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Research Article

IN VITRO AND *IN VIVO* ANALYSIS OF BACTERIOCIN FROM HOSPITAL ACQUIRED METHICILLIN RESISTANT *STAPHYLOCOCCUS AUREUS*

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ABSTRACT
Antimicrobial peptides are considered as the evolutionary ancient and effective defensive weapons. The AMPs serves a fundamental role in the successful evolution of complex multicellular organisms and has widespread in both animal and plant kingdoms. Both gram-positive and gram-negative bacteria secrete antimicrobial peptides. Antimicrobial peptides are small, positively charged, amphipathic molecules (which have both hydrophobic and hydrophilic regions) of variable amino acid composition and length (6-100 amino acids). These peptides have broad-spectrum with potentially high antimicrobial activity against both human and veterinary pathogens. Based on their secondary structure, AMPs are grouped into four major classes: b-sheet, a-helical, loop, and extended peptides an essential requirement for any antimicrobial agent is that it has selective toxicity for the microbial target, which is an important feature of AMPs. Bacteriocins are ribosomally synthesized and extracellulary released bioactive peptides or peptide complexes which have bactericidal or bacteriostatic effect. The bacteriocins are proteinaceous compounds that mainly inhibit closely related species; bacteriocins have ability to inhibit the actions of unrelated genera. There are several large categories of bacteriocin which are only phenomenologically related, these include the bacteriocins from gram-positive bacteria, the colicins, the microcins and the bacteriocins from Archae. The bacteriocins produced by *Staphylococcus* spp. which is collectively known as staphylococcins, staphylococci are capable of inhibiting other Gram-positive bacteria, but not Gram negative. Phage typing is a valuable tool and has established its importance in finding the transmission of hospital staphylococci from personnel and patients to newly admitted patients. Phage group II of staphylococci has been identified as one of etiologic agents in exfoliative dermatitisin newborns (Ritter's disease), toxic epidermal necrolysis in older individuals, staphylococcal scarlatiniform rash, and bullous impetigo, the clinical features of these syndromes may overlap and they share a common etiology. The clinical manifestations represent a spectrum of a single syndrome which they refer to as the Staphylococcal Scalded Skin Syndrome (SSSS).

Key Words: *AMP, Antimicrobial Peptides, Bacteriocins, Proteinaceous Compounds, Staphylococcin, Ritters Disese Ssss: Staphylococcal Scalded Skin Syndrome (SSSS)*

INTRODUCTION
Antimicrobial peptides are considered as the evolutionary ancient and effective defensive weapons. The AMPs serves a fundamental role in the successful evolution of complex multicellular organisms and has widespread in both animal and plant kingdoms. (Zasloff, 2002) Both gram-positive and gram-negative bacteria secrete antimicrobial peptides. Antimicrobial peptides are small, positively charged, amphipathic molecules (which have both hydrophobic and hydrophilic regions) of variable amino acid composition and length (6-100 amino acids). These peptides have broad-spectrum with potentially high antimicrobial activity against both human and veterinary pathogens. Antimicrobial peptides are stored in their structure, function and thus it is attractive to speculate that synthetic antimicrobial peptides which might be used in prevention or treatment of infections (Brogden *et al.,* 2003). The diversity of the antimicrobial peptides discovered so far has been broadly categorized under the basis of their secondary structure. Based on their secondary structure, AMPs are grouped into four major classes: b-sheet, a-helical, loop, and extended

peptides (Giuliani *et al.,* 2007) An essential requirement for any antimicrobial agent is that it has selective toxicity for the microbial target, which is an important feature of AMPs.

