# Efficacy of Indolicidin, Cecropin A (1-7)-Melittin (CAMA) and Their Combination Against Biofilm-Forming Multidrug-Resistant Enteroaggregative *Escherichia coli*

Jess Vergis[1] · S. V. S. Malik[1] · Richa Pathak[1] · Manesh Kumar[1] · R. Sunitha[1] · S. B. Barbuddhe[2] · Deepak B. Rawool[1]

## Abstract

The present study examined the anti-biofilm efficacy of two short-chain antimicrobial peptides (AMPs), namely, indolicidin and cecropin A (1-7)-melittin (CAMA) against biofilm-forming multidrug-resistant enteroaggregative *Escherichia coli* (MDR-EAEC) isolates. The typical EAEC isolates re-validated by PCR and confirmed using HEp-2 cell adherence assay was subjected to antibiotic susceptibility testing to confirm its MDR status. The biofilm-forming ability of MDR-EAEC isolates was assessed by Congo red binding, microtitre plate assays and hydrophobicity index; broth microdilution technique was employed to determine minimum inhibitory concentrations (MICs) and minimum biofilm eradication concentrations (MBECs). The obtained MIC and MBEC values for both AMPs were evaluated alone and in combination against MDR-EAEC biofilms using crystal violet (CV) staining and confocal microscopy-based live/dead cell quantification methods. All the three MDR-EAEC strains revealed weak to strong biofilm-forming ability and were found to be electron-donating and weakly electron-accepting (hydrophobicity index). Also, highly significant ($P < 0.001$) time-dependent hydrodynamic growth of the three MDR-EAEC strains was observed at 48 h of incubation in Dulbecco's modified Eagle's medium (DMEM) containing 0.45% D-glucose. AMPs and their combination were able to inhibit the initial biofilm formation at 24 h and 48 h as evidenced by CV staining and confocal quantification. Further, the application of AMPs (individually and combination) against the preformed MDR-EAEC biofilms resulted in highly significant eradication ($P < 0.001$) at 24 h post treatment. However, significant differences were not observed between AMP treatments (individually or in combination). The AMPs seem to be an effective candidates for further investigations such as safety, stability and appropriate biofilm-forming MDR-EAEC animal models.

**Keywords** Antimicrobial peptide · Biofilm · Enteroaggregative *E. coli* · Multidrug resistance

## Introduction

Enteroaggregative *Escherichia coli* (EAEC) are implicated in endemic as well as epidemic diarrhoeal episodes globally [1, 2]. Pathogenicity of EAEC is described in three distinct stages, namely, an initial adherence to the intestinal mucosal surface, biofilm formation and induction of inflammatory response resulting in the release of toxins [1]. The biofilm-forming ability of EAEC is an important attribute for the pathogenicity and is well correlated with its persistent infection and resistance to conventional antimicrobial therapy. This colonization leads to long-term carrier status facilitating antibiotic pressure, leading to an alarming rate of multidrug resistance among EAEC strains [1, 3]. The pathogen causes chronic inflammation and damage to the intestinal epithelium, thereby causing malnutrition and intellectual deficits in infants [1], while intestinal changes and chronic diarrhoeal episodes are notable features in animal EAEC infections [2]. Generally, antimicrobial therapy is recommended to ensure early resolution and to halt the progression to dehydration, hypovolaemic shock and death [3]. Fluoroquinolones are the drugs of choice for symptomatic EAEC infections; however, these are no longer effective due to high level of drug resistance observed among EAEC strains across the globe [3, 4]. Further, due to the physiological properties of biofilm, bacteria within the

communities become highly resistant to many of the conventional therapies, hence exhibiting much higher antimicrobial resistance levels compared with those normally observed during 'planktonic' growth [5, 6]. Moreover, bacterial cells embedded within biofilm can withstand nutrient deprivation and pH changes. The 'persisters' within the microbial biofilm are primarily induced at high rate under adverse environmental conditions and often constitute dormant, non-dividing cells [7]. This protective nature of microbial biofilms not only limit the efficacy but also provide refractoriness to antimicrobial agents and biocides, especially in assuring the microbiological safety [6]. Therefore, intensive research is directed towards the identification of novel and unconventional anti-biofilm therapies, including adjunctive or preventive approaches [8, 9].

Antimicrobial peptides (AMPs) are evolutionarily conserved molecules found in organisms ranging from prokaryotes to humans that have been heralded as promising alternatives to the currently available antibiotics [10, 11]. The complex and often multimodal antimicrobial actions of AMPs are refractory to develop perdurable resistance by the microbes which offers notable advantage of AMPs over conventional antibiotics [11, 12]. Intriguingly, synthetic short-chain cationic peptides with potential anti-biofilm activity have recently been documented [13, 14]. In particular, indolicidin, a tridecapeptide peptide isolated from the cytoplasmic granules of bovine neutrophils, was reported to exhibit antimicrobial activity against Gram-negative and Gram-positive bacteria, fungi, HIV-1 virus and protozoa [5, 6]. Moreover, cecropin A (1-7)-melittin (CAMA), a cecropin-melittin hybrid peptide with 15 amino acid residues, is composed of the cationic region of 'cecropin A' and the hydrophobic as well as non-haemolytic region of the bee venom peptide 'melittin'. CAMA exhibited potential antimicrobial effect against Gram-negative and Gram-positive bacteria, fungi, *Leishmania* spp. [15]. However, studies addressing the anti-biofilm potential of these peptides against multidrug resistant (MDR)-EAEC are lacking. The objective of the present study was to evaluate the anti-biofilm efficacy of two short-chain antimicrobial peptides with different mechanisms of actions against biofilm-forming MDR-EAEC isolates.

## Materials and Methods

### Bacterial Strains

The typical EAEC isolates from the laboratory repository were re-validated by PCR assay (chromosomal genes: *fim*A, *aai*A, *ast*A, *pil*S, *ecp*, *irp*2 and plasmid-encoded genes: *cvd*432, *aaf*II, *agg*3A, *agg*R, *agg*A) (Supplementary Material S1) [16] and confirmed using the HEp-2 cell adherence assay (Supplementary Material S2) [17]. The isolates

were tested for antibiotic susceptibility as per the Clinical and Laboratory Standards Institute (CLSI) guidelines [18]. Three MDR-EAEC strains with GenBank accession numbers, KY941936.1 (MDR 1), KY941937.1 (MDR 2) and KY941938.1 (MDR 3) were included in the study. Furthermore, in order to detect the extended spectrum beta-lactamase (ESBL)-producing ability of these isolates, a double disc test using antibiotic discs of cefotaxime and ceftazidime with clavulanic acid (BD Difco, USA) was employed [18]. *E. coli* ATCC 25922 used as quality control strain was provided by the Department of Veterinary Public Health, College of Veterinary and Animal Sciences, Pookode, India.

### Antimicrobial Peptides

Two short-chain AMPs with different mechanisms of action, namely, indolicidin and CAMA retrieved from Biofilm active AMPs (BaAMPs) [19] were synthesized commercially (Shanghai Science Peptide Biological Technology, China). The peptides were resuspended in PBS (final stock concentration of 10 mg/mL) and stored at −20 °C until further use. The amino acid sequences and physico-chemical properties of the AMPs [19] are provided in Supplementary Table 1.

