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Synthetic peptides, SG-15 and GG-15: As an efflux pump inhibitors to combat AMR

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Abstract
The present study aimed to designing of synthetic peptides as efflux pump inhibitors and determination of their efficacy along with some antibiotics. Two synthetic peptides SG-15 and GG-15 were designed on the basis of protein sequence and structure of AcrAB-TolC a commonly expressed efflux pump of *Escherichia coli* (*E. coli*). Efficacy of both the peptides were evaluated by determination of their synergistic effect with three antibiotics namely; Ampicillin, Gentamicin and Cephalothin through minimum inhibitory concentration of on 10 *E. coli* isolates obtained from mastitic milk samples of cattle. Designed synthetic peptides of present study were proven potent efflux pump inhibitors as SG-15 was able to decrease in MIC of an antibiotics upto 8-16 folds. Discovery of such agents are becoming hope in the treatment of newly emerging multidrug resistant strains of several common pathogenic bacteria.

Keywords: Efflux pump inhibitors (EPI), SG-15 and GG-15

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 large 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 major role in human health, as antibiotic-resistant bacteria can be transmitted between humans and animals through contact, food products and from the environment (Landers *et al.*, 2012) [16]. New Delhi Metallo β-Lactamase-1 (NDM-1) and Expanded Spectrum β-Lactamases (ESBL) producing gram-negative bacteria (Ghatak *et al.*, 2013) [12] isolated in milk samples obtained from cattle with mastitis have been reported (Eisenberger *et al*., 2018) [10]. Vancomycin-resistant *Staphylococcus aureus* (VRSA) strains in samples obtained from surgical site (Bhattacharyya *et al.*, 2016) [5] and multidrug resistant *E. coli* was also isolated from the milk sample with clinical mastitis (Todorvic *et al*., 2018) [26].
There are different intrinsic mechanisms for antimicrobial resistance present in bacteria such as overexpression of multidrug efflux pumps which pump out the applied antibiotic from bacterial cell wall (Webber and Piddock, 2003) [29], genetic mutations and horizontal transfer of drug resistance genes (Schmieder and Edwards, 2012) [21].
These efflux pumps are proteinaceous transporters found in prokaryotic as well as eukaryotic cells for performing various physiological functions. Efflux pumps of efflux superfamilies 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) [24].
Several drugs have been tried to inhibit the mechanism of such pumps including PhenylalanylArginyl β–naphthylamide (PAβN), globomycin, carbonyl cyanide m-chlorophenylhydrazone (CCCP) and quinolones (Pages *et al*., 2005). Peptides for blocking of efflux pumps, can be easily designed, synthesized and modified suitably (Poulsen and Deber, 2012; Lamers *et al*., 2013) [20]. The current research aims at screening of newer synthetic peptides for blocking of bacterial efflux pumps.
Efflux pumps are proteinacious structure and synthetic peptides are most widely used to study the relationship between structure and activity of biologically active protein. A specific advantage is that they can be generated as exact copies of protein fragments as well as in diverse chemical modifications according to the need (Groß *et al.*, 2016) [13]. Designing of synthetic peptides against efflux pumps can be done by several computational software such

as, Peptide Builder (Tien *et al*., 2013) [25], NHLBI-Ab*Designer* (Pisitkun *et al*., 2011) [19] and pepsequencer (Schutkowski *et al.*, 2005) [22] named a few. Keeping in view the need to search suitable peptides administered along with antibiotics to block the efflux pumps helping antibiotics to function properly and to prevent economic loss due to futile treatment strategies against serious bacterial infections in animals, the present research study has been conducted.

Materials and Methods

Sampling and study area

In the present investigation out of 28 milk sample 10 isolates of *Escherichia coli* from mastitis milk of cattle were used. The samples were collected from the RAJUVAS clinics. 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 conformation

The procedure for isolation and identification of bacterial culture was followed as per the standard protocols (Carter *et al.*, 1994) [6]. 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 organism isolated colonies were further streaked on to Eosin Methylene Blue (EMB) agar (Edward and Ewing 1986). Besides this MALDI-TOF MS was used for species level conformation also used as per the method described by Singhal *et al.*, 2015 [23].

Antibiotic sensitivity assay

Antibiotic susceptibility testing was done as per the disc diffusion method (Bauer *et al.*, 1966) [3] following the guidelines of Clinical Laboratory Standard Institute (CLSI) against 23 antibiotics of different classes. 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 includes pencillins, cephalosporins, carbapenems and monobactums.

Ethidium bromide-agar (Et-br) cartwheel method for evaluation of efflux activity

Fluorometric determination of ethidium bromide efflux kinetics in E. coli can be 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 [7, 17]

For confirmation of efflux activity in isolates this experiment was further repeated with adding of Carbonyl Cyanide m-Chlorophenylhydrazone (CCCP),is a known efflux pump inhibitor acts by inhibition of proton motive forces by was mixed with Et-br in TSA.