Bacteriocins are ribosomally synthesized and extracellulary released bioactive peptides or peptide complexes which have bactericidal or bacteriostatic effect (Klaenhammer, 1993).The bacteriocins are proteinaceous compounds that mainly inhibit closely related species, bacteriocins have ability to inhibit the actions of unrelated genera such as Clostridia, Listeria, enter pathogenic bacteria and gram-negative. Bacteriocins are categorized in several ways, including producing strain, common resistance mechanisms, and mechanism of killing. There are several large categories of bacteriocin which are only phenomenologically related, these include the bacteriocins from gram-positive bacteria, the colicins, the microcins and the bacteriocins from Archae (Cascales *et al.,* 2007). The bacteriocins can be classified into Class I, Class IIa/b/c, and Class III (Cotter *et al.,* 2006). Bacteriocins are of interest in medicine because they are made by non-pathogenic bacterium that normally colonizes in the human body. Loss of these harmless bacteria and the use of antibiotic, may allow opportunistic pathogenic bacteria to invade the human body. Genetically encoded immunity to the action of its own bacteriocin was exhibited by the producer cell (Nes *et al.,* 1996). This genetically encoded immunity is heterogeneous compounds that have variable molecular weights, biochemical properties and inhibitory spectra (Sullivan *et al.,* 2002). Most bacteriocins mechanisms of action are diverse, but their target is mostly bacterial membrane (Klaenhammer, 1993). Several uncharacterized substances with bacteriocin-like activity have been identified and are referred to as bacteriocin-like inhibitory substances (BLIS/BLS) (Daw and Falkiner, 1996). The bacteriocins produced by *Staphylococcus* spp. which is collectively known as staphylococcins, in 1885 Babes reported the first citation about bacteriocin-like antagonism between Gram positive bacteria and described that staphylococci could inhibit the growth of other staphylococci on solid medium. However, the term staphylococci was created later in 1946, after the description that bioactive substances produced by staphylococci are capable of inhibiting other Gram-positive bacteria, but not Gram negative (Fredericq, 1946).

Phage typing is a valuable tool and has established its importance in finding the transmission of hospital staphylococci from personnel and patients to newly admitted patients (Arora *et al.,* 1999). It is a successful method in characterization of *Stap. Aureus* and can be applied to study the spread, origin and outbreak of staphylococcal infection. Phage group II of staphylococci has been identified as one of etiologic agents in exfoliative dermatitisin newborns (Ritter's disease), toxic epidermal necrolysis in older individuals, staphylococcal scarlatiniform rash, and bullous impetigo (Melish and Glasgow, 1972 and Melish and Glasgow 1973) the clinical features of these syndromes may overlap and they share a common etiology. Melish and Glasgow (1973) have suggested that these clinical manifestations represent a spectrum of a single syndrome which they refer to as the Staphylococcal Scalded Skin Syndrome (SSSS). The exfoliative toxin (ET) produced by the group II staphylococci has a subsequent result of the action of an extracellular protein which was been purified and found as acidlabile, heat stable, and antigenic (Arbuthnott *et al.,* 1984, Kappral and Miller, 1971 and Melish *et al.,* 1972). Recently, Kondo *et al.,* (1973) isolated four proteins from phage group II staphylococci capable of producing exfoliation in newborn mice.

MATERIALS AND METHODS

Isolation and Identification of the Bacterial Strain

The wound swabs were collected from various laboratories in and around the city; the samples were processed within 24hrs after collection. The samples collected were plated onto the Mannitol Salt agar plates in duplicates and incubated at 37ºC for 24hrs. The identification of isolates as *Staphylococcus aureus* was based on the colony morphology, Gram's staining, and catalase and coagulase tests. The gram positive coccal isolates identified by microscopy were further identified with DNA'se. *S. aureus* ATCC 25923 was used as a standard control strain. The standard procedure was followed to test the antimicrobial susceptibility of the strains using Oxacillin discs (5µg). (Clinical Labortory Standard's

Institute, 2006) A zone of inhibition less than 10 mm was indicative of methicillin resistance. Further the isolates were confirmed by 16S rRNA sequencing, PCR was performed to amplify the 16S ribosomal RNA was determined by direct sequencing. 16S rRNA was separated by gel electrophoresis.