### Biofilm Formation by MDR-EAEC Isolates

#### Congo Red Binding Assay

The biofilm-producing ability of MDR-EAEC isolates was assessed using Congo red binding assay [20]. The inoculated Congo red agar (CRA) plates showing black streaks or colonies with a dry crystalline consistency were regarded as strong to moderate biofilm producers, whereas the presence of pink or red colonies were regarded to be weak biofilm producers [20].

#### Microbial Adhesion to Solvents

The hydrophobicity index was determined by microbial adhesion to solvents using three solvents (n-hexadecane, chloroform and diethyl ether) [21]. The suspension of MDR-EAEC cells grown overnight in nutrient broth ($OD_{600}$ nm of 0.80) was prepared in 1.5 mM sodium chloride ($A_0$); 2.4 mL of this suspension was overlaid by 0.4 mL of each solvents. After vigorous mixing, phases were allowed to separate for 15 min at room temperature and the $OD_{600}$ nm of aqueous phase ($A_1$) was measured. The percentage of hydrophobicity was calculated [21] as, hydrophobicity $(\%) = [1 - (A_1/A_0)] \times 100$.

### Time-Dependent Biofilm-Forming Ability in Different Media

Time-dependent biofilm-forming ability of the test isolates in different media was assessed using microtitre plate assay [16, 22]. DMEM and nutrient broth each supplemented with either 0.45% D-glucose or 0.45% D-mannose were used. *E. coli* ATCC 25922 and *E. coli* DH5α were used as positive and negative biofilm controls, respectively. The optical density values obtained for the test cultures with each respective medium and time were statistically analysed to determine the optimal medium and time requirement for biofilm formation and also to classify the test cultures as high, moderate and weak biofilm producers [16].

## Minimum Inhibitory Concentration (MIC), Minimum Bactericidal Concentration (MBC) and Fractional Inhibitory Concentration Index (FICI) of AMPs

The MIC and MBC values of AMPs against 'planktonic' MDR-EAEC cells were determined using a micro-broth dilution method [18]. The lowest dilution of AMP revealing no visible growth by naked eye were designated as MIC of respective AMP, while MBC value for each AMP was determined at the end of 24 h incubation period. Approximately 10 μL of seeded inoculum was drawn from each well having no visible growth and were placed on eosin methylene blue agar (HiMedia, Mumbai, India). The lowest concentration that produced at least 99.9% killing of the initial inoculum of planktonic cells was considered as the MBC for the respective AMP [23].

The FICI was assessed by the checkerboard micro dilution assay [24]. It was calculated by comparing the MIC of each individual AMPs against the concentrations achieved by the AMP combination and was graded as synergy (FICI ≤ 0.50), additiveness (0.50 ≤ FICI ≤ 1.0), indifference (1.0 ≤ FICI ≤ 4.0) and antagonism (FICI ≥ 4.0) [24].

$$\text{FICI} = \text{FIC}_A + \text{FIC}_B = [\text{MIC } (A_{\text{comb } A/B})/\text{MIC } (A_{\text{alone}})] + [\text{MIC } (B_{\text{comb } A/B})/\text{MIC } (B_{\text{alone}})]$$

### Inhibition of Biofilm Formation by AMPs

#### Microtitre Plate Assay

The efficacy of AMP to inhibit the MDR-EAEC biofilm formation was assessed using microtitre plate assay [25]. In brief, 200 μL of each MDR-EAEC (ca. $5 \times 10^5$ CFU/200 μL) isolate grown in 96-well flat-bottom microtitre plates in DMEM containing 0.45% D-glucose having MIC concentrations of each AMPs and their combinations were incubated at 37 °C and monitored for inhibition of biofilm formation at 24 and 48 h post incubation. After each incubation period, the plates were subjected to CV staining. The respective test culture in DMEM medium served as biofilm formation control, while sterile DMEM medium was kept as media control. The assays were performed thrice independently.

### Live/Dead Staining

The efficacy of AMP to inhibit the biofilm formation by MDR-EAEC was assessed in the form of live/dead staining [14]. In brief, circular polystyrene coverslips (15 mm diameter, 1 mm thick) were inserted into each well (12-well plate), which were later amenable for confocal microscopy for live/dead cell count. The MDR-EAEC were cultured and treated as described in the 'Microtitre plate assay' section. The seeded plates were then incubated at 37 °C and monitored for biofilm inhibition at 24 and 48 h post incubation using BacLight kit (Invitrogen, USA). Before staining, at each incubation interval, the coverslips were removed from the wells, rinsed with sterile water to remove any planktonic cells and dried at 50 °C for 20 min. A solution of live/dead bacterial stain was prepared (3:1000 dilution) using 1.5 μL each of SYTO 9 and propidium iodide solution in 997 μL of sterile water. Approximately 60 μL of this BacLight solution was dispensed on each coverslip and incubated in the dark for 25 min at room temperature. After incubation, the excess stain was washed off with sterile PBS and the wells were air-dried. The stained biofilms were examined separately using the red and green filters of confocal microscope (Olympus FV 100, Japan) and the captured images were merged using the Olympus Fluoview v 3.0a software.

### Minimum Biofilm Eradication Concentration (MBEC)

The MBEC of AMPs was estimated against preformed biofilm (48 h) formed by the MDR-EAEC [25]. In brief, the preformed biofilms in 96-well microtitre plates were added with twofold serial dilutions of each AMPs (200 μL) ranging from 512 to 0.125 mg/L prepared in DMEM medium containing 0.45% D-glucose. The seeded plates were incubated at 37 °C for 24 h, later the supernatant was gently aspirated and the plates were washed twice using PBS. The wells were scrapped and the scraped biofilm material was collected with the help of PBS (1 mL), mixed thoroughly for 5 min to disrupt the biofilm and 100 μL of it was spread on to EMB agar plates. The colonies were counted after 24 h incubation at 37 °C. The lowest concentration of AMP that prevented the bacterial re-growth was designated as MBEC for each respective AMP under study [25].

### Eradication of MDR-EAEC Biofilm Formation by AMPs

The efficacy of AMPs to eradicate the preformed biofilms (48 h) of MDR-EAEC was assessed by CV staining and also by live/dead staining. The preformed, washed and air-dried biofilms of MDR-EAEC strains in 96-well microtitre plates

were supplemented with 200 μL of fresh DMEM having 1× MBEC of either individual AMPs and/or its combinations. The seeded plates were then incubated at 37 °C for 24 h and later subjected to CV staining. To assess the eradication of preformed biofilm, biofilms (48 h) produced on circular polystyrene coverslips in 12-well format as described earlier were exposed to 1× MBEC of either individual AMPs and/or its combinations prepared in DMEM medium containing 0.45% D-glucose for 24 h at 37 °C. After incubation, the treated preformed biofilms were then subjected to live/dead staining.

### Quantification of Live/Dead Cells

The confocal images were subjected to quantification of live and dead cell analysis using Fiji ImageJ software ver. 1.51s [26]. The varying proportions of green (live bacteria) as well as red and yellow (dead bacteria) bio-volumes from the analysed stacks deducting the background score were plotted in intensity histogram and interpreted as function of red/green intensity.

### Statistical Analysis

All the experiments were repeated individually and independently thrice and the data obtained is reflected as mean ± standard deviation for each assay. The GraphPad Prism 5.01 software (GraphPad Software Inc., San Diego, CA) was used for statistical analysis. A one-way analysis of variance with the Bonferroni multiple comparison test was used to analyse the data.

