MOLECULAR AND MICROBIOLOGICAL STUDIES ON VANCOMYCIN RESISTANT *STAPHYLOCOCCUS AUREUS* (VRSA) STRAINS ISOLATED FROM BURNED PATIENTS

ABSTRACT:
The present investigation covered a total of seventy burned patients, 40 from Sidnawy Hospital, Zagazig University, Egypt, and 30 patients from King Fahd Hospital, Al Qassim, Saudi Arabia. The incidence of total microorganisms isolated from swab, blood, urinary and sputum cultures indicated that Gram-positive bacteria were the most predominant microorganisms representing 72 out of 121 total isolates (59.50%), while 49 isolates were Gram-negative (40.50%). The most common pathogenic bacterial group isolated from patients was *Staphylococcus aureus*, followed by *K. pneumonia* and *P. aeruginosa*, whereas, *S. epidermidis* and *Micrococcus* showed the lowest incidence. The antibiotics sensitivity against fifty seven *S. aureus* isolates indicated high resistance to ampicillin (63.15%) and methicillin (71.92%), intermediate to amoxicillin, and high susceptibility to imipenem and vancomycin except that, the four isolates no. 12, 30, 50 and 95 were multiresistant. These isolates were resistant to 11 antibiotics which include imipenem and vancomycin. Identification of the four isolates was confirmed molecularly using 16S rDNA gene sequence. The determined minimum inhibitory concentrations (MICs) of vancomycin antibiotic indicated differences in antibiotic resistance among the four tested *S. aureus* strains. Multiplex PCR amplification of *mecA* and *vanA* genes of Vancomycin resistant *S. aureus* and Bactericidal effect of essential oils

KEY WORDS:
Bacterial infections in burned patients, *mecA* and *vanA* genes of Vancomycin resistant *S. aureus* and Bactericidal effect of essential oils

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INTRODUCTION:  
Infection in the burned patient is a leading cause of morbidity and mortality and remains one of the most challenging concerns for the burn unit (Cochran et al., 2002). Burn is one of the most common and devastating forms of trauma. Patients with serious thermal injury require immediate specialized care in order to minimize morbidity and mortality. Significant thermal injuries induce a state of immuno-suppression, which predisposes infectious complications in burned patients (Church et al., 2006).

Antimicrobial resistance can increase complications and costs associated with procedures and burn treatment. An infected wound complicates the postoperative course and results in prolonged stay in the hospital.
and delayed recovery (Li, 1989). The pathogens that infect the burns are primarily Gram-positive bacteria such as methicillin-resistant S. aureus (MRSA), enterococci, group A beta-hemolytic Streptococcus, coagulase negative Staphylococcus, and Gram-negative bacteria such as Pseudomonas aeruginosa, and Klebsiella species. These latter pathogens are notable for their increasing resistance to a broad array of different antimicrobial agents. In addition, burn wounds are commonly infected with fungal pathogens (Gladwin and Trattler, 2007).

Antibiotic resistance in bacteria is a serious problem facing society today. There are many reasons for this problem, one of which is an overuse of antibiotics. Through evolution, tougher bacteria that cannot be killed by antibiotics are surviving and reproducing. Methicillin-resistant S. aureus (MRSA) become an endemic organism in many burn units (Stefani and Agodi, 2008).

Vancomycin resistance has been reported in clinical isolates of both coagulase-negative staphylococci and Staphylococcus aureus. The emerging threat of widespread vancomycin resistance poses a serious public health concern given the fact that vancomycin has long been the preferred treatment of antibiotic-resistant Gram-positive organisms (Dorman and Deans, 2000).

Sinsimer et al. (2005) stated that drug resistance, particularly vancomycin and methicillin resistance, in S. aureus continues to emerge as a significant public health threat in both the hospital and community settings. In addition to the limited treatment options, S. aureus strains acquire and express numerous virulence factors that continue to increase its ability to cause a wide spectrum of human disease. As a result, empirical treatment decisions are confounded and there is a heightened need for a diagnostic test (or assay) to rapidly identify antibiotic resistance and specific virulence determinants and indicate the appropriate treatment. Multiplex molecular beacon probes with real-time PCR were used for the rapid detection of drug resistance-determining genes and virulence factors in S. aureus.

