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KRP Niranjana
PG and Research, Department of Advanced Zoology and Biotechnology, Loyola College (Autonomous), Nungambakkam, Chennai, Tamil Nadu, India

M Raja
PG and Research, Department of Advanced Zoology and Biotechnology, Loyola College (Autonomous), Nungambakkam, Chennai, Tamil Nadu, India

Corresponding Author:
M Raja
PG and Research, Department of Advanced Zoology and Biotechnology, Loyola College (Autonomous), Nungambakkam, Chennai, Tamil Nadu, India

Antimicrobial efficacy of a synthetic antimicrobial peptide against mammary pathogenic *Escherichia coli* (MPEC)

KRP Niranjana and M Raja

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Abstract
Mastitis is caused by bacteria acquired from the environment and is more predominant in milk yielding cows. Although generally self-limiting, bovine mastitis is a condition with multiple etiologies. Coliform mastitis is caused by *Escherichia coli* which are the common inhabitants of the gastrointestinal tract. E coli infection of the udder is due to faecal contamination and indicated poor hygiene and maintenance of dairy cattle. In general, treatment of mastitis involves identification of bacteria and their sensitivity to commonly used panels of antibiotics. Antibiotic therapy leads to rapid resolution of symptoms but longer duration of treatment is often practiced to prevent recurrence. Such routine use of antibiotics in dairy cattle selects for resistant bacteria. Antimicrobial peptides (AMPs) are increasingly viewed as potential alternatives to conventional antibiotics and are less prone to elicit resistance in pathogens. In this study a synthetic AMP, Cecropin A (CecA) is shown to be effective against mammary pathogenic *Escherichia coli* (MPEC) that are isolated from udder infections. The antibacterial activity of the CecA was demonstrated with a minimum inhibitory concentration (MIC) of 75 ug/ ml. The CecA was also shown to be non-haemolytic and non-cytotoxic at the MIC. Our results demonstrate the potential of this CecA peptide for therapeutic use against extended spectrum - multidrug resistant *Escherichia coli*.

Keywords: *Escherichia coli*, betalactamse resistant, cecropin, coliform mastitis

Introduction
Bovine mastitis manifests as an inflammation of the udder tissue parenchyma, giving rise to various alterations in milk composition, including physical, chemical, and bacteriological changes, along with pathological modifications in the glandular tissue. While mastitis can stem from diverse factors such as trauma, physiological changes, allergies, and others, infectious causes predominate. More than 140 species of microorganisms have been identified as contributors to bovine mastitis, underscoring its complex etiology.This condition imposes substantial economic burdens, attributable to expenses related to diagnostic procedures, veterinary interventions, discarded milk, labor costs, and indirect impacts like reduced reproduction rates and premature culling of affected animals (Heikkilä *et al*., 2018; Romero *et al*., 2018; Hogeveen *et al*., 2019) [1, 2, 3].

The multifaceted repercussions of bovine mastitis highlight the urgency of effective management strategies to mitigate economic losses and ensure the well-being of dairy herds. *Escherichia coli* constitutes a highly diverse group of commensal inhabitants within the gastrointestinal tract. However, due to the remarkable genomic adaptability of this organism (Méric *et al*., 2016; Blum *et al*., 2017; Denamur *et al*., 2021) [4, 5, 6], it has given rise to pathogenic strains capable of inducing various diseases. Among these, bovine mastitis can occur when the teat skin becomes contaminated with fecal matter. Mammary pathogenic E. coli (MPEC) has been identified as a newly recognized pathotype responsible for instigating mastitis in dairy animals (Shpigel *et al*., 2008; Richards *et al*., 2015) [7, 8].

Treatment of coliform mastitis is challenging and often involves long term use of antibiotics. Such long term use of antibiotics often leads to the rise of antimicrobial resistance. Hence alternatives to antibiotics are often explored. Insects are one of the major sources of

antimicrobial peptides (Yi *et al*., 2014) [9]. Cecropin A (CeA), is an insect antimicrobial peptide, that was proven to have robust antimicrobial efficacy against a wide range of bacteria, Encompassing both gram-positive and gram-negative strains (Steiner *et al*., 1981) [10]. This characteristic renders it an appealing candidate for substitution in lieu of conventional antimicrobials.

