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Invitro Efficacy Testing of Bacteriocin against Multidrug Resistant *Pseudomonas aeruginosa*

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A B S T R A C T

Bacteriocin is a protein substance possessing antimicrobial activities during the growth by great number of Gram positive and Gram negative bacteria (Zacharof and Lovitt). It will destabilize the cytoplasmic membrane and kills bacteria. Various studies reported antibacterial activity of bacteriocin against Listeria and other Gram positive bacteria. Hence, there is a need to know the effectiveness of bacteriocin against drug resistant Gram negative bacteria especially *Pseudomonas aeruginosa*, the leading cause of Hospital Acquired Infection (HAI). Objective of the research was to find the efficacy testing of bacteriocin against multidrug resistant *Pseudomonas aeruginosa*, to isolate and identify multidrug resistant *Pseudomonas aeruginosa*. To extract and purify bacteriocin from lactobacillus acidophilus and to perform invitro efficacy testing of bacteriocin against multidrug resistant *Pseudomonas aeruginosa*. Conventional identification and antimicrobial susceptibility was performed based on conventional procedure. The study was done at the Clinical Microbiology Laboratory of Saveetha Medical College and hospital during the period of January to June 2020. Total 60 samples were collected from both male and female and from all the age groups. Among that 60 samples 39% were wound swab, 29% were urine, 5% were pus, 3% were tissue culture, 3% of samples were tracheal culture. The strains of *Pseudomonas aeruginosa* collected for this study shows resistance to cefepime, ceftazidime, ciprofloxacin, meropenem, imipenem, gentamicin, cotrimaxazole and piperacillin-tazobactam. Bacteriocins were protein in nature and simple proteins to particles resembling components of bacteriophage. The zone of inhibition formation is strong in vitro and can act against the pathogenesis of *Pseudomonas aeruginosa*. Disinfectants have ineffective in controlling the nosocomial infection in the spread of *pseudomonas aeruginosa*. Bacteriocins not only can support the disinfectants, it can also be combined with antibiotics when purified and processed.

Introduction

Pseudomonas aeruginosa is a Gram-negative, non-fermentative, encapsulated, rod shaped bacterium that can cause disease in plants, animals, and humans. *Pseudomonas aeruginosa* was discovered by a French bacteriologist and chemist, CarleGessard in 1882, it includes 0.5 to 0.8 um by 1.5 to 3 um rod shaped bacterium having one single polar flagellum and it is actively motile. *Pseudomonas aeruginosa* is multidrug resistant pathogen. It is associated with serious illness such as hospital acquired infection and various sepsis syndromes. *Pseudomonas aeruginosa* is an important community acquired pathogen (Gerhald P. Bodey *et al.,* 1983). *Pseudomonas aeruginosa* is typically an opportunistic pathogen that seldom causes disease in healthy subjects. Normally it causes infection by disturbing the physical barriers (skin or mucous membranes). It causes nearly 20% of infections in most hospitals. It is responsible for 10% of all hospital acquired infections. Infections caused by *Pseudomonas aeruginosa* is severe and life threatening. Occasionally, *Pseudomonas aeruginosa* can colonize moist areas of human body sites such as the perineum, axilla, ear, nasal mucosa and throat (Waheedullah *et al.,* 2017). Patients admitted in hospital for longer period are colonized by *pseudomonas aeruginosa* and are high risk of developing infection due to virulence factors such as protease, phospholipase, biofilm forming ability, quorum sensing etc., *Pseudomonas aeruginosa* strains may be transmitted from patient to patient and sometimes lead to outbreaks among cystic fibrosis patients attending the same hospital. Pseudomonas infection is especially prevalent among patients with burn wounds, cystic fibrosis, acute leukemia, organ transplants and intra venous drug addiction. The most serious infection includes malignant external otitis, endophthalmitis, endocarditis, meningitis, pneumonia and septicaemia. *Pseudomonas aeruginosa* is difficult organism to control with antibiotics or disinfectants. It is intrinsically resistant to antimicrobial agents due to low permeability of its cell wall and multiple other mechanisms. There are three basic mechanism by which organisms resists the action of antimicrobial agents, restricted uptake and efflux; drug inactivation and changes in targets (Lambert *et al.,* 2001). Silver nanoparticles could be used as a potential alternative therapy to reduce severity of disease due to *Pseudomonas aeruginosa* infections (Navindrakumari Palaniswamy *et al.,* 2014). Recent studies reported high effectiveness of herbal extracts against *Pseudomonas aeruginosa* (Meseret Mitiku *et al.,* 2014). Oregano combined with marjoram, thyme, or basil also had an additive effect against *Pseudomonas aeruginosa* (Guiterrez *et al.,* 2008). The history of bacteriocins begins with the Belgian scientist Andre Gratia. In 1925, as an early result of a search for bacteria with antimicrobial property; Gratia described the activity of colicin the first known bacteriocin. Bacteriocins did not receive the same level of attention as antibiotics, as a lack of understanding of their biology leads to difficulty in their production and low consistency in controlling their microbial growth (Zacharof and Lovitt, 2012). Currently, bacteriocin use is most often associated with the food industry. Many bacteriocins are produced by Gram- positive bacterias such as *Lactococcus* Sp. Nisin is the most widely used of these bacteriocins, acting as a food preservative, and has GRAS(Generally Recognized As Safe) status from the FDA (Food And Drug Administration)and is approved as preservative (food additive) in the European Union (E234). Bacteriocins are ribosomally synthesized antimicrobial peptides and have drawn attention in recent years due to their potential therapeutic applications in treating bacteria, including multiple drug resistant bacteria. Bacteriocins from lactic acid bacteria have been in use for a while as natural preservatives in food industry. Although bacteriocins were originally found to be produced by Lactobacillus only, it was subsequently shown to be produced by different species and multiple strains (Garima Sharma *et al.,*). Bacteriocins are generally safe, and stable, and have therapeutic potential as broad-spectrum antibiofilm agents (Singh *et al.,* 2012).

