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Research Article

MOLECULAR EVALUATION OF ANTISTAPHYLOCOCCAL SECONDARY METABOLITES OF BACTERIAL ISOLATES FROM HOSPITAL WASTEWATER ENVIRONMENT

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ABSTRACT

Objective: Antibiotics revolutionized medicine in the 20th century, and have together with vaccination led to the near eradication of diseases such as tuberculosis in the developed world. Their effectiveness and easy access led to overuse, especially in livestock raising, prompting bacteria to develop resistance. The lantibiotics form a particular group among the antimicrobial peptides and are characterized by unique structural features. They are produced by a group of bacteria against some gram-positive bacteria. The present study was designed to screen for the lantibiotic producing organisms from the hospital samples.

Methods: The bacterial isolates were identified based on Bergey's manual of bacteriology and screened for the epidermin gene by polymerase chain reaction amplification.

Results: Of the 21 isolates screened, only 10 of them showed positive amplification for the epigene. Based on the biochemical characteristics, the isolates obtained were identified and labeled.

Conclusion: Further study on the purification of the compound need to be done. Some bacterial samples may not have the epidermin gene which does not show amplification, this confers that the epidermin gene is absent in certain organisms.

Keywords: Antibiotic resistance, Lantibiotics, Epidermin gene, Gram-positive bacteria.

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INTRODUCTION

Antibiotics, also called antibacterials, are a type of antimicrobial drugs used in the treatment and prevention of bacterial infection [1]. They may either kill or inhibit the growth of bacteria. A limited number of antibiotics also possess antiprotozoal activity. Antibiotics are not effective against viruses such as the common cold or influenza, and may be harmful when taken inappropriately [2].

The emergence of resistance of bacteria to antibiotics is a common phenomenon [3]. Emergence of resistance often reflects evolutionary processes that take place during antibiotic therapy [4,5]. The antibiotic treatment may select for bacterial strains with physiologically or genetically enhanced capacity to survive high doses of antibiotics. Under certain conditions, it may result in the preferential growth of resistant bacteria while growth of susceptible bacteria is inhibited by the drug. Antibacterial resistance may impose a biological cost, thereby reducing fitness of resistant strains, which can limit the spread of antibacterial-resistant bacteria, for example, in the absence of antibacterial compounds [6-8].

This article treats the terms as synonymous and according to the most widespread definition of antibiotics being a substance used against bacteria [3,9]. Lantibiotics are a class of peptide antibiotics produced by a large number of gram-positive bacteria such as *Streptococcus* and *Streptomyces* to attack other gram-positive bacteria and contain the characteristic polycyclic thioether amino acids lanthionine or methyllanthionine, as well as the unsaturated amino acids dehydroalanine and 2-aminoisobutyric acid [10]. It is composed of two alanine residues that are cross-linked on their β-carbon atoms by a thioether (monosulfide) linkage [11].

The lantibiotics are a class of more extensively modified bacteriocins, also called Class I bacteriocins [12,13]. (Bacteriocins for which disulfide bonds are the only modification to the peptide are Class II bacteriocins. Lantibiotics are well studied because of the commercial use of these bacteria in the food industry for making dairy products such as cheese [14,15]. Nisin and epidermin are members of a family of lantibiotics that bind to lipid II, a cell wall precursor lipid component of target bacteria and disrupt cell wall production [16]. All lantibiotics that have been characterized with respect to the stereochemistry of the thioether linkage contain (2*S*, 6R)-lanthionines, with many family members also containing (2*S*, 3*S*, 6*R*)-3-methyllanthionines [17-19]. In addition, they generally (but not always) contain the unsaturated amino acids 2, 3-dehydroalanine and (*Z*)-2, 3-dehydrobutyrine [20]. In all, no <15 different posttranslational modifications have been identified in lantibiotics and it is likely that other modifications remain to be discovered. These modifications release the peptides from the structural and functional constraints typically imposed on naturally occurring ribosomal peptides [21].

