Antimicrobial susceptibility profile of aerobic bacteria collected from diabetic foot ulcer infections, In Sulaimani Province

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ABSTRACT

This study was planned to determine the frequency and in-vitro susceptibility pattern of aerobic bacterial isolates from diabetic foot infections against different antimicrobial agents. A total of 62 isolated bacteria comprising of 44 (71%) Gram-positive bacteria and 18 (29%) Gram-negative bacteria were isolated from 50 specimens and the majority of infections were predominantly due to 38 (76%) mono-bacterial and 12 (24%) mixed-bacterial. All bacterial isolates were tested for their in-vitro susceptibility to antibiotics using disc diffusion technique and broth method [(MIC) minimum inhibitory concentration]. Anti-microbial susceptibility results showed that vancomycin showed activity against Gram-positive bacteria while both gentamycin and ciprofloxacin had overall anti-microbial activity against Pseudomonas aeruginosa and Escherichia coli. Whereas Serratia spp. susceptible to tested antibiotic except for streptomycin. Broth macrodilution susceptibility testing demonstrated that 35/62 (56.4% %) aerobic bacterial isolates exhibited a MIC of 1.5g/mL for leaf extract of Salix alba, and 62/62 (100%) were susceptible with a MIC of ≤ 3 g/mL.

1- INTRODUCTION

Diabetic foot ulceration are complex, chronic wounds, which have a major long-term impact on the morbidity, mortality and quality of patients’ lives and one of the main medical, communal, financial problems throughout the world (Islam et al., 2013). They need urgent control of the infectious process by suitable debridement and antimicrobial drug use to prevent potentially devastating complications (Lipsky et al., 2012). Most lesions in formerly untreated patients (acute infections) are infected by aerobic Gram-positive cocci (frequently as monomicrobial infections) while chronic infections and previously treated...
infections are often polymicrobial, usually with the addendum of aerobic or facultative anaerobic Gram-negative bacilli (Charles et al., 2015). Antimicrobial resistance is one of our greatest serious public health threats (WHO, 2014). The most significant cause of antimicrobial resistance is overusing or misuse of antibiotics (Ventola, 2015). Patients having diabetic foot ulcer infections face more serious problems including multidrug resistant microorganisms (Trivedi et al., 2014; Lipsky et al., 2012). The appearance and spread of multi-drug resistant strains with diabetic foot ulcer has made treatment much more complicated. Therefore, use of suitable antibiotics is necessary to avoid the risk of severity in foot infections of diabetic patients (Neto, et al., 2012). It is important to be most familiar with the common pathogens to prescribe empiric antibiotics. Salix plants have long been used for treatment of diseases. S. alba is in family salicacea, commonly known as white willow. S. alba was described to contain many bioactive compounds which possess well antibacterial properties. Consequently, this study was designed with the objectives to determine the bacterial profile and to assess the in vitro antimicrobial susceptibility profile of bacterial isolates from patients with diabetic foot ulcer (DFU) to varieties of commonly used drugs and chloroform leaf extract of Salix alba.

2- Materials and methods

2.1. Ethics Statement

Ethical approval was granted from the Sulaimani teaching hospitals and the University of Sulaimani. Informed assent was obtained from each diabetic foot ulcer patient regarding demographic characteristics.

2.2. Study design

Total of 50 diabetic patients admitted in the Sulaimani teaching hospitals, Kurdistan region - Iraq, had the ulcer in their foot during September 2014 to October 2015 were included in this study.

2.3. Isolation and identification technique

All specimens were selected from new admitted diabetic foot ulcer patients, Figure 1. In order to avoid the isolation of normal microbial flora, debridement was performed to
clean the wounds and to obtain specimens by tissue biopsy, wound curettage, or aspiration techniques (Zubair et al., 2011). Gram staining was routinely done on specimens to get information about the types of organisms present. The specimen was inoculated onto appropriate aerobic planting media, such as blood agar, MacConkey agar and Nutrient agar and the plates were incubated overnight at 37°C. Any significant colony grown after 24h was isolated and identified according to morphological, cultural and conventional biochemicals (catalase, oxidase, and coagulase). All the strains were confirmed with the Vitek 2 Compact automated system by using Gram-Negative and positive (GN-and GP ID) cards according to the manufacturer’s instructions (bioMerieux, Marcy l’Etoile, France).