## Results

The PCR profile of the EAEC isolates are given as Supplementary Table 2. All the three typical MDR-EAEC pathotype isolates were resistant to four or more classes of antibiotics. Moreover, all the three MDR-EAEC isolates were ESBL producers by a double disc test. The antibiotic susceptibility results of the isolates are summarized in Supplementary Table 3.

### Biofilm Formation of MDR-EAEC Isolates

#### Congo Red Binding Assay

MDR-2 and MDR-3 exhibited black streaks and colonies with dry crystalline consistency, suggestive of moderate to strong biofilm-forming strains, while MDR-1 produced pink or red colonies that is indicative of weak biofilm former (Supplementary Fig. 1).

#### Microbial Adhesion to Solvents

A solvent-dependent microbial adhesion was observed for all the three MDR-EAEC strains (Supplementary Fig. 2). All the three strains displayed maximal affinity for the acidic solvent (chloroform) and a low affinity for the basic solvent (diethyl ether) despite having similar van der Waal's properties (Supplementary Fig. 2). With apolar solvent (n-hexadecane), a moderate affinity (ca. 40%) was observed. Overall, the results suggested that all the three MDR-EAEC strains were electron-donating (basic) and weakly electron-accepting (acidic).

#### Time-Dependent Biofilm-Forming Ability of MDR-EAEC in Different Media

A highly significant ($P < 0.001$) biofilm-forming ability was observed among all the three MDR-EAEC isolates and positive biofilm control (E. coli ATCC 25922) in DMEM containing 0.45% D-glucose when compared with negative biofilm control (E. coli DH5α). Moreover, the results observed for biofilm-forming ability in all the other media (plain DMEM,

**Fig. 1** Biofilm biomass of MDR-EAEC test isolates. The results of biofilm-forming assay expressed as numericals of optical density values at 595 nm with respect to the control. Error bars indicate the standard deviation observed between three independent experiments
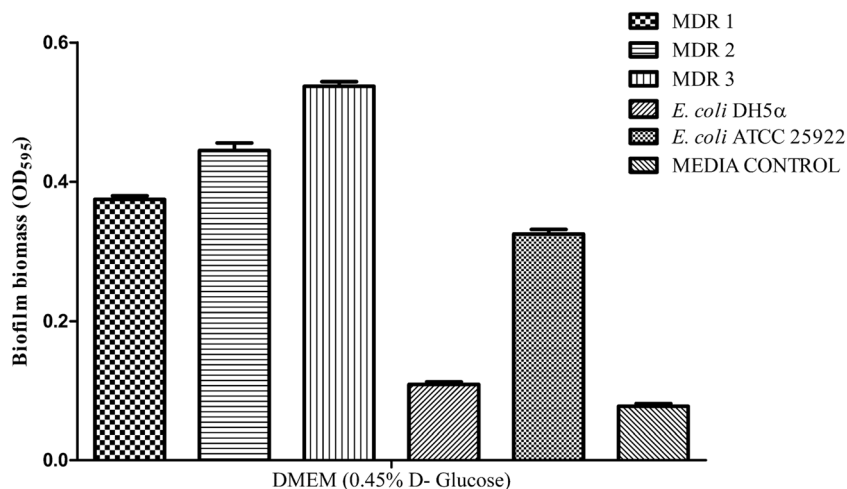
**Table 1** In vitro antibacterial and anti-biofilm activity of AMPs against MDR-EAEC isolates

| NCBI GenBank Accession No. | Indolicidin | | | Cecropin A (1-7)-melittin | | | FICI |
|---|---|---|---|---|---|---|---|
| | MIC (mg/L) | MBC (mg/L) | MBEC (mg/L) | MIC (mg/L) | MBC (mg/L) | MBEC (mg/L) | |
| KY941936.1 (MDR 1) | 32.0 | 64.0 | 64.0 | 4.0 | 4.0 | 16.0 | 1.0 |
| KY941937.1 (MDR 2) | 32.0 | 64.0 | 64.0 | 2.0 | 4.0 | 16.0 | 1.0 |
| KY941938.1 (MDR 3) | 32.0 | 32.0 | 128.0 | 2.0 | 2.0 | 16.0 | 1.0 |

DMEM containing 0.45% D-mannose; nutrient broth; nutrient broth containing 0.45% D-glucose and nutrient broth containing 0.45% D-mannose) were not significantly comparable. Further, all the three MDR-EAEC isolates exhibited a highly significant ($P < 0.001$) biofilm-forming ability at 48 h of incubation than at 24 h and 72 h. Hence, DMEM containing 0.45% D-glucose was considered as an optimal medium for biofilm formation on polystyrene surface at 48 h of incubation (Supplementary Fig. 3a, b). Further, on employing the classification criteria [19], all the three MDR-EAEC strains were moderate biofilm producers, as evidenced from $OD_{595}$ values ranging from 0.375 to 0.537 (Fig. 1).

## Determination of MIC, MBC and FICI of AMPs

The MBC values for both the AMPs were either equal or twofold greater than the MIC values (Table 1). However, higher MIC and MBC values were observed for indolicidin than CAMA (Table 1) against MDR-EAEC strains. The MIC values for the antibiotics (ampicillin, ceftazidime and ciprofloxacin) used against *E. coli* ATCC 25922 were within the prescribed CLSI range (data not shown).

When indolicidin and CAMA were used together in combination against MDR-EAEC strains, an FICI value equal to 1.0 was obtained.

