

Fourier Transform Infrared Spectroscopy Typing an *Enterococcus* sp.

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Abstract

Enterococcus species are one of the leading causes of nosocomial infections, which are difficult to treat specially with the rise of its Vancomycin resistant. Studies of *Enterococcus* isolates are essential for epidemiological investigation. Typing *Enterococci* is often based on the traditional phenotypic as well as genotypic methods. In this study Fourier-transform infrared (FTIR) spectroscopy is used as a novel phenotypic approach to the typing of *Enterococci*. FTIR spectroscopy results compared to antibiotic susceptibility testing and PCR amplification of Vancomycin gene results; the analysis showed that, 6 isolates were positive for Van gene (4 of VanA, 1 of VanB and 1 VanA plus VanB). Three of VanA and VanA plus VanB were resistant to all antibiotic tested (Ampicillin, Teicoplanin and Vancomycin) and VanB was found to be sensitive. FTIR spectroscopy (first derivatives) divided the isolates into 8 groups. 3 groups of VanA (4 isolates), one of VanB (one isolate), one of VanA plus VanB (one isolate) and the other 13 *Enterococcus* isolates were divided into 3 clusters. The study demonstrated that FTIR spectroscopy has good discriminative capacity and high reproducibility as compared to other techniques.

Keywords

Enterococci, Antibiotic Susceptibility Testing, FTIR Spectroscopy, PCR, VRE Typing

1. Introduction

Enterococci is an opportunistic human pathogens, the two most important species of this group are *E. faecalis*

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and *E. faecium*, which are considered as the leading causes of nosocomial infections. It also causes: endocarditis, septicemia, intra-abdominal, pelvic and urinary tract infections [1]. These infections are often difficult to treat due to the increased antibiotic resistance associated with this organism [2]-[4]. The recent increase of vancomycin-resistant, *vre enterococci* strains in clinical isolates is especially a cause of serious concern, because the types of glycopeptide antibiotics often remains the last treatment available in life-threatening infections [5]. This situation is further complicated by the fact that *Enterococci* have developed a number of mechanisms for the transfer of resistance genes [6]. In general, this has resulted in an increased need for rapid and accurate identification of *Enterococci* as a means of effectively assisting infection control and epidemiological investigations.

For most clinical microbiological laboratories, the primary method of identifying *Enterococcus* strains relies on the traditional phenotypic characterization. However, it is a challenging procedure that can take several days to accomplish because of the phenotypic and biochemical similarities between many *Enterococci* [7]. Molecular genetic techniques have been successfully used to identify *Enterococci* at the species level [8]-[11], but it is difficult to adapt them for use in routine laboratories due to their high costs and the requirement for highly skilled personnel.

An extensive approach for investigation of bacterial cells of different bacterial species and strains has been undertaken by Neumann and his co-worker [12]-[15]. They have shown that FT-IR absorption spectra are highly specific fingerprints of microbial cell.

Attempts to apply Infrared (IR) Spectroscopy technology to biology began as early as the 1910s, when the use of IR spectroscopy for the analysis of biological samples was first suggested. By the late 1940s, the technique was being successfully explored for the study of biological materials and in fact, IR spectroscopy has become an accepted tool for the characterization of biomolecules [14] [15]. The earlier applications of FT-IR in microorganisms date back to the 1950s [16]-[19]. The use of infrared (IR) spectroscopy as a means of differentiating and characterization of bacteria was extensively reported [20]-[22]. The different studies undertaken by Neumann and co-workers of bacterial cells of different species and strains has shown that FT-IR absorption spectra are highly specific fingerprints of microbial cells. They showed that the FTIR spectra of microorganisms display specifically a complete phonetic and a genetic fingerprint of the cells under study, as all cell components depend on the expression of smaller or larger parts of the genome. This is why the specificity of the technique is very high, allowing highly accurate differentiations of microorganisms at quite different taxonomic levels, even down to the subspecies, strain and/or serogroup/serotype level.

FT-IR spectra of intact microbial cells are highly specific fingerprint-like signature. It is a valuable tool for the identification of biological samples of interest [23], microorganisms and in strains characterization, medical applications, pharmaceutical industry and in drinking water control [24].

In the present work, the Fourier Transform Infrared spectroscopy [FTIRS] was used to type *Enterococcus* sp. that was isolated from a hospital in an attempt to develop a standardized procedure for the differentiation and characterization of *Enterococci*.

