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Synthetic peptides SG-15 shows synergistic effect with some antibiotics against *Escherichia coli* isolates from Mastitic cow milk

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Abstract
Antibiotics have been used in large amount for treating mild to severe microbial infection since their introduction in early 20th century but in present scenario a worrying and serious condition is the emergence of multidrug resistant strains of most common infectious microbes due to huge and unmonitored use of antibiotics. There is an urgent need to discover some therapeutic agents that can increase the potency of commonly used antibiotics. The present study aimed to designing and *in vitro* testing of synthetic peptides on the basis of protein sequence and structure of AcrAB-TolC, is a commonly expressed efflux pump of *Escherichia coli* (*E. coli*). SG-15 was designed as inner membrane protein (AcrB) blocker. A 10 *E. coli* isolates were obtained from 28 clinical mastic milk samples. Proteomic based characterization of isolates performed using VITEK MS RUO (Research Use Only) and genotypic characterization was done using PCR. After proteomic and genotypic confirmation of isolates antibiotic susceptibility testing was done as per the disc diffusion method and efflux activity was estimated by cartwheel method. More than half of the isolates exhibited resistance to multiple antibiotics and all the isolates showed varying degree of efflux activity. After that activity of synthetic peptide SG-15 tested alone and also in combination with antibiotics namely gentamicin, ampicillin, and cephalothin from which some combination exhibited significant synergistic effect.

Keywords: *Escherichia coli*, efflux activity, SG-15, synergistic effect

Introduction
Antimicrobial resistance in bacterial pathogen is a global health threat and need efforts to improve this worldwide challenge associated with high morbidity and mortality (Velez *et al.*, 2016) [28]. Resistance of important bacterial pathogens to common antimicrobial therapies and emergence of multidrug-resistant bacteria are increasing at an alarming rate (Akova, 2016) [1]. The declining effectiveness of antibiotics imposes potentially huge health and economic burdens on societies and antibiotic resistance is the next great global challenge and significant action to combat it is required.
The use of antibiotics in food animals play a vital role in human health, as antibiotic-resistant bacteria can be transmitted between humans and animals through contact, food products and also from the environment (Landers *et al.*, 2012) [15]. New Delhi Metallo β-Lactamase-1 (NDM-1) and Expanded Spectrum β-Lactamases (ESBL) producing gram-negative bacteria (Ghatak *et al.*, 2013) [13] have been isolated from mastitis milk samples of cattle (Eisenberger *et al.*, 2018) [11]. Vancomycin-resistant *Staphylococcus aureus* (VRSA) from surgical site (Bhattacharyya *et al.*, 2016) [6] and multidrug resistant *E. coli* from the milk sample with clinical mastitis has also been reported (Todorvic *et al.*, 2018) [26].
There are different intrinsic mechanisms for antimicrobial resistance present in bacteria among which expression of efflux pump is one of the commonest mechanisms in which extrusion out of the applied antibiotic from bacterial cell wall happens (Webber and Piddock, 2003) [29], other intrinsic mechanisms of resistance are genetic mutations and horizontal transfer of drug resistance genes (Schmieder and Edwards, 2012) [23].
E. coli is a common universal inhabitant of gastrointestinal tract and can act as a commensal or a pathogen, being commensal bacteria, it is always exposed to antibiotic stress when an individual is treated with an antibiotic such exposures also increase the chances of *E. coli* to become multidrug resistant (MDR). Most *E. coli* strains are harmless, but some pathogenic strains (enter pathogenic *E. coli*) can cause bloody diarrhea, gastroenteritis, dysentery and

urinary tract infections in humans and animals (Levine, 1987) [16]. The occurrence of multidrug resistant bacteria is a serious problem in the treatment of bacterial infections (Bolhuis *et al.*, 1997) [7]. Increased use of antibiotics has also raised their involvement in transfer of resistance genes to gut bacteria (Van-den, *et al.*, 2001) [27]. Therefore, it is important to study resistance profiles of *E. coli*. The prevalence of resistance in commensal *E. coli* is an indicator for the selective pressure by antibiotics used and resistance to be expected in commensal bacteria.

