were performed in triplicate for all 6 isolates. The dashed grey line indicates the lower limit of quantification (Log 0.7 cfu/ml).

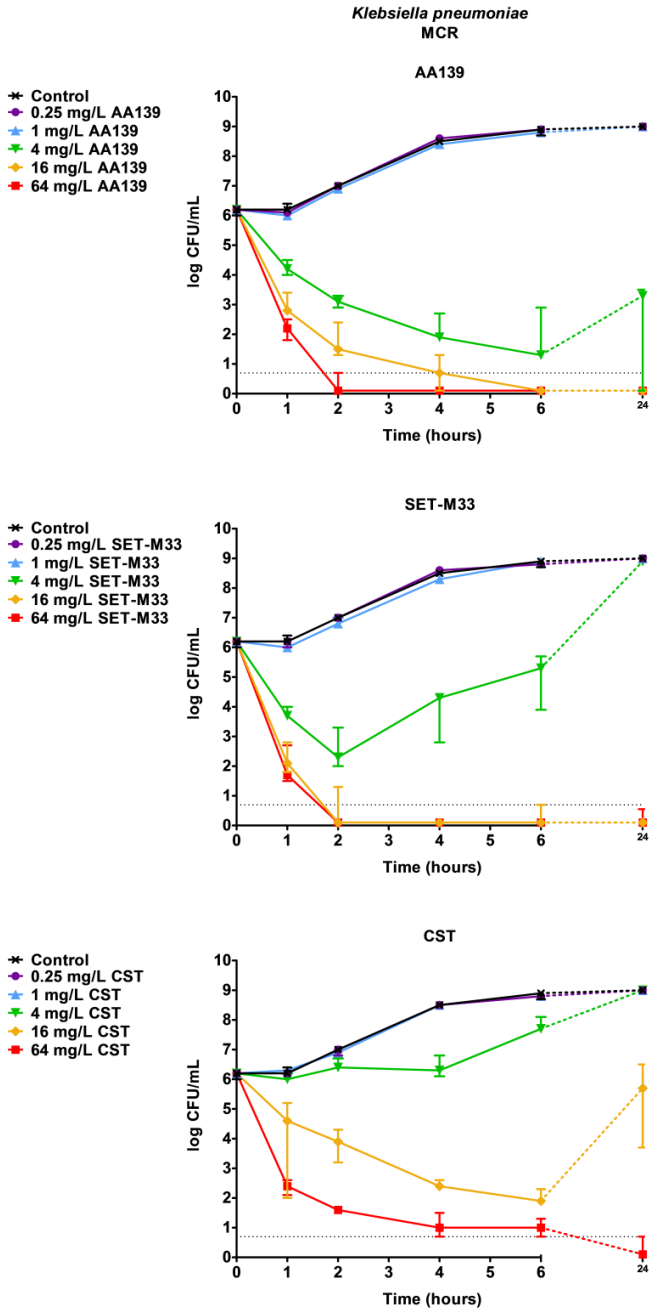

Figure 3. Concentration- and time-dependent bactericidal activity of AA139, SET-M33 and colistin (CST) against an MCR-producing *K. pneumoniae* ESBL isolate. Shown here are the median and range from triplicate experiments. The dashed grey line indicates the lower limit of quantification (Log 0.7 cfu/mL).

Table 1. MIC values (mg/L) of *K. pneumoniae* groups differing in antibiotic resistance profile

Antibiotic resistance profile	Number of isolates	Ceftazidime	Cefotaxime	Meropenem	Tigecycline	AA139	SET-M33	Colistin
Wildtype	10	0.5 0.25 - 1	0.09 <0.06 - 1	0.06 0.03 - 0.5	*2* 1 - *2*	4 4 – 4	8 8 - 16	1 0.5 - 2
ESBL	10	**64** *2* - **128**	**512** **128** - **>512**	0.06 0.03 - 0.13	*2* 1 - **16**	4 4 – 4	8 4 - 16	0.5 0.5 - **16**
KPC	10	**512** **128** - **>512**	**512** **128** - **>512**	**32** **16** - **128**	**4** *2* - **8**	4 2 - 16	16 4 - 16	1.5 0.5 - **64**
OXA-48-like	10	**256** 0.5 - **512**	**512** *2* - **>512**	2 1 - **>128**	*2* 1 - **8**	4 4 – 8	16 8 - 32	1 0.5 - **>64**
NDM	10	**>512** **>512** - **>512**	**>512** **512** - **>512**	**96** **16** - **>128**	**8** *2* - **16**	4 2 – 8	8 8 – 16	**3** 0.5 - **32**

MIC assays were performed in triplicate for each isolate, shown are the median values and range. MIC values are interpreted as susceptible, intermediate (italic), or resistant (bold) according to EUCAST guidelines. Interpretation has not been performed for the novel antimicrobial peptides AA139 and SET-M33.

Table 2. MIC values (mg/L) of *K. pneumoniae* groups differing in colistin susceptibility

Colistin susceptibility	Number of isolates	Ceftazidime	Cefotaxime	Meropenem	Tigecycline	AA139	SET-M33	Colistin
Colistin-susceptible	37	**128** 0.25 - **>512**	**512** <0.06 - **>512**	0.5 0.03 - **128**	*2* 1 - **16**	4 2 – 8	8 4 - 16	0.5 0.5 - 2
Colistin-resistant	13	**>512** 1 - **>512**	**512** *2* - **>512**	**64** 0.13 - **>128**	**4** *2* - **16**	4 2 - 16	16 8 - 32	**32** **4** - **>64**

MIC assays were performed in triplicate for each isolate, shown are the median values and range. MIC values are interpreted as susceptible, intermediate (italic), or resistant (bold) according to EUCAST guidelines. Interpretation has not been performed for the novel antimicrobial peptides AA139 and SET-M33.

Table 3. MIC values (mg/L) of selection of multidrug-resistant *K. pneumoniae* isolates

Selection	Isolate name	Antibiotic resistance profile	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**

MIC assays were performed in triplicate for each isolate, shown are the median values. MIC values for colistin are interpreted as susceptible or resistant (bold) according to EUCAST guidelines. Interpretation has not been performed for the novel antimicrobial peptides AA139 and SET-M33.

Table 4. Change in susceptibility to AMPs of *K. pneumoniae* isolates

AMP concentration (mg/L)	MIC (mg/L) after 24 hours exposure to AMP					
	Colistin-susceptible isolates			Colistin-resistant isolates		
	AA139	SET-M33	Colistin	AA139	SET-M33	Colistin
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 assays were performed in triplicate for each isolate. Shown are the median MIC values of 3 isolates pooled together. MIC values for colistin are interpreted as susceptible or resistant (bold) according to EUCAST guidelines. Interpretation has not been performed for the novel AMPs AA139 and SET-M33.

AMP, antimicrobial peptide; nd, not determined (no regrowth in the original TKK).

Table 5. Change in susceptibility to AMPs of MCR-producing *K. pneumoniae* isolate

AMP concentration (mg/L)	MIC (mg/L) after 24 hours exposure to AMP		
	MCR-producing *K. pneumoniae*		
	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

MIC assays were performed in triplicate, shown are the median values. MIC values for colistin are interpreted as susceptible or resistant (bold) according to EUCAST guidelines. Interpretation has not been performed for the novel AMPs AA139 and SET-M33.

AMP, antimicrobial peptide; nd, not determined (no regrowth in the original TKK).