2.4. Collection and extraction method of leaves of *Slix alba*

Leaf of *S. alba* collected between September and October 2015, at Topzawa-Dokan in the north of Iraq, because during this season leaves are almost completely developed (Szafranek et al., 2008). The healthy leaves were washed with demineralized water to remove the dust particles, dried, grinded and extracted with chloroform in Soxhlet equipment for 72 h, and then filtered by using whatman filter paper.

**Figure 1:** Neuropathic ulceration of the foot in a diabetic patient
2.5. Susceptibility testing

2.5.1. Disk diffusion method

Antimicrobial susceptibility testing of aerobic bacteria was performed using the disk diffusion method as recommended by the Clinical and Laboratory Standards institutes (Jeane B et al., 2015). Antimicrobial disks used were amikacin (30µg), amoxicillin (30µg), ampicillin (10µg), carbenicillin (100µg), cefuroxime (30µg), cefotaxime (30µg), gentamycin (30µg), erythromycin (15µg), tetracycline (30µg), penicillin (30µg), streptomycin (10µg), ciprofloxacin (30µg), rifampicin (30µg) and vancomycin (30µg). The 0.5 McFarland turbidity was prepared from pure colonies in brain heart infusion then streaked the swab over the entire surface of the Mueller Hinton agar plate. The antibiotic discs were applied with sterile forceps onto the surface medium. The inoculated agar plates were incubated at 37 °C for 24 h. The diameter of this zone was measured and compared with CLSI guidelines for zone sizes.

2.5.2. Determination of minimum inhibitory concentrations (MICs) of Salix alba leaf extract.

The MIC values of bacterial isolates were tested against extracted leaf. MICs were determined by using the broth macrodilution method (Szafranek et al., 2008). Briefly, 100 µL of adjusted bacterial suspensions equivalent to a 0.5 McFarland standard were added to a twofold serial dilution of extracted leaf that diluted in Mueller-Hinton broth. Based on the previously described method of Qureshi et al. (Qureshi et al., 2015), the antimicrobial activity of the extracts of Salix alba was studied in different concentrations (0.5, 1, 1.5 and 3 mg/mL) against six bacterial isolates. The results were observed after 24-hour incubation at 37°C. The minimum concentration of the extract inhibiting bacterial growth compared to the control culture was considered as the MIC. Duplicate tests were performed for each concentration.
3- Results

A total of 62 pathogenic bacteria were isolated from 50 specimens of patients with diabetes foot ulcer. The age of the patients ranged from 31-60 (mean age 50± 7) years, Table 1.

Table 1: Demographics of Diabetic Foot Patients

<table>
<thead>
<tr>
<th>Gender</th>
<th>Age</th>
<th>Frequency</th>
<th>Percentage %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>31-40</td>
<td>41-50</td>
<td>51-60</td>
</tr>
<tr>
<td>Male</td>
<td>9</td>
<td>15</td>
<td>28</td>
</tr>
<tr>
<td>Female</td>
<td>6</td>
<td>11</td>
<td>22</td>
</tr>
<tr>
<td>Total</td>
<td>15</td>
<td>26</td>
<td>50</td>
</tr>
</tbody>
</table>

Gram-positive bacteria were the most frequently isolated pathogens 44 (71%) including Staphylococcus aureus 18 (40.9%), Streptococcus pyogenes 16 (36.3%), Staphylococcus epidermidis 8 (18.1%), and Leuconostoc mesenteroides 2 (4.5%). Gram-negative bacteria accounted for 18 (29%) of all bacterial isolates. Pseudomonas aeruginosa was the most frequently isolated Gram-negative 10 (55.5%), followed by Escherichia coli 6 (33.3) and Serratia spp. 2 (11.1%), Figure 2. In the current study, mono-bacterial were isolated from 38 (76%) patients and mixed-bacterial infections were notice in 12 (24%) patients.
Figure 2: Frequency of bacteria isolated from diabetic foot patients.

