INTRODUCTION

Enterococci are part of the normal intestinal flora of humans and animals. They are considered important human pathogens. The genus Enterococcus includes more than 17 species, although only a few cause clinical infections in humans. Most of the times, these infections are difficult to treat because of the high rate of intrinsic and acquired resistance of enterococci to multiple antimicrobials\(^1,2\). Enterococcus faecalis and Enterococcus faecium are the most frequently isolated species from clinical specimens. About 90-95% of enterococcal infections in humans are caused by these two species and the remaining 5-10% are caused by other members of the genus\(^3\).

The mainstay of the treatment of enterococcal infection over the years was penicillin with gentamicin due to their synergistic action. By 1979, resistance to high-level gentamicin was reported due to genetically acquired mechanisms. Today acquired resistance has rendered many of the circulating strains of enterococci resistant to other available therapeutic options as well. Presently many circulating strains are reported to have acquired resistance to most of the remaining therapeutic options including vancomycin and linezolid which are thought to be antibiotics of last resort in enterococcal infections\(^4,5\). Vancomycin-resistant enterococci (VRE) first appeared in
Europe in the late 1980s. Nowadays, six types of acquired vancomycin resistance in enterococci are known; however, only Van A and to a lesser extent Van B are mainly prevalent. VRE are resistant to most of the commonly used antibiotics, providing a selective advantage over other intestinal flora organisms thus posing a major therapeutic challenge.

The Vitek 2 system is an automated system that provides rapid and accurate identification and antimicrobial susceptibility testing (AST) results for most clinical isolates including enterococci. Biochemical reactions are used for identification purpose, and minimum inhibitory concentrations (MICs) are determined by applying an algorithm to the growth kinetics monitored by the Vitek 2 system.

The objective of this study is to determine the diagnostic accuracy of in-house biochemical testing for identification of Enterococcus species isolated from various clinical specimens against gold standard i.e., automated Vitek 2 system. This study also includes the antimicrobial susceptibility of enterococci against various antimicrobials.

**METHODOLOGY**

This cross-sectional comparative was carried out at the department of Microbiology, Armed Forces Institute of Pathology, Rawalpindi, from April 2017 to March 2018.

A total of 218 isolates from various clinical specimens suspected to be Enterococcus spp. (gram positive cocci, catalase negative, Lancefield group D) were checked by in-house biochemical testing and Vitek 2 compact system.

The in-house tests included a set of 3 parameters.

Bile esculin agar [prepared by adding 5.0 grams of bile esculin agar base to 100 mL distilled water, then dispensed in individual bottles (5 mL each) in a slanting position to obtain agar with a slant] to differentiate group D and non-group D streptococci (group D streptococci and enterococci (formerly group D streptococci) cause esculin hydrolysis and are tolerant to the presence of bile, whereas non-group D streptococci do not). This provides a way to presumptively identify group D streptococci.

About 6.5% saline broth [prepared by adding 1.5 grams of nutrient broth and 6.5 grams of sodium chloride in 100 mL distilled water, then dispensed in individual bottles (5 mL each)] to differentiate salt-tolerant enterococci from group D streptococci (enterococci cause turbidity in the broth because they are salt-tolerant whereas group D streptococci do not).

1% arabinose [prepared by adding 1 gram arabinose to 100 mL buffered peptone water, 1 mL Andrade indicator is added to see for color change] to differentiate Enterococcus faecium (medium turns pink because of arabinose fermentation) from Enterococcus faecalis (does not ferment arabinose).

The in-house biochemical testing can help identify only 2 species of enterococci i.e., E. faecalis and E. faecium. The rest of the species cannot be identified. So the results of this in-house testing were compared with those of Vitek 2 compact, automated ID/AST instrument (bioMerieux Diagnostics). The antimicrobial susceptibility testing for the isolates was also performed against various antimicrobials via disk diffusion method, as per CLSI guidelines.

The Vitek 2 system (bioMerieux) is an automated system that consists of a filling-sealer unit, a reader-incubator, a computer control module, a data terminal, and a multicopy printer. The system detects bacterial growth and metabolic changes in the microwells of thin plastic cards by using a fluorescence-based technology. Different microwell cards contain antibiotics or biochemical substrates. We used the ID-GPC card of the Vitek 2 system for identification and the AST-P516 card of the Vitek 2 system for the antimicrobial susceptibility testing of enterococci.