In this study, we evaluated the effectiveness of cecropin A (CecA) sourced from the greater wax moth *Galleria mellonella* for its antimicrobial capabilities against mammary pathogenic *Escherichia coli*. This CecA peptide has already been demonstrated to disrupt the biofilm of uropathogenic *Escherichia coli* (Kalsy *et al*., 2020) [11]. We conducted a comparative analysis of the antimicrobial efficacy of this antimicrobial peptide (AMP) with that of antibiotics to which the organism demonstrated susceptibility *in vitro*.

Materials and Methods

Bacterial strain and Cecropin A

The mammary pathogenic*E coli*was isolated from an infected cattle udder.Bacterial cultures were cultured aerobically in Luria broth (LB) (Himedia, India) and maintained at a temperature of 37°C. Solid cultures were prepared on Luria agar (LA) plates. To ensure extended viability, bacterial strains were cryopreserved at -80 °C in LB medium containing 30% glycerol.CecA is 39 amino acids long (KWKIFKKIEKAGRNIRDGIIKAGPAVSVVGEAATIYKT G) and was commercially synthesized with carboxy terminal amidation (GL Biochem, China).

Antimicrobial sensitivity testing

Antibacterial susceptibility testing was performed by disc diffusion method according to the criteria of the Clinical and Laboratory Standards Institute (CLSI) version 2017. From a pure culture 3-5 selected colonies of bacteria were taken and transferred to a tube containing 5ml nutrient broth and mixed gently until a homogenous suspension was formed and incubated at 37°C until the turbidity of the suspension become adjusted to a McFarland 0.5. A sterile cotton swab was used and the excess suspension was removed by gentile rotation of the swab against the internal surface of the tube. The swab was then used to distribute the bacteria evenly over the entire surface of Mullen Hinton agar. The inoculated plates were left at room temperature to dry for 3 to 5 min and a set of antibiotic discs containingampicilin (10 µg), ciprofloxacin (5 µg), gentamicin (10 µg), amoxicillin-clavulonate (30 µg), chloroamphenicol (30 µg), tetracycline (30 µg) and CecropinA (75 µg) were dispensed on the surface of the inoculated Muller-Hinton plate. All standard drugs concentrations were selected based on recommendation of CLSI guidelines. The inoculated plate was incubated at 37°C for 24 hours before reading.

Minimum Inhibitory Concentration (MIC)

Logarithmic growth phase bacterial cultures in 10 ml LB were diluted to a final OD600 of ~0.002 and MICs were determined using the broth microdilution method in 96-well microtiter plates with an assay volume of 200 µl. Briefly, serial dilutions of CecA was transferred to the microtiter plate and incubated overnight at 37 °C. The absorbance at 600 nm was measured to determine growth inhibition as an indicator of antimicrobial activity. The MIC was defined as the minimum concentration at which no bacterial growth was observed in each well.

Hemolytic assay

The hemolytic potential of Cec-A was determined with assay volumes of 100 µl. Sheep erythrocytes were harvested by centrifugation at 1500 × g for 3 min at 4 °C and washed three times in phosphate-buffered saline (PBS). A suspension of the erythrocytes was prepared with a dilution actor of 1:10 in PBS. Test compounds with protease inhibitor cocktail (Sigma-Aldrich) that selectively inhibits cysteine and serine proteases but not metalloproteases or aspartic proteases, were incubated with the erythrocyte suspension for 1 h at 37 °C in a 96-well plate. Percentage hemolysis of the test compounds was calculated in comparison to 10% Triton X-100 (positive control).