Virulence factors are the mechanisms and strategies used by bacteria to initiate and establish infections (Roth and Hughes, 2004). The risk of invasive burn wound infection is influenced by the extent and depth of the burn injury, various host factors, and the quantity and virulence of the microbial flora colonizing the wound. Common burn wound pathogens such as S. aureus produce a number of virulence factors that are important in the pathogenesis of invasive infection. Virulence factors mediate a number of processes, including adhesion, nutrient acquisition, immune system evasion, leukocyte killing, tissue destruction, and bloodstream invasion. Also carries many intrinsic and acquired antimicrobial resistance traits that make infected burn wounds difficult to treat (Laupland et al., 2005).

Staphylococcus aureus also has a diverse array of virulence factors that facilitate adherence to host tissues, immune system evasion, and destruction of host cells and tissues, including coagulase, protein A, leukocidins, hemolysins, and superantigens. Resistance to methicillin in Staphylococcus aureus, and more recently emergence of resistance to glycopeptides and oxazolidinones, also complicate the treatment of burn wound infections and sepsis caused by this highly virulent organism (Meka et al., 2004). The broad range of infections caused by S. aureus is related to a number of virulence factors that allow it to adhere to surfaces, invade or avoid the immune system, and cause harmful toxic effects to the host. It has also been shown that not every S. aureus strain produces every virulence factor, or does not produce each to the same degree (Todar, 2008).

A large number of essential oils and their constituents were investigated for their antimicrobial properties against series of bacteria (Dapkevicius et al., 2002; Miura et al., 2002). Thyme and tea tree oils were the best antiseptic substance available for external wounds, it is valuable in the treatment of infections, also it has been traditionally used for a number of applications including skin problems such as burns, eczema and cuts bruises, treatment of circulation problems, joint inflammation, respiratory infections, tonsillitis, pharyngitis, bronchitis, cough, excellent in treating cold (Aqil et al., 2005). Thyme oil as a strong antioxidant, it had antibacterial activity against 25 different genera of bacteria (Shan et al., 2007).

The essential oil of thyme and tea tree showed a wide antibacterial activity against microorganisms that had developed resistance to antibiotics as methicillin resistant S. aureus (MRSA) and vancomycin resistant S. aureus (VRSA) (Yarwood et al., 2004). Tea tree oil have the ability to reduce or stop the production of certain virulence factors, this may be another mechanism by which tea tree oil worked to prevent or clear S. aureus infections (Nostro et al., 2004). A combination of plant extracts with antibiotics help to minimize concentrations and reduce sensory impact. Furthermore, these combinations may also control some bacteria that are known to show consistently high resistance to antimicrobials (Aqil et al., 2005).

The aim of present investigation was to study the distribution and incidence of different bacterial groups in wounds, blood,
urine and sputum of the burned patients. Studying susceptibility of the bacterial isolates to different antibiotics and some essential oils either separately or combined and their effects on production of virulence factors. Also, using molecular studies with PCR for 16S rDNA and the genes confirming methicillin (mecA) and vancomycin (vanA) resistance in tested strains were detected.

**MATERIAL AND METHODS:**

This study was conducted on seventy burned patients. Forty patients admitted to Sidnawy Hospital, Zagazige University, Egypt in the period from February to August 2010 and 30 patients from King Fahd Hospital, Al Qassim, Saudi Arabia in the period from September 2010 to March 2011.

**Isolation, purification, and identification of bacteria:**

Samples were collected from wounds, blood, respiratory and urinary tract of infected patients as pus, blood, sputum and urine samples, respectively. Swabs of the different collections were streaked on the surface of Nutrient agar, cystine lactose electrolyte deficient medium (C.L.E.D), MacConkey agar, Mannitol salt agar and Blood agar media (Murray et al., 1999).

Conventional methods for the identification and characterization of pure isolates were employed according to Holt et al. (1994) and Murray et al. (1999), including Gram staining and biochemical reactions.

**Antibiotic susceptibility of tested S. aureus:**

Susceptibility of the tested isolates to fifteen antibiotics (ciprofloxacin, cefotaxime, amoxicillin, ampicillin/subbactam, chloromphenicol, rifampicin, erythromycin, tetracycline, oxycciline, cephardin, vancomycin, ampicillin, imipenem, methicillin and cefepime) was carried out using disk diffusion technique according to NCCLS (1999).

The minimum inhibitory concentrations (MICs) and Minimum bacteriocidal concentration (MBCs) of vancomycin 250 mg/L (a member of glycopeptide family) against the selected S. aureus strains were carried out using the standard agar dilution susceptibility test (Goldstein et al., 1978). In agar dilution method test, different antibiotic concentrations were incorporated in melted agar medium seeded with tested bacteria and the growth was determined as CFU/ml after 24 hr. The lowest concentration of drug, which completely inhibits the growth, disregarding a single colony or faint haze of growth, was defined as the minimum inhibitory concentration of the drug (Wenger et al., 1976).

