Environment Conservation Journal 25 (1):112-119, 2024

Journal homepage:https://www.environcj.in/

Environment Conservation Journal

ISSN 0972-3099 (Print) 2278-5124 (Online)

Fatty acid profiling of enterococcal isolates by Fames analysis with reference to antibiotic resistance from clinical samples collected in the Chandrapur region

Ashwini S. Muttawar✉
Department of Microbiology, Institute for Higher Learning Research & Specialized Study, Sardar Patel Mahavidyalaya, Chandrapur (M.S), India
Vijay S. Wadhai
Department of Microbiology, Institute for Higher Learning