Cytotoxicity assay

Cytotoxic potential of Cec-A was evaluated using the baby hamster kidney fibroblast cell line BHK-21. The cells were grown in the presence of Dulbecco's modified Eagle's medium (DMEM) supplemented with 4.5 g/l glucose, 110 mg/l sodium pyruvate and L-glutamine, and 10% fetal bovine serum (FBS). The cells were maintained at 37 °C with a 5% CO2 atmosphere. Cec-A with the protease inhibitor was dissolved in water and diluted in DMEM. One day before each experiment, cells were plated at a density of 8×10^4 cells/ ml in 96-well plates. The cells were rinsed with PBS before adding 100 µl of the Cec-A, and incubated for 3.5 hours at 37 °C. The Cec-A was removed and cells were rinsed with PBS. Then, 50 µl of MTT reagent (5 mg/ml) was added and incubated for 2 h at 37 °C in the CO2 incubator. The MTT solution was then discarded, and 100 µl of isopropanol was added. The plates were placed on a shaker to solubilize the formations of purple crystal formazan. The absorbance was measured using a microplate reader at a wavelength of 570 nm. Cells exposed to DMEM without test compounds were used as a negative control and DMEM only was used as a blank reference. Percentage cytotoxicity of the test compounds was calculated in comparison to the negative control.

Results and Discussion

Mammary pathogenic *Escherichia coli* were isolated from a cow with mastitis. The pus was collected and cultured. The single colony was picked and stored as glycerol stocks. Antimicrobial sensitivity testing was carried out as per standard procedure. The zone of inhibition is given in Table-1. For subsequent studies, the glycerol stock was revived and plated on LB agar plate and single colony was picked and cultured (Figure-1). Antibiotic sensitivity testing on the isolated E coli revealed that the organism was sensitive to gentamicin and tetracycline but resistant to ampicillin, amoxycillin-clavulanate and ciprofloxacin, indicating the production of beta-lactamase. Further investigation into the nature of beta lactamase production revealed that the bacterium is an extended spectrum beta-lactamase (ESBL) producer. Infections from such ESBL organisms are difficult to treat in a clinical setting and also are prone to acquire additional plasmid-based resistance genes for other antibiotics. Insect antimicrobial peptides are small and cationic/basic with activities against bacteria and/or fungi, and some AMPs also show activities against some parasites and viruses. Considering the emergence of antibiotic resistance in bacterial species, this study tested the efficacy of an insect antimicrobial peptide Cecropin A (CecA) from the greater wax moth *Galleria mellonella* that has been previously shown to eradicate biofilms formed by uropathogenic *Escherichia*

coli strains.

In our experiments, Cecropin-A exhibited potent antibacterial activity against *E. coli* at a minimum inhibitory concentration of 75µg/ml which is comparable to that of Gentamicin for which the organism has no resistance mechanism. This potent concentration (75µg/ml) of the Cecropin-A is also shown to be non-cytotoxic to baby hamster kidney cells and did not cause haemolysis in sheep red blood cells. This indicates that it can safely be tested using *in vivo* models of E coli infection. Our findings are consistent with that of the findings of Kalsy *et al*., 2020 [11] who has demonstrated the peptides antimicrobial property against uropathogenic *E coli*.

However, antimicrobial peptides are highly prone to enzymatic degradation by proteases, which limits their use *in vivo*. However, the antimicrobial peptides have been shown to be protected against proteolytic degradation when they were encapsulated into LNCs or cubosomes (Boge *et al*., 2017, 2019) [12]. Our preliminary investigations show that Cecropin-A has a potential to be used as a therapeutic agent against *Escherichia coli* infections of the udder.

