Haryana Vet. (June, 2023) **62(1),** 123-127 ISSN 0033-4359 (P) **Research Article**

ANTIBACTERIAL EFFICACY OF CECROPIN A (1-7)-MELITTIN ANTIMICROBIAL PEPTIDE AGAINST MULTI-DRUG RESISTANT *SALMONELLA* TYPHIMURIUM

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ABSTRACT

The present study evaluated the antibacterial activity of Cecropin A (1-7)-melittin (CAMA), an antimicrobial peptide against multidrug-resistant (MDR) *Salmonella* Typhimurium. The observed minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values of CAMA against MDR- *S.* Typhimurium was 64 and 128 µM, respectively. Further, CAMA was assessed for its stability at high-end temperatures, various proteolytic enzymes (trypsin, proteinase K, and lysozyme), and varying pH (pH 2, 4, 6 and 8). However, CAMA was found to be stable even at high-end temperatures, various proteolytic enzymes, physiological concentrations of salts, and varying pH (pH 2, 4, 6 and 8), except for a two-fold increase in MIC, which was observed after exposure to trypsin enzyme. Also, CAMA was evaluated for its safety against sheep erythrocytes, mammalian cell lines (RAW 264.7 and HEp-2 cells), and beneficial gut lactobacilli (*L. acidophilus* and *L. rhamnosus*). CAMA was found non-hemolytic on sheep erythrocytes, revealed minimal cytotoxicity in RAW 264.7 and HEp-2 cells, and was tested safe against beneficial gut lactobacilli. This study suggested that CAMA could be a potential therapeutic candidate against MDR- *S.* Typhimurium; however, further intracellular killing ability in appropriate *in vitro* and *in vivo* models should be undertaken to address its therapeutic utility against MDR strains of *S.* Typhimurium.

Keywords: Antimicrobial peptide; Antimicrobial resistance; *Salmonella* Typhimurium; Cecropin A (1-7)- Melittin

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The emergence of pathogenic *Salmonella* Typhimurium, armed with multiple antibiotic resistance, in particular, presents a considerable threat to public health and food safety (Toor *et al.*, 2018; Zeng *et al.*, 2021). The use of antimicrobials creates selection pressure for the emergence and dissemination of antimicrobial resistance (AMR), which has been documented in *S.* Typhimurium infections worldwide. Infact AMR infections are expected to cause 10 million deaths annually by the year 2050 if no new antimicrobial approaches are implemented (Diaz *et al.*, 2022). AMR is an imminent threat to public health, and therefore alternative antibiotic strategies are urgently needed to combat the AMR crisis (Dijksteel *et al.*, 2021).

Antimicrobial peptides (AMPs), also known as host defense peptides (HDPs) have the potential to be developed as a new generation of antimicrobials (Muñoz-Flores *et al.*, 2022). Cationic Antimicrobial Peptides (cAMPs) appear to be promising candidates to overcome resistance, which mainly kill microorganisms via non-receptor mediated membrane damage (Mookherjee *et al.*, 2020). Most cAMPs are relatively short, commonly consisting of 10–50 amino acids, displaying an overall positive charge ranging from +2 to +11, and contain a substantial proportion (typically, 50%) of hydrophobic residues (Grimsey *et al.*, 2020). Cecropin A (1-7)-melittin (CAMA), is a hybrid peptide with 15 amino acid residues, and is composed of the cationic region of 'cecropin A' and the hydrophobic as well as a non-hemolytic region of the bee venom peptide 'melittin'. CAMA exhibited potential antimicrobial effect against Gram-negative and Gram-positive bacteria, fungi, *Leishmania* spp. (Majidiani and Fasihi-Ramandi, 2021). The antibacterial activity of CAMA is through interaction with the cell surface and lipopolysaccharides (LPS) permeability, resulting in inhibition of macromolecular biosynthesis and ultimately cell death (Pei *et al.*, 2021). The efficacy of CAMA against different multidrug resistant (MDR) pathogens has been explored, *viz.*, *Acinetobacter baumannii* (Rishi *et al.*, 2018), *Entamoeba histolytica* (Abhari *et al.*, 2019), MDR-enteroaggregative *Escherichia coli* (Vergis *et al.*, 2019), *Legionella pneumophila* (Birteksoz-Tan *et al.*, 2019). However, limited studies have been evaluated the antibacterial efficacy of CAMA against various *Salmonella* serovars (Abhari *et al.*, 2019; Kühnle *et al.*, 2020; Gourkhede *et al.*, 2020; Wuersching *et al.*, 2021; Nishanth *et al.*, 2022). Moreover, the antibacterial efficacy of CAMA has so far not been systematically documented against MDR- *S.* Typhimurium strains. In this study, we have evaluated the *in vitro* antibacterial efficacy of CAMA against multidrug-resistant (MDR) strains of *S.* Typhimurium and also explored the *in vitro* safety and stability aspects of

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this hybrid AMP.