**Molecular characterization of VRSA strains:**

**-Identification by using 16S rDNA:**

Identification of selected VRSA isolates was confirmed by using 16S rDNA genes sequence. The DNA was extracted from bacteria according to Sambrook and Russel (2001).

**-Detection of van A gene and mec A gene:**

For detection of mec and van genes, four primer systems were designed, optimized and used for this purpose.

The mec and van genes were amplified by polymerase chain reaction (PCR) using primer systems listed in table 1 (Sinsimer et al., 2005; Zhang et al., 2005).

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Primer Name</th>
<th>Size (bp)</th>
<th>Primer sequences</th>
<th>Tm (°C)</th>
<th>Product size (bp)</th>
<th>Concentration (μmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>mecA1</td>
<td>FmecA1</td>
<td>24</td>
<td>TGCTCAGGATCTGGCTATCCACCC ACCACCAATTTTGCTGCGAGTT</td>
<td>68.5</td>
<td>818</td>
<td>0.4</td>
</tr>
<tr>
<td></td>
<td>RmecA1</td>
<td>23</td>
<td>ACCACCAATTTTGCTGCGAGTT</td>
<td>62.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>mecA2</td>
<td>FmecA2</td>
<td>24</td>
<td>GGCTCAAATCTGGATCCACCC ACCACCAATTTTGCTGCGAGTT</td>
<td>68.5</td>
<td>818</td>
<td>0.2</td>
</tr>
<tr>
<td></td>
<td>RmecA2</td>
<td>23</td>
<td>ACCACCAATTTTGCTGCGAGTT</td>
<td>62.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>vanA1</td>
<td>FvanA1</td>
<td>20</td>
<td>AGTCGACAAATTGGATATGGG GGGATAAACGACTGGTATGAC</td>
<td>53.9</td>
<td>945</td>
<td>0.4</td>
</tr>
<tr>
<td></td>
<td>RvanA1</td>
<td>20</td>
<td>GGTTGCGATCCATAGCGGATCG</td>
<td>58.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>vanA2</td>
<td>FvanA2</td>
<td>20</td>
<td>GCTCGAATTTGTGAGGCTCCTGCT</td>
<td>56.4</td>
<td>945</td>
<td>0.2</td>
</tr>
<tr>
<td></td>
<td>RvanA2</td>
<td>20</td>
<td>TCGTATATTGGTCTGGCGAT</td>
<td>54.3</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Tm: melting temperature of primer

The thermocycler program composed of 4 min. at 95°C and then 30 cycles of 1 min at 94°C, 1 min at 40-55°C and 90 seconds at 72°C, followed by an addition 10 min. at 72°C. The optimal parameters and the annealing temperature in particular, had to be determined empirically depending on the fragment to be amplified. The annealing temperature was set at 46°C.

**Determination of virulence factors produced by vancomycin resistant S. aureus strains:**

The tested VRSA strains were screened for their capability to produce specific virulence factors, namely, hemolysin, protease and lecithinase enzymes on blood agar, casein agar and egg yolk agar media, respectively, at 35°C for 24 h using an agar well diffusion assay according to Klaenhammer (1988).
Antimicrobial activity of some essential oils against the VRSA strains:

The standard 9 volatile plant oils (Thyme, Cinnamon, Lemmon grass, Clove, Garlic, Olive, Tea tree, Peppermint and Nigella sativa) were used in this research and purchased from Sekam Company, Egypt.

The antimicrobial activity of tested oils against VRSA strains were carried out using well agar diffusion technique (Dorman and Deans, 2000).

The MICs of thyme oil and tea tree oil as the most potent oils against tested bacteria were determined in nutrient agar as described previously by Goldstein et al. (1978).

Influence of combination between vancomycin and (thyme or tea tree oil) against VRSA strains:

Different concentrations of vancomycin and thyme or tea tree oil were added in the nutrient agar medium seeded with tested bacteria and the growth was determined as CFU/ml after 24 h as previously detected.

Effect of tea tree oil on the virulence factors of the VRSA strains:

The tested S. aureus strains treated with different concentration of tea tree oil were examined for their capability of producing extracellular degrading enzymes as a virulence factors. Tween 80 (v/v) was included in all dilutions to enhance oil solubility. Determination of the enzymatic activities of different staphylococci was performed using agar diffusion assay according to Klaenhammer (1988) as previously mentioned.

