Antibacterial Activity \textit{Pseudomonas} sp. Isolated Rhizosphere against Methicillin Resistance \textit{Staphylococcus aureus} from Clinical Samples

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Abstract—These methicillin-resistant \textit{Staphylococcus aureus} (MRSA) is a substantial public health problem worldwide, causing significant morbidity and mortality. Methicillin-resistant \textit{S. aureus} is the leading cause of skin and soft tissue infection in patients reporting to emergency departments for treatment. The different types of biosurfactants with antimicrobial activity include lipopeptides, glycolipids, phospholipids and lipopolysaccharides. A few lipopeptides have been reported to produce by Gram-negative bacteria like \textit{Pseudomonas}. The aim of this study was to antibacterial activity of a compound from \textit{Pseudomonas} sp. against MRSA strains from clinical samples. The \textit{Pseudomonas} sp. was isolated from Rhizosphere in North Iran. The antimicrobial activity of cell-free supernatant and partially purified bacteriocin was determined by well diffusion method. A total of 100 \textit{S. aureus} isolated from clinical specimens. The bacteria were primarily identified by colony morphology, microscopy of Gram’s stain and routine biochemical tests and antibiotic disk sensitivity tests (Cefoxitin; Ceftriaxox; Amoxicillin; Cefotaxime; Co-trimoxazole; Penicillin; Tetracyclin; Azitromycin). Results of this study showed the isolate \textit{Pseudomonas} sp. broadest antimicrobial spectrum against MRSA isolated clinical samples. However, the spectrum of inhibitory activity of these bacteria suggests a potentially useful means for controlling the growth of food-borne pathogens bacteria such: \textit{S. aureus} and MRSA isolated food samples.

Index Terms—MRSA, rhizosphere, antibiotic activity, clinical sample.

I. INTRODUCTION

\textit{Staphylococcus aureus} is a major cause of serious hospital, and community-acquired bacteremia worldwide and is associated with a high morbidity and mortality [1]. The emergence of methicillin-resistant \textit{S. aureus} (MRSA) strains since 1961 has complicated the treatment of \textit{S. aureus} infections, and glycopeptides (vancomycin or teicoplanin) are, in many cases, the only therapeutic alternative. In recent years, new anti-staphylococcal antibiotics, such as linezolid or daptomycin, have been developed, but their cost and the absence of large clinical trials demonstrate clear superiority over vancomycin maintains glycopeptides as a first-line option when infection due to MRSA is suspected or diagnosed [2], [3].

In the 1960s and 1970s, replacement of anti-biotic-resistant bacteria with another kind but similar antibiotic susceptible bacteria was used to inter-rup the \textit{S. aureus} outbreaks in nurseries in order to reduce recurrent furunculosis in persistent carriers. Unfortunately, a “nonpathogenic” strain, \textit{S. aureus} 502A, was not always nonpathogenic and its use fell out of favor [4], [5].

One non-antibiotic strategy to combat the bacterial infections involves the selection and promotion of endogenous barrier flora to interfere with pathogenic bacterial adhesion [4].

Currently, MRSA strains account for many of the staphylococcal infections and reports of MRSA strains are increasing worldwide [6]. There are also several reports from Iran showing the prevalence of methicillin resistance among clinical isolates of \textit{S. aureus} [7], [8]. Since the first isolates of MRSA were identified in the United Kingdom in 1961, MRSA has been a primary cause of health care–associated infections throughout Europe, Asia, Australia, and the United States [9], [10].

Methicillin-resistant \textit{Staphylococcus aureus} (MRSA) is a formidable bacterial pathogen responsible for a variety of infections commonly seen in patients of all ages [11], [12]. The MRSA strains associated with hospitals are referred to as hospital-acquired MRSA (HA-MRSA) and are the most common cause of hospital-acquired infections [13], [14]. Methicillin-resistant \textit{S. aureus} is the leading cause of skin and soft tissue infection in patients reporting to emergency departments for treatment [15], with a rising rate in primary care clinics [16] and intensive care units. Invasive MRSA related conditions most commonly reported include septic shock (56%), pneumonia (32%), endocarditis (19%), bacteremia (10%), and cellulitis (6%) [17].

The rhizosphere, representing the thin layer of soil surrounding plant roots and the soil occupied by the roots, supports large active groups of bacteria [18]. Several rhizobacteria have been used extensively as biological agent to control many soil borne plant pathogens [19]. The different types of biosurfactants with antimicrobial activity include lipopeptides, glycolipids, phospholipids and lipopolysaccharides [20] A few lipopeptides have

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been reported to produce by Gram-negative bacteria like Pseudomonas [21].

II. MATERIALS AND METHODS

A. Clinical Specimens

One hundred swab samples were collected in 2014 on mannitol salt broth medium from different hospital Tehran (IRAN). Samples were transported in an ice box (at 4 °C) Microbiological examination was performed within 24 h.