Bacteriocin is an antagonistic substance produced by another organism living in the same ecological niche

and they are considered to be antagonistic competitive inhibition. The production of these substances is a natural defense biological strategy. Bacteriocins are the protein antibiotics produced by bacteria against the closely related strains. However, their broad spectrum inhibitory activity is now well established against different bacteria, certain viruses, fungi and even protozoa (José Luis *et al.,* 2007).

Materials and Methods

The study was performed for a period of six months from January 2020 to June 2020, at Saveetha Medical College and Hospital, located at Thandalam, Kancheepuram. All samples received in the Clinical Microbiology Laboratory were processed in sequence as follows: Microscopy-Gram staining, culture, identification of bacteria by Bio-chemical test, Antibacterial susceptibility test of bacteriocin. 60 strains of *Pseudomonas aeruginosa* were isolated.

Bacteriocin extraction – Ammonium sulfate precipitation method: *Pseudomonas aeruginosa* SA188 was grown in BHI at 29 ^{o}C for 18 hours and the cells were separated by centrifugation at 6000 X g for 30 minutes at 4 ^{o}C. The cell free supernatant (CFS) was adjusted to pH 7.0 and was filter sterilized by a 0.45 µm pore size membrane filter. This crude bacteriocin preparation was partially purified by 70% Ammonium sulfate precipitation at 4 ^{0}C. The precipitate was sedimented by centrifugation at 6000 X g for 45 minutes at 4 ^{0}C. The resulting pellet was suspended in 50mM Sodium Phosphate Buffer of pH 7.0 and referred as partially purified bacteriocin preparation (12). The activity units (AU) of both crude and the partially purified bacteriocin preparations were determined by paper disc diffusion assay.

Bacteriocin efficacy disc diffusion assay (Bhuvaneshwari *et al.,* 2018)

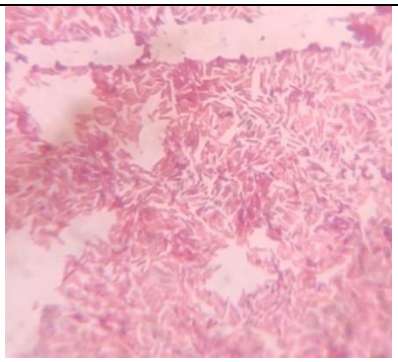
Disc diffusion assay was performed in Muller Hinton agar. The muller Hinton agar plates should be of 3-4mm thickness. Lawn culture of the inoculum was made on Mueller Hinton Agar after checking the turbidity standard. The turbidity standard for lawn culture is 0.5 µm in density checker. *Pseudomonas aeruginosa (ATCC 27853)* was used as control strain for doing lawn culture to test the bacteriocin efficiency. The filter paper disc was impregnated with prepared bacteriocin and was used as bacteriocin disc. The disc was placed in the lawn culture with a distance of 24 mm in a sterile glass plate of 100 mm in diameter. Totally 6 disc were placed, in the order of 5 in the periphery and 1 in the centre. Then the plates were incubated at 37 ^{o}C for 24 hours and the zone of inhibition was measured. Out of 6 disc atleast 4 discs should be having a inhibitory zone size of 21mm.

Results and Discussion

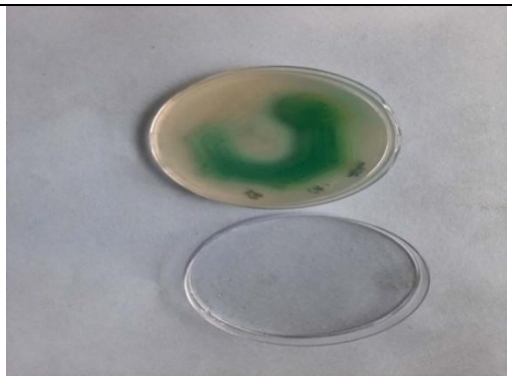
The prospective study was conducted at Clinical Microbiology Laboratory of Saveetha Medical College and Hospital during the period of mid of January 2020 to mid of January 2020.Ethical clearance was obtained. Out of 60 samples received 54% samples were from male and 46% samples were from female. Among 60 samples 30% samples were from patients above the age group of 50 years.25% samples were from the patients of age group 41 -50 years. 37% of samples were from age group of 21 -40 years.8% of samples were from patients of age group 0-20.

