The Incidence of Methicillin Resistance Staphylococcus Aureus in Tobacco Users

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Abstract:
Objectives: This study was carried out to detect methicillin resistant staphylococcus aureus among tobacco users in the Port Sudan city, Sudan, using PCR technique to help reduce morbidity and mortality among tobacco users during the period from February to June, 2018.

Materials and Method: 100 (one hundred) Mouth swabs were obtained randomly from tobacco users. The specimen was cultured on blood agar with novobiocin disc and mannitol salt agar for primary isolation of Pathogen. Identification of isolate was done by colonial morphology, gram stain and biochemical tests. Modified Kirby-Bauer disc diffusion method was adopted to determine the resistance rate of staphylococcus aureus to Methicillin disc. Then mecA gene detection was done used PCR technique.

Results: A 70 (seventy) staphylococcus aureus was isolated from the mouth swab, and 14 (fourteen) MRSA was isolated out of this 70 (seventy) staphylococcus aureus, 1 (one) out of this were positive for mecA gene. MRSA isolate was found to be high among tobacco consumer for long period using rather than those who tobacco consuming for short period. The result revealed that the antimicrobial resistance of S. aureus for Methicillin was (20%).

Conclusion: This study concluded that there is correlation between tobacco use and duration of use and increasing susceptibility to MRSA infection

Key words tobacco users, MRSA, staphylococcus aureus.

INTRODUCTION

Tobacco is a major global health problem and one of the main causes of death overall worldwide.[1] The consuming of tobacco is common overall world-wide, but people of many regions, including southern parts of United States, Scandinavian countries, southern African countries and the Sudan in northeast Africa, have a long history of tobacco use.[2] Tobacco in the Sudan known as toombak and made up from fermented ground tobacco mixed with sodium carbonate.[3] The Snuff is usually placed in the buckle lower or labial vestibules of the oral cavity, in long period users leading to cause various diseases including periodontal diseases, cancer of the oral cavity and pharynx.[3-5] Periodontal diseases is a chronic infectious disorders caused primarily by bacteria.[4] Tobacco is containing a numbers of carcinogens substance that make the tobacco is most important risk factor for cancer.[2]
Antimicrobial resistance is a major worldwide health problem concern, and drug resistant 
*Staphylococcus aureus* is a serious threat.[6] *staphylococcus aureus* is the most clinically significant species responsible for skin, soft tissue, bacteremia and endocarditis and this infections ranging from relatively mild to life-threatening condition.[7,8] Methicillin resistant *staphylococcus aureus* (MRSA), a potential dangerous type of staph bacteria that causes severe morbidity and mortality worldwide.[9] The first strains of MRSA emerged during the 1960s.[10] Unlike the penicillin resistance in *Staphylococcus aureus*, this resistance was mediated by hyper production of beta-lactamases, modification of normal PBPs, Methicillin resistance is due to the acquisition of gene encoding a unique penicillin-binding protein PBP2a , its encoded by the meCA gene.[11,12]

The Centers for Disease Control and Prevention (CDC) has concluded that MRSA is an important etiological agent that responsible for both hospital and Community onset disease and remains to be done to further decrease risks of developing this infection.[13] The harmful effect of tobacco use lead to health problem in the mouth and developed to be complicated, infection of Staph in tobacco users characterized by the presence of ulcer in the lip that is red and warm to the touch. The harmful effect of MRSA infection is un-response to treatment and leading to cause fatal condition. MRSA infection among tobacco users is not investigated and researched enough; the purpose of this study was to examine the relationship among MRSA infection and tobacco using.

**MATERIALS AND METHODS**

A cross-sectional study was conducted from February to June, 2018 among tobacco users in the Port Sudan City. Port Sudan is a district of the Red Sea state, which is located in the north-east of the Republic of Sudan. Tobacco consumer adult male aged 19 years old to 55 years old were included into study. Data were collected by structured questionnaire.

**EXPERIMENTAL WORK**

**SAMPLE COLLECTION**

A multistage random sampling technique was used to select the study location. One hundred samples were obtained from various tobacco users and collected from the ulcerated area from lip by using swabs. The specimens were transported to the laboratory directly and inoculated onto plates of Blood Agar with Novobiocin disc and Mannitol Salt Agar (MSA) and then incubated aerobically at 37 °C for 24hours.

**BACTERIAL IDENTIFICATION**

The isolated *Staphylococcus aureus* identified by phenotypic method such as colony morphology and by performing Gram’s stain and biochemical tests.

**COLONIAL MORPHOLOGY**

**MANNITOL SALT AGAR**

Yellow colony (mannitol ferment), circular, small in size, smooth, convex, moist.