The overall mechanism of action of lantibiotic peptides can be phased into 3 steps, (1) binding of the peptide to the membrane, (2) insertion of the peptide into the membrane, (3) membrane permeabilization [22]. Lantibiotics show substantial specificity for some components (e. g., lipid II) of bacterial cell membranes, especially of gram-positive bacteria. Type A lantibiotics kill rapidly by pore formation, whereas Type B lantibiotics inhibit peptidoglycan biosynthesis [23]. Type A lantibiotics exert bactericidal activity toward a broad range of gram-positive bacterial strains, including *Streptococci*, *Bacillus*, *Listeriae*, *Clostridia*, and *Staphylococci*. The antibacterial effect is strong: The addition of nM concentrations of a lantibiotic is sufficient to kill bacterial cells. In contrast, other well-known antibacterial compounds, such as melittin, magainin, or cecropin, are active in mM concentrations [24].

Fungal cells, yeast cells, or human cells are very insensitive to lantibiotics, even when treated with mM concentrations of the peptides.

In general, gram-negative bacteria are also insensitive to lantibiotics. It has been shown that their outer membrane functions as a barrier for the lantibiotics [25]. When the outer membrane of the gram-negative strains *Escherichia coli* or *Salmonella* species was weakened by treatment with ethylenediamine tetraacetic acid (EDTA) or osmotic shock, the susceptibility of the cells toward nisin or Pep5 strongly increased. The primary target for the activity of the lantibiotics appears to be the bacterial cytoplasmic membrane. The peptides interfere with the membrane function of sensitive cells by increasing the permeability of the bilayer for small molecules and disrupting the membrane potential, resulting in cell death. Furthermore, other mechanisms could be involved in their biological activity, since it has been reported that lantibiotics also inhibit outgrowth of bacterial spores, activate autolytic enzymes and might inhibit cell wall biosynthesis [26]. However, these mechanisms seem to be secondary effects, since these processes are relatively slow and require relatively high concentrations of lantibiotics.

Since the lantibiotics act on the bacterial cytoplasmic-membrane, they first have to pass the bacterial cell wall. Many non-lantibiotic bacteriocins, such as lactococcin appear to interact with membrane-associated receptor proteins, before their membrane perturbing activity. In contrast, a specific proteinaceous receptor for lantibiotic species on the outer surface of bacteria has not been found so far [27]. This study was designed to isolate the strains from hospital environment and to screen for the epidermin genes using traditional polymerase chain reaction **(**PCR).

METHODS

This study was conducted in the Department of Microbiology, Indian Academy Degree College. Sampling was done at 5 hospital premises (Poly Vista Clinic, Shree Vijay Laxmi Hospital, Cratis Hospital, Avhieta Hospital and Good Shepherd Hospital) in and around Bengaluru. About 10 samples were collected from each area and then cultured for isolation.

Isolation of bacteria from the sample

The samples were serially diluted with sterile distilled water and the tubes labeled as 10^{-3} and 10^{-4} are plated onto the Mueller Hinton Agar medium and then incubated at 37°C for 24 hrs. The colonies obtained were further subcultured to obtain pure colonies. The pure colonies obtained were used for the experimental study [28].

Biochemical characterization

Isolated bacteria were recultured and identified on basis of Bergey's Manual of Systematic Bacteriology. Gram staining and biochemical test such as indole test, sugar utilization test, methyl red test, citrate utilization Test, Voges-Proskauer test, starch hydrolysis, catalase test, and oxidase test were carried out to find the enzymatic activity of isolated organism [29].

DNA isolation

A volume of 2 ml of culture was centrifuged at 8000 rpm, 10 minutes and the pellet formed was suspended in 567 µl of Tris-EDTA (TE) buffer. About 30 µl of 10% safety data sheet and 3 µl of 20 mg/ml proteinase k was added to the contents and mixed thoroughly and incubated for 1 hr at 37°C. 100 µl of 5 M NaCl was added and mix thoroughly. The mixture was then added with 80 µl hexadecyltrimethlyammonium bromide/NaCl solution and incubated for 10 minutes at 65°C. Following incubation, approximately equal volume (0.7-0.8 ml) of chloroform/isoamyl alcohol [24:1] was added and centrifuged for 8 minutes at 8000 rpm. The aqueous supernatant was transferred to a fresh tube and added with equal volume of phenol/chloroform/isoamyl alcohol [25:24:1] and the contents were spinned down in a microcentrifuge for 5 minutes. 0.6 µl isopropanol was added to precipitate the DNA. The pellet was suspended in TE buffer until further use.