MATERIALS AND METHODS

Bacterial strains and antimicrobial peptides

The three characterized MDR-field isolates of *S.* Typhimurium maintained in the repository of the Food Microbiology Laboratory, ICAR-NRCM, Hyderabad, were used in the present study. For antimicrobial susceptibility testing, *Escherichia coli* ATCC 25922 was used as the quality control strain. The sequence of CAMA peptide (KWKLFKKIGAVLKVLC) (BaAMPs database) was outsourced for its synthesis (Shanghai Science Biological Technology, China). The lactobacilli strain used for safety assays in current study was *L. rhamnosus* (MTCC 1408) and *L. acidophilus* (MTCC 10307).

Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

The antibacterial activity of CAMA was evaluated by determining the MIC and MBC values against the field isolates of MDR-*S.* Typhimurium (n=3) using a micro broth dilution assay [CLSI, 2022] as described previously (Gourkhede *et al.*, 2020; Nishanth *et al.*, 2022).

In vitro stability assays

The CAMA was evaluated for its stability at high-end temperatures (70º C and 90º C), proteases enzymes (trypsin, proteinase-K, and lysozyme) and varying pH (2, 4, 6, 8) as described earlier (Gourkhede *et al.*, 2020; Nishanth *et al.*, 2022).

In brief, in the thermostability assay CAMA was subjected to high-end temperatures at 70º C and 90º C for 5, 15, and 30 min, keeping an untreated peptide at room temperature as a control for each time interval.

The susceptibility of CAMA to the proteases enzyme (trypsin, proteinase-K, and lysozyme) was investigated by incubating the CAMA with individual enzymes (1:100 w/w) at 37º C for 30 sec, 2-, 5-, 15- or 30-min.

The pH stability of CAMA was evaluated by exposing it to different pH concentrations (pH 2, 4, 6 and 8) in CA-MH broth inoculated with MDR- S. typhimurium (n=3). CA-MH broth adjusted at different pH (pH 2, 4, 6 and 8) inoculated with corresponding strains of MDR- *S.* Typhimurium served as control.

In vitro safety assays

The safety of CAMA was evaluated by using haemolytic assay (sheep RBCs), cytotoxicity assay (HEp-2 and RAW 264.7 cell lines) and efficacy against beneficial gut lactobacilli (*Lactobacillus rhamnosus* and *L. acidophilus*) as described earlier (Gourkhede *et al.*, 2020; Nishanth *et al*., 2022).

In the haemolytic assay, CAMA was incubated with sheep erythrocytes for 60 min and hemoglobin release was measured at 540 nm.

In the cytotoxicity assay, the monolayer of HEp-2 (human laryngeal epithelioma) and RAW 264.7 (murine macrophage) cell lines were treated with CAMA individually. Cytotoxicity was determined by using an MTT cell proliferation assay kit (Himedia, India) and absorbance was measured at 590 nm.

The adverse effects of CAMA on beneficial gut lactobacilli were tested by co-incubating both CAMA with *L. rhamnosus* and *L. acidophilus* strains at 37°C for 24 hrs and then determining the lactobacilli count on MRS agar.

Statistical analysis

All the experiments were carried out three independent times in triplicates. The results were analyzed by using GraphPad Prism v. 5.01 (GraphPad Software Inc., California, USA). The cytotoxicity assay was analyzed by using one-way analysis of variance (ANOVA) with Bonferroni multiple comparison post-test, while the association of CAMA on beneficial gut lactobacilli was measured by paired two-tailed 't' test. The p-value 0.05 was considered significant, whereas 0.05was considered non-significant.

RESULTS AND DISCUSSION

AMPs have interestingly grabbed attention as a novel alternative to antibiotics against MDR pathogens (Magana *et al.*, 2020). The present study has been undertaken to evaluate the antimicrobial activity of CAMA, a hybrid peptide against MDR *S.* Typhimurium strains (n=3).

Determination of MIC and MBC

The MIC values observed for CAMA against all the isolates of MDR- *S.* Typhimurium was 64 µM. The MBC values obtained for CAMA were two-fold than the MIC concentrations *viz.*, 128 µM. The CAMA showed effective antibacterial activity in *in vitro* studies. Earlier researchers have also reported similar antimicrobial activity of CAMA against various pathogens (MIC ranges 4-8 µg/ml) (Abhari *et al.*, 2019; Vergis *et al.*, 2019; Birteksoz-Tan *et al.*, 2019; Eshtiaghi *et al.*, 2021). The antibacterial activity of CAMA is mainly through interaction with cell surface and LPS permeability, resulting in inhibition of macromolecular biosynthesis and ultimately cell death (Pei *et al.*, 2021).