**BLOOD AGAR**

Beta-hemolytic, creamy colony, circular, small in size, smooth, convex, moist and sensitive to Novobiocin disc.

**GRAM STAIN**

The aim of present stain is to distinguish between gram positive and gram negative bacteria. Thin smear of the isolates was prepared on a clean glass slides by using sterile loop. The slides dried by air and then heat fixed by passing it through the flame and gram stain was performed, by covering the smear with crystal violet stain for 30 second and washed with water. Flood with lugol’s iodine for 30 second and then washed with water, then decolorized by 95% alcohol for 3 second and washed with water. The slide was counter stained with safranin stain for 1 minute.
and then washed with water. The slides were air dried and then examined under oil immersion lens 100 x.

**BIOCHEMICAL TESTS**

**CATALASE TEST**
This test was performed to distinguish between *Staphylococci* from *Streptococci*. Catalase is an enzyme that breakdown hydrogen peroxide into water and oxygen. A small amount of colony taken by wooden stick and immersed in 3% H₂O₂ Solution in test tube. The production of gas bubbles indicated as a positive test.

**COAGULASE TEST**
This test was used to differentiate *staphylococcus aureus* from coagulase-negative *staphylococci*. *Staphylococcus aureus* produces two forms of coagulase bound coagulase and free coagulase. Bound coagulase, otherwise known as "clumping factor", can be detected by carrying out a slide coagulase test, and free coagulase can be detected by using a tube coagulase test. The coagulase test was performed by the fibrinogen slide method; two drops of saline were putted onto the slide labelled with sample number, Test (T) and control (C). The two saline drops are emulsified with the tested organism by wooden stick. A drop of plasma (rabbit plasma anti coagulated with EDTA was used) placed on the inoculated saline drop corresponding to test, and mixed well, then the slide was rocked gently for about 10 seconds. The positive, macroscopic clumping was observed in the plasma within 10 seconds.

**DANse TEST**
This test was performed to confirm staphylococci aureus colonies. A loop was sterilized and allowed to cool, then a part from the colony was taken and inoculated as a line in DANse media and incubated at 37 °C for 18h, in the second day 1% N HCL was added in surface of media. The clear zone around the colony indicated as a positive test.

**ANTIBIOTIC SUSCEPTIBILITY TESTING**
This test was performed to determine the sensitivity of isolates to antibiotics. The resistance of *staphylococcus aureus* against Methicillin was observed by disk diffusion method in this study. Suspension from *staphylococcus aureus* in broth medium with a turbidity equivalent to 0.5 McFarland stander (0.5 mL barium chloride + 99.5 mL of sulfuric acid) was prepared, from each inoculated suspension was cultured on Mueller Hinton agar by cotton swab and the Disc of Methicillin (5μg) was applied to the surface of agar plate. Then the plates were incubated at 37°C for 18hours. Zone of inhibition was measured by a ruler in millimeter. The results were interpreted according to CLSI guidelines 2013. An inhibition zone diameter of mm was reported as methicillin resistant and ≥ 14 mm was reported as methicillin sensitive.

**MOLECULAR STUDY USING POLYMERASE CHAIN REACTION**

**DNA EXTRACTION**
The targeting DNA was extracted from bacterial cell by boiling method. To an Eppendorf tube 200μl of DW was added, then by using sterile loop two colonies was taken, and putted into the Eppendorf tube, then was dissolved by shaking and then 10μl from lysosome enzyme solution was added to Eppendorf tube and incubated at 37 °C in water bath for 30 minute. The Eppendorf tube then was transmitted to another water bath for boiling, boiled at 100 °C for 30 minute, then was centrifuged at 12.000 rpm for 15 minutes, with caution all the supernatant was transferred to new Eppendorf tube by using Automatic pipette and was stored at -20°C for PCR technique.

**PCR AMPLIFICATION AND PRODUCT DETECTION**

**PRIMES DESIGN**
Forward and reverse primers targeting mecA gene were designed used software program.

Volume 9, Issue 01, 2019
PCR AMPLIFICATION AND PRODUCT DETECTION

All extracted 16 MRSA samples were amplified by using PCR technique. Ready-mixed master mix tubes used with 5μl volume, 5μl of DNA template was added to each PCR tube, then 2μl of forward and reverse primers were added, and 13μl of distilled water, then was mixed well by gentle vortexing. PCR protocol was run: First DNA was initially denatured at 94 °C for 5 minutes, followed by 30 seconds, then primer annealing at 55°C for 30 seconds, and extension at 72°C for 30 seconds and final extension for 5 minutes. This process repeated for 40 cycles. Then PCR product along with 50 bp ladder control was detected using agarose gel diffusion (1%), and examined by gel documentation machine.