PCR amplification

The gene selected for the primer designing was *epidermin* gene with an amplicon size of 321 bp. The specific primers were designed using primer 3 plus software, and the designed oligonucleotides were synthesized in sigma corporation USA and ordered from Eurofins, Bangalore. The *epi* gene was amplified by PCR using purified genomic DNA as a template. Oligonucleotide primers were synthesized to amplify the intact region of *epi* gene. The forward primer, ATCGATAAGCCATCGCAAGA 3' and the reverse primer, 5' GTCGTTTGTCGAACATGTGG 3', were purchased from Eurofins, Bangalore. These primers correspond to the gene *epi* and thus, the final PCR product was 321 bp.

The PCR mixture comprised ×10 reaction buffer with MgCl2 (1.5 mM), 2 µl of dNTP mix (2.5 mM), 2 µl each of forward and reverse primers (10 picomoles/µl each primer), 0.3 µl of Taq DNA polymerase (5 U/µl), and 50 ng/µl of template DNA in a total volume of 50 µl. The PCR was performed with the following cycling profile: Initial denaturation at 94°C for 2 minutes, followed by 35 cycles of 50 seconds denaturation at 94°C, annealing at 55°C for 30 seconds, and extension at 72°C for 1 minute. The time for the final extension step was increased to 6 minutes. The PCR products amplified were then qualitatively analyzed on 1% agarose gel.

Table 1: Biochemical characteristics of the 10 isolates isolated from sample area 1

Colony	Catalase	Oxidase	Indole	MR-VP	Citrate	Glucose fermentation	Mannitol fermentation	Organism
PV1	Negative	Negative	Negative	VP positive	Negative	Gas formation and color change	Negative	*Aeromonas* spp.
PV2	Negative	Negative	Negative	MR positive	Negative	Negative	Gas formation and color change	*Staphylococcus aureus*
PV3	Negative	Negative	Negative	VP positive	Negative	Gas formation and color change	Negative	*Aeromonas* spp.
PV4	Negative	Positive	Negative	VP positive	Negative	Gas formation and color change	Negative	*Lactobacillus fermenti*
PV5	Negative	Negative	Negative	MR positive	Negative	Negative	Gas formation and color change	*Staphylococcus aureus*
PV6	Negative	Negative	Negative	VP positive	Negative	Color change	Color change	*Lactobacillus casei*
PV7	Negative	Negative	Negative	MR positive	Negative	Negative	Gas formation and color change	*Staphylococcus aureus*
PV8	Negative	Positive	Negative	VP positive	Negative	Gas formation and color change	Negative	*Lactobacillus fermenti*
PV9	Negative	Negative	Negative	MR positive	Negative	Color change	Gas formation and color change	*Lactobacillus casei*
PV10	Negative	Negative	Negative	MR positive	Negative	Color change	Color change	*Lactobacillus casei*

MR: Methyl red, VP: Voges-Proskauer, PV:

RESULTS AND DISCUSSION

Culture isolates

The bacterial isolates obtained from different sampling areas were subcultured and identified based on their cultural characteristics. Both Gram-positive and Gram-negative strains were observed from the isolates. The biochemical characteristics obtained are shown in Tables 1-5 and were identified and labeled.