2. Materials and Methods

2.1. Strains Collection and Growth Conditions

A total of 19 clinical isolates of *Enterococcus* sp. were collected from different body sites of various hospital wards. A standard strain of *E. faecalis* (ATCC-51299) was also included. The *Enterococci* isolates were stocked in proper media and stored at -70°C .

2.2. Antibiotics Susceptibility Testing

Antibiotic susceptibilities were determined by the disk diffusion method on Mueller-Hinton medium (Difco Laboratories), according to the methods outlined by the Clinical and Laboratory Standards Institute, formerly the National Committee for Clinical Laboratory Standard [25]. The antibiotics used are: ampicillin 10 μg teicoplanin 30 μg and vancomycin 30 μg all from oxoid limited England.

2.3. Molecular Identification of *Vre* Resistant Genes by Multiplex PCR

DNA Extraction

DNA was extracted using rapid lysis method [26]; Bacteria were harvested from blood agar plates, one loopful

by using a 1 μL loop. Cells were re-suspended in 50 μL of lysostaphin, 100 $\mu\text{L}/\text{mL}$ in water; (Sigma chemical Co. USA). Cells suspension were incubated at 37°C for 10 minutes. A 50 μL of proteinase K solution 100 $\mu\text{L}/\text{mL}$, (Sigma, USA) and 150 μL buffer 0.1 M Tris buffer (pH 7.5) were added and cell suspensions were incubated for an additional 10 min at 37°C, and then placed in a boiling water bath for 5 minutes.

2.4. Multiplex Pcr

Multiplex polymerase chain reaction or Multiplex pcr was carried out with 25 μL reaction volume, each reaction contained 2.5 μL of 10X buffer, 0.5 μL of dNTP (Invitrogen, United Kingdom), 0.2 μL of VanA primer 5'-CATG AATAGAATAAAAAGTTGCAATA-3', 5'-CCCCTTTAACGCTAATACGATCAA-3', and 0.1 μL of VanB primer, 5'-GTGACAAACCGGAGGCGAGGA-3', 5'-CCGCCATCCTCCTGCAAAAAA-3', 0.25 μL Taq DNA polymerase (Invitrogen; United Kingdom) and 3.0 μL . Thermocycling DNA was performed in PTC-150 DNA engine with an initial 5 min. denaturation at 95°C, followed by 30 cycles of denaturation at 95°C, for 30 seconds, annealing at 55°C, for 1 min. and extension at 72°C, for 1 min, followed by a final extension step at 72°C, for 5 min.

2.5. FTIR Spectra of *Enterococcus* sp.

The *Enterococcus* sp. was grown under standardized conditions on blood agar plates. A single isolated colony was carefully removed from previously prepared culture using a sterile 1 μL disposable loop and suspended in 200 μL sterile physiological saline solution (0.85% NaCl). Then 120 μL of the suspension was evenly spread over the entire surface of a silicon window 32 mm dia, and allowed to dry for 30 min at 39°C, leaving a transparent film suitable for FTIR absorbance transmission measurements. It is reported that first derivatives of infrared spectra were recorded in fingerprint region 900 - 700 cm^{-1} [27]. Spectral comparisons were performed using (Origin pro 7.5 software). Four essential measurements were performed to obtain accurate results and yield highest reproducibility. Firstly, by running FTIR spectroscopy scan in the range 1200 - 400 cm^{-1} to check for the presence of the absorbance-transmittance spectrum. Secondly, the time effect of keeping the isolate for certain period of time in saline solution was inspected, this measurement was repeated at nine days time intervals for the radiation range 900 - 400 cm^{-1} . Thirdly, sources of contamination of the prepared samples were investigated. Fourthly, the silicon window spectrum was compared with its data sheet specification to assign the exact background signal value.

3. Results and Discussion

Disc diffusion test was carried out for the three antibiotics and results are summarized in **Table 1**. Fifteen isolates were found sensitive to vancomycin, and 4 resistant. For ampicillin, 12 were sensitive and 7 are resistant. For teicoplanin, 15 isolates were sensitive, 2 intermediate and 2 resistant. The minimum inhibitory concentration obtained for *Enterococcus* isolates are shown in **Table 1**. There were 15 sensitive to vancomycin and 4 were resistant. For teicoplanin there were 15 sensitive and 4 resistant. For ampicillin 12 isolates were found to be sensitive and 7 resistant. Three genotypes were obtained for *Enterococcus* sp., VanA, VanB, VanA plus B as shown in **Table 1**.