Antibiotic resistance profile:

The results of antibiotics resistance studies are summarized in Table 2. Antimicrobial susceptibility results showed that Gram-positive bacteria were mainly resistant to rifampicin (63.6%), streptomycin (59%) and erythromycin (56.8.3%). The same rate of resistance (54.5%) was found to amikacin, penicillin, amoxicillin, ampicillin and tetracycline. Further, 47.7% of isolates were found susceptible to gentamycin, carbenicillin, cefuroxime, cefotaxime and ciprofloxacin. On the other hand, 93.2% of Gram-positive isolates were vancomycin-susceptible, the exception being 6.8%. Among the Gram-negative organisms, *P. aeruginosa* was resistant to most antimicrobial agents tested except for constant susceptibility to ciprofloxacin and gentamycin. *E. coli*, showed 100% sensitivity to vancomycin, gentamycin, ciprofloxacin and amikacin and 33% resistant to ampicillin, cefuroxime, and cefotaxime, while penicillin showed 83.3% resistance and 50% to the amoxicillin, carbenicillin, erythromycin and tetracycline. One *Serratia*
spp. isolate was susceptible to tested antibiotic except for natural resistance.

Table 2: Antimicrobial resistance pattern of gram-positive and gram-negative aerobes isolated in diabetic foot ulcer infections.

<table>
<thead>
<tr>
<th>Antimicrobial Agents</th>
<th>S. aureus n=18</th>
<th>S. pyogenes n=16</th>
<th>S. epidermidis n=8</th>
<th>L. mesenteroides n=2</th>
<th>P. aeruginosa n=10</th>
<th>E. coli n=6</th>
<th>Serratia spp. n=2</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amikacin</td>
<td>15</td>
<td>0</td>
<td>8</td>
<td>1</td>
<td>7</td>
<td>0</td>
<td>0</td>
<td>31</td>
</tr>
<tr>
<td>Amoxicillin</td>
<td>15</td>
<td>0</td>
<td>8</td>
<td>1</td>
<td>7</td>
<td>3</td>
<td>0</td>
<td>34</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>15</td>
<td>0</td>
<td>8</td>
<td>1</td>
<td>7</td>
<td>2</td>
<td>0</td>
<td>33</td>
</tr>
<tr>
<td>Carbenicillin</td>
<td>15</td>
<td>0</td>
<td>5</td>
<td>1</td>
<td>7</td>
<td>3</td>
<td>0</td>
<td>31</td>
</tr>
<tr>
<td>Cefuroxime</td>
<td>15</td>
<td>0</td>
<td>6</td>
<td>0</td>
<td>7</td>
<td>2</td>
<td>0</td>
<td>30</td>
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<tr>
<td>Cefotaxime</td>
<td>15</td>
<td>0</td>
<td>6</td>
<td>0</td>
<td>7</td>
<td>2</td>
<td>0</td>
<td>30</td>
</tr>
<tr>
<td>Gentamycin</td>
<td>15</td>
<td>0</td>
<td>6</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>21</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>15</td>
<td>4</td>
<td>6</td>
<td>0</td>
<td>7</td>
<td>3</td>
<td>0</td>
<td>35</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>15</td>
<td>0</td>
<td>8</td>
<td>1</td>
<td>7</td>
<td>3</td>
<td>0</td>
<td>34</td>
</tr>
<tr>
<td>Penicillin</td>
<td>15</td>
<td>0</td>
<td>8</td>
<td>1</td>
<td>7</td>
<td>5</td>
<td>0</td>
<td>36</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>15</td>
<td>4</td>
<td>6</td>
<td>1</td>
<td>7</td>
<td>NT</td>
<td>1</td>
<td>34</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>15</td>
<td>0</td>
<td>5</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>21</td>
</tr>
<tr>
<td>Rifampicin</td>
<td>15</td>
<td>5</td>
<td>8</td>
<td>0</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>28</td>
</tr>
<tr>
<td>Vancomycin</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>NT</td>
<td>0</td>
<td>NT</td>
<td>3</td>
</tr>
</tbody>
</table>

NT: Not tested

Leaf extract of S. alba susceptibility testing by the broth macrodilution method

In all experiments, positive controls without leaf extract yielded growth of the organism in 24hrs. Susceptibility testing demonstrated that 9/18 (50%) S. aureus, 9/16 (56.25%)
Streptococcus pyogenes, 6/8 (75%) S. epidermidis, 2/2 (100%) L. mesenteroides, 5/6 (83.3%) E. coli and 4/10 (40%) P. aeruginosa exhibited a MIC of 1.5 mg/mL for leaf extract. We observed that all bacterial isolates were susceptible to 3 mg/mL leaf extract, Figure 3.