B. Confirmation of Saureus

The samples collected on mannitol salt broth media were incubated at 37°C for 24 h and then inoculated onto mannitol salt agar media and incubated at 37°C for 24 h. Selected isolates with colony morphology, Gram stain reactions and biochemical characteristics (ie, Coagulase, DNase, Manitol utilization).

C. Confirmation of MRSA and Antibiotic Resistance Testing

All isolates of S. aureus that demonstrated any level of Oxacillin (Methicillin) and Cefoxitin (FOX) resistance. All cultures were grown on Mueller-Hinton agar plates at 37 C for 18 hours in the presence of the following antibiotics: ampicillin(10μg), Cefotaxime (30 μg), Cefoxitin (30μg), Penicillin (10U), Oxacillin (1.0μg), Trimethoprim-sulfamethoxazole (SXT; 1.25μg/23.75μg), Tetracycline (30μg), Azitrmycin(15μg) and Ceftriaxon(30μg) (PT Diagnostic Systems). The zone of inhibition (in mm) around each disk was documented and compared with a standard interpretivechart [22].

D. Isolation of Pseudomonas sp. from Rhizosphere Soil

1g of rhizosphere soil sample was suspended in 99 ml of sterile saline solution. Samples were serially diluted (10⁵) and 0.1 ml of sample was spreaded on King’s B agar plates and incubated at 37°C for 24 h. The isolated bacteria was confirmed by morphological and biochemical characteristics, based on Bergeys’ Manual of Systematic Bacteriology.

E. Purification of Protein

A 18-h-old culture of the bacteriocinogenic LAB strain was centrifuged (∼9000g, 20 min, 4°C) and the peptic fraction precipitated from the cell-free supernatant with 70% saturated ammonium sulphate [23]. Some bacteriocins can precipitate at lower ammonium sulphate concentrations, or even in a small range of saturation, then is important to assay which is the concentration of salt that precipitates the peptide of interest. The suspension was incubated overnight at 4°C and agitated with a magnetic stirrer. Salted-out proteins were precipitated by centrifugation (∼10000g for 20 min) and dissolved in a small volume of 10 mM phosphate buffer (pH 7.0) or distilled water. The suspension can be desalted by dialysis with phosphate buffer at 4°C during 12h by using benzoylated membranes (molecular weight cut off 1200; Sigma-Aldrich) or with dialysis cassettes with cut-off of 2000 to 3500 (Pierce Biotechnology, Inc).

Since most bacteriocins have a size smaller than 10000 Da, the use of regular dialysis bags with cut-off of 10000-12000 Da is inappropriate for this procedure.

F. Evaluation of the Antibacterial Effect by the Well Diffusion Technique

The antimicrobial activity of cell-free supernatant and partially purified protein was determined by well diffusion method [24]. Screening of Antibacterial Activity of Pseudomonas sp. isolated from rhizosphere soil by Agar well diffusion method. Antibacterial activity of Pseudomonas sp. isolated from rhizosphere soil was tested against target bacterial pathogens of health significance like MRSA isolated by in vitro techniques using Muller-Hinton agar plates at 37 °C for 24 h. MRSA suspensions of 10⁷ colony forming units (CFU)/ml were grown to log phase, and the well diffusion were treated with the antibacterial compounds. The plates were incubated at 37°C for 24 h, and the size of the inhibition halo diameter was evaluated (mm). The antibacterial effect was determined by measuring the size of inhibited halos formed around clinical samplese wells.

G. Identification Pseudomonas sp. Isolated from Soil by 16S rRNA Gene Sequence

Chromosomal DNA used for polymerase chain reaction (PCR) was prepared by using phenol-chloroform method [25]. The DNA fragments containing 16S rDNA were amplified from chromosomal DNA with primers pairs 27F (5- AGATTTGATCMTGGCTCAG -3) and 1492R (5- GGTACCTTGTAGACTT - 3) [26]. PCR reactions were performed in a DNA thermal cycler (Biorad, USA) in a total volume of 50 µl containing Master mix (Takara, Japan).

Amplification consisted of a 1 min denaturation step at 94°C, a minute annealing step at 58°C and a minute extension step at 72°C. The first cycle was preceded by incubation for 5 min at 94°C. After 35 cycles, there was a final 10 min extension at 72°C. Negative controls containing no DNA template were included in parallel. PCR products were separated in a 1.5% (w/v) agarose gel and were subsequently visualized by ultraviolet (UV) illumination after ethidium bromide staining.

III. RESULTS

A. Antibiogram Profile MRSA Isolated Clinical Samples

The one hundred S. aureus isolated from clinical sample twenty (20%) isolate confirm MRSA. Antimicrobial sensitivity test of the MRSA isolates recorded high resistance rate to different antibiotics (Table I). The current data showed that only 10% of MRSA isolates were susceptible to SXT. In the current study, 20 out of 20 isolates (100%) of MRSA were multidrug resistant. All were resistant to penicillins; amoxicillin; Cefoxitin; Tetracycline; Oxacillin; Ceftriaxon; Azitrimycin and Cefotaxime. The multi-drug resistance in MRSA isolates might be due to the antibiotic’s selective exposure.