The FTIR spectra of *Enterococci* samples were obtained in the range 4000 - 500 cm^{-1} which includes the known fingerprint region *i.e.*, 900 - 700 cm^{-1} . The results were repeated for each sample over different times and the results were highly reproducible over the indicated time period as shown in **Figure 1**. The resulting 8 different spectra groups were designated as G1-G8. Isolates which showed almost identical absorbance peaks and line shapes were considered similar. All isolates had absorbance peak either 725 or 738 cm^{-1} and most of the groups had an absorbance peak of 830 cm^{-1} except in group G2 and group G6 which was absent, they had absorbance peak of about 889 cm^{-1} .

Another absorbance peak about 950 cm^{-1} in the outer fingerprint region was present in six groups and was absent in G2 and G6 (**Table 2** and **Table 3**). For easier comparison of isolates, first-derivative FTIR spectra plotted to show similarities and differences between isolates.

Five different types with different spectra (different absorbance peaks) were obtained of the genotypes VanA, VanB and VanA plus B, one group of VanB (1 isolate), one of VanA plus B (1 isolate) and 3 of VanA (4 isolates) as shown in **Figures 2-4** and **Table 2**. Interestingly VanB (isolate No. 44404) spectrum was similar but

Table 1. Results for enterococcal isolates regarding zone diameter, Minimum inhibitory concentration, and genotypes.

Sample No.	Disk diffusion results/zone diameter (mm)			Minimum inhibitory concentration ($\mu\text{g/ml}$)			Genotypes
	Ampicillin	Teicoplanin.	Vancomycin	Ampicillin	Teicoplanin.	Vancomycin	
<i>E. faecalis</i> ATCC 51299	20	16	15	<2.0	<2.0	32	
48198	0	10	0	256	256	256	VanA
4898	0	0	0	16	128	256	VanA
41720	8	20	17	32	16	32	VanA
44932	22	18	17	<2.0	<2.0	<2.0	
44404	27	21	19	<2.0	<2.0	<2.0	VanB
45805	22	20	18	<2.0	<2.0	<2.0	
43191	25	19	17	<2.0	<2.0	<2.0	
48278	0	0	0	256	256	256	VanA+B
45511	0	12	0	256	256	256	VanA
44218	0	20	18	64	<2.0	<2.0	
41509	24	19	18	<2.0	<2.0	<2.0	
47892	22	17	17	<2.0	<2.0	<2.0	
47808	21	19	17	<2.0	<2.0	<2.0	
47781	22	20	19	<2.0	<2.0	<2.0	
45416	22	19	17	<2.0	<2.0	<2.0	
41838	0	19	20	256	<2.0	<2.0	
45148	22	18	17	<2.0	<2.0	<2.0	
47891	23	19	17	<2.0	<2.0	<2.0	
44083	28	22	20	<2.0	<2.0	<2.0	

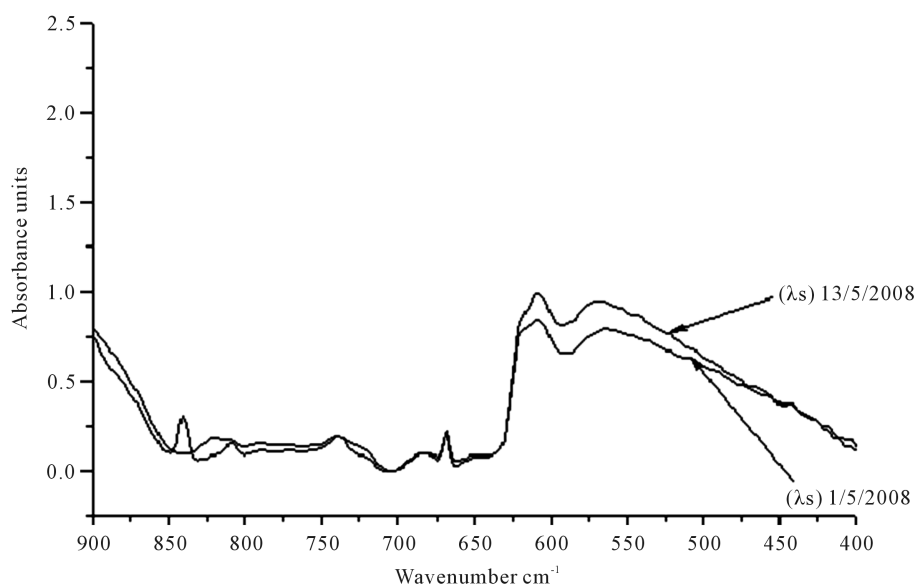
**Figure 1.** Repetitive FTIR spectra measurements for one isolate over a period of nine days.