Identification of Bacteriocinogenic Strain

The phage typing was done to identify the toxin production of the isolates.

Stab Overlay Assay

The 24hrs culture of *Staphylococcus aureus* was stabbed in pre-poured Brain Heart Infusion agar plates, incubated at 37°C for 24 hrs, after incubation the colonies on the stabbed area was scrapped off and the plates was exposed to chloroform vapours for 15 minutes. Then 3 ml of BHI soft agar containing 0.1 ml of 24hrs old indicator organism was poured over the plates and incubated at 37ºC. After overnight incubation, plates were examined for zone of inhibition around the stabbed area.

Isolation of Bacteriocin by Ammonium Sulphate Precipitation

The Brain Heart infusion broth (100ml) was prepared and was inoculated MRSA and incubated for 24 hrs at 37°C. After incubation, the broth was centrifuged at 10,000 rpm for 10 mins. The supernatant was taken and the pellet was stored, to the supernatant (100 ml), 56.105 grams of Ammonium sulphate, which gives 80% concentration, was added and dissolved completely, and then it was kept at 4°C for 24 hrs. The precipitate was obtained by centrifugation at 10,000 rpm for 10 mins. The pellet was solubilised in 200 ml of 50 mM Sodium Phosphate buffer at pH 7 and the supernatant was also stored separately (Harris 1989).

Dialysis

The supernatant containing bacteriocin got by Ammonium Sulphate Precipitation test was partially purified by resuspending in 10ml of 10mM sodium citrate buffer (pH7.0) using Dialysis membrane. Sodium Citrate buffer (pH 7.0) was prepared and dialysis membrane was cut and the supernatant was loaded in the dialysis bag and was tightly tied with the help of thread. The dialysis bags were hanged and dipped inside the sodium citrate buffer and the whole set up was placed on a magnetic stirrer with a magnetic bead inside the buffer and it was kept at 4°C for 4-5 hrs.

Molecular Weight Determination by SDS-Page

The molecular weight of the isolated bacteriocin was determined by Sodium Deodecyl Sulphate – Polyacrylamide Agarose Gel Electrophoresis (SDS-PAGE) using 12% gel following electrophoresis. The separating gel was set in the glass plate by overlaying 0.5ml of 10% SDS solution. Then the stacking gel was poured and the comb was inserted and was allowed to set. The samples which were concentrated by salt out process and the samples got from dialysis were loaded along with the protein marker, conducted at 15mV-30mV for 40 min and the gel was stained with coomassie brilliant blue for about 30mins and was destained thereafter until the stains were removed. Then the gel was kept in white illuminator and molecular weight was observed. After that the gel was transferred to phosphate buffer and stored at 4ºC (Alessandra *et al.*, 2007).

Agar Well Diffusion Method of Bacteriocin against MRSA

Pre-poured Brain Heart Infusion agar plates were overlaid with 0.1ml (2×10^8 CFU/ml) of test culture. Wells (7mm in diameter) were cut into these agar plates and 100µl of the partially purified bacteriocin was added, and kept for incubation at 37ºC for 24hrs. The zones of inhibition were measured in mm in diameter. Inhibition was scored positive if the width of the clear zone around the well was observed (Iqbal *et al.*, 1999).

Invivo Analysis of Bacteriocin to Detect Staphylococcal Scalded Skin Syndrome (SSSS) by Intraperitoneal Injection in Mice

Equipment: Syringe and 23-27g, ½-1 inch needle, preferably with a short bevel.

Volume: The volume injected IP into an adult mouse should not exceed 2ml.

The mouse is grasped and held in dorsal recumbency in a head down position. The injection is made in the lateral aspect of the lower quadrant. The use of a short bevel needle inserted through the skin and musculature and immediately lifted against the abdominal wall, aids in avoiding puncture of the

abdominal viscera. Immobilizing the left leg is also essential in reducing the risk. Rapid injection, especially with a large syringe, may cause discomfort and tissue damage and should be avoided.