These efflux pumps are proteinaceous transporters exist in prokaryotic as well as eukaryotic cells for performing various physiological functions. Efflux pumps of efflux super families such as MFS, MATE, SMR and RND are localized in the cytoplasmic membrane and derive energy for extruding of various substrates by the proton motive forces. Among the efflux pumps, only ABC transporters derive energy by ATP hydrolysis (Sun, *et al*., 2014) [25]. Several drugs have been tried to inhibit the mechanism of such pumps including Phenylalanyl Arginyl β–naphthylamide (PAβN), globomycin. In the present era of AMR antimicrobial peptides (AMP) have fetched much attention by researchers to combat the AMR. Peptides for blocking of efflux pumps, can be easily designed, synthesized and modified suitably and has attracted attention of several researchers (Poulsen and Deber, 2012; Lamers, *et al.*, 2013) [20, 14]. The current research aims at screening of newer synthetic peptides for blocking of bacterial efflux pumps.

Materials and methods

Sampling and study area: In the present investigation a total 28 milk samples have been collected from which 10 isolates of *E. coli* were obtained. The samples were collected from the Veterinary Clinical Complex, CVAS, Bikaner. The samples were collected aseptically and placed in sterile container, taking all precautions to avoid contamination. The research has been conducted at the department of veterinary microbiology and biotechnology, RAJUVAS, Bikaner in the year 2018.

Isolation and species level confirmation: The procedure for isolation and identification of bacterial culture was followed as per the standard protocols (Carter, *et al.*, 1994) [8]. For primary cultivation each isolate was streaked on MacConkey agar plates in primary, secondary, and tertiary fashion in order to obtain isolated colonies of bacteria. After the revival of organism isolated colonies were further streaked on to Eosin Methylene Blue (EMB) agar (Edward and Ewing 1986) [12].Proteomic based microbial identification done with VITEK MS RUO (Research Use Only).

Antibiogram of *E. coli* Isolates from Mastitic Milk: Antibiotic susceptibility testing was done as per the disc diffusion method (Bauer, 1966) [3]. In brief, the isolates were inoculated in sterile 5 ml nutrient broth, incubated for 18 hrs at 37ºC and then the opacity was adjusted to 0.5 McFarland opacity standards with normal saline solution (Quinn, *et al.*, 1994) [21]. Each inoculum was then spread over the surface of Mueller- Hinton agar plates with sterilized swab. Plates were allowed to dry for 10 minutes at room temperature and the antibiotic discs were carefully placed on the surface and plates were incubated for 24hrs at 37ºC and the zone of inhibition around each disc was measured in millimeters. Following the guidelines of Clinical Laboratory Standard Institute (CLSI) 23 antibiotics of different classes have been used. The antibiotics tested were belonging to various group i.e., β-lactam antibiotics, aminoglycosides, polypeptide, phenicoles, quinolones, tetracyclines, sulphonamides, RNA synthesis inhibitors, macrolides and lincosamides. β-lactam antibiotics including pencillins, cephalosporins, carbapenems and monobactams were used.

Designing of synthetic peptides (SG-15)

Basis of designing of peptides was efflux pump protein AcrAB-TolC, of bacterium *E. coli*, a tripartite protein in which TolC is the outer membrane protein and AcrB is the inner membrane transporter whereas AcrA is the periplasmic membrane fusion protein. PeptideSG-15 was taken from 807-821 of AcrB protein with the help of software AbDesigner. Designed peptides were synthesized from BioChem Group Labs. Designing was similar to investigation of Bellmann-Sickert *et al.* 2013 and Poulsen *et al.* 2012 [20].

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

On the basis of obtained antibiogram and CLSI guidelines three antibiotics viz. Ampicillin, Cephalothin and Gentamicin were selected for synergistic study with designed peptides. MIC of three selected antibiotics, designed peptides and CCCP were determined using broth micro dilution method (Wiegand *et al.*, 2008) [30]. The bacterial culture was grown overnight in MHB for 18hrs at 37ºC and then the opacity was adjusted to 0.5 McFarland opacity standards with normal saline solution (Quinn *et al.*, 1994) [21] so that the suspension contained around 10^7 -10^8 CFU/ml. Further diluted culture up to 5×10^5 CFU/ml was used for inoculation in plate. Antibiotic dilutions were made one concentration higher than required. For example, if a final dilution of 100 µg/mL was required then 200 µg/mL concentration of antibiotic was prepared to compensate the equal volume of inoculum. In brief initially 75 µl of Müeller Hinton broth was dispense into all wells of the microtitre plate except positive control wells. To the first well 75 µl of highest concentration of the antibiotic/ antimicrobial peptide to be used was added. Using the multichannel pipette, serial two-fold dilutions were prepared. To each well 75 µl 10^4 to 10^5 cfu/mL of test organism were added. Inoculated and inoculated wells of antibiotic-free broth were included as controls to check the adequacy of the broth to support the growth of the organism and the sterility. Plates were incubated for 16-18 hours at 37ºC. MIC was determined as the lowest concentration of the antibiotic at which there was no visible growth. After determining the MIC of antibiotics against MDR isolates for combination study different concentration of antibiotics in 50µl with different concentration of peptides prepared in 25 µl were used in checkerboard pattern. Stock solution and working solution of Cephalothin and CCCP were prepared with DMSO whereas SG-15, Gentamicin and Ampicillin were dissolved in sterile distilled water.