Table 2. Comparison of the absorbance peaks of the genotypes VanA, VanB, VanA plus B and *E. faecalis* ATCC 51299 VanB.

Phenotype	Sample No	Absorbance Peaks in the Range (1000 - 500 cm ⁻¹)				
ATCC 51299 VanB	ATCC 51299	950.11	829.56	738.06	725.72	610.87
Van B G1	44404	949.94	828.92	738.01	727	610.22
VanA G2	41720	-	889.77	738.38	618.19	-
	4898	-	889.28	738.25	611.17	541.28
VanA G3	48198	950.36	829.02	738.15	610.66	508.49
VanA G4	45511	950.06	830.23	724.74	-	518.32
VanA+B G5	48278	950.66	828.69	738.15	610.68	-

Table 3. Comparison of the absorbance peaks of the non vancomycin resistant *Enterococcus* isolates.

Phenotype	Sample No.	Absorbance Peaks in the Range (1000 - 500 cm ⁻¹)				
G6	43191	545.06	611.44	738.47	892.21	-
G6	45148	544.57	610.42	738.55	897.94	-
G6	47891	-	611.03	738.30	890.80	-
G6	44218	-	612.08	738.43	889.16	-
G7	47892	511.86	-	724.75	830.48	949.57
G7	44932	520.05	-	724.82	830.36	949.94
G7	44083	514.74	-	724.85	830.36	949.87
G7	47808	509.83	-	724.88	830.23	950.33
G7	41838	518.42	-	724.77	830.48	950.14
G8	45805	523.09	610.31	738.16	829.14	950.21
G8	47781	508.54	610.72	738.23	828.09	950.82
G8	45416	531.7	610.92	738.18	828.69	950.62
G8	41509	519.74	615.90	738.77	830.14	950.03

not identical to ATCC which is also VanB genotype as shown in **Figure 2**. **Figure 3** shows three different groups of VanA, isolates numbers: 45511, 41720 and 48198. **Figure 4** shows spectra of the isolates numbers: 44404, 45511 and 48278, *i.e.* comparing the spectra of VanB, VanA, VanA+B groups respectively. The rest of *Enterococcus* isolates were typed into another three different groups, G6 four isolates, G7 five isolates and G8 four isolates as shown in **Figure 5**, **Figure 6** and **Figure 7** respectively.

Enterococcus sp. is considered the second most common bacteria responsible for nosocomial infections with increasing ampicillin and vancomycin resistance [28]. VanA and VanB are among the glycopeptide resistance determinants encountered in clinical settings [29]. In this study we aimed to explore the vancomycin resistant of *Enterococcus* specimens isolated from a hospital. The isolates were typed using antibiotic susceptibility testing, vancomycin resistant gene and FTIR spectroscopy. Three genotypes of vancomycin resistant were found to be circulating in the hospital which is considered an alarming signal since the genes coding for vancomycin resistance is located on conjugative transposon [30]. It is found that antibiotic susceptibility testing was not discriminative enough compared to FTIR which has proven to be very useful typing technique. The reproducibility