**Fig. 2** Inhibition of MDR-EAEC biofilm by AMPs alone and combination at **a** 24 h and **b** 48 h by CV staining. MDR-EAEC biofilm inhibition at **a** 24 h and **b** 48 h expressed as numericals of optical density of biofilm formed with respect to control; error bars indicate the standard deviation between strains obtained in three independent experiments. Biofilm and negative control bars indicate corresponding MDR-EAEC and DH5α biofilms, respectively, without any AMP. AMP-1 and AMP-2 indicate indolicidin and CAMA, respectively (**$P < 0.01$; ***$P < 0.001$)
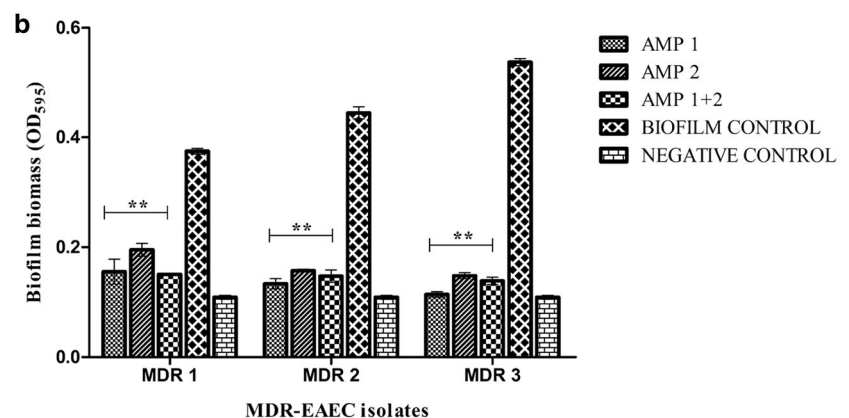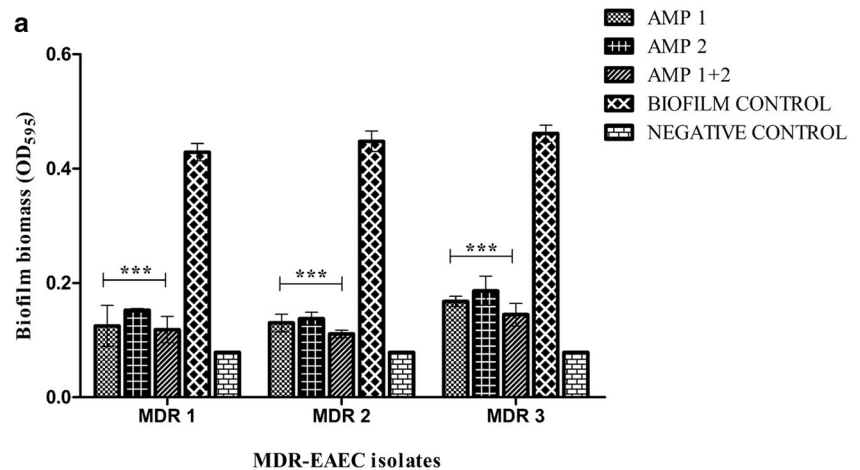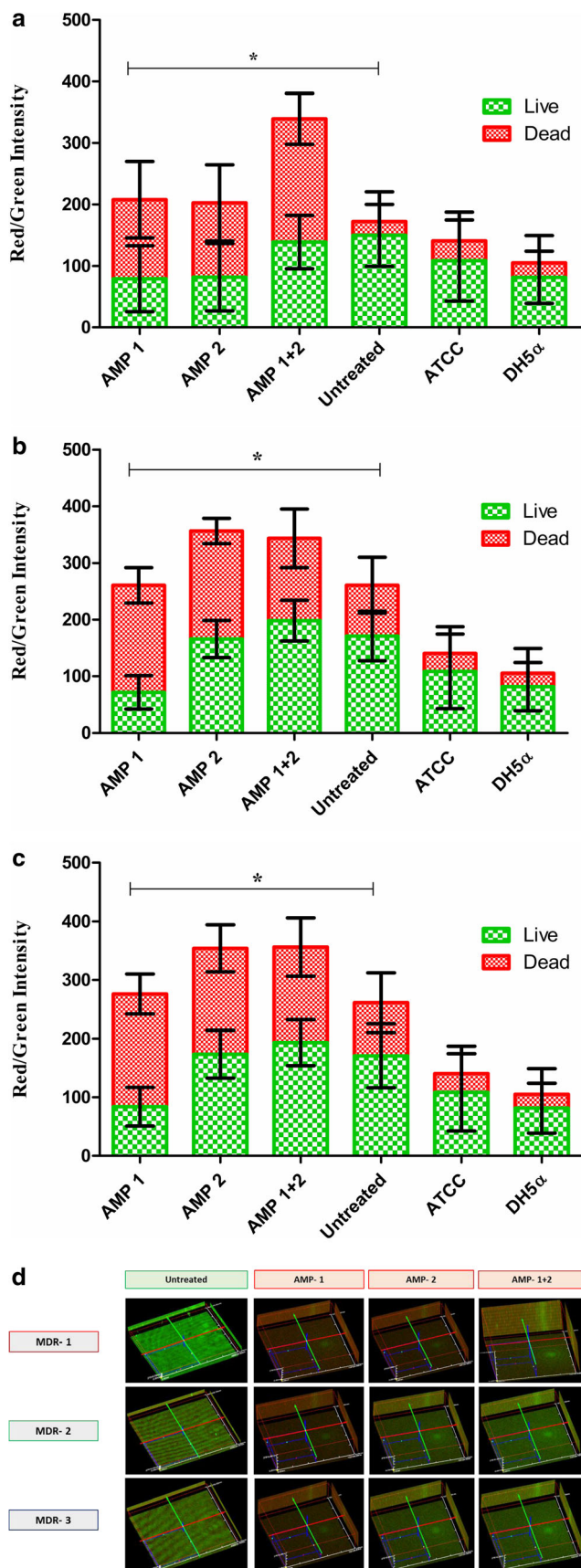
◀ **Fig. 3** Red/green intensity plot of MDR-EAEC biofilm inhibition by AMPs alone and in combination at 24 h. MDR-EAEC biofilm inhibition (**a** MDR-1, **b** MDR-2 and **c** MDR-3) at 24 h expressed as RG intensity of biofilm formed and confocal imaging (**d**) with respect to control; error bars indicate the standard deviation between strains obtained in three independent experiments. Biofilm and negative control bars indicate corresponding untreated MDR-EAEC and DH5α biofilms, respectively, without any AMP. AMP-1 and AMP-2 indicate indolicidin and CAMA, respectively (*$P < 0.05$)

## In Vitro Efficacy of AMPs Against MDR-EAEC Biofilm Formation

### Inhibition of MDR-EAEC Biofilm Formation

In CV staining, a highly significant reduction in the biofilm biomass was observed at 24 h ($P < 0.001$) and 48 h ($P < 0.01$) on treatment with both the AMPs (individual and in combination) as compared to their respective controls (Fig. 2). Similar reduction in biofilm mass formed by MDR-EAEC isolates was observed when evaluated with confocal microscopy, however, initially at 24 h, significant ($P < 0.05$) inhibition was observed (Fig. 3; Supplementary Fig. 4), which after 48 h, resulted in highly significant ($P < 0.01$) inhibition of the biofilm biomass (Fig. 4; Supplementary Fig. 5). Significant differences ($P > 0.05$) were not observed between AMP treatments, i.e. either employed individually or in combinations.

### Minimum Biofilm Eradication Concentration

The MBEC values of indolicidin and CAMA obtained against the MDR-EAEC strains (Table 1) were either equal to or greater than the MBC values. Moreover, the higher MBEC values were observed for indolicidin than CAMA (Table 1) against MDR-EAEC strains.

### Eradication of MDR-EAEC Biofilm Formation by AMPs

A highly significant ($P < 0.001$) reduction in the bacterial biomass was observed on treating the AMPs (individual and in combination at 1× MBEC concentration) on preformed biofilm of MDR-EAEC, as evidenced by both CV staining (Fig. 5) and confocal microscopy (Fig. 6; Supplementary Fig. 6). Hence, it was observed that indolicidin and CAMA eliminated the preformed biofilms of MDR-EAEC. However, significant differences ($P > 0.05$) were not observed between the AMP treatments.