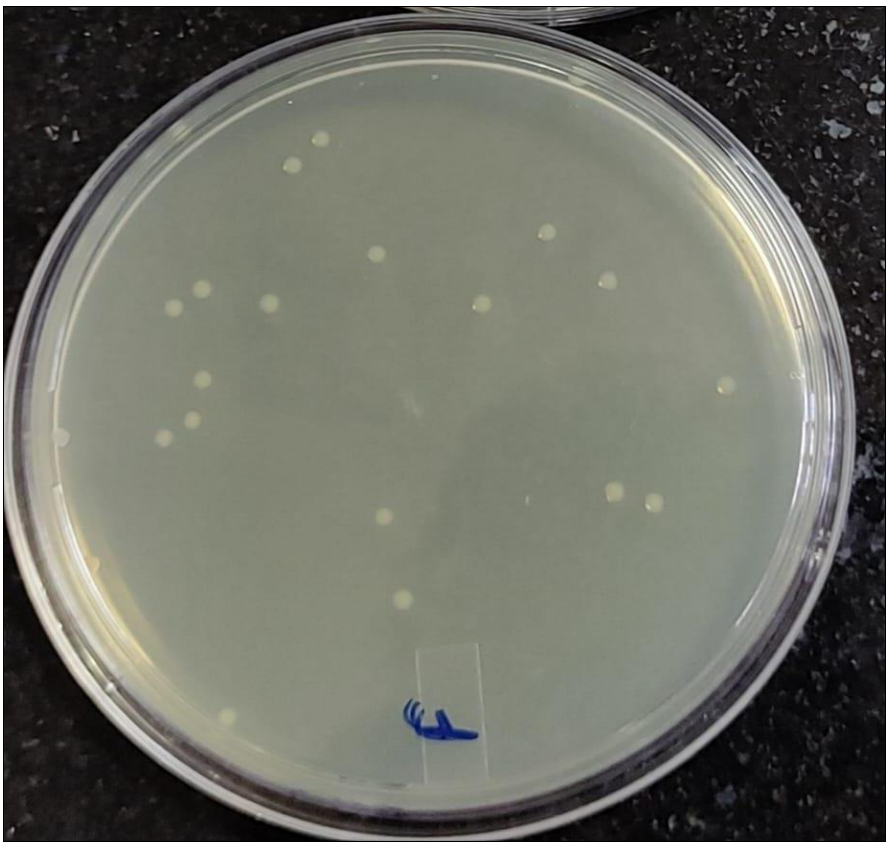

Fig 1: *Escherichia coli* colonies on a Luria Bertani agar plate

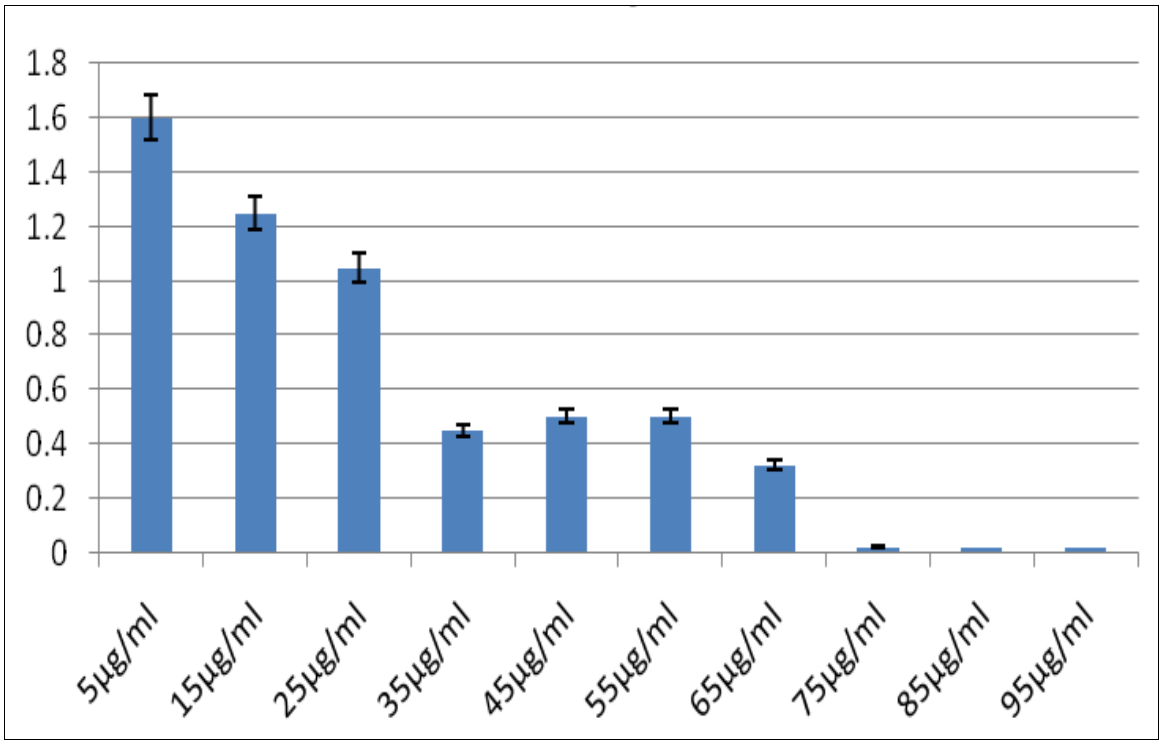

Fig 2: Cecropin-A exhibited complete antibacterial activity against *E. coli* at a minimum inhibitory concentration of 75µg/ml