B. Antimicrobial Activity Cell Free Supernatant and Purified Protein

The antimicrobial activity of CFCS and purified protein (PP) from CFCS were tested against MRSA by well diffusion assay (Fig. 1). The CFCS and PP exhibited an antibacterial effect on a broad range of MRSA strains.

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Concentration(μg)</th>
<th>Resistant</th>
<th>Sensitive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ampicillin</td>
<td>10</td>
<td>20</td>
<td>0</td>
</tr>
<tr>
<td>Cefoxitin</td>
<td>30</td>
<td>20</td>
<td>0</td>
</tr>
<tr>
<td>Oxacillin</td>
<td>1</td>
<td>20</td>
<td>0</td>
</tr>
<tr>
<td>SXT</td>
<td>1.25/23.75</td>
<td>18</td>
<td>2</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>30</td>
<td>20</td>
<td>0</td>
</tr>
<tr>
<td>Penicillin</td>
<td>10</td>
<td>20</td>
<td>0</td>
</tr>
<tr>
<td>Ceftriaxon</td>
<td>30</td>
<td>20</td>
<td>0</td>
</tr>
<tr>
<td>Azitrimycin</td>
<td>15</td>
<td>20</td>
<td>0</td>
</tr>
<tr>
<td>Cefotaxime</td>
<td>30</td>
<td>20</td>
<td>0</td>
</tr>
</tbody>
</table>

**Table I. Prevalence of Antibiotic Resistance Among MRSA Isolates**

In the current study, it showed that CFCS and PP *Pseudomonas* sp. isolated from rhizosphere broadest antibacterial spectrum against MRSA isolated clinical specimens. All isolates were sensitive to Cell free supernatant and purified protein *Pseudomonas* sp. (Table II).

**Table II. Antimicrobial Activity Spectrum of the Cell-Free Culture Supernatant and Partially Purified Protein of Pseudomonas sp.**

<table>
<thead>
<tr>
<th>Code isolate</th>
<th>Diameter of Zones Inhibition (mm)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Cell free Supernatant(100μl/well)</td>
</tr>
<tr>
<td>Clin1</td>
<td>18</td>
</tr>
<tr>
<td>Clin2</td>
<td>17</td>
</tr>
<tr>
<td>Clin3</td>
<td>17</td>
</tr>
<tr>
<td>Clin14</td>
<td>17</td>
</tr>
<tr>
<td>Clin25</td>
<td>18</td>
</tr>
<tr>
<td>Clin57</td>
<td>16</td>
</tr>
<tr>
<td>Clin59</td>
<td>17</td>
</tr>
<tr>
<td>Clin63</td>
<td>16</td>
</tr>
<tr>
<td>Clin67</td>
<td>18</td>
</tr>
<tr>
<td>Clin69</td>
<td>19</td>
</tr>
<tr>
<td>Clin74</td>
<td>17</td>
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<tr>
<td>Clin75</td>
<td>16</td>
</tr>
<tr>
<td>Clin78</td>
<td>17</td>
</tr>
<tr>
<td>Clin83</td>
<td>18</td>
</tr>
<tr>
<td>Clin86</td>
<td>18</td>
</tr>
<tr>
<td>Clin91</td>
<td>19</td>
</tr>
<tr>
<td>Clin96</td>
<td>18</td>
</tr>
<tr>
<td>Clin98</td>
<td>16</td>
</tr>
</tbody>
</table>
IV. DISCUSSION

Over the past few years, studies concerning bacteriocins produced by LAB have received an increasing interest because of the potential use of bacteriocins as food preservatives [27]. Bacteriocin-producing isolate *Pseudomonas* sp. was isolated from by rhizosphere Soil, direct plating method. The isolate *Pseudomonas* sp. showed the broadest antimicrobial spectrum against MRSA isolated clinical samples. However, the spectrum of inhibitory activity of these bacteria suggests a potentially useful means for controlling the growth of food-borne pathogens bacteria such: *S. aureus* and MRSA isolated food samples.

According to the study Laine et al (1996) have found that *Pseudomonas* sp. Antimicrobial activity against food poisoning bacteria and moulds [28]. We suggestion that purified protein of *Pseudomonas* sp. could be used in the food preservation.

REFERENCES


Hanieh Asli Kousha received the B.S in Biology from Azad University of central Tehran (2012) and the M.S in Microbiology from the Islamic Azad University of Tonekabon Branch, 2014. Both activities and interest of Hanieh Asli Kousha are in environmental microbiology and food microbiology, especially in probiotics. Hanieh Asli Kousha has 5 ISI articles in international journals and has participated in an international congress.