For determination of MBC 10µl of media from wells where there was no visible growth in the MIC experiment and drop was plated on to Mueller Hinton agar plates and incubated for 16-18 hours at 37ºC. The MBC was read as the lowest Concentration from where no colony formation occurred at plate.

Ethidium bromide-agar (Et-Br) cartwheel method for evaluation of efflux activity: Fluor metric determination of ethidium bromide efflux kinetics in *E. coli* was done with the help of ethidium bromide (Et-br) which is a substrate for

efflux pump. Accumulation and efflux of Et-br can be studied under limiting energy supply (absence of glucose and low temperature) and in the presence and absence of the efflux pump inhibitors. The test was performed as per the method of Martins *et al.*, 2013 [17]. In brief, the bacterial cultures in log phase were swabbed on Tryptone soya agar (TSA) plates containing 5 different concentrations of Et-br (0.5, 1, 1.5, 2 and 2.5mg/L) in wheel pattern and incubated at 37ºC for 16-18 hours and fluorescence was detected under UV light. The effect of Carbonyl Cyanide 3-Chloro Phenylhydrazone (CCCP), a well characterized efflux pump inhibitor was taken as positive control for this experiment. For this CCCP was used at a concentration of about 12.5µM in TSA plates with all 5 concentrations of Et-br and reduction in fluorescence is recorded after incubation.

Result and Discussion

Isolation and identification of *E. coli*: A total 10 isolates of *E. coli* were isolated from 28 milk samples of cattle with clinical mastitis on the basis of cultural characteristics and biochemical tests. *E. coli* isolates revealed characteristic rose pink colonies (lactose fermenting type) on MacConkey agar plates further these pink colonies were streaked on Eosin Methylene blue (EMB) agar on which all ten isolates of *E. coli* produced greenish metallic sheen colonies on EMB agar and after that with help of MALDI-TOF-MS isolates were confirmed as *E. coli* from moderate to extensive probability.

Antibiotic sensitivity assay: Result for antibiogram study was interpreted as sensitive(S), resistant (R), and intermediate (I). Among cell wall synthesis inhibitor class of antibiotics oxacillin, member of penicillin group showed highest resistance (100%) which was similar for the results of Rajala-schultz *et al.*, 2004 [22]. More than half of the isolates exhibited resistance to multiple antibiotics which is quite similar to findings of Nontongana *et al.* 2014 [18].