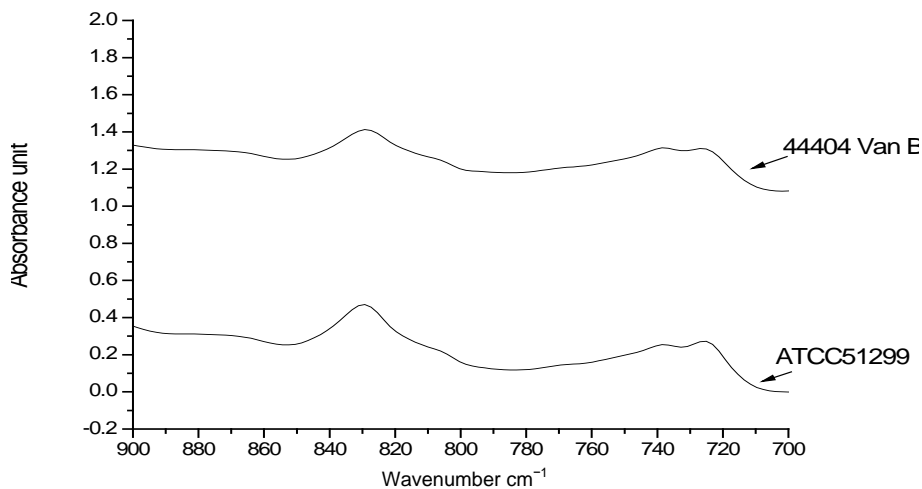


Figure 2. Spectra of ATCC 51299 VanB and VanB isolate number 44404 in the range 900 - 700 cm^{-1} .

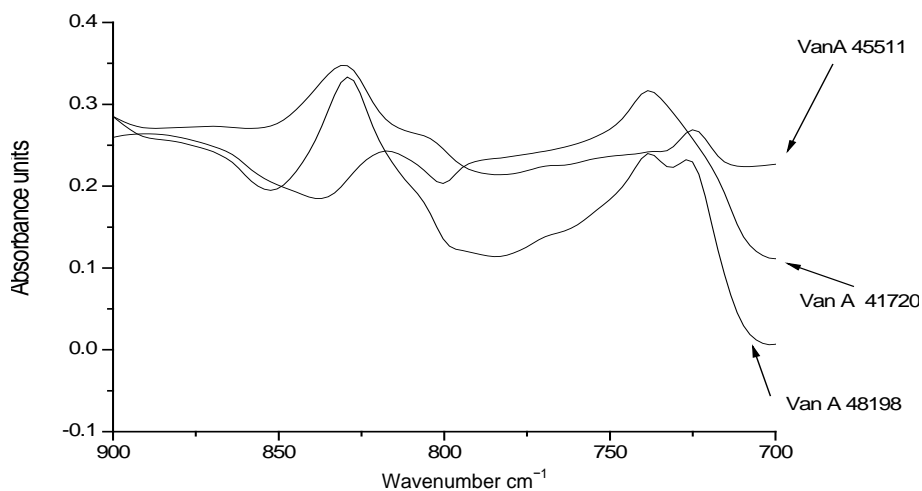


Figure 3. Spectra of VanA type of the samples numbers: 45511, 41720 and 48198 taken in the fingerprint region 900 - 700 cm^{-1} .

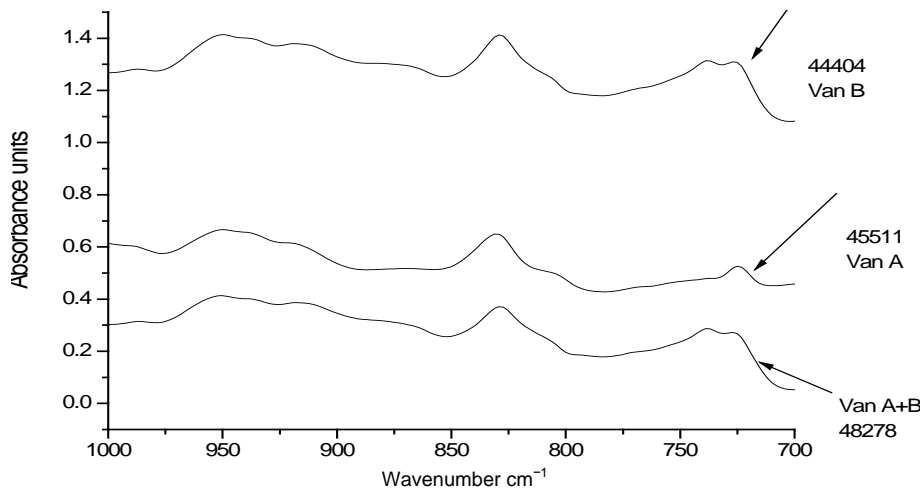


Figure 4. Spectra of VanB, VanA and VanA plus B isolates over the range 1000 - 700 cm^{-1} .