Figure 3: Result susceptibility testing of bacterial isolates to chloroform leaf extract of S. alba.

4- Discussion

With the increasing prevalence of diabetes mellitus, there is rising problem of infections, particularly foot ulcer infections. Diabetic foot ulcers may cause up to 20% of all diabetic hospital admissions (Perim et al., 2015). In our study mono-bacterial infections were reported in 76% foot infections while mixed-bacterial infections were seen only 24% cases. Several previous studies have reported the similar results (Shankar et al., 2005 and Murali, et al., 2014), whereas other studies have revealed contrasting results with polymicrobial infections being more predominant (Neto et al., 2012; Zubair et al., 2011). These discrepancies are due to differences in diabetic foot infections, severe diabetic foot infections regularly yield polymicrobial isolates, while mild infections are often monomicrobial (Citron et al., 2007). We observed Gram-
positive aerobic bacteria were most often isolated which is in accordance with the earlier studies (Zubair, et al., 2011; Perim et al., 2015). *S. aureus* and coagulase-negative staphylococci were the most frequently isolated bacteria and Gram-negative bacilli such as *P. aeruginosa, E. coli* and *Serratia* spp. These results are agreeable with the findings of other studies (Zubair et al., 2011; Szafranek, et al., 2008; Perim et al., 2015). Several studies have demonstrated that Gram-negative bacteria were isolated more frequently than Gram-positive bacteria (Raja 2007; Singh et al., 2009). These contrarieties could be partially due to differences in the causative agents occurring over time, geographical variations, or the kinds and severity of infection incorporated in the studies (Zubair et al., 2011). The in-vitro susceptibility data herein presented were interpreted as authentic, since the negative controls used in culture-based experiments remained negative. The antimicrobial susceptibility test showed vancomycin as the more active antibiotic against Gram-positive bacteria. These observations are important for patients’ management. This result agrees with the results previously reported in a similar study conducted in Brazil on 89 bacterial isolates (Szafranek, et al., 2008). We found similar results that all *S. pyogenes* strains were susceptible to most antibiotics of the penicillin family, amikacin, ciprofloxacin and vancomycin (Singh et al., 2009; Rajalakshmi and Amsaveni, 2013). Gentamycin and ciprofloxacin were noticed to be the most effective antibiotics against gram-negative bacteria in our study. This is in accordance with the reports of Abdulraza et al. (2005). Our results showed that the majority of *P. aeruginosa* isolates were resistant to a large range of penicillin groups. These results were comparable to those achieved by Zubair et al. (2011). We found the *Serratia* spp. isolates to be susceptible to most of the tested antibiotics except amoxicillin, ampicillin, penicillin, and streptomycin. Kang et al. (2013) report similar finding from studies conducted in Korea (Kang et al., 2013). From a total of 62 bacterial isolates, 56.4% (26 Gram-positive bacteria and 9 Gram-negative bacteria) were susceptible to
leaf extract of *S. alba* with a MIC of 1.5 mg/mL whereas all isolates (100%) were susceptible with a MIC of ≤ 3 mg/mL. Chloroform leaf extract was found to be more active against Gram-positive bacteria (59%) than Gram-negative bacteria (50%), this may attribute to the differences in their cell wall structure. Gram-negative organisms are considered to be more resistant due to their outer membrane acting as a barrier to many environmental substances, including antibiotics. A similar finding has recently been reported by Tan *et al.* (2015), generally, ylang-ylang oil and different extracts of *Cananga odorata* demonstrated significant antibacterial activities against Gram-positive bacteria than Gram-negative bacteria (Tan *et al.*, 2015). As such, our results contradict previous finding that Gram-positive bacteria are less susceptible than Gram-negative bacteria to different concentration of leaf extract (Parekh and Chanda, 2007). In conclusion, we have demonstrated that Gram-positive bacteria are the most common pathogens related with diabetic foot infections in Sulaimani. Those pathogens are regularly resistant to commonly used antimicrobial drugs, nevertheless remain susceptible to vancomycin. Gentamycin and ciprofloxacin have a good *in vitro* activity against Gram-negative bacteria. It was obviously observed that chloroform leaf extract of *S. alba* had good antibacterial activity against Gram-positive bacteria.

**Acknowledgments**

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**Reference:**