RESULTS

BACTERIAL IDENTIFICATION AND SENSITIVITY TESTING

The result showed that 70 out of 100 sample (70%) gave positive result for *staphylococcus aureus* (able to grow on mannitol salt agar and the gram stain reaction is gram positive cocci arrangement in cluster). (Table 1) Biochemical tests were used to confirm the identification of *staphylococcus aureus* isolated like; Catalase, Coagulase and DNase, were 100% positive. Fourteen samples were resistant to Methicillin and 56 were sensitive. The percent of methicillin resistant was 20% (Table 2) and the maximum zone of inhibition of methicillin against *staphylococcus aureus* was 6 mm and most frequent zone of inhibition was 8 mm, and the incidence of MRSA among tobacco users different age groups slightly higher in older age who used tobacco for long period of time (Fig. 1).

MOLECULAR DETECTION OF mecA GENE

One sample out of 14 methicillin resistant *staphylococcus aureus* was positive for mecA gene used MA1 and MA2 (Table 3), with band length of 210 bp (Fig. 2).

<table>
<thead>
<tr>
<th>Age</th>
<th>Mean Period of tobacco use</th>
<th>No of Sample taken</th>
<th>No of S.aureus isolate</th>
<th>NO of Other isolate</th>
<th>Percentage S.aureus</th>
<th>Percentage of Other</th>
</tr>
</thead>
<tbody>
<tr>
<td>19 - 29</td>
<td>4.7</td>
<td>20</td>
<td>14</td>
<td>6</td>
<td>14%</td>
<td>6%</td>
</tr>
<tr>
<td>30 - 39</td>
<td>7.9</td>
<td>17</td>
<td>11</td>
<td>6</td>
<td>11%</td>
<td>6%</td>
</tr>
<tr>
<td>40 - 49</td>
<td>13.5</td>
<td>18</td>
<td>14</td>
<td>4</td>
<td>14%</td>
<td>4%</td>
</tr>
<tr>
<td>50 - 59</td>
<td>28.2</td>
<td>23</td>
<td>15</td>
<td>8</td>
<td>15%</td>
<td>8%</td>
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<td>60 - 69</td>
<td>32</td>
<td>22</td>
<td>16</td>
<td>6</td>
<td>16%</td>
<td>6%</td>
</tr>
<tr>
<td>Total</td>
<td>86.3</td>
<td>100</td>
<td>70</td>
<td>30</td>
<td>70%</td>
<td>30%</td>
</tr>
</tbody>
</table>

| Table 2 Percentage of sensitivity against Methicillin disc for *staphylococcus* isolates. |
|---------------------------------|-----------------|-----------------|
| Sensitivity against Methicillin disc | Percentage of Sensitive | Percentage of Resistance |
| Sensitive                      | 56              | 80%             |
| Resistance                     | 14              | 20%             |

<p>| Table 3 Oligonucleotide primer used in the study. |</p>
<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
<th>Target</th>
</tr>
</thead>
<tbody>
<tr>
<td>MA1</td>
<td>5'-TGCTATCCACCCTCAACAGG-3'</td>
<td>MecA gene</td>
</tr>
<tr>
<td>MA2</td>
<td>5'-AACGTTGTAACCACCCCAAGA-3'</td>
<td>MecA gene</td>
</tr>
</tbody>
</table>
DISCUSSION
This study is first study carried in the Port Sudan city, the technique to detect an incidence of MRSA among tobacco users. No similar studies were carried out at the population in Sudan or other near places. The most studies that have been carried out were detection of MRSA among cigarette smoker’s and nasal carrier. The study shows total incidence of 67 for *staphylococcus aureus* and MRSA was among tobacco users in the Port Sudan city.

CONCLUSION
There is a correlation of tobacco using and the period of using with MRSA infection. Alarming and highlights the need for adjusted infection control measures to prevent MRSA.

RECOMMENDATIONS
I. Perform campaigns awareness to community about the effect of toombak use.
II. Ask the government to make some strict rules about toombak sale in order to reduce the number of tobacco users.
III. Conduct a further study on *staphylococcus aureus* isolation and Methicillin and vancomycin sensitivity testing.

ACKNOWLEDGMENT
We would like to express our thanks and appreciate tobacco user’s patient to be part of study. Special thanks to Microbiology department of Alahlia College, Sudan.

REFERENCES


Author Contributions: All authors were responsible for the structure of this paper and all authors approved the final versions for submission.

Conflicts of Interest: No conflict of interest.

Statement of originality of work: The manuscript has been read and approved by all the authors, the requirements for authorship have been met, and that each author believes that the manuscript represents honest and original work.

Source of support: Nil

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Volume 9, Issue 01, 2019