Designing of synthetic peptides

The peptides were designed with help of software AbDesigner which is known software for production of siglec-15 antibodies for treatment of bone loss-related diseases and the Siglec-15 has been patented by U.S. patent and trademark office (Tremblay *et al.*, 2017) [27] further docking of these peptides and efflux pump protein of AcrAB-TolC complex is done with help of a computational software pepATTRACT 2.0 This tool is used for designing of antinflammatory peptides that are used as therapeutic agents for inflammation related diseases (La *et al.*, 2018) [14].

Determination of Minimum inhibitory concentration (MIC) and Minimum bactericidal concentration (MBC)

The efficacy of both peptide along with antibiotics was checked by the determination of MIC by broth dilution method (Wiegand *et al.*, 2008) [30]. MIC and MBC were determined for the antibiotic alone or in combination with designed Peptides and CCCP separately and effects on lowering of MIC of antibiotics were compared.

This experiment was carried out by microtitre plate method as follows

a. Firstly Prepared the desired test antibiotic solution then dilute in double strength Müeller Hinton broth to 2X the top concentration desired in the test (e.g. if the highest test concentration is 256µg/mL, dilute to 512µg/mL)
b. 75 µl of Müeller Hinton broth was dispensed into all wells of the microtitre plate except positive control wells.
c. Mixing of the antibiotics done by sucking up and down 5-8 times using the pipette was done and followed by withdrawl 75 µl of higher antibiotic dilution and added to column 1.
d. Mixing was done by sucking and transferring it to column 2. Repeat the procedure upto column 10 and Discard 75µl from column 10.
e. The bacteria inoculums were prepared to the size of 104 to 105 CFU/mL, by diluting it using broth solution.
f. 75 µl of the test organism suspension diluted 1:100 was added to each well.
g. Inoculated and uninoculated wells of antibiotic-free broth were included as a control to check the adequacy of the broth to support the growth of the organism and the sterility. Wells having inoculated broth were marked as positive control whereas wells having uninoculated broth were marked as negative control.
h. The plates were incubated in 37 °C or other desired temperature for 12-18 hours. The reading of results could be made manually using a black card or electronically with an ELISA reader.
i. After incubation on next day the MIC was determined as the lowest concentration of the antibiotic at which there was no visible growth.

Table 1: Diluents and solvents used for respective antibiotics and efflux pump inhibitors

Antibiotics/ synthetic peptides	Solvents	Diluents
Cephalothin	DMSO	Mueller-Hinton broth
Gentamicin and ampicillin	Sterile distilled water	Mueller-Hinton broth
SG-15	Sterile distilled water	Mueller-Hinton broth
GG-15	Sterile distilled water	Mueller-Hinton broth
CCCP	DMSO	Mueller-Hinton broth

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 the by MALDI-TOF MS isolates were confirmed as *E.coli* from moderate to extensive probability.

Table 2: Identification of *E. coli* isolates by MALDI-TOF MS

Sample no.	% Probability	Pathogen detected
BG-1	85.40	*E. coli*
BG-2	97.50	*E. coli*
BG-3	82.60	*E. coli*
BG-4	95.30	*E. coli*
BG-5	92.50	*E. coli*
BG-6	86.20	*E. coli*
BG-7	78.90	*E. coli*
BG-8	88.50	*E. coli*
BG-9	91.60	*E. coli*
BG-10	81.40	*E. coli*

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 shown highest resistance (100%) which was similar for the results of Rajala-schultz *et al*., (2004). More than half of the isolates exhibited resistance to multiple antibiotics which is quite similar to findings of Nontongana *et al.* (2014).

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 CCCP into Et-br plates resulted in reduced intensity of fluorescence compared to plates containing only Et-br hence it has been proven that all isolates having varying degree of efflux activity.

Designing of synthetic peptides

Synthetic peptides have been designed on the basis of amino acid sequence and structure of efflux pump protein AcrAB-TolC, which is tripartite pump protein highly expressed for efflux activity in *E. coli.* It was done by help of a software namely AbDesigner. Designing of two 15 amino acid containing peptides one for inner membrane protein inhibition and another for outer membrane protein inhibition of pump protein. The 15 amino acid sequences for (i) SG-15 is SRWEYGSPRLERYNG from 807-821 and (ii) GG-15 comprising of GTQYDDSNGGQNKVG from 271-285 AA sequence of AcrAB-TolC protein. 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].

Comparison of MIC and MBC values of selected antibiotics alone and in synergistically with synthetic peptides

A total three antibiotics were selected namely: Gentamicin, Cephalothin and Ampicillin. Gentamicin, which interrupting protein synthesis and reported as substrate for efflux pump of *E. coli.* Cephalothin, a beta-lactam antibiotic and Ampicillin is amino penicillin also a member of beta-lactam antibiotics acts by inhibition of cell wall synthesis. Beta-lactum antibiotics are considered as one of the important substrate extruded by efflux pump of *E. coli* (Anes *et al.*, 2015) [2]. CCCP was used as standard efflux pump inhibitor in this experiment so that effects of peptides were compared with it.