Each organism suspension was prepared from the growth of pure cultures of bacteria cultivated for 18 to 24 h on blood agar. The suspensions were prepared in sterile saline (0.45% NaCl)
Clinical Specimens Against Vitek 2

Table-I: The scheme of in-house biochemical testing for enterococcal identification.

<table>
<thead>
<tr>
<th>Biochemical Parameters</th>
<th>Group D Streptococci</th>
<th>Enterococci</th>
<th>Non-Group D Streptococci</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Enterococcus Faecium</td>
<td>Enterococcus Faecalis</td>
</tr>
<tr>
<td>Bile esculin (hydrolysis)</td>
<td>Positive</td>
<td>Positive</td>
<td>Positive</td>
</tr>
<tr>
<td>6.5% NaCl (turbidity)</td>
<td>Negative</td>
<td>Positive</td>
<td>Positive</td>
</tr>
<tr>
<td>1% arabinose (fermentation)</td>
<td>Negative</td>
<td>Positive</td>
<td>Negative</td>
</tr>
</tbody>
</table>

Table-II: Distribution of clinical specimens included in study.

<table>
<thead>
<tr>
<th>Specimen</th>
<th>Number of Samples</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urine</td>
<td>81 (37.2%)</td>
<td></td>
</tr>
<tr>
<td>Pus</td>
<td>43 (19.3%)</td>
<td></td>
</tr>
<tr>
<td>Pus swab</td>
<td>29 (13.3%)</td>
<td></td>
</tr>
<tr>
<td>Stool</td>
<td>16 (7.4%)</td>
<td></td>
</tr>
<tr>
<td>Blood</td>
<td>15 (6.9%)</td>
<td></td>
</tr>
<tr>
<td>Fluid</td>
<td>10 (4.6%)</td>
<td></td>
</tr>
<tr>
<td>Tissue</td>
<td>9 (4.2%)</td>
<td></td>
</tr>
<tr>
<td>Drain fluid</td>
<td>5 (2.4%)</td>
<td></td>
</tr>
<tr>
<td>Cerebrospinal fluid</td>
<td>4 (1.9%)</td>
<td></td>
</tr>
<tr>
<td>Pleural fluid</td>
<td>4 (1.9%)</td>
<td></td>
</tr>
<tr>
<td>Bile</td>
<td>2 (0.9%)</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>218 (100%)</td>
<td></td>
</tr>
</tbody>
</table>

RESULTS

Two hundred and eighteen isolates were included in the study, out of which 127 (58.3%) samples were from male patients and 91 (41.7%) samples were from female patients, with age of patients ranging from 13 to 69 years.

Isolates included in the study were from various clinical specimens as shown in table-II.

Keeping Vitek 2 as gold standard, in-house biochemical testing was found to have a good diagnostic accuracy for enterococcal identification. Out of the total 218 isolates, Vitek 2 reported 202 as enterococci and 16 as non-enterococci. In-house biochemical testing reported 207 as enterococci and 11 as non-enterococci. Comparing the results of in-house testing with gold standard i.e., Vitek 2 system, the statistical data was calculated and found as follows:

- Sensitivity: 100%
- Specificity: 68.75%
- Positive predictive value: 97.58%
- Negative predictive value: 100%
- Accuracy: 97.71%

Antimicrobial susceptibility testing was performed for the enterococcal isolates against various antimicrobials as per CLSI guidelines, using disk diffusion method. The resistance of isolates against various antimicrobials is shown in percentage in the figure.

DISCUSSION

In this study, we determined the diagnostic accuracy of in-house biochemical testing for identification of enterococci isolated from various clinical specimens. The in-house biochemical testing used 3 parameters i.e., bile esculin agar, 6.5% saline broth and 1% arabinose. These three param-
Clinical Specimens Against Vitek 2

1. Enterococcus faecium: 135 (62%)
2. Enterococcus faecalis: 72 (33%)
3. Enterococcus avium: 8 (5.9%)
4. Enterococcus casseliflavus: 2 (1.5%)
5. Aerococcus viridans: 1 (0.7%)
6. Enterococcus hirae: 1 (0.7%)
7. Enterococcus raffinosus: 1 (0.7%)
8. Kocuria kristinae: 1 (0.7%)
9. Lactococcus garvieae: 1 (0.7%)
10. Pediococcus pentosaceus: 2 (1.5%)

Out of the total 218 isolates, the in-house biochemical testing reported:

1. 135 isolates (62%) to be Enterococcus faecium
2. 72 isolates (33%) to be Enterococcus faecalis.
3. 11 isolates (5%) to be non-enterococci.