RESULTS AND DISCUSSIONS

Isolation and Identification of Staphylococcus Aureus

The isolates formed golden yellow mannitol fermenting colonies in Mannitol Salt Agar in the Nutrient Agar the colonies appear to be slightly yellow in colour. Under microscopic examination, the isolates appeared as Gram positive cocci in clusters. The isolates release oxygen when 3% hydrogen peroxide was added, which shows the isolates were positive for catalase, clumped the blood cells and did not re-emulisify thereafter shows the isolates were coagulase positive and were DNAse positive. It has been known that coagulase is not an enzyme but an extracellular substance (Monica, 2002). Among the pathogens, methicillin (oxacillin) resistant *S. aureus* is one of the most important causes of nosocomial infections worldwide and can cause outbreaks that are difficult to control (Salmenlinna, 2002). Species level confirmation of the isolate by 16s rRNA confirmed the isolate as *Stap. Aureus* and the sequence obtained were deposited in the GenBank under the accession number No.JQ958397

Identification of Bacteriocinogenic Strain

The strains were characterized by phage-typing and was identified that the phage group II of type 55 and 71 were capable of producing bacteriocin as a toxin. This phage group tend to be associated with particular skin diseases, a rare neonatal disease called the Staphylococcal Scalded Skin Syndrome (SSSS) and related milder, but more common form is pemphigus neonatorum are generally caused by strains belonging to phage group II (Dancer *et al.,* 1990).

Stab Overlay Assay

Zone of inhibition formed around the test organism was measured; there was no change in the size of zone even after the exposure to chloroform. The zone was measured which suggest that indicator organism is sensitive to test strain. In the case of staphylococcin it was resistant and its activity retained even after 20-30 mins exposure to chloroform vapours (Firdous *et al.,* 2007).

Ammonium Sulphate Precipitation Test

The partial purification of bacteriocin was done by Ammonium sulphate precipitation. The precipitate was recovered by centrifugation at 10,000 rpm for 10 mins; the pellet was solubilised 50mM sodium phosphate buffer of pH 7.0, which is known as crude preparation. Voet *et al.,* 1999 reported that the procedure is used for the concentration of proteins; this technique was used because it is the most commonly used reagent for salting out of proteins due to high solubility which permits the achievement of solution with high ionic strength.

Dialysis

The supernatant obtained from ammonium sulphate precipitation was subjected to dialysis, in which the supernatant was filled in the dialysis membrane, tied and hanged in the sodium citrate buffer of pH 7.0 with the magnetic beads inside. The setup was kept at 4ºC for 4-5 hrs. After the incubation, the retantate was collected in a sterile container and stored at 4ºC for further studies.

Molecular Weight Determination of the Bacteriocin by SDS-Page

Molecular weight determination of the partially purified bacteriocin by SDS-PAGE revealed the homogeneity of a single protein. Single protein bands were observed when stained with coomassie brilliant blue and it clearly indicated the purity of the protein. By comparing the substances with the known molecular weight, the bacteriocin obtained comes under the low molecular weight protein category.

Agar Well Diffusion Method

Zone of inhibition was formed around the well contained the partially purified bacteriocin was measured.

Invivo Analysis of Bacteriocin to Detect Staphylococcal Scalded Skin Syndrome (SSSS) by Intraperitoneal Injection in Mice

The crude bacteriocin from the phage group II was injected to the mice, after 4 hrs of injection scaly appearance in the skin of mice was observed. This is due to the bacteriocin produced by strain, which belongs to phage group II. Not all exfoliative toxin producing phage group II staphylococci produce exfoliative toxin if the genes for bacteriocin and exfoliative toxin are on the same plasmid, the plasmid could contain either one or both of these genes depending upon the specific staphylococcal strain (Warren *et al.,* 1975).

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