## Discussion

Multidrug resistance developed as a result of the 'antibiotic selection pressure' makes the pathogens impervious to varied
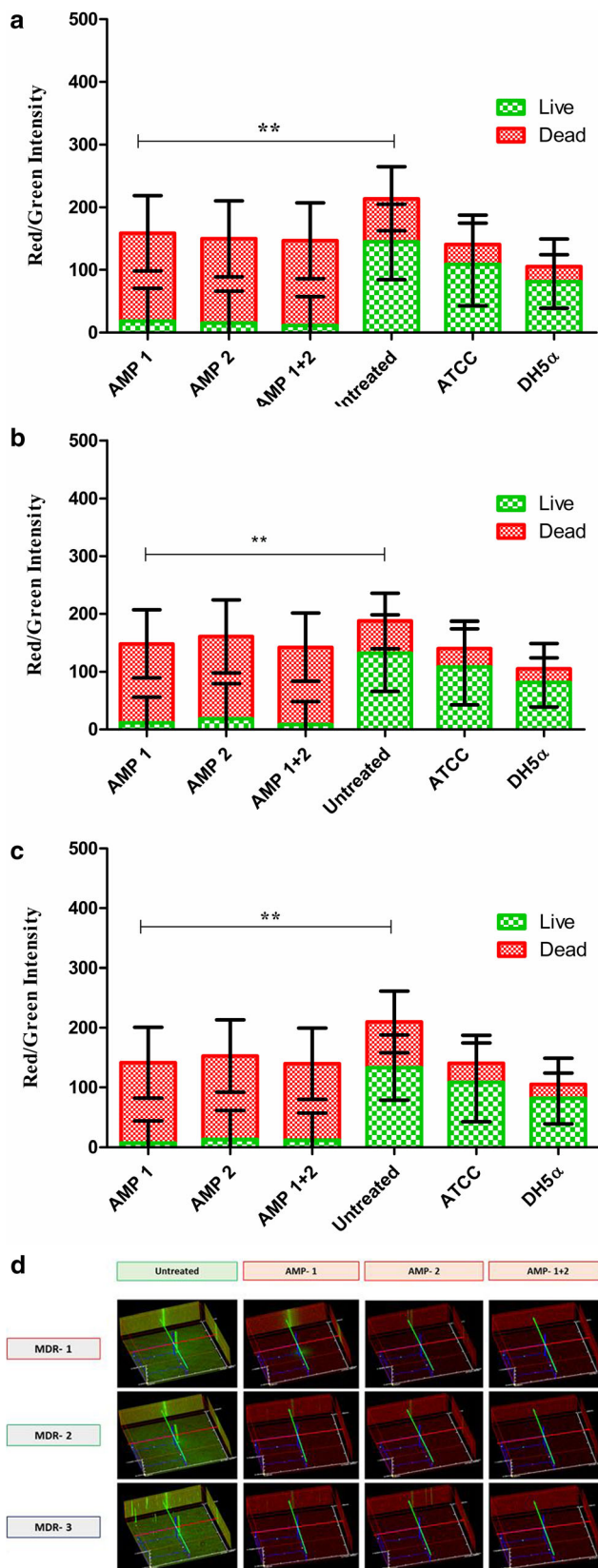
◀ **Fig. 4** Red/green intensity plot of MDR-EAEC biofilm inhibition by AMPs alone and in combination at 48 h. MDR-EAEC biofilm inhibition (**a** MDR-1, **b** MDR-2 and **c** MDR-3) at 48 h expressed as RG intensity of biofilm formed and confocal imaging (**d**) with respect to control; error bars indicate the standard deviation between strains obtained in three independent experiments. Biofilm and negative control bars indicate corresponding untreated MDR-EAEC and DH5α biofilms, respectively, without any AMP. AMP-1 and AMP-2 indicate indolicidin and CAMA, respectively ($**P < 0.01$)

antibiotics of first-line empirical therapy (fluoroquinolones and beta-lactams) have been evident globally among the EAEC isolates [1, 3]. In India, majority of the MDR-EAEC strains have been recovered from the diarrhoeic children less than 5 years of age [28, 29].

It has been estimated that up to 80% of microbial infections in the living system involve biofilm formation, greatly contributing to morbidity and mortality [8]. Biofilms are encased in a protective extracellular matrix containing water, polysaccharides, proteins, extracellular DNA and lipids [5] and thereby creating difficulty in therapeutic attainment of concentrations of antimicrobial agents [5]. Biofilm-forming ability of the MDR-EAEC strains in this study was examined using Congo red binding assay. Congo red, an amyloid dye, stains the exopolysaacharide and is considered to be a qualitative indication of curli production of Gram-negative bacilli. Curli and other amyloid fibres play significant role in modulating the viscoelastic properties of bacterial biofilms [30, 31], hence extensively used to score the production of polysaccharides and other extracellular factors responsible for biofilm formation.

The capacity of bacterial cell surfaces to adhere onto various clinical, food and industrial surfaces were estimated by microbial adhesion to solvents (MATS) test, a measure of hydrophobicity index [32, 33]. The maximal affinity of MDR-EAEC strains to acidic solvent, chloroform, as compared to the basic solvent diethyl ether clearly emphasizes the hydrophobic nature of cell surface. Moreover, the adhesion of the EAEC strains was higher to monopolar solvent (chloroform) than to the apolar solvent (n-hexadecane) with similar van der Waal's properties which could be suggestive of the fact that the strains are of strongly electron-donating (basic) in nature and weakly electron-accepting (acidic), as reported earlier [21, 32, 33]. The microbial affinity to the surfaces would be a result of interplay of electrostatic, van der Waal's and Lewis acid–base interactions as the microbes adhere to the surfaces.

In the present study, the bacterial cells were observed to bind at a significantly higher degree to the hydrophobic surfaces (SS 304 and PS) than the hydrophilic glass surface. We used tissue coated PS microtitre plates to provide optimum cell attachment. Moreover, PS plates have more hydrophobic and rougher surfaces for enhanced attachment and which eventually leads to maximum biofilm formation by the

classes of antibiotics specifically recommended for their empirical therapy [27]. Of late, multidrug resistance towards the
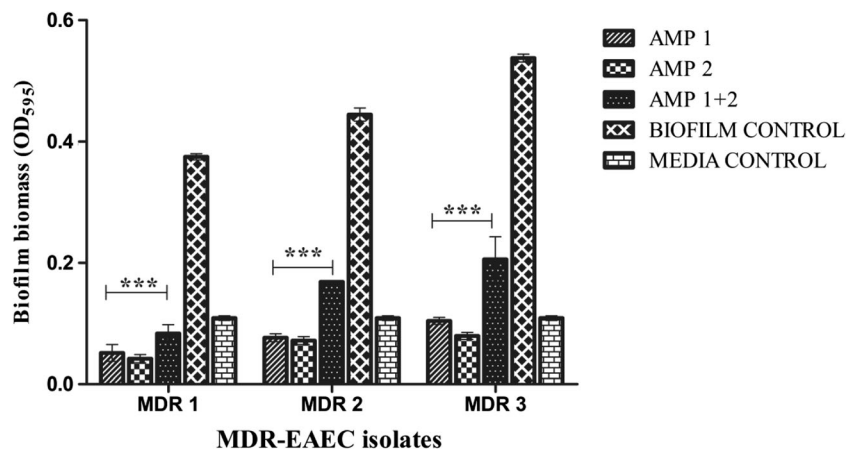
**Fig. 5** Effect of treating MDR-EAEC preformed biofilms (48 h) with AMPs alone and in combination for an additional 24 h by CV staining. Eradication of MDR-EAEC preformed biofilm expressed as numericals of optical density of biofilm formed with respect to control; error bars indicate the standard deviation between strains obtained in three independent experiments. Biofilm and negative control bars indicate corresponding MDR-EAEC and DH5α biofilms, respectively, without any AMP. AMP-1 and AMP-2 indicate indolicidin and CAMA, respectively ($***P < 0.001$)

pathogen [34]. Bacterial surface appendages (proteins and other cell organelles) with adhesive properties as well as factors contributing to the charge of the cell surface depends on the growth media and mostly on the adherence properties of the EAEC strains [34]. Moreover, as reported in an earlier study [35], the presence of 0.45% D-glucose was observed to be a strong signal for biofilm formation of EAEC and thereby, promoting the formation of biofilm significantly among EAEC strains.Taken together, of the different surfaces, growth media and time employing three MDR-EAEC strains studied here, a significant hydrodynamic growth of MDR-EAEC strains was observed on PS surface in DMEM containing 0.45% D-glucose at 48 h.

