Recent Research in Science and Technology 2014, 6(2): 07-09
ISSN: 2076-5061
Available Online: http://recent-science.com/

Indolicidin – Antibacterial activity against bacterial pathogens isolated from ocular infections

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Abstract
Indolicidin is a novel/ promising antimicrobial peptide (a 13 amino acid cationic antimicrobial residue present in the form of cytoplasmic granules of bovine neutrophils) and observed with a broad spectrum of antimicrobial activity against bacteria, fungi, protozoa & even viruses. In the present study, *Escherichia coli* was transformed with pET 21a+ plasmid carrying indolicidin gene and was expressed. The crude extracts of indolicidin samples induced with varying IPTG concentrations (5mM and 20 mM/ ml of the medium) in Min A medium were checked for antibacterial activities against clinically important ocular bacterial pathogens such as *E. coli*, *Klebsiella* sp*,, Pseudomonas* sp*., Acenitobacter* sp., *Staphylococcus aureus,* coagulase negative *Staphylococcus aureus, Streptococcus viridans, S. pneumoniae and S. pyogens* and its activity was evaluated.

Keywords: Indolicidin, antimicrobial activity, pET 21a+ plasmid, IPTG

INTRODUCTION

The antimicrobial activities of cationic antimicrobial peptide like indolicidin against bacteria, fungi, protozoa and against viruses are currently being investigated across the globe. It is a short peptide with 38% of tryptophan and 28% of proline. In the mammals, indolicidin performs a significant role in defense and inhibits the invading gram-negative and gram-positive bacteria (Selsted *et al*., 1992 and Subbalakshmi *et al*., 1996), viruses (Robinson *et al.* 1998) and fungi (Ahmad *et al.* 1995, Zhang *et al.,* 2001 and Lee e*t al*. 2003) more effectively. Considering the alarming levels of reports on the emergence of multidrug resistant bacteria /fungal/ viral pathogens, development and study of alternative drugs such as indolicidin has become the order of the day. Availability of such alternative drugs for treatment would enable the health care providers to treat and manage diseases in a better way while concurrently avoiding any the emergence of drug resistant pathogens. In the above said context, a pilot scale study was carried out and selected *E.coli* host cells were transformed with plasmids carrying indolicidin gene and expressed. The crude extracts containing indolicidin was evaluated for its antibacterial activities against frequently isolated bacterial pathogens causing various eye infections.

MATERIALS AND METHOD

Host cells and Indolicidin plasmid

Four *E. coli* host cells [C41 (DE3), C43 (DE3), C41 (DE3) & P LYS C43 (DE3)] were employed for expressing indolicidin gene carried by the plasmid pET 21a+. All the strains were grown in Luria-Bertaini (LB) broth at 37°C. *E. coli* was inoculated on 100 ml of LB medium and shaker incubated at 37^{0}C for 4-6 hours. After incubation, the cells were collected and were re-suspended in 10 ml of cold 0.1M $CaCl_2$ and further incubated for 20 minutes at cold condition. The cells were again collected and treated with 5ml of 0.1M $CaCl_2$ and 15% of glycerol and the content was dispersed in microtubes and stored at -80°C.

Transformation of E.coli cells

With 50 µl of the competent test *E. coli* cells, 2 µL of the plasmid vector was mixed and incubated in ice for 30 minutes. The cells were subsequently kept in water bath at 42^{o}C for 45 seconds initially and further incubated for 2 minutes in ice. To this, 950 µl of LB broth medium was added and incubated in a shaker incubator at 250 rpm for 1 hour. The culture thus obtained was then inoculated into the LB ampicillin medium and incubated for 37^{o}C for overnight. The colonies formed were considered to be the transformants/ recombinants.