The 218 isolates were identified using the automated Vitek 2 system as gold standard along with the in-house biochemical identification. Vitek 2 system led to identification of 202 isolates as seven enterococcal species including E. faecium, E. faecalis, E. avium, E. casseliflavus, E. gallinarum, E. hirae, E. raffinosus, and 16 isolates turned out to be non-enterococci.

1. Enterococcus faecium: 121 (55.6%)
2. Enterococcus faecalis: 67 (30.8%)
3. Enterococcus avium: 14 (6.1%)
4. Enterococcus casseliflavus: 2 (0.9%)
5. Aerococcus viridans: 1 (0.7%)
6. Enterococcus hirae: 1 (0.7%)
7. Enterococcus raffinosus: 1 (0.7%)
8. Kocuria kristinae: 1 (0.7%)
9. Lactococcus garvieae: 1 (0.7%)
10. Pediococcus pentosaceus: 2 (1.0%)

The frequencies of various Enterococcus species in our setup thus turned out to be:

1. Enterococcus faecium: 121 (59.9%)
2. Enterococcus faecalis: 67 (32.2%)
3. Enterococcus avium: 14 (6.1%)
4. Enterococcus casseliflavus: 2 (0.9%)
5. Aerococcus viridans: 1 (0.7%)
6. Enterococcus hirae: 1 (0.7%)
7. Enterococcus raffinosus: 1 (0.7%)
8. Kocuria kristinae: 1 (0.7%)
9. Lactococcus garvieae: 1 (0.7%)
10. Pediococcus pentosaceus: 2 (1.0%)

The most frequently isolated species in our setup was found to be Enterococcus faecium, followed by Enterococcus faecalis. Sixteen isolates were found to be non-enterococci, however 5 out of
these were diagnosed as enterococci by in-house biochemical testing.

Antimicrobial susceptibility testing was performed for the enterococcal isolates against various antimicrobials as per CLSI guidelines, using disk diffusion method.

Penicillin susceptibility was performed for a total of 201 isolates. One hundred forty seven (73.1%) were found to be resistant and 54 (26.9%) were found to be susceptible. Ampicillin susceptibility was performed for 202 isolates which showed 138 (68.3%) isolates to be resistant and 64 (31.7%) to be susceptible. Thus, comparing the susceptibility of penicillin and ampicillin, out of the total 147 penicillin resistant isolates, 137 were resistant to ampicillin as well, but 10 isolates were susceptible to ampicillin; and all of the 54 isolates susceptible to penicillin were also susceptible to ampicillin. 93.2% of penicillin resistant isolates were resistant to ampicillin as well. A study conducted by Grayson et al showed that certain characteristics of VRE like its colonization strategy, persistence in the environment, and genome plasticity, make it a major nosocomial pathogen worldwide, typically in immunocompromised patients. In a study by Matar et al conducted in cancer patients, it was seen that VRE fecal colonization was documented in 4.7% of patients screened.

Figure: Resistance of isolates against various antimicrobials.

Vancomycin susceptibility was performed for 204 isolates, 128 (62.7%) turned out to be susceptible and 76 (37.3%) turned out to be resistant to vancomycin. Teicoplanin susceptibility was performed for 203 isolates, 130 (64%) turned out to be susceptible whereas 73 (36%) were found to be resistant to teicoplanin. Other than the species showing intrinsic resistance to vancomycin because of van C gene (E. gallinarum, E. casseliflavus), susceptibility of vancomycin and teicoplanin was compared. Out of the total 76 vancomycin resistant enterococci (VRE), 71 (93.4%) were found resistant to teicoplanin as well, however 5 (6.6%) VRE isolates were found susceptible to teicoplanin. All of the vancomycin susceptible isolates were found susceptible to teicoplanin. The isolates which show resistance to both vancomycin and teicoplanin are likely to have van A gene whereas van B gene confers resistance to vancomycin and not to teicoplanin. Thus, van A seems to be much more prevalent in our setup than van B gene on the basis of susceptibility pattern, however this study does not include the molecular detection of vancomycin resistance genes, so this assumption cannot be confirmed. A study by O’Driscoll et al showed that certain characteristics of VRE like its colonization strategy, persistence in the environment, and genome plasticity, make it a major nosocomial pathogen worldwide, typically in immunocompromised patients. In a study by Scheetz et al established an ecological link between linezolid consumption and increasing incidence of enterococci with decreased susceptibility to linezolid.