Characteristically, EAEC strains enhance mucous secretion from the mucosa, with trapping of the bacteria in a bacterium-mucus biofilm [1]. This mucus-containing biofilm may explain why the individuals infected with EAEC develop mucoid stools, malnutrition and persistent colonization with prolonged diarrhoea [1, 2]. Earlier studies reported grading pattern for EAEC biofilm [16, 22], wherein EAEC strains were graded as weak (OD595 < 0.323), moderate (OD595 = 0.324–0.648) or strong biofilm formers (OD595 > 0.648). All the three MDR-EAEC strains employed in this study were found to be moderate biofilm producers, as observed earlier [16].

AMPs exhibit direct antimicrobial activity and carry immunomodulatory properties [36] and are interesting compounds for the development of novel therapeutics with anti-biofilm activity [9]. Several recent studies have explored the effect of AMPs and are discovered from all kingdoms of life such as bacteria, fungi and protozoa [10]. There were 221 AMPs documented in the regularly updated biofilm active AMPs (BaAMPs) [19]. In the present study, we investigated the anti-biofilm activity of two short-chain cationic peptides with high hydrophobicity residues, indolicidin and CAMA on biofilm-forming MDR-EAEC strains. Earlier studies have proven that short-chain (12–50 amino acid) peptides with cationic amino acids and high proportion of hydrophobic residues (~ 50%) were effective against biofilm-forming bacteria [13, 37]. Indolicidin has been isolated from the cytoplasmic granules of bovine neutrophils [38]. In Gram-negative bacteria, the antimicrobial activity of indolicidin is primarily due to the disruption of cytoplasmic membrane by channel formation and particularly in *E. coli*, it inhibits DNA replication leading to bacterial filamentation [38]. CAMA, a cecropin-melittin hybrid peptide with 15 amino acid residues, is composed of the cationic region of 'cecropin A' and the hydrophobic as well as non-haemolytic region of bee venom peptide 'melittin' and acts mainly by way of membrane disruption due to the formation of 'toroidal' pores and/or detergent-like mechanism [15].

We evaluated the anti-biofilm efficacy of indolicidin and CAMA by focussing on preventing biofilm formation, dispersing existing ones, reducing biomass and/or killing microbial cells within biofilms [9]. We also explored the combinatorial use of AMPs, which could result in a rapid increase of anti-biofilm activities [39, 40]. The combination of indolicidin and CAMA exhibited an additive interaction with the FICI value equal to 1, against the biofilm-forming MDR-EAEC isolates. This additive combination of AMPs would be potential for the treatment of biofilm-related infections. Earlier researchers also suggested similar additive or synergistic interactions while using other AMPs in combinations against various biofilm-forming pathogens especially, *Staphylococcus aureus* and *Pseudomonas aeruginosa* [24, 41, 42].

While assessing the biofilm biomass, CV stains surface negatively charged molecules and EPS matrix; however, it fails to differentiate viable or dead cells or extracellular matrix [6] and provides a semi-quantitative biofilm biomass determination assay. We observed clusters of dead (red) and live
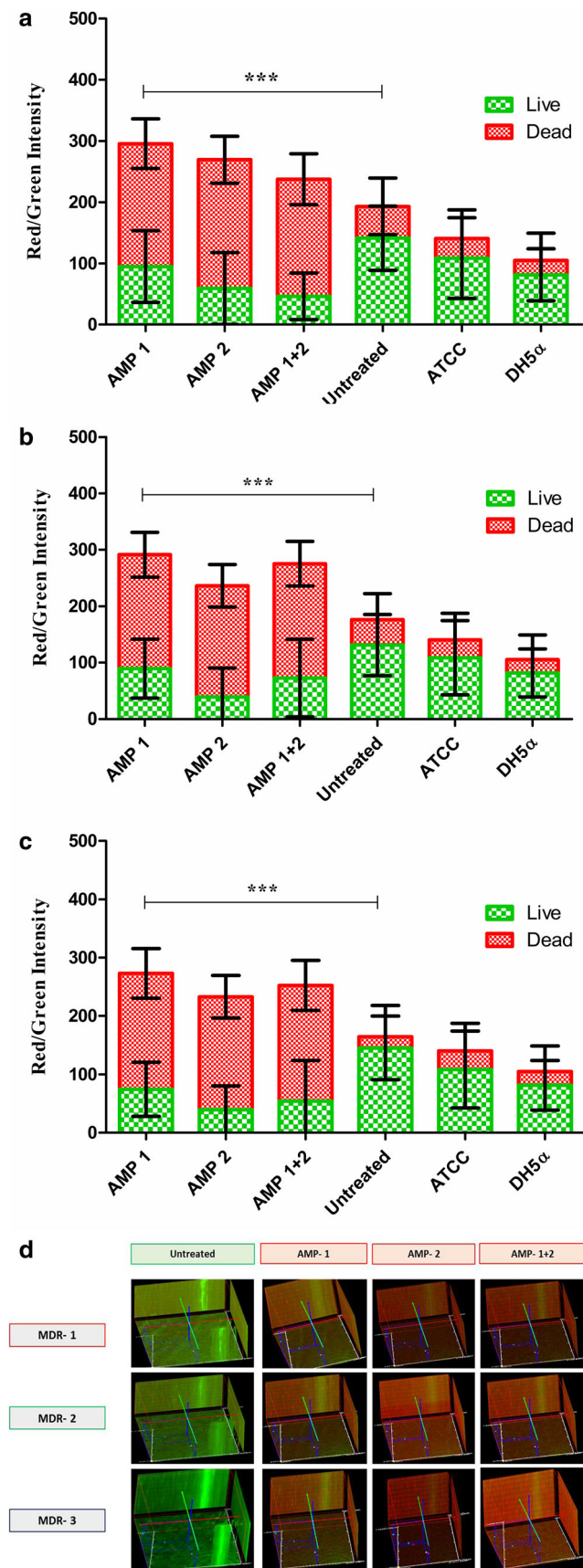
◀ **Fig. 6** Red/green intensity plot of MDR-EAEC preformed biofilms (48 h) treated with AMPs alone and in combination for an additional 24 h. Eradication of MDR-EAEC (**a** MDR-1, **b** MDR-2 and **c** MDR-3) preformed biofilm expressed as RG intensity of biofilm formed and confocal imaging (**d**) with respect to control; error bars indicate the standard deviation between strains obtained in three independent experiments. Biofilm and negative control bars indicate corresponding untreated MDR-EAEC and DH5α biofilms, respectively, without any AMP. AMP-1 and AMP-2 indicate indolicidin and CAMA, respectively ($***P < 0.001$)

(green) bacteria and also a sub-population of bacteria with compromised cell membrane (yellow) by confocal microscopy in the biofilms treated with indolicidin and CAMA. Earlier studies reported the usage of double staining method to distinguish bacterial cells with compromised membrane integrity [42] and determined biofilm biomass quantitatively.

A significant inhibition of the initial biofilm formation by the AMPs at 24 h as compared to the control was observed by CV staining and live/dead cell quantification. Furthermore, the CV staining and confocal microscopy performed at 48 h demonstrated that the interaction of peptides (at 1× MIC concentration) inhibited the initial biofilm biomass formation. It could hence be well established that for a short duration of time, AMPs act as coating agents covering either biomaterial surfaces, bacterial surfaces or both [43, 44].