The MIC and MBC values of Gentamicin when tested with *E. coli* ranged between 4-8 µg/ml and 8-32 µg/ml respectively. When Gentamicin used in combination with peptide SG-15, MIC and MBC value was reduced by 4-8 fold and 2 fold respectively and when Gentamicin used in combination with peptide GG-15 there was 2 fold decrease in MIC no change in MBC values whereas on combining of both the peptides i.e. SG-15 and GG-15, there was significant decrease in MIC value of 8-16 fold but only 2 fold reduction in MBC. On comparison of effect of peptides with CCCP results were almost similar. These results were quite similar to results of (Coutinho *et al.*, 2008) [8]. They have used Chlorpromazine and *Menthaarvensis* to reduce MIC of Gentamicin and the MIC values were reduced to 1/16.

The MIC and MBC values of Cephalothin when tested with *E. coli* ranged between 16-32 µg/ml and 64-256 µg/ml respectively. When Cephalothin used in combination with peptide SG-15, MIC and MBC value were 8-16 and 8-32 fold decreased respectively and when Cephalothin used in combination with peptide GG-15 there was 2-4 fold decrease in MIC and 2-8 fold decrease in MBC whereas on combining of both the peptides i.e. SG-15 and GG-15, there was significant decrease in MIC value of 16-32 fold and in MBC 8-32 fold reduction. On comparison of effect of peptides with CCCP results were almost similar.

The MIC and MBC values of ampicillin when tested with *E. coli* ranged between 4-16 µg/ml and 16-64 µg/ml respectively. Combination of ampicillin and peptide SG-15 was able to reduce MIC and MBC by 2-4 fold but combination with peptide GG-15 given no change in MIC and MBC whereas on combining of both the peptides i.e. SG-15 and GG-15 with ampicillin, there was 2-4 fold decrease in both MIC and MBC which is similar to SG-15 that means there was no effect of GG-15 (outer membrane protein blocker). On comparison of effect of peptides with CCCP results were almost similar. This may be due to involvement of only inner membrane protein AcrB in extrusion of antibiotic which is involved by taking antibiotics or other substrate in its binding pocket and extrude it by peristaltic mechanism (Edward *et al.*, 2003) [9] On comparison of effect of peptides with CCCP results were similar except for 4 isolates.

All the results of MIC and MBC were suggesting that SG-15 was more effective as compared to GG-15 this may be due to AcrB acts by selection of substrate for pump and binding of substrate in pocket form structure and SG-15 was designed as inner membrane protein blocker and blocking of AcrB was more effective than blocking of TolC. Efficacies of synthetic peptides as well as CCCP were differing for different antibiotics it may be due to positive or negative interaction of antibiotics and these efflux pump inhibitors and this interaction may be based on structure of antibiotic or nature of antibiotic. Peptides were more efficient in terms of concentration required as peptides were shown their effect at 1µg/ml whereas CCCP were effective at 10µg/ml dissolved in distilled water and DMSO respectively. Peptides are considered good therapeutic agents because they are having high safety level, tolerability, predictable metabolism and standard synthetic protocols (Fosgerau and Hoffmann, 2015) [11].

The current study indicated that all the isolates taken for the study from cattle milk with clinical mastitis had exposed to a large number of antibiotics by showing resistance to more than half of antibiotics and having efflux activity which is major innate cause of antimicrobial resistance. Synthetic peptides SG-15 and GG-15 had a variable effect in combination with antibiotics. All three antibiotics in combination with both the peptides gave a synergistic effect against *E. coli* but, the extent of effect varied between different antibiotics. When peptides were used in combination with Cephalothin gave more synergistic effect which is followed by Gentamicin and Ampicillin whereas Ampicillin and GG-15 combination had given no effect.

Conclusion

Efflux pumps are one of the important innate causes of antimicrobial resistance so there is urgent need to discover new efflux pump inhibitors which will safer and easy to incorporated with antibiotics than existing efflux pump inhibitors. Peptides are more safe and tolerable compounds and can be easily synthesized. In the present study two synthetic peptides SG-15 and GG-15 were designed for blocking of AcrAB-TolC efflux pump of *E. coli*. The efficacy of both these peptides was tested on 10 pathogenic *E. coli* isolates from cattle milk with clinical mastitis. The three antibiotics *i.e.* Gentamicin, Cephalothin and Ampicillin in combination with SG-15 gave a synergistic effect against *E. coli* but, the extent of effect varied between different antibiotics but efficacy of peptides were found working at par with established efflux pump blocker named carbonyl cyanide m-chlorophenylhydrazone(CCCP). The designed peptides have shown potent efflux inhibition activity, possibly by blocking the commonly expressed efflux pump, AcrAB-TolC of *E. coli*. These peptides can be incorporated with antibiotics to reduce antibiotic resistance due to extrusion by efflux pumps and further this kind of peptides can be searched from natural sources also.

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