PCR amplification

A total of 21 bacterial cultures were selected and screened, their genomic DNA isolated and amplified through PCR (Fig. 1). Of the 21 bacterial samples, only 10 showed positive amplification towards epidermin gene which are (*Staphylococcus aureus, Lactobacillus fermenti*, *Streptococcus* sp., *Lactobacillus casei*, *Bacillus badius, Bacillus larvae*, *S. aureus*, *B. popilliae, Bacillus megaterium*, *Bacillus* sp.). From 10 bacterial cultures, 5 organisms showed maximum amplification for epidermin gene (*L. fermenti*, *Streptococcus* sp., *B. badius, B. larvae, Bacillus* sp.).

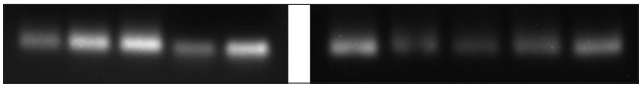

Fig. 1: Left: Gel showing the polymerase chain reaction amplification of epi gene on 2% agarose gel. Molecular marker (100 bp) is used. Lane 1: *Staphylococcus aureus,* Lane 2: *Lactobacillus fermenti*, Lane 3: *Streptococcus* sp. Lane 4: *Lactobacillus casei*, Lane 5: *Bacillus badius*; Right: Lane 1: *Bacillus larvae*, Lane 2: *S. aureus,* Lane 3: *Bacillus popilliae,* Lane 4: *Bacillus megaterium*, Lane 5: *Bacillus* sp

DISCUSSION

Antibiotic resistance in bacteria is high and is increasing in many countries worldwide causing great concern for public health [30]. During the past decade, there have been an increasing number of reports both antibiotic and antibiotic resistance genes in different environmental settings. Antibiotic resistant bacteria and genes encoding antibiotic resistance are commonly detected in wastewater, often at higher rates and concentrations compared to surface water [31]. Wastewater can also provide favorable conditions for the growth of a diverse bacterial community, which constitutes a basis for the selection and spread of antibiotic resistance. Wastewater samples were collected and organisms were isolated which are process for DNA isolation without any contamination of RNA. Further, it is processed for PCR amplification. A total of 21 samples were processed for PCR

Table 2: Biochemical characteristics of the 10 isolates isolated from sample area 2

Colony	Catalase	Oxidase	Indole	MR-VP	Citrate	Mannitol fermentation	Starch hydrolysis	Organism
SVL1	Negative	Positive	Negative	MR positive	Negative	Negative	Positive	*Bacillus hadius*
SVL2	Negative	Positive	Negative	VP positive	Negative	Negative	Negative	*Bacillus larvae*
SVL3	Negative	Positive	Negative	MR positive	Negative	Negative	Positive	*Bacillus hadius*
SVL4	Negative	Negative	Negative	VP positive	Positive	Negative	Positive	*Bacillus licheniformis*
SVL5	Negative	Negative	Negative	VP positive	Negative	Negative	Positive	*Bacillus pohmyxa*
SVL 6	Negative	Positive	Negative	VP positive	Negative	Color change	Negative	*Bacillus larvae*
SVL7	Negative	Positive	Negative	VP positive	Positive	Negative	Positive	*Bacillus coagulans*
SVL8	Negative	Positive	Negative	VP positive	Negative	Negative	Positive	*Bacillus coagulans*
SVL9	Negative	Negative	Negative	MR positive	Positive	Negative	Positive	*Bacillus megaterium*
SVL10	Negative	Positive	Negative	MR positive	Positive	Negative	Negative	*Bacillus azotoformans*

MR: Methyl red, VP: Voges-Proskauer, SVL: ???

Table 3: Biochemical characteristics of the 10 isolates isolated from sample area 3