Table 1: Antibiogram obtained for *E. coli* isolates

Class of antibiotic	Antibiotics	EC- 1	EC-2	EC-3	EC-4	EC-5	EC-6	EC-7	EC-8	EC-9	EC-10
1st Gen. Cephalosporin	Cephalothin (CEP)	S	R	R	R	S	S	S	R	S	S
2nd Gen. Cephalosporin	Cefuroxime (CXM)	R	R	I	R	R	R	R	R	R	R
3rd Gen. Cephalosporin	Ceftazidime (CAZ)	S	S	R	R	I	I	R	R	R	R
4th Gen. Cephalosporin	Cefipime (CPM)	S	S	S	S	S	S	S	S	S	S
Penicillinase stable	Oxacillin (OX)	R	R	R	R	R	R	R	R	R	R
Aminopenicillin	Ampicillin (AMP)	S	S	I	S	S	S	S	I	S	S
Carboxypenicillin	Ticarcillin (TI)	S	S	R	S	S	S	S	S	S	S
Polypeptide	Polymyxin-B (PB)	S	S	S	S	S	S	S	S	S	S
Monobactums	Azetreonam (AT)	S	S	S	S	R	I	R	R	R	I
Carbapenems	Meropenam (MRP)	S	S	I	I	S	S	S	S	S	S
Glycopeptides	Vancomycin (VA)	R	R	R	R	R	R	R	R	R	R
1st gen. aminoglycoside	Kanamycin (K)	R	R	R	S	S	R	R	R	S	S
2nd gen. aminoglycoside	Gentamicin (GEN)	S	S	R	R	S	S	I	S	S	R
3rd gen. aminoglycoside	Amikacin (AK)	S	R	R	S	S	S	S	S	R	R
Natural tetracycline	Tetracycline (TE)	R	R	R	R	R	R	R	R	R	R
Lincosamides	Clindamycin (CD)	R	R	R	R	R	R	R	R	R	R
Macrolides (50-S)	Erythromycin (E)	R	R	R	R	R	R	R	R	R	R
Phenicoles (50-S)	Chloramphenicol (C)	R	S	R	S	S	S	S	R	R	R
2nd gen. quinolone	Ciprofloxacin (CIP)	S	S	R	S	S	S	S	S	S	S
3rd gen. quinolone	Levofloxacin (LE)	S	S	S	S	S	S	S	S	S	S
RNA synthesis inhibitor	Rifampicin (RIF)	R	R	R	R	R	R	R	R	R	R
Sulphonamide	Nitrofurantoin (NIT)	R	R	R	R	R	R	S	R	S	S
Combination of sulpha + trimethoprim	Co-trimoxazole (COT)	R	R	R	R	R	R	R	R	R	R

S=Sensitive, I=Intermediate, R=Resistant

Efflux activity of *E. coli* isolates by cartwheel method: The results of this method presented in the form of intensity of florescence given by the isolates which were interpreted as "-", "+", "++", "+++" and "++++". All the *E. coli* isolates produced varying degree of fluorescence at different concentration of Et-br. By the addition of peptides into Et-br plates resulted in reduced intensity of fluorescence compared to plates containing only Et-br. CCCP was taken as negative control for this experiment as it is a well-established efflux pump inhibitor.

Table 2: Intensity of Florescence given by *E. coli* isolate on Et-br plates

Isolates	Concentration of Et-br									
	0.5mg/l	1.0mg/l	1.5mg/l	2.0mg/l	2.5mg/l	0.5mg/l	1.0mg/l	1.5mg/l	2.0mg/l	2.5mg/l
	Et-br alone	With CCCP	Et-br alone	With CCCP	Et-br alone	Et-br alone	With CCCP	Et-br alone	With CCCP	Et-br alone
EC-1	+	-	+	-	++	+	++	+	+++	++
EC-2	-	-	-	-	+	-	+	+	++	+
EC-3	+	-	+	-	++	+	+++	+	++++	++
EC-4	+	-	+	+	+	-	++	+	++++	+++
EC-5	+	-	+	-	+	+	++	+	+++	++
EC-6	-	-	+	-	+	+	++	++	+++	++
EC-7	-	-	+	-	+	+	++	+	++	+
EC-8	+	-	+	+	+	+	+++	++	+++	++
EC-9	-	-	+	+	+	+	++	+	+++	++
EC-10	+	-	-	-	+	+	+	+	++	+

- No florescence, + mild florescence, ++ moderate florescence, +++ strong florescence and ++++ strongest florescence