We evaluated the anti-biofilm potential of the individual compounds and their combination on matured biofilm established in 48 h using MBEC concentration. MBEC concentrations were found to be equal to or greater than MBC values which might be due to the moderate biofilm-forming pattern of MDR-EAEC strains. Earlier workers demonstrated varying ranges of MBEC concentration, which could either be equal to MIC levels or even several fold higher [40, 45]. In the present study, a 24-h treatment with the individual AMPs and their combination resulted in significant eradication of the preformed biofilms of MDR-EAEC at MBEC concentrations. The eradication of mature biofilms could be achieved either through the direct killing of embedded bacteria or by detachment of live bacteria from biofilms by non-bactericidal peptides [39, 40]. To the best of our knowledge, the release of live bacteria from biofilms by AMPs had not been reported. Interestingly, the inhibition as well as eradication levels of biofilm formation using individual AMPs or their combination were not significantly different, probably due to the differences in the mechanism of action of indolicidin and CAMA.

## Conclusions

In conclusion, we evaluated for the first time the anti-biofilm potential of two short-chain AMPs and their combination against three biofilm-producing MDR-EAEC isolates.

Interestingly, the peptides could inhibit the initial biofilm formation and also eliminate the preformed biofilm of MDR-EAEC strains as evidenced by CV staining and quantification by confocal microscopy. Moreover, the inhibition as well as eradication levels of biofilm formation using individual AMPs or their combinations were not significantly different. Based on the in vitro assays, both these AMPs could be suggested as effective therapeutic candidates for future investigations. Further, cytotoxicity as well as stability assays of the AMPs could be performed before their trials in suitable in vivo models against biofilm-forming MDR-EAEC strains.

## Compliance with Ethical Standards

**Conflict of Interest** The authors declare that they have no conflict of interest.

## References

1. Jensen BH, Olsen KE, Struve C, Krogfelt KA, Petersen AM (2014) Epidemiology and clinical manifestations of enteroaggregative *Escherichia coli*. Clin Microbiol Rev 27:614–630. https://doi.org/10.1128/CMR.00112-13

2. Rogawski ET, Guerrant RL, Havt A, Lima IF, Medeiros PH, Seidman JC et al (2017) Epidemiology of enteroaggregative *Escherichia coli* infections and associated outcomes in the MAL-ED birth cohort. PLoS Neglected Trop Dis 11:e0005798. https://doi.org/10.1371/journal.pntd.0005798

3. Ali MM, Ahmed SF, Klena JD, Mohamed ZK, Moussa TA, Ghenghesh KS (2014) Enteroaggregative *Escherichia coli* in diarrheic children in Egypt: molecular characterization and antimicrobial susceptibility. J Infect Develop Countr 8:589–596. https://doi.org/10.3855/jidc.4077

4. Kong H, Hong X, Li X (2015) Current perspectives in pathogenesis and antimicrobial resistance of enteroaggregative *Escherichia coli*. Microb Pathog 85:44–49. https://doi.org/10.1016/j.micpath.2015.06.002

5. Hall CW, Mah TF (2017) Molecular mechanisms of biofilm-based antibiotic resistance and tolerance in pathogenic bacteria. FEMS Microbiol Rev 41:276–301. https://doi.org/10.1093/femsre/fux010

6. Lin S, Yang L, Chen G, Li B, Chen D, Li L, Xu Z (2017) Pathogenic features and characteristics of food borne pathogens biofilm: biomass, viability and matrix. Microb Pathog 111:285–291. https://doi.org/10.1016/j.micpath.2017.08.005

7. Lebeaux D, Ghigo JM, Beloin C (2014) Biofilm-related infections: bridging the gap between clinical management and fundamental aspects of recalcitrance toward antibiotics. Microbiol Mol Biol Rev 78(3):510–543. https://doi.org/10.1128/MMBR.00013-14

8. Batoni G, Maisetta G, Esin S (2016) Antimicrobial peptides and their interaction with biofilms of medically relevant bacteria. Biochim Biophys Acta Biomembr 1858:1044–1060. https://doi.org/10.1016/j.bbamem.2015.10.013

9. Ribeiro SM, Felicio MR, Boas EV, Goncalves S, Costa FF, Samy RP et al (2016) New frontiers for anti-biofilm drug development. Pharmacol Therapeutics 160:133–144. https://doi.org/10.1016/j.pharmthera.2016.02.006

10. Yazici A, Ortucu S, Taskin M, Marinelli L (2018) Natural-based antibiofilm and antimicrobial peptides from microorganisms. Curr Top Med Chem doi 18:2102–2107. https://doi.org/10.2174/1568026618666181112143351

11. Kumar P, Kizhakkedathu J, Straus S (2018) Antimicrobial peptides: diversity, mechanism of action and strategies to improve the activity and biocompatibility *in vivo*. Biomolecules 8:4. https://doi.org/10.3390/biom8010004

12. Zasloff M (2002) Antimicrobial peptides of multicellular organisms. Nature. 415:389–395. https://doi.org/10.1038/415389a

13. Anunthawan T, de la Fuente-Núñez C, Hancock RE, Klaynongsruang S (2015) Cationic amphipathic peptides KT2 and RT2 are taken up into bacterial cells and kill planktonic and biofilm bacteria. Biochim Biophys Acta Biomembr 1848:1352–1358. https://doi.org/10.1016/j.bbamem.2015.02.021

14. De Zoysa GH, Cameron AJ, Hegde VV, Raghothama S, Sarojini V (2015) Antimicrobial peptides with potential for biofilm eradication: synthesis and structure activity relationship studies of battacin peptides. J Med Chem 58:625–639. https://doi.org/10.1021/jm501084q

15. Silva T, Claro B, Silva BF, Vale N, Gomes P, Gomes MS et al (2018) Unravelling a mechanism of action for a cecropin A-melittin hybrid antimicrobial peptide: the induced formation of multilamellar lipid stacks. Langmuir 34(5):2158–2170. https://doi.org/10.1021/acs.langmuir.7b03639

16. Vijay D, Dhaka P, Vergis J, Negi M, Mohan V, Kumar M, Doijad S, Poharkar K, Kumar A, Malik SS, Barbuddhe SB, Rawool DB (2015) Characterization and biofilm forming ability of diarrhoeagenic enteroaggregative *Escherichia coli* isolates recovered from human infants and young animals. Comp Immunol Microbiol Infect Dis 38:21–31. https://doi.org/10.1016/j.cimid.2014.11.004

17. Cravioto A, Gross RJ, Scotland SM, Rowe B (1979) An adhesive factor found in strains of *Escherichia coli* belonging to the traditional infantile enteropathogenic serotypes. Curr Microbiol 3:95–99 https://link.springer.com/article/10.1007/BF02602439

18. Wayne PA (2018) Performance standards for antimicrobial susceptibility testing, 28th edn. Supplement M100. Clinical and Laboratory Standards Institute, USA

19. Di Luca M, Maccari G, Maisetta G, Batoni G (2015) BaAMPs: the database of biofilm-active antimicrobial peptides. Biofouling 31:193–199. https://doi.org/10.1080/08927014.2015.1021340

20. Freeman DJ, Falkiner FR, Keane CT (1989) New method for detecting slime production by coagulase negative staphylococci. J Clin Pathol 42:872–874. https://doi.org/10.1136/jcp.42.8.872