Colony	Catalase	Oxidase	Indole	MR-VP	Citrate	Mannitol fermentation	Starch hydrolysis	Organism
CR1	Negative	Positive	Negative	VP positive	Positive	Negative	Positive	*Bacillus coagulans*
CR2	Negative	Positive	Negative	VP positive	Positive	Negative	Negative	*Bacillus azotoformans*
CR3	Negative	Positive	Negative	VP positive	Negative	Colour change	Negative	*Streptococcus* spp.
CR4	Negative	Negative	Negative	VP positive	Negative	Negative	Positive	*Bacillus* sp.
CR5	Negative	Positive	Negative	VP positive	Positive	Negative	Positive	*Bacillus coagulans*
CR6	Negative	Positive	Negative	VP positive	Positive	Negative	Positive	*Bacillus coagulans*
CR7	Negative	Positive	Negative	VP positive	Positive	Negative	Positive	*Bacillus coagulans*
CR8	Negative	Negative	Negative	VP positive	Negative	Negative	Negative	*Bacillus* sp.
CR9	Negative	Positive	Negative	MR positive	Positive	Negative	Negative	*Bacillus azotoformans*
CR10	Negative	Positive	Negative	VP positive	Positive	Negative	positive	*Bacillus coagulans*

MR: Methyl red, VP: Voges-Proskauer, CR: ???

Table 4: Biochemical characteristics of the 10 isolates isolated from sample area 4

Colony	Catalase	Oxidase	Indole	MR-VP	Citrate	Glucose fermentation	Mannitol fermentation	Starch hydrolysis	Organism
AVI	Negative	Negative	Negative	VP positive	Negative	Negative	Negative	Negative	*Bacillus* sp.
AV2	Negative	Positive	Negative	VP positive	Negative	Color change	Negative	Negative	*Aeromonas* spp.
AV3	Negative	Negative	Negative	MR positive	Negative	Negative	Negative	Negative	*Bacillus popilliae*
AV4	Negative	Positive	Negative	VP positive	Negative	Negative	color change	Positive	*Lactobacillus casei*
AV5	Negative	Negative	Negative	VP positive	Negative	Negative	Negative	Positive	*Bacillus polymyxa*
AV6	Negative	Positive	Negative	VP positive	Negative	Negative	Negative	Positive	*Streptococcus* sp.
AV7	Negative	Positive	Negative	MR positive	Negative	Negative	Negative	Positive	*Bacillus badius*
AY8	Negative	Negative	Negative	MR positive	Negative	Negative	Negative	Positive	*Bacillus badius*
AY9	Negative	Positive	Negative	MR positive	Positive	Negative	Negative	Positive	*Bacillus megaterium*
AV10	Negative	Negative	Negative	MR positive	Positive	Negative	Negative	Negative	*Bacillus azotoformans*

MR: Methyl red, VP: Voges-Proskauer, AV: ???

Table 5: Biochemical characteristics of the 10 isolates isolated from sample area 5

Colony	Catalase	Oxidase	Indole	MR-VP	Citrate	Glucose fermentation	Mannitol fermentation	Organism
GS1	Negative	Negative	Negative	VP positive	Negative	Color change	Color change	*Streptococcus* sp.
GS2	Negative	Negative	Negative	MR positive	Negative	Color change	Color change	*Aeromonas* spp.
GS3	Negative	Negative	Negative	MR positive	Negative	Color change	Color change	*Aeromonas* spp.
GS4	Negative	Positive	Negative	VP positive	Negative	Color change	Color change	*Aeromonas sobria*
GS5	Negative	Negative	Negative	VP positive	Negative	Color change	Color change	*Streptococcus* spp.
GS6	Negative	Positive	Negative	MR positive	Negative	Color change	Color change	*Lactobacillus casei*
GS7	Negative	Positive	Negative	MR positive	Negative	Color change	Color change	*Lactobacillus casei*
GS8	Negative	Positive	Negative	MR positive	Negative	Color change	Color change	*Lactobacillus casei*
GS9	Negative	Positive	Negative	MR positive	Negative	Color change	Color change	*Lactobacillus casei*
GS10	Negative	Positive	Negative	MR positive	Negative	Color change	Color change	*Lactobacillus casei*

MR: Methyl red, VP: Voges-Proskauer, GS: ???

amplification out of which 10 isolates showed positive amplification. Totally 5 organisms gave maximum amplification for epidermin gene. Some bacterial samples may not have the epidermin gene which does not show amplification this confers that the epidermin gene is absent in certain organisms. Further the experiment can be planned to purify the compound of interest and use it as an antibacterial against certain gram-positive organisms.

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