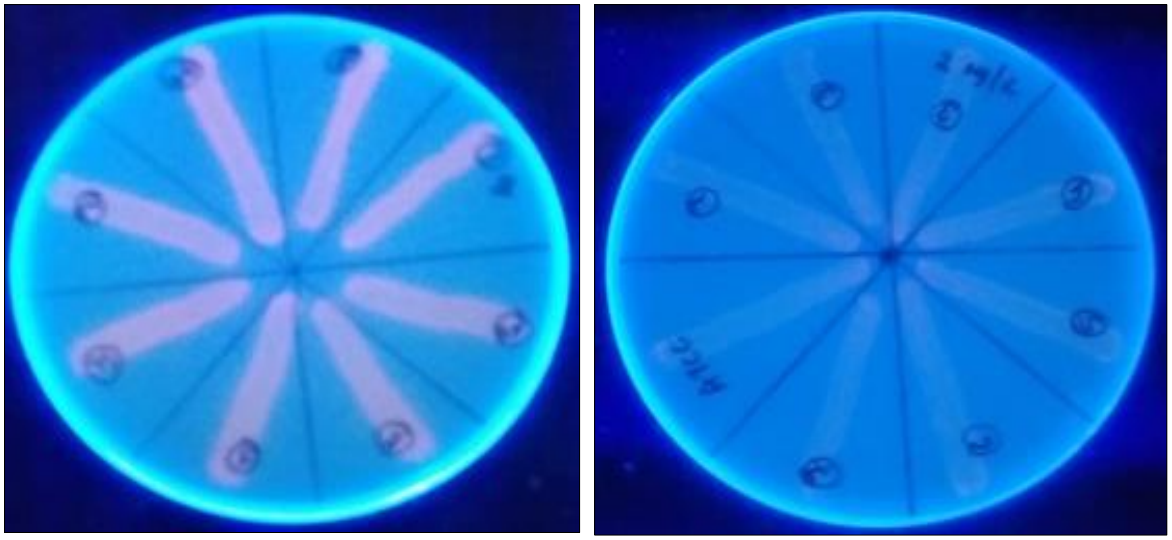

Fig 1: Efflux activity on plates containing Et-br, 2mg/L (A) Containing only Et-br showing +++ fluorescence whereas (B) Containing CCCP showing only + fluorescence

Determination of MIC and MBC: Three antibiotics namely Gentamicin, Cephalothin, and Ampicillin were selected for this combinational study. Gentamicin, comes under protein synthesis inhibitor class of antibiotics and also reported substrate for efflux pump of *E. coli* Cephalothin, comes under beta-lactam antibiotic group and Ampicillin, amino penicillin and also comes under beta lactam group which is cell wall synthesis inhibitor group. Beta lactam antibiotics are also the common substrate for efflux pump of *E. coli* (Anes *et al.*, 2015) [2]. Effects of both the peptides were compared with CCCP, a standard efflux pump inhibitor. Efflux pump inhibitory as well as synergistic effects of peptides with antibiotics were tested against *E. coli* isolates. The MIC and MBC for all these antibiotics were determined alone as well as in the presence of synthetic peptides. The MIC values of Gentamicin when tested with *E. coli* ranged between 4-8 µg/ml and combination of SG-15 with Gentamicin reduced the MIC of Gentamicin by 4-8-fold and GG-15 was able to reduce MIC by 2-fold. Effects of peptides were almost similar to effect of CCCP and these results were utterly similar with results of (Coutinho *et al.*, 2008) [10]. The MIC values of Cephalothin were ranged between 16-32µg/ml and in combination of SG-15 and cephalothin 8-16-fold decreased MIC was found. The MIC value for Ampicillin against *E. coli* isolates were found 4-16 µg/ml and combination of SG-15 with ampicillin have reduced MIC by 2-4 folds There was no inhibition recorded when peptide tested alone against *E. coli* isolates but were found able to lower the MIC values of antibiotics.

When gentamicin tested with *E. coli* for MBC was found 8-32 µg/ml and in combination of peptide SG-15 values were found 2-fold reduced. The MBC values for cephalothin ranged between 64-256µg/ml but when cephalothin was used in combination with peptide SG-15, MBC values were found upto 8- 16-fold decreased. For Ampicillin MBC values were found between 16-64µg/ml and when it was used with peptide SG-15 there was 2-4-fold reduction in MBC values were recorded.

Conclusion

Peptides are considered good therapeutic agents as they are having high safety level, tolerability, predictable metabolism and a standard synthesizing protocol (Fosgerau and Hoffmann, 2015). The current study indicated that synthetic peptide SG-15 had shown a variable effect in combination with antibiotics. The extent of effect varied with different antibiotics and peptide designed from inner membrane protein exhibited greater antimicrobial effect in combination with antibiotics than peptide designed from outer membrane protein of efflux pump protein sequence of *E. coli*. Due to lowered resistance emergence chances for peptides by bacteria these can become a potent alternative to available antibiotics to which microbes are generating resistance very rapidly as well as use of combination of such peptides with

conventional antibiotics may also be a great approach to enhance efficacy of antibiotics as well as to reduce the required dose of antibiotics.

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