21. Bellon-Fontaine MN, Rault J, Van Oss CJ (1996) Microbial adhesion to solvents: a novel method to determine the electron-donor/electron-acceptor or Lewis acid-base properties of microbial cells. Colloids Surf B: Biointerfaces 7:47–53. https://doi.org/10.1016/0927-7765(96)01272-6

22. Wakimoto N, Nishi J, Sheikh J, Nataro JP, Sarantuya JA, Iwashita M et al (2004) Quantitative biofilm assay using a microtiter plate to screen for enteroaggregative *Escherichia coli*. Am J Trop Med Hyg 71:687–690. https://doi.org/10.4269/ajtmh.2004.71.687

23. Wayne PA (1999) Methods for determining bactericidal activity of antimicrobial agents: approved guideline. M26-A. USA: National Committee for Clinical Laboratory Standards

24. Jorge P, Grzywacz D, Kamysz W, Lourenço A, Pereira MO (2017) Searching for new strategies against biofilm infections: colistin-AMP combinations against *Pseudomonas aeruginosa* and *Staphylococcus aureus* single-and double-species biofilms. PLoS One 12:e0174654. https://doi.org/10.1371/journal.pone.0174654

25. Ceri H, Olson ME, Stremick C, Read RR, Morck D, Buret A (1999) The Calgary Biofilm Device: new technology for rapid determination of antibiotic susceptibilities of bacterial biofilms. J Clin Microbiol 37:1771–1776

26. Schindelin J, Arganda-Carreras I, Frise E, Kaynig V, Longair M, Pietzsch T, Preibisch S, Rueden C, Saalfeld S, Schmid B, Tinevez JY, White DJ, Hartenstein V, Eliceiri K, Tomancak P, Cardona A (2012) Fiji: an open-source platform for biological-image analysis. Nat Methods 9:676–682. https://doi.org/10.1038/nmeth.2019

27. Davies J, Davies D (2010) Origins and evolution of antibiotic resistance. Microbiol Mol Biol Rev 74(3):417–433. https://doi.org/10.1128/MMBR.00016-10

28. Ballal M, Devadas SM, Chakraborty R, Shetty V (2014) Emerging trends in the etiology and antimicrobial susceptibility pattern of enteric pathogens in rural coastal India. Int J Clin Med 5:425–432. https://doi.org/10.4236/ijcm.2014.57058

29. Dhaka P, Vijay D, Vergis J, Negi M, Kumar M, Mohan V, Doijad S, Poharkar KV, Malik SS, Barbuddhe SB, Rawool DB (2016) Genetic diversity and antibiogram profile of diarrhoeagenic *Escherichia coli* pathotypes isolated from human, animal, foods and associated environmental sources. Infect Ecol Epidemiol 6: 31055. https://doi.org/10.3402/iee.v6.31055

30. Cegelski L, Pinkner JS, Hammer ND, Cusumano CK, Hung CS, Chorell E, Åberg V, Walker JN, Seed PC, Almqvist F, Chapman MR, Hultgren SJ (2009) Small-molecule inhibitors target *Escherichia coli* amyloid biogenesis and biofilm formation. Nat Chem Biol 5(12):913–919. https://doi.org/10.1038/nchembio.242

31. Reichhardt C, Jacobson AN, Maher MC, Uang J, McCrate OA, Eckart M et al (2015) Congo red interactions with curli-producing *E. coli* and native curli amyloid fibers. PLoS One 10(10):e0140388. https://doi.org/10.1371/journal.pone.0140388

32. Doijad SP, Barbuddhe SB, Garg S, Poharkar KV, Kalorey DR, Kurkure NV, Rawool DB, Chakraborty T (2015) Biofilm-forming abilities of *Listeria monocytogenes* serotypes isolated from different sources. PLoS One 10:e0137046. https://doi.org/10.1371/journal.pone.0137046

33. Perpetuini G, Tittarelli F, Schirone M, Di Gianvito P, Corsetti A, Arfelli G et al (2018) Adhesion properties and surface hydrophobicity of *Pichia manshurica* strains isolated from organic wines. LWT Food Sci Technol 87:385–392. https://doi.org/10.1016/j.lwt.2017.09.011

34. Donlan RM, Costerton JW (2002) Biofilms: survival mechanisms of clinically relevant microorganisms. Clin Microbiol Rev 15:167–193. https://doi.org/10.1128/CMR.15.2.167-193.2002

35. Sheikh J, Hicks S, Dall'Agnol M, Phillips AD, Nataro JP (2001) Roles for *Fis* and *Yaf*K in biofilm formation by enteroaggregative *Escherichia coli*. Mol Microbiol 41:983–997. https://doi.org/10.1046/j.1365-2958.2001.02512.x

36. Fjell CD, Hiss JA, Hancock RE, Schneider G (2012) Designing antimicrobial peptides: form follows function. Nat Rev Drug Discov 11:37–51. https://doi.org/10.1038/nrd3591

37. de la Fuente-Núñez C, Reffuveille F, Mansour SC, Reckseidler-Zenteno SL, Hernández D, Brackman G, Coenye T, Hancock REW (2015) D-enantiomeric peptides that eradicate wild-type and multidrug-resistant biofilms and protect against lethal *Pseudomonas aeruginosa* infections. Chem Biol 22:196–205. https://doi.org/10.1016/j.chembiol.2015.01.002

38. Falla TJ, Karunaratne DN, Hancock RE (1996) Mode of action of the antimicrobial peptide indolicidin. J Biol Chem 271:19298–19303. https://doi.org/10.1074/jbc.271.32.19298

39. Segev-Zarko LA, Saar-Dover R, Brumfeld V, Mangoni ML, Shai Y (2015) Mechanisms of biofilm inhibition and degradation by antimicrobial peptides. Biochem J BJ20141251 468:259–270. https://doi.org/10.1042/BJ20141251

40. Mataraci E, Dosler S (2012) *In vitro* activities of antibiotics and antimicrobial cationic peptides alone and in combination against methicillin resistance *Staphylococcus aureus* biofilms. Antimicrob Agents Chemother 56:6366–6371. https://doi.org/10.1128/AAC.01180-12

41. Grassi L, Maisetta G, Esin S, Batoni G (2017) Combination strategies to enhance the efficacy of antimicrobial peptides against bacterial biofilms. Front Microbiol 8:2409. https://doi.org/10.3389/fmicb.2017.02409

42. Mishra B, Wang G (2017) Individual and combined effects of engineered peptides and antibiotics on *Pseudomonas aeruginosa* biofilms. Pharmaceuticals 10:58. https://doi.org/10.3390/ph10030058

43. Riool M, de Breij A, Drijfhout JW, Nibbering PH, Zaat SA (2017) Antimicrobial peptides in biomedical device manufacturing. Front Chem 5:63. https://doi.org/10.3389/fchem.2017.00063

44. Mas-Moruno C, Su B, Dalby MJ (2019) Multifunctional coatings and nanotopographies: toward cell instructive and antibacterial implants. Adv Healthcare Mat 8(1):1801103. https://doi.org/10.1002/adhm.201801103

45. Liu J, Ling JQ, Zhang K, Wu CD (2013) Physiological properties of *Streptococcus mutans* UA159 biofilm-detached cells. FEMS Microbiol Lett 340:11–18. https://doi.org/10.1111/1574-6968.12066

Ⓐ Springer