RESULTS AND DISCUSSION:

Distribution of collected bacterial isolates:

Burn patients are ideal hosts for opportunistic infections. Infections remain the leading cause of death among patients who are hospitalized for burns (Tancheva and Hadijski, 2005). Gram-positive organisms of particular concern include methicillin-resistant S. aureus (MRSA) and coagulase negative Staphylococci. Methicillin-resistant S. aureus was first seen in the United States in the late 1960s and has become an endemic organism in many burn units. It has been argued that no extraordinary efforts be made to control its spread; however this view has been increasingly challenged in the era of vancomycin-resistant Enterococcus (VRE) (Zaid, 2001). In present investigation the incidence of total bacteria isolated from Egyptian and Saudi Arabian hospitals is shown in table 2 which indicate that Gram-positive bacteria were the most predominant. They represented 72 out of 121 total isolates (59.50%), while 49 isolates were Gram-negative bacteria (40.50%). Many investigators found that initially there is a colonization by Gram-positive organisms in wounds which is replaced later by Gram-negative organisms (Ahmad and Aqil, 2006).

Table 2. Distribution of collected bacterial isolates according to their Gram's stain reaction and source of isolation from Egyptian and Saudi Arabian Hospitals

<table>
<thead>
<tr>
<th>Locality</th>
<th>Source of isolation</th>
<th>Gram positive isolates</th>
<th>Gram negative isolates</th>
<th>Total No.</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Egypt</td>
<td>Wound culture</td>
<td>22</td>
<td>46.8</td>
<td>18</td>
<td>54.5</td>
</tr>
<tr>
<td></td>
<td>blood culture</td>
<td>17</td>
<td>36.2</td>
<td>9</td>
<td>27.3</td>
</tr>
<tr>
<td></td>
<td>Urinary &amp; sputum culture</td>
<td>8</td>
<td>17</td>
<td>6</td>
<td>18.2</td>
</tr>
<tr>
<td>Saudi Arabia</td>
<td>Wound culture</td>
<td>13</td>
<td>52</td>
<td>8</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>blood culture</td>
<td>6</td>
<td>24</td>
<td>5</td>
<td>31.25</td>
</tr>
<tr>
<td>Both hospitals</td>
<td>Total</td>
<td>25</td>
<td>60.98</td>
<td>16</td>
<td>39.02</td>
</tr>
</tbody>
</table>

Results in figure 1 showed that the most common pathogenic bacterial group isolated from patients was Staphylococcus aureus, followed by K. pneumonia and P. aeruginosa, whereas, S. epidermidis and Micrococcus showed the lowest incidence. These results are on line with Tiwari and Sen (2006). They found that S. aureus was the most common isolate from blood of burned patients followed by P.aeruginosa, and Coagulase Negative Staphylococci (CONS). On the other hand, Biswajit et al. (2008) reported that Gram-negative organisms were found to be more prevalent. Pseudomonas aeruginosa was found to be the most common isolate followed by Staphylococcus aureus, Staphylococcus epidermidis, Klebsiella and Salmonella.

Antibiotic susceptibility of bacterial isolates:

The occurrence of antibiotic-resistant bacteria (ARB) is a pressing public health problem worldwide. Many bacteria have become and continue to be resistant nearly against all antimicrobial agents (Xi et al., 2009). The antibiotic sensitivity measure the susceptibility of microorganisms to a range of
potential antimicrobial agents (Sahloff and Martin, 2002). The susceptibility of S. aureus, K. pneumonia, P. aeruginosa, S. epidermidis and Micrococcus isolates to different antibiotics was studied in the present investigation. According to standard evaluation of inhibition zone diameter of NCCLS (1999), all tested isolates exhibited variable effects against tested antibiotics.

The activities of fifteen different antibiotics against fifty seven S. aureus isolates which were recovered from different clinical specimens were determined. The results in table 3 suggested that high resistance was against ampicillin (63.15%) and methicillin (71.92%), high intermediate to amoxicillin, while high susceptibility to imipenem and vancomycin except the four multiresistant isolates no. 12, 30, 50, and 95 which were resistant against 11-12 antibiotics includes imipenem and vancomycin (Table 4). The reduced susceptibility to vancomycin by S. aureus is hypothesised to result from changes in peptidoglycan synthesis (Jacobs and Archer, 2003). There is a visible irregularly shaped and thickened cell wall in these vancomycin intermediate S. aureus VISA strains due to increased amounts of peptidoglycan. Evidently, there is a decrease in crosslinking of the peptidoglycan strands resulting in the exposure of more D-alanyl-D-lanine residues (Walsh and How, 2002). Extensive use of glycopeptides (vancomycin or teicoplanin) allowed selection of resistant isolates of S. aureus as recorded by Rybak and Akins (2001). The staphylococcal cell wall is a dynamic structure important for maintaining cell integrity and critical in host-pathogen interactions (Tomasz, 2006).