Out of 60 samples, 39% samples were wound swab, 29% samples were urine, 5% samples were pus, 3% of the samples were tissue culture, 3% of the samples were of tracheal culture, 12% of the samples were sputum, 4% of the samples were from blood, 3% of the samples were obtained from ear swab, 1% of the sample was from bronchial wash, 1% of the sample was from endotracheal culture. Out of 60 pathogenic strains, 90% strains showed zone of inhibition greater than and equal to 24 mm, 7% strains showed zone of inhition 23 mm, 3% of strains showed zone of inhibition 20 mm.

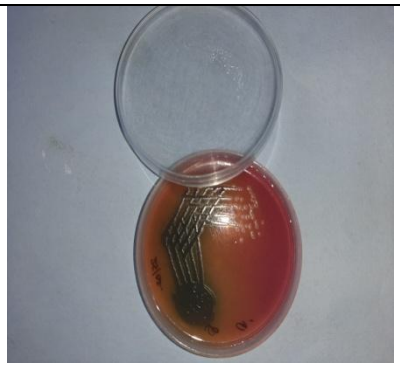
Fig.1 (a) Gram staining of *pseudomonas aeruginosa*
(b) *Pseudomonas aeruginosa* showing green pigments in nutrient agar
(c) *Pseudomonas aeruginosa* in Macconkey
(d) Bacteriocin efficiency
(e) Antibacterial susceptibility testing of Bacteriocin against *Pseudomonas aeruginosa*



(a)



(b)



(c)

(d)

(e)

In this present study 60 samples were collected from patients with *Pseudomonas aeruginosa*. The samples were collected from the period of mid of January 2020 to June 2020.The samples received to Clinical Microbiology Laboratory of Saveetha Medical College and Hospital was included. Out of 60 samples, 54% were collected from male and 46% were collected from female patients.

Among 100 samples that were included in this study, 39% sample was wound swab, 29% of the sample was urine, 12% of the sample was sputum, 5% of the sample was from pus, 4% of the sample was from blood and the rest of the samples were ear swab (3%), bronchial wash (1%) and finally endo-tracheal sample (1%).

In this study 30% of the samples from patients of age group above 50 years,25% of samples from patients of age group 41-50,37% of samples from the age group of 21 -40,8 % of samples from the age group of 0-20.

The *pseudomonas aeruginosa* strains collected for this study showed resistance to Cefepime (19%), followed by Ceftazidime (28%), Ciprofloxacin (26%), Meropenem (25%), Imipenem (26%), Gentamicin (22%), Co-trimaxazole (16%) and Piperacillin-tazobactam (21%). In a study conducted by Piyush Tripathi *et al.,* there was similar co-relation of antibiotic resistant pattern of *Pseudomonas aeruginosa* which showed a slightly higher resistance to drugs, Cefefpime (36.27%), followed by Ceftazidime (35.30%), Ciprofloxacin (31.37%), Gentamicin (28.43%), Meropenem (20.59%) and Levofloxacin (32.35%). Bacteriocins are antibiotic substances produced by many species of bacteria which are thought to be inhibitory for strains of the same or closely related species. Bacteriocins are protein in nature and range from simple proteins to particles resembling bacteriophage components.

In a study conducted by slim smaoui (Slim Smaoui *et al.,* 2010), shows the supernatant culture of the strain inhibits the growth of all tested pathogenic including the four Gram-negative bacteria *(Salmonella enterica* ATCC43972, *Pseudomonas aeruginosa* ATCC49189, *Hafnia sp*. And *Serratia sp)*.

In a study conducted by Valerie Aloush (Valerie Aloush *et al.,* 2006) the problem of antibiotic resistance in *Pseudomanas aeruginosa* is on the increase. A study in Boston Hospital revealed 22 cases of MDR *Pseudomonas aeruginosa* that were related to denovo emergence of resistance during treatment.

In a study conducted by Annabel H. A Parret (2002) *Pseudomonas aeruginosa* frequently produce proteinaceous, narrow spectrum antibacterial bacteriocins known as pyocins, the bacteriocins of different types inhabiting diverse nitches.

In a study conducted by Yvon Michel-Briand, Christine baysse (Yvon Michel-Briand and Christine Baysse), pyocins are produced by more than 90% of *Pseudomonas aeruginosa* strains, they povoke a depolarization of cytoplasmic membrane in relation with pore formation. Considering that Disinfectants have been ineffective in controlling the nosocomial infection spread of *Pseudomonas aeruginosa* as per the literature review, bacteriocins can be used to support the disinfectants. Bacteriocins can not only support disinfectants; they can also support antibiotics when made in synergism. So, further studies can be done in the area of using bacteriocins to support antibiotics in vivo because of the emergence of multidrug resistant and extreme drug resistant *Pseudomonas aeruginosa*. Based on this study the exact molecule responsible for antibacterial activity will be decided and purified using column chromatography.

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