IPTG induced production of Indolicidin & confirmation on SDS-PAGE

E.coli thus obtained was grown in Min A medium and indolicidin was expressed at lab scale in the presence of 5 mM and 20 mM / ml isopropyl thiogalacto pyranoside (IPTG) as an inducer. The overnight IPTG grown culture was centrifuged, supernatant was estimated for the total protein, and was used as the indolicidin sample for antimicrobial evaluations. The sodium deodecyl sulfate – Polyacrylamide Gel Electrophoresis was employed to confirm the presence of indolicidin peptide as a discrete band in the sample.

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Analyses of antibacterial activity of sample containing indolicidin

The antimicrobial activities of the crude indolicidin extract obtained from media expressed with 5 mM / 20 mM / ml IPTG were analyzed against *E. coli*, *Klebsiella* sp*,, Pseudomonas* sp*., Acenitobacter* sp., *Staphylococcus aureus,* coagulase negative *Staphylococcus aureus, Streptococcus viridans, S. pneumoniae and S. pyogens* isolated from patients who suffered from bacterial eye infections. Precisely, the crude samples (4ml of the sample + 4 ml of the test culture & 6ml of the sample + 2 ml of the test culture) were incubated with fresh cultures of the test bacteria (with suitable test controls) after one and half an hour and 3 hours of incubation. Subsequently, the treated cells were gram stained and their cell morphology was studied under the microscope. Any change from the actual morphologies of the gram stained bacterial cells and confirmation of distorted cell morphology was considered to be positive for antibacterial activity by Indolicidin. The findings were simultaneously compared with the standard controls (untreated gram stained bacterial cells).

RESULTS AND DISCUSSION

The normal growth of *E.coli* (transformed) was noted on ampicillin containing LB agar. Similarly, a 2 kDa band representing the indolicidin peptide was confirmed on SDS- PAGE gel. A total protein concentration of 4 mg/ml was estimated in Min A medium by the transformed *E. coli* (no IPTG used as inducer) after one day of incubation while the same was calculated to be 7mg/ml when grown with IPTG at 5 mM concentration. Whereas a total of 7. 8 mg/ml of protein was obtained when IPTG was used at 20 mM concentration. This further confirmed maximum production of indolicidin by the transformed cells when the IPTG concentration was increased. The crude preparation of indolicidin containing sample was checked for its antibacterial activities against a total of 9 ocular bacterial pathogens (*E. coli*, *Klebsiella* sp*,, Pseudomonas* sp*., Acenitobacter* sp., *Staphylococcus aureus,* coagulase negative *Staphylococcus aureus, Streptococcus viridans, S. pneumoniae and S. pyogens*) by assessing the differences in the normal morphological features of the organism before and after treatment with the indolicidin sample. While remarkable activity was noted against *E. coli* and *Nocardia* spp. (many of the cells showed altered cell morphology after incubation with the preparations of indolicidin), the indolicidin activity as observed to be optimal against the other pathogens tested. Again, the degree of antimicrobial activity was noted to vary between the samples obtained from 5mM and 20 mM IPTG grown indolicidin samples as many of the treated cells lost their integrity of cell morphology (Table 1& 2). Indolicidin peptide has been shown to permeabilize both the outer and the cytoplasmic membrane of *E. coli*, but is distinct from other amphiphilic peptides in that the membrane permeabilization does not lead to lysis (Subbulakshmi *et al*., 1996). Indolicidin against Gram-negative species is associated with a better ability to permeabilize both the outer and the inner membrane (Rozek *et al*., 2000). Antimicrobial peptides show a broad range of activity against gram positive and gram negative bacteria, fungi, mycobacteria and some enveloped viruses (Rinaldi *et al*., and Kinnunen *et al*., 2002).