Table 3. Antibiotic susceptibility profile for tested Staphylococcus aureus isolates

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>S: No. of susceptible isolates</th>
<th>S: %</th>
<th>I: No. of intermediate isolates</th>
<th>I: %</th>
<th>R: No. of resistant isolates</th>
<th>R: %</th>
</tr>
</thead>
<tbody>
<tr>
<td>CIP (ciprofloxacin)</td>
<td>37</td>
<td>64.9</td>
<td>1</td>
<td>1.7</td>
<td>19</td>
<td>33.3</td>
</tr>
<tr>
<td>CTX (ceftaxime)</td>
<td>20</td>
<td>35.08</td>
<td>8</td>
<td>14.03</td>
<td>29</td>
<td>50.87</td>
</tr>
<tr>
<td>AX (amoxycillin)</td>
<td>17</td>
<td>29.8</td>
<td>10</td>
<td>17.5</td>
<td>30</td>
<td>52.6</td>
</tr>
<tr>
<td>SAM (ampicillin/sulbactam)</td>
<td>23</td>
<td>40.35</td>
<td>8</td>
<td>14.03</td>
<td>26</td>
<td>45.61</td>
</tr>
<tr>
<td>C (chlorompenicol)</td>
<td>32</td>
<td>56.14</td>
<td>8</td>
<td>14.03</td>
<td>17</td>
<td>29.82</td>
</tr>
<tr>
<td>RF (rifampicin)</td>
<td>37</td>
<td>64.91</td>
<td>1</td>
<td>1.75</td>
<td>19</td>
<td>33.33</td>
</tr>
<tr>
<td>E (erythromycin)</td>
<td>36</td>
<td>63.15</td>
<td>4</td>
<td>7.01</td>
<td>22</td>
<td>38.59</td>
</tr>
<tr>
<td>TE (tetracyline)</td>
<td>31</td>
<td>54.38</td>
<td>4</td>
<td>7.01</td>
<td>22</td>
<td>38.59</td>
</tr>
<tr>
<td>OX (oxycline)</td>
<td>27</td>
<td>47.36</td>
<td>2</td>
<td>3.50</td>
<td>28</td>
<td>49.12</td>
</tr>
<tr>
<td>CE (cephadin,.)</td>
<td>17</td>
<td>29.82</td>
<td>11</td>
<td>19.29</td>
<td>29</td>
<td>50.87</td>
</tr>
<tr>
<td>VA (vancomycin)</td>
<td>51</td>
<td>89.47</td>
<td>1</td>
<td>1.75</td>
<td>5</td>
<td>8.77</td>
</tr>
<tr>
<td>Am (ampicillin)</td>
<td>14</td>
<td>24.56</td>
<td>7</td>
<td>12.28</td>
<td>36</td>
<td>63.15</td>
</tr>
<tr>
<td>IPM (imipenem)</td>
<td>49</td>
<td>85.96</td>
<td>4</td>
<td>7.01</td>
<td>4</td>
<td>7.01</td>
</tr>
<tr>
<td>MET (methicillin)</td>
<td>12</td>
<td>21.05</td>
<td>4</td>
<td>7.01</td>
<td>41</td>
<td>71.92</td>
</tr>
<tr>
<td>FEP (cefpime)</td>
<td>28</td>
<td>49.12</td>
<td>9</td>
<td>15.78</td>
<td>20</td>
<td>35.08</td>
</tr>
</tbody>
</table>

S: Sensitive, R: Resistant, I: Intermediate

Table 4. Evaluation of inhibition zone for the VRSA strains against different antibiotic