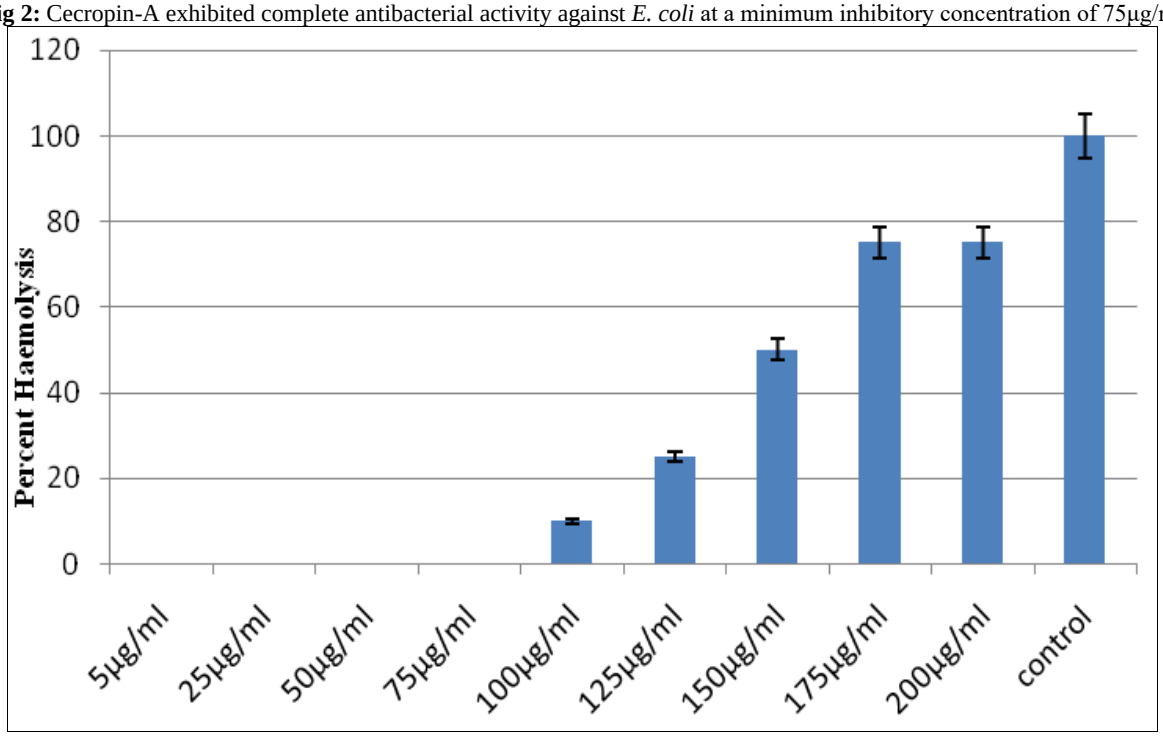

Fig 3: Cecropin-A did not cause haemolysis at the concentration of 75µg/ml

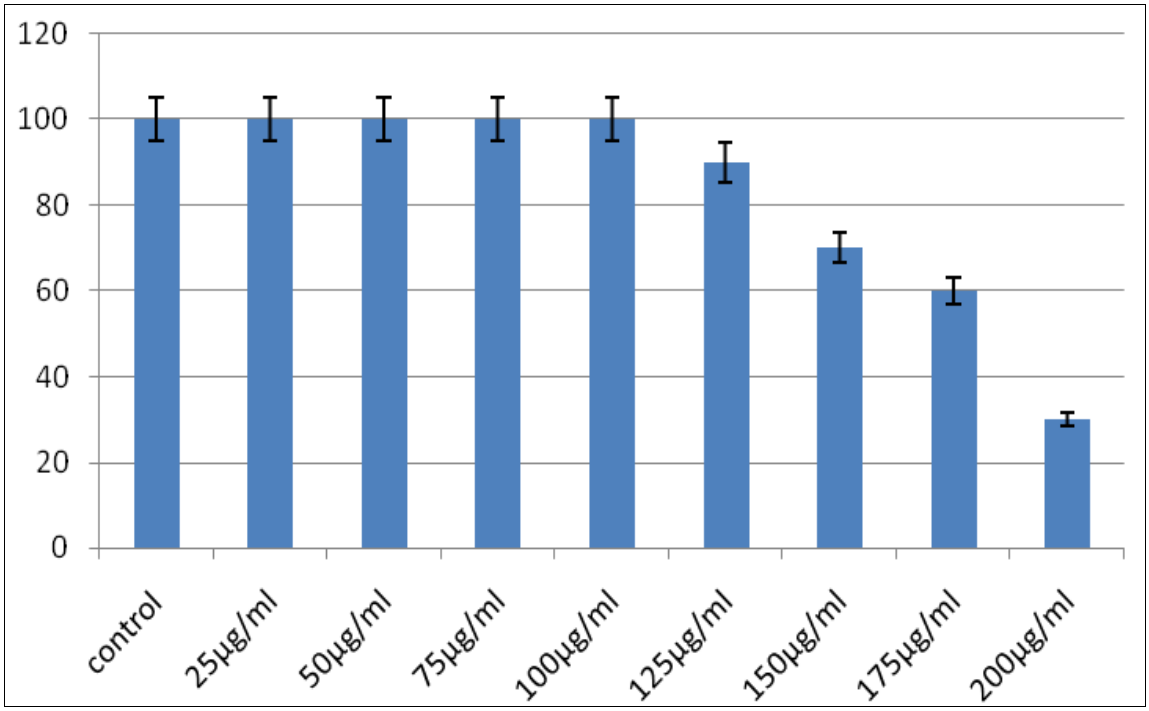

Fig 4: Cecropin-A was not cytotoxic to BHK21 cells at the minimum inhibitory concentration of 75µg/ml

Table 1: Antimicrobial sensitivity profile of the mammary pathogenic E coli isolate

S. No.	Antibiotic	Disc content	Zone diameter	Interpretation
1	Ampicilin	10 µg	< 8	Resistant
2	Ciprofloxacin	5 µg	< 8	Resistant
3	Gentamicin	10 µg	>18	Sensitive
4	Amoxicillin-clavulonate	30 µg	< 8	Resistant
5	Chloroamphenicol	30 µg	15	Intermediate
6	Tetracycline	30 µg	>17	Sensitive
7	Cefpodoxime	10 µg	20	Resistant
8	CecropinA	75 µg	>22	Sensitive

Conclusion

Antibiotic-resistant bacteria are a major concern to both human and animal health. Mastitis causing bacteria can considerably affect quality milk production and can also render the animal unproductive. There is no effective treatment for antibiotic-resistant bacteria, and the search for

alternatives is being pursued. Treatment of mammary pathogenic *E coli* infection is difficult because this bacterium can form biofilms that are resistant to antibiotics. In this study, we have investigated the potential antibacterial activity of synthetic Cecropin A. Our results demonstrate that the synthetic Cecropin A has a potent antibacterial activity against extended spectrum beta lactamase resistant *E coli* that colonise the bovine mammary gland.

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