In vitro stability study

The clinical application of AMPs is hard to interpret

Table 1. *In vitro* stability of CAMA against MDR-*S.* Typhimurium on exposure to high-end

Temp. Incubation Time (Min)	70º C MIC/ MBC (µM)			90º C MIC/ MBC (µM)		
	MDR-1	MDR-2	MDR-3	MDR-1	MDR-2	MDR-3
5	64/128	64/128	64/128	64/128	64/128	64/128
15	64/128	64/128	64/128	64/128	64/128	64/128
30	64/128	64/128	64/128	64/128	64/128	64/128

temperatures

Table 2. *In vitro* stability of CAMA against MDR-*S.* Typhimurium on exposure to proteases enzymes

MIC/ MBC(µM)	Incubation Time (Min)	MDR-1	MDR-2	MDR-3
	Trypsin			
	1	128/128	128/128	128/128
	5	128/128	128/128	128/128
	15	128/128	128/128	128/128
	30	128/128	128/128	128/128
	Proteinase K			
MIC/ MBC(µM)	1	64/128	64/128	64/128
	5	64/128	64/128	64/128
	15	64/128	64/128	64/128
	30	64/128	64/128	64/128
	Lysozyme			
MIC/ MBC(µM)	1	64/128	64/128	64/128
	5	64/128	64/128	64/128
	15	64/128	64/128	64/128
	30	64/128	64/128	64/128

due to the lack of stability and safety studies. AMPs were further subjected to check stability at the higher temperature because feed is often treated to higher temperatures (50º C and 90º C) during the feed pelleting process (EFSA, 2017). Also, AMPs often get exposed to various proteolytic enzymes, physiological salts, and different pH inside the host body (Kumar *et al.*, 2018).

In the present study, CAMA was found to be stable as antibacterial activity of CAMA retained unaltered even after exposure to 70º C and 90º C for 5, 15 and 30 min, respectively (Table 1). A two-fold rise in MIC and MBC values of CAMA was observed on exposure to trypsin (Table 2), however, on exposure to proteinase K and lysozyme, the antimicrobial activity of CAMA against MDR- *S.* Typhimurium was unaltered. Similarly, at different pH, CAMA exhibited stable antimicrobial activity against MDR-*S.* Typhimurium (Table 3).

The stability of AMPs against proteolytic enzymes could be improved by altering the amino acid sequence of AMPs (Kumar *et al.*, 2018). In earlier studies, researchers demonstrated that AMPs containing tryptophan and arginine residues in their sequence help to improve their antimicrobial activity under challenging salt conditions

Table 3. *In vitro* stability of CAMA against MDR-*S.* Typhimurium on exposure to different pH

pH Concentration	MDR-1MIC/ MBC (µM)	MDR-2MIC/ MBC (µM)	MDR-3MIC/ MBC (µM)
2	No growth of bacterial culture observed		
4	64/128	64/128	64/128
6	64/128	64/128	64/128
8	64/128	64/128	64/128

Table 4. *In vitro* cytotoxicity of CAMA in cell lines

AMP Concentration	RAW 264.7 cell line (% cytotoxicity)	HEp-2 cell line (% cytotoxicity)
MIC (1X)	8.2	26
MIC (2X)	11.7	29
MIC (4X)	68.7	66

(Mohamed *et al.*, 2017). Also, the CAMA were found stable at different pH concentrations suggesting their stability in various parts of the gastrointestinal tract in the body.

***In vitro* safety assay**

Along with stability, the safety of AMPs to blood cells, various cell lines, and beneficial gut flora are equally important. Therefore, to check the safety aspects of CAMA, in vitro haemolytic assay, cytotoxicity assay, and adverse effects against beneficial gut lactobacilli were investigated.