<table>
<thead>
<tr>
<th>Isolate no.</th>
<th>C</th>
<th>CTX</th>
<th>AX</th>
<th>SAM</th>
<th>C</th>
<th>RF</th>
<th>E</th>
<th>TE</th>
<th>OX</th>
<th>CE</th>
<th>VA</th>
<th>AM</th>
<th>IPM</th>
<th>MET</th>
<th>FEP</th>
<th>No. of resistant antibiotics</th>
</tr>
</thead>
<tbody>
<tr>
<td>12</td>
<td>S</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>I</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>R</td>
<td>R</td>
<td>12</td>
</tr>
<tr>
<td>30</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>R</td>
<td>12</td>
</tr>
<tr>
<td>50</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>R</td>
<td>S</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>11</td>
</tr>
<tr>
<td>95</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>R</td>
<td>R</td>
<td>12</td>
</tr>
</tbody>
</table>

S: Sensitive, R: Resistant, I: Intermediate

In addition, Bonfiglio et al. (1998) concluded that under certain conditions, S. aureus can evade the host regulatory mechanisms that keep it in its normal state, erupting into a florid infection that can rapidly develop into a serious and even life threatening condition. S. aureus is also
notorious for its ability to develop broad antibiotic resistance. The new strain resistant to vancomycin, VRSA, is resistant to all known antibiotics. There are many different mechanisms by which microorganisms can express resistance to antibiotics, and some are very well documented: 1) they may produce enzymes that destroy the active drug, 2) they can change membrane permeability, 3) they express altered structural targets, 4) they express metabolic pathways that bypass the reaction inhibited by the drug, or 5) they express altered enzymes that are less affected by the drug (Salyers and Whitt, 2001).

The MICs and MBCs of vancomycin antibiotic against multiresistant S. aureus isolates:

The MICs and MBCs of vancomycin antibiotic against multiresistant S. aureus isolates were determined. The results in table 5 showed that, the highly resistance for vancomycin antibiotic was observed by S. aureus 12 and 50 than the other strains, this is online with Chang et al. (2003) and Proft et al. (2000). The difference in antibiotic resistance was related to the individual mechanisms of each strain and the appearance of different antibiotic resistant Staphylococci over the past years has been regarded as an inevitable genetic response to antibiotics (Sanders et al., 1984). Moreover, the peptidoglycan from different S. aureus strains is highly conserved, with almost identical high-performance liquid chromatography (HPLC) muropeptide patterns across strains, suggesting that the composition, and consequently the antibiotic resistance are not only species specific but also strain specific (Tomasz, 2006).

Table 5. Values of MICs and MBCs (µg/ml) of Vancomycin against the multiresistant S. aureus strains

<table>
<thead>
<tr>
<th>Tested bacteria</th>
<th>MIC (µg/ml)</th>
<th>MBC (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. aureus (12)</td>
<td>63</td>
<td>63</td>
</tr>
<tr>
<td>S. aureus (30)</td>
<td>31.25</td>
<td>63</td>
</tr>
<tr>
<td>S. aureus (50)</td>
<td>63</td>
<td>125</td>
</tr>
<tr>
<td>S. aureus (95)</td>
<td>31.25</td>
<td>63</td>
</tr>
</tbody>
</table>

The present data revealed that vancomycin was extended and selected for the further studies. Murphy et al. (2003) denoted that vancomycin is the drug of choice for therapy of the common causes of hospital acquired infections caused by antibiotic resistant S. aureus. Moreover, the change in resistance breakpoints for MIC of vancomycin against infected S. aureus over 20 years ago was associated between vancomycin MIC, vancomycin treatment failure and also increases the heteroresistant strains (Tenover and Moellering, 2007).

Molecular characterization of VRSA isolates: Identification of selected isolates by 16S rDNA:

According to standard evaluation of inhibition zone diameter of NCCLS (1999), four S. aureus isolates number (12, 30, 50, and 95) were selected as the vancomycin resistant isolates. The PCR amplification of 16S rRNA gene confirmed the identity (95%) of the selected isolates as S. aureus 12 (ZWFR 12), S. aureus 30 (ZWFR 30), S. aureus 50 (ZWFR 50) and S. aureus 95 (ZWFR 95) (Fig. 2). The partial nucleotide sequences of amplified gene (s) were submitted in DDBJ/EMBL/GenBank at web server (http://www.ddbj.nig.ac.jp/sub/ref8-e.html) under accession number (s) 12 [AB674512], 30 [AB674513], 50 [AB674514] and 95 [AB674515], respectively. Several genomic targets had been effectively used for identification of antibiotic resistant S. aureus as previously studied by Walker (1998) and Stefani and Agodi (2008).
Fig. 3. The phylogenetic tree of S. aureus no. 12 [AB674512].

Fig. 4. The phylogenetic tree of S. aureus no. 30 [AB674513].