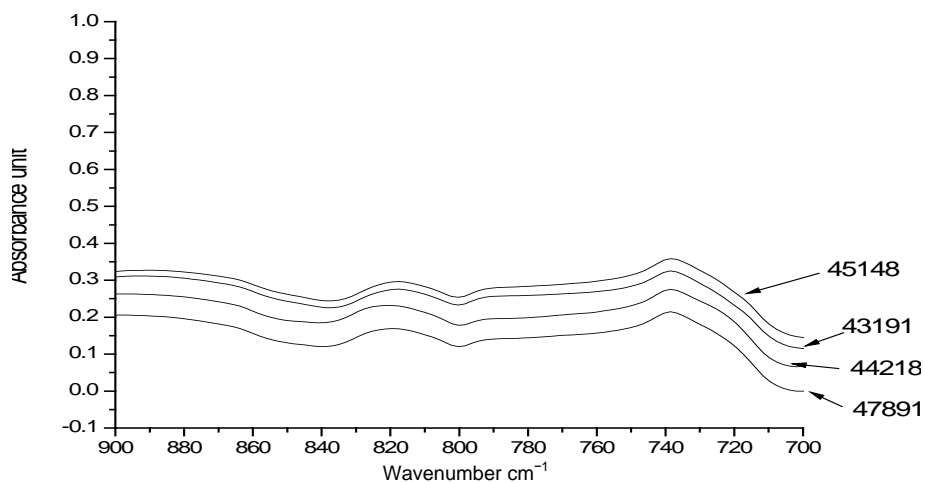


Figure 5. Comparisons between spectra of the group G6 samples in the fingerprint region 900 - 700 cm^{-1} .

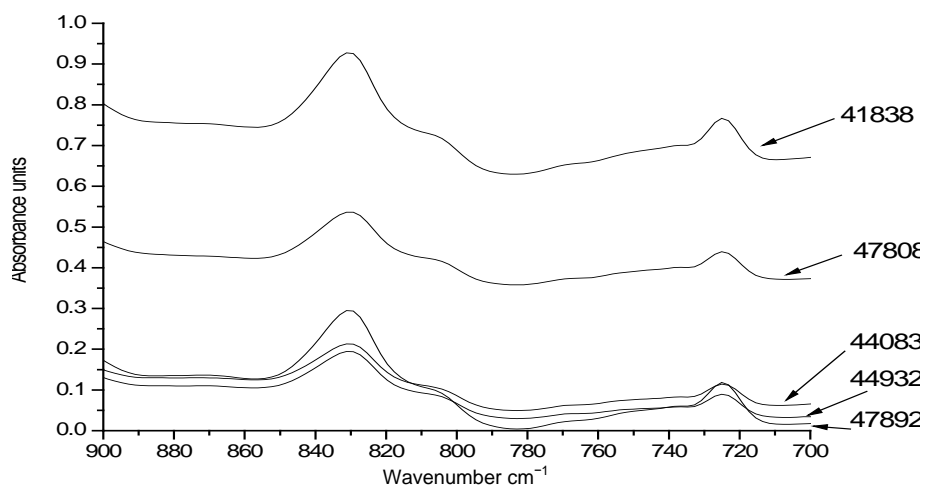


Figure 6. Comparisons between spectra of the group G7 samples in the fingerprint region 900 - 700 cm^{-1} .

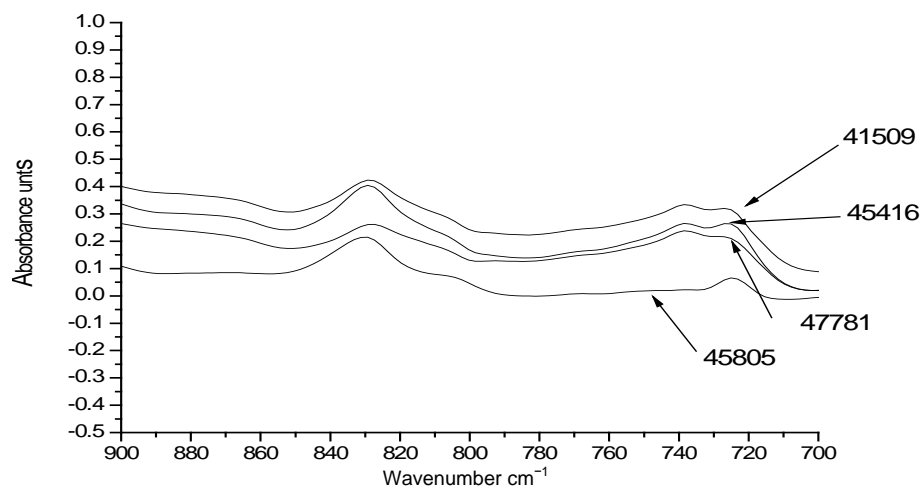


Figure 7. Comparisons between spectra of the group G8 samples in the fingerprint region 900 - 700 cm^{-1} .