Haemolysis of sheep RBCs was estimated at 1X, 2X, and 4X MIC concentrations for CAMA (17-30), where CAMA was found to be non-hemolytic at all three MIC concentrations, suggesting its safety on sheep erythrocytes. Also, CAMA revealed minimum cytotoxicity at 1X and 2X MIC, however, slightly higher cytotoxicity at 4X MIC was observed (Table 4; Fig. 1). Overall, in comparison to positive control i.e. triton-X, CAMA revealed significantly ($p < 0.0001$) less cytotoxicity to both the cell lines (Fig. 1). The cytotoxic effect of AMPs mainly depends on its characteristic like hydrophobicity and amphipathicity. Cytotoxicity of AMPs can be reduced by altering the amino acid sequence of AMPs, as certain amino acids responsible to increase hydrophobicity which indirectly helps in reducing cell cytotoxicity (Kumar *et al.*, 2018). Further, safety against beneficial gut flora was tested, because they are very crucial in maintaining gut homeostasis and increasing intestinal epithelial cells (Ageitos *et al.*, 2017). Overall, a non-significant ($P > 0.05$) antimicrobial effect was observed when tested against *L. acidophilus* and *L. rhamnosus* (Fig. 2). CAMA showed no adverse effect on beneficial gut flora.

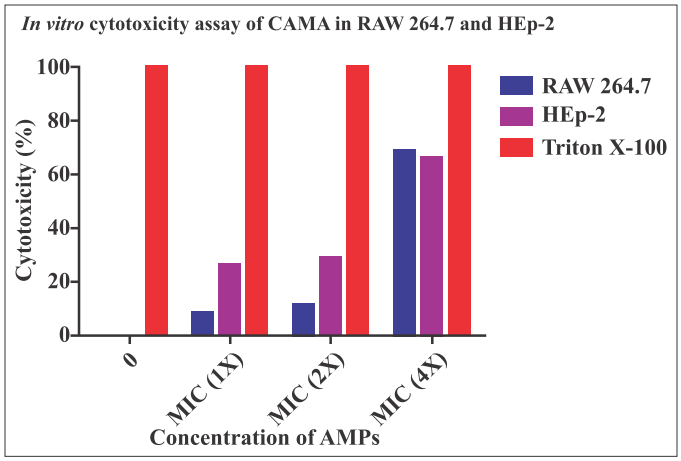

Fig. 1. *In vitro* cytotoxicity assay of CAMA in RAW 264.7 and HEp-2 cells. Cell lines were incubated with three different concentrations (1X, 2X, and 4X MIC) of CAMA for 24 h in the CO_2 incubator. Cytotoxicity was monitored as a percentage of viable cells using MTT assay and measuring the absorbance at 590 nm. Error bars indicate the standard deviation observed between three independent experiments. Triton X-100 served as a positive control exhibiting 100 % cytotoxicity.

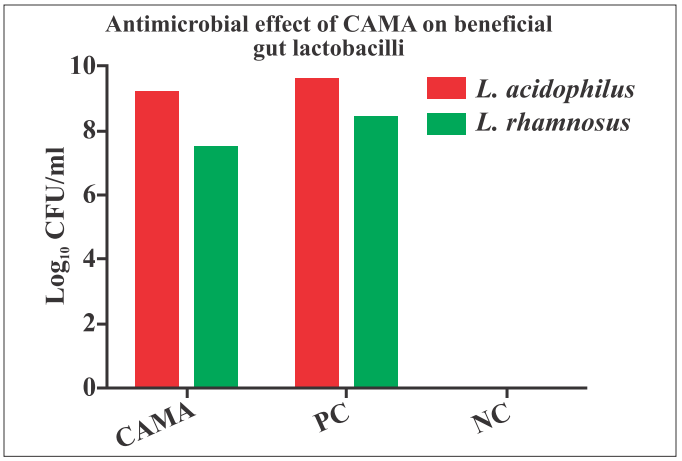

Fig. 2. Antimicrobial effect of CAMA on beneficial gut lactobacilli. CAMA was co-incubated with *L. acidophilus* and *L. rhamnosus*, respectively in MRS broth for 24 h post-incubation. CFU counts were determined for *L. acidophilus* and *L. rhamnosus* on MRS agar plate. The data obtained from three independent experiments were expressed as log_{10} CFU/ml. Error bars indicate the standard deviation between strains obtained in three independent experiments. Untreated *L. acidophilus* and *L. rhamnosus* served as a positive growth control (PC), while the media (MRS broth) served as a negative control (NC).

CONCLUSION

To conclude, the antibacterial efficacy of CAMA was evaluated against MDR- *S.* Typhimurium. CAMA exhibited effective antibacterial activity and was found stable at high temperatures, against various proteolytic enzymes, and different pH concentrations. Also, it was found safe on sheep erythrocytes, cell lines (RAW 264.7 and HEp-2), and against beneficial gut lactobacilli. Further, to explore its utility from a public health perspective, in-depth antimicrobial efficacy studies of AMPs like intracellular killing ability and antibiofilm activity against *Salmonella* strains are warranted in appropriate *in vitro* and/or *vivo* models.

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