Fig. 5. The phylogenetic tree of S. aureus no. 50 [AB674514].
The other *S. aureus* 30 and 95 strains were vanA gene negative. These results may due to presence of another gene for vancomycin resistance as van C or van R or others as suggested by Heggers *et al.* (2009). Also, to understand the mechanisms and potential impacts of vancomycin resistance in *S. aureus*, a clear understanding of the organism’s cell wall is required. Actually, thickening of the cell wall of Vancomycin-resistant staphylococci has been found to be associated with complex re-organization of cell wall metabolism with extra cell wall material showing reduced peptidoglycan cross-linking of D-Ala-D-Ala termini of side chains (Walsh and Howe, 2002; Biswajit *et al.*, 2008; de Niederhäusern *et al.*, 2011). These data may explain another resistant mechanism against vancomycin by the tested *S. aureus* strains.

**Specific virulence factors of the VRSA strains:**

The selected *S. aureus* strains were screened for their capability of producing degrading enzymes as a certain virulence factors by using well agar diffusion method. Data in Table 6 revealed that four examined strains produced the tested virulence factors including hemolysins, licithinases and proteases. *S. aureus* is notorious not only for its ability to rapidly acquire antibiotic resistance, but also for the wide variety of factors it uses in its defense against host immunity (Jansen *et al.*, 2007). These results are in accordance with data reported in other studies of Bamberger and Boyd (2005) who reported that most of isolated *S. aureus* strains were found to produce extracellular enzymes: licticinase, gelatinase and protease.
Table 6. The virulence factors (VF) s of selected VRSA strains using different volumes 10, 20, & 30 μl of the supernatant

<table>
<thead>
<tr>
<th>Bacterial isolates</th>
<th>Lecithinase</th>
<th>Hemolysin</th>
<th>Proteinase</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 μl</td>
<td>20 μl</td>
<td>30 μl</td>
<td>10 μl</td>
</tr>
<tr>
<td>12 S. aureus</td>
<td>20</td>
<td>24</td>
<td>26</td>
</tr>
<tr>
<td>30 S. aureus</td>
<td>10</td>
<td>12</td>
<td>15</td>
</tr>
<tr>
<td>50 S. aureus</td>
<td>22</td>
<td>24</td>
<td>28</td>
</tr>
<tr>
<td>95 S. aureus</td>
<td>18</td>
<td>21</td>
<td>27</td>
</tr>
</tbody>
</table>

Susceptibility of the VRSA to certain essential oils

Volatile oils had been widely used for their properties as antibacterial, antifungal, antiviral and insecticidal activities (Perry et al., 2003; Silva et al., 2003). Depending on oil type and concentration, they exhibit cytotoxic effects on living cells but are usually non genotoxic (Bakkali et al., 2008). The present investigations study the effect of different essential plant oils on growth of the tested S. aureus strains. The results represented in figure 8 illustrated that, thyme and tea tree oils had the highest antibacterial activity against tested S. aureus strains followed by clove and lemon grass. While, cinnamon oil had the lowest antibacterial activity against the tested strains. The results are in agreement with El-Aidy (2008) who found that thyme and tea tree oil were the most effective oil against E. coli, S. aureus, P. aerugenosa and K. pneumonieae.

Fig. 8. Effect of essential oils against the tested vancomycin resistant S. aureus strains

Effect of vancomycin separately or combined with thyme and tea tree oil on the VRSA strains:

The combination of antimicrobial agents are considered to be synergetic if the effect of combination is greater than the effect of either agent alone or greater than the sum of the effect of individual agents. Antagonism result occurred if the combination provides more effect than the effect of either agent alone or more than the sum the effect of individual agents (Cappelletty and Rybak, 1996). In the present study, the results in table 7 demonstrated that the combination between vancomycin with thyme oil, also vancomycin with tea tree oil gave a synergetic effect against the VRSA strains. The complete inhibition of microbial growth was achieved when 50% of MIC of vancomycin with 50% of MIC of thyme oil or 50% of MIC of tea was supplemented to the growth medium. This finding was in agreement with Mostafa (2005) and Abd El-Rahman (2008). Also, El-Aidy (2008) found that combination between impenem antibiotic and thyme oil gave highly synergetic effect against E. coli and S. aureus as 27 and 30 mm, respectively. Rational for the use of antimicrobial combination was to decrease emergence of resistance isolates, decreased dose-related toxicity of antimicrobial using the other agent (s) (Mandell and Sande, 1980) and to polymicrobial infection (it is one of the reasons for broad-spectrum coverage).
It has been observed previously that the suppression of extracellular protein synthesis occurs only with antibiotics that inhibit protein synthesis and that antibiotics with other modes of action may in fact have a stimulatory effect (Abo-Ghalia et al., 2004). Although tea tree oil is not an antibiotic, it inhibits the synthesis of the extracellular enzyme protease.