of FTIR technique was tested by subjecting the same isolates to the test on different separated times. According to FTIR results, the 19 enterococcus isolates were divided into 8 clusters. Predominant absorbance peaks were present in all spectra, such as the peaks that arise at 725 or 738 cm^{-1} and it was noted that most of the groups had an absorbance peak at 830 cm^{-1} except for groups 2 and 6, the absorbance peak was at 889 cm^{-1} . Another noticeable absorbance peak at 950 cm^{-1} was present in six of the eight groups and absent in groups 2 and 6. This could be explained that groups 2 and 6 might be of different species. We suggest that further biochemical investigation might be needed for confirmed identification.

FTIR spectroscopy studies to bacteria employ traditional windows such as Zinc selenide (ZnSe) in preparing the adhesive culture [31]. In this work, silicone window have been used, with transmittance scale 97% - 103% T. This high specification allows absorption of incident radiation energy to occur by the adhesive culture rather by window, and hence, an accurate measurement is obtained. Results were analyzed by comparing both the line shapes of the FTIR spectra of species in the first derivative (see **Figures 1-7**) and the corresponding values in wave numbers of absorbance peaks in the fingerprint regions (see **Table 2** and **Table 3**). Scans were performed in the mid IR range *i.e.* 4000 - 500 cm^{-1} , although the range of concern is 1000 - 500 cm^{-1} , in which the fingerprint region *i.e.* 900 - 700 cm^{-1} lies. This region needs studies since, some specific spectra patterns are not yet assigned to cellular components or functional groups [27]. In this study we look for minute differences between Enterococci isolates, other studies shown that they have high degree of similarity among [32].

Figure 1 shows the results of the arbitrary samples (AS), taken over a time span of nine days. The shift between spectra might be attributed to the difference in lab temperature at the different times of measurements; this effect is reported in many types of bacteria [33] [34] and needs further studies in Enterococci type. The nearly matching spectra indicate an almost equal concentration of the samples. **Figure 2** shows a spectrum comparison of the standard strain ATCC 512299 VanB and the isolated strain 44404 in the range 1000 - 500 cm^{-1} . It indicates close line shape similarity of spectra but not quite identical especially in the fingerprint region. This is expected, since they both belong to the same genotype of VanB, but their site of collection is different. **Table 2**, shows for the previously mentioned samples, that their absorbance peaks in the range 1000 - 500 cm^{-1} is almost the same.

In **Table 2** & **Table 3**, absorption lines 738 or 724 cm^{-1} seems common among all samples in the fingerprint region. This observation indicates that Enterococci bacteria species have common organic compounds which identify its type. To confirm this conclusion, two different genera of bacteria other than enterococcus were measured by FTIR spectroscopy and the output absorbance peaks *i.e.* 738 or 724 cm^{-1} was absent in the fingerprint region (data not shown). Further studies have to be carried out with many genera of bacteria to establish a firm stands for this conclusion. The organic compound which is related to the absorbance peak value 724 cm^{-1} could be dimethylene, which has rocking vibration mode, and the value 829.66 cm^{-1} is related to the S-H band stretching vibration of the sulfite group [35]. The absorbance peak 950 cm^{-1} belongs to ammonia molecules at A1 symmetric, while the absorbance peak 610 cm^{-1} defines the sulfite ions again [35]. Therefore, FTIR was able to differentiate between VanA isolates and categorize them into 3 different groups, see **Figure 3** and **Table 2**, in which the isolates 47120 & 4898 were considered as group G2.

4. Conclusion

FTIR spectroscopy is a methodology that fingerprints the whole cell and is able to detect subtle compositional changes that cannot be revealed by conventional phenotypic or even genotypic methods. Contrary, antibiotic susceptibility testing was not discriminative enough when compared to FTIR. The later technique was used in this study to type Enterococcus clinical isolates and has proven very useful. It is easy to carry and allows the analysis of large number of strains, although it requires a high degree of standardization and complex data processing. In conclusion, certain physical and chemical properties do exist that can be determined by other means to study different molecules and organic compounds constituting part of the cell structure. This observation indicates that Enterococci bacteria species have organic compounds which can be used to identify its type and it is believed that FTIR has great potential in such clinical microbiology application.

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