Linezolid susceptibility was performed for 204 isolates. One hundred and eighty one (88.7%) were susceptible, 22 (10.8%) were resistant and 1 (0.5%) was intermediate. The results of a study conducted by Scheetz et al established an ecological link between linezolid consumption and increasing incidence of enterococci with decreased susceptibility to linezolid.

Ciprofloxacin susceptibility was performed for 198 isolates. One hundred seventy one (86.4%) were found to be resistant, 25 (12.6%) were susceptible and 2 (1%) were intermediate. Levofloxacain susceptibility was performed for 198 isolates. One hundred and sixty four (82.8%) were found to be resistant, 31 (15.7%) were susceptible
and 3 (1.5%) were intermediate. The susceptibility of ciprofloxacin and levofloxacin was compared for 195 isolates. Out of the total 170 ciprofloxacin resistant isolates, 162 (95.3%) were also resistant to levofloxacin, 6 (3.5%) were susceptible to levofloxacin, 2 (1.2%) were found to be intermediate. Twenty five ciprofloxacin susceptible isolates were also found to be susceptible to levofloxacin. Schouten et al showed that over 90% of E. faecalis isolates were susceptible to sparfloxacin, trovafloxacin, and moxifloxacin. The activities of these towards E. faecium, however, were much lower.

One hundred and ninety eight isolates were tested for nitrofurantoin susceptibility. Ninety five (48%) were found susceptible, 86 (43.4%) were resistant and 21 (10.6%) were intermediate. Zhanel et al tested the activity of nitrofurantoin against 300 isolates of E. faecium, E. faecalis, and E. gallinarum. No isolates tested were resistant to nitrofurantoin, including vancomycin-resistant isolates. This study concluded that nitrofurantoin may provide effective treatment of urinary tract infections caused by VRE.

E. faecalis isolates were tested for fosfomycin susceptibility. About 58.2% were found susceptible, 41.8% turned out to be resistant (however fosfomycin was reported only for the urinary isolates). Butcu et al conducted a study that showed 2.3% of E. faecalis strains to be resistant to fosfomycin.

Tetracycline susceptibility was performed for 199 isolates. One hundred forty nine (74.9%) were found to be resistant, 48 (24.1%) were susceptible and 2 (1%) were intermediate. Doxycycline susceptibility was performed for 198 isolates. Ninety five (48%) were found to be resistant, 68 (34.3%) were susceptible and 35 (17.7%) were intermediate. The susceptibility of tetracycline and doxycycline was compared for 196 isolates. Out of the total 147 tetracycline resistant isolates, 95 (63.8%) were also resistant to doxycycline, 35 (23.5%) were intermediate, and 17 (11.4%) were found to be susceptible. All the 47 tetracycline susceptible and the 2 tetracycline intermediate isolates were found susceptible to doxycycline. Reinert et al described antimicrobial susceptibility among bacterial isolates associated with hospital infections. In this study, tigecycline was found to be the only antimicrobial to maintain activity against all Gram-positive isolates including E. faecium and E. faecalis.

One hundred ninety two isolates were tested for erythromycin susceptibility. One hundred forty eight (77.1%) were found resistant, 34 (17.7%) were susceptible and 10 (5.2%) were intermediate.

CONCLUSION

The in-house biochemical testing can identify the 2 most frequent enterococcal species involved in human enterococcal infections (E. faecium and E. faecalis), therefore in resource-limited settings, can be quite a useful method for identification of enterococci. However, it requires overnight incubation and cannot identify other enterococcal species and non-enterococcal species.

Vitek 2 is an automated system that is easy to handle and provides a faster (4 to 15 h) and reasonably accurate identification of the most commonly isolated Enterococcus species along with the rarely isolated species and gives accurate AST results. It improves the work flow of the clinical microbiology laboratory by significantly reducing the handling time.

Enterococcal isolates from various clinical specimens in our setup showed least resistance to linezolid, followed by teicoplanin and vancomycin. Nitrofurantoin and fosfomycin have less than 50% resistance for urinary isolates.

CONFLICT OF INTEREST

This study has no conflict of interest to be declared by any author.

REFERENCES

Clinical Specimens Against Vitek 2