In our investigation on the virulence factors (VFs) production (lecithinase, hemolysin and protease) by S. aureus (12, 30, 50, and 95) under treatment of tea tree oil as more efficient oil indicated that, S. aureus (12, 30, 50, and 95) showed lower levels of lecithinase activity when cultured with 0.3% tea tree oil (Table 8). The four S. aureus (12, 30, 50, and 95) strains, produced lower levels of hemolysin and protease were produced in the presence of 0.6% tea tree oil as compared to control cells. In general, these experiments demonstrate reduction in levels of protease, hemolysin and lecithinase after growth for 24 h in the presence of 0.3 and 0.6% tea tree oil. The suppression of toxins and enzymes is an important part in the treatment and management of S. aureus (Chang et al., 2003). A thorough and complete understanding of the infections of these S. aureus products and components is necessary to apply the correct treatment and to prevent infections (Edwards-Jones and Foster, 2002). These results are in accordance with data reported in study of Iwalokun et al. (2003) who stated that the exposure to tea tree oil may alter cell permeability leading to the leakage of extracellular proteins. It is also hypothetically possible that the presence of tea tree oil affects the outermost part of the cell wall in such as way as to cause the extracellular proteins to become less highly associated with the cell wall and therefore more extracellular proteins is found free in the supernatant. Increased expression of VFs after treatment with sub-inhibitory levels of antimicrobial agents has been reported previously, although infrequently (Bisognano et al., 1997).

However, more studies are still needed to explore the definite mechanisms by which these isolates acquire resistance to vancomycin which may open the door to overcome this problem.

CONCLUSION:

Gram positive and Gram negative bacteria were of high distribution in burned patients. The presence of different pathogenic bacterial groups including S. aureus, P. aeruginosa, K. pneumonia and Micrococcus sp. and also of antibiotic multiresistant S. aureus represent significant public health problems. Molecular studies with PCR for 16S rDNA and van A and mec A genes were considered as tools for rapid detection of vancomycin resistant S. aureus strains. The essential plant oils as thyme and tea tree oils were the most potent against the tested S. aureus strains. The tested vancomycin resistant S. aureus VRSA strains exhibited significant reduction in levels of protease, hemolysin and lecithinase after growth for 24 h in the presence of 0.3 and 0.6% tea tree oil.

ACKNOWLEDGEMENT:

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REFERENCES:


Table 8. The effect of tea tree oil on virulence factors of tested isolates

<table>
<thead>
<tr>
<th>Diameter of inhibition concentration</th>
<th>Lecithinase</th>
<th>Hemolysin</th>
<th>Protease</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>12</td>
<td>30</td>
<td>50</td>
</tr>
<tr>
<td>0%</td>
<td>20</td>
<td>18</td>
<td>25</td>
</tr>
<tr>
<td>0.1%</td>
<td>18</td>
<td>7</td>
<td>12</td>
</tr>
<tr>
<td>0.3%</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>0.6%</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 7. Effect of Van, Thyme, & Tea tree oils separate or combined on the tested VRSA strains

<table>
<thead>
<tr>
<th>Tested strain</th>
<th>Inhibition zone (mm)</th>
<th>Thyme oil (30µg)</th>
<th>Thyme oil (30µg)</th>
<th>Tea tree oil (30µg)</th>
<th>Tea tree oil (30µg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. aureus 12</td>
<td>10.0</td>
<td>8.2</td>
<td>15.0</td>
<td>8.0</td>
<td>18.0</td>
</tr>
<tr>
<td>S. aureus 30</td>
<td>11.0</td>
<td>7.5</td>
<td>18.5</td>
<td>8.5</td>
<td>19.5</td>
</tr>
<tr>
<td>S. aureus 50</td>
<td>9.0</td>
<td>8.0</td>
<td>17.0</td>
<td>7.5</td>
<td>16.5</td>
</tr>
<tr>
<td>S. aureus 95</td>
<td>10.0</td>
<td>9.0</td>
<td>19.0</td>
<td>9.0</td>
<td>19.0</td>
</tr>
</tbody>
</table>


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ization and concomitant subtyping of staphylococcal
cassette chromosome mec types I to V in

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