Table 1. Antibacterial activities of indolicidin
(expressed at 5 mM IPTG concentration in Min A medium)

Bacterial pathogen	Cell morphology after treatment with crude preparations of indolicidin sample	Level of antibacterial activity
E. coli	Normal pink colored gram negative rods in control. After treatment with indolicidin most of the cells were normal. However, some of the cells were abnormally long and elongated and some with small filamentous structure. The cells were permeabilized.	+
Pseudomonas sp.,	Control - Pink colored Gram negative slender rods. After treatment with indolicidin most of the cells were normal. However, few cells were amorphous & were permeabilized / filamentous and some noted with elongated structures.	-
Coagulase negative *Staphylococcus aureus*	Control- Normal gram positive cocci Some cocci were enlarged & amourphous. The actual clumps changed in to long elongated chains.	-
Staphylococcus aureus	Control- Gram positive oval shaped cocci. Normal cells were more while permeabilized / collapsed cells were scanty in number.	-
Nocardia sp.,	Control- Violet colored gram positive rods. Majority of the cells were amorphous. The cells were long elongated and permeabilized.	+
Streptococcus pneumoniae	Control- Gram positive cocci. Majority of the cells were enlarged /scanty number of cells. Cells formed clumped chains.	-
Coagulase Negative *Staphylococcus aureus*	Control- Gram positive Cocci. After treatment with indolicidin most of the cells were normal. However, few cells were amorphous & were permeabilized.	-
Pseudomonas sp.,	Control- Normal gram positive slender rods. After treatment with indolicidin most of the cells were normal. However, a very few cells were enlarged and amorphous & were permeabilized.	--
Coagulase Negative *Staphylococcus aureus*	Control- Gram positive cocci Most of the cell structures were normal, and scanty number of cells were enlarged and permeabilized.	-

Table 2. Antibacterial activities of indolicidin (expressed at 20 mM IPTG concentration in Min A medium)

Bacterial pathogen	Cell morphology after treatment with crude preparations of indolicidin sample	Level of antibacterial activity
E. coli	Normal pink colored gram negative rods in control. After treatment with indolicidin, cells were abnormally long and elongated and some with small filamentous structure. The cells were permeabilized and amorphous.	+
Pseudomonas sp.,	Control - Pink colored Gram negative slender rods. After treatment with indolicidin the cells were amorphous & some were permeabilized / filamentous and some noted with elongated structures	+
Coagulase Negative *Staphylococcus aureus*	Control- Normal gram positive cocci Less number of cell were present and majority of them enlarged & amorphous. The clusters changed in to long elongated chains.	+
Staphylococcus aureus	Control- Gram positive oval shaped cocci. Majority of the cells were permeabilized / collapsed & were scanty in number.	+
Nocardia sp.,	Control- Violet colored gram positive rods. Majority of the cells were amorphous. The cells were long elongated and permeabilized	++
Streptococcus pneumoniae	Control- Gram positive cocci. Majority of the cells were enlarged scanty number of cells. Cells were permeabilized and amorphous.	-
Coagulase Negative *Staphylococcus aureus*	Control; Gram positive Cocci. After treatment with indolicidin most of the cells were normal. However, some of the cells were amorphous & were permeabilized.	+
Pseudomonas sp.,	Control; Normal gram positive slender rods. After treatment with indolicidin majority of the cells were enlarged and amorphous & were permeabilized.	+
Coagulase Negative *Staphylococcus aureus*	Control; Gram positive cocci Most of the cell structures were normal, and scanty numbers of cells were enlarged and permeabilized and amorphous.	-

'- -'= Very less activity, '-'= Less activity, '+'= Activity and '++' = High activity.

CONCLUSION

The *E. coli* based expression of indolicidin and its use against test bacterial pathogens isolated from patients suffering from ocular infections revealed that the IPTG used in this study at 5 mM & 20 mM concentrations could induct differentially and allow the expression of indolicidin at higher levels especially when IPTG was used at 20mM concentration and that there was a noticeable indolicidin activity almost against of the nine (although activity was good with *Nocardia* spp.) test pathogens.

ACKNOWLEDGEMENT

The authors sincerely thank the University Grants Commission (UGC), New Delhi for having funded this research work [Major Research Project (On-going) F. No. 41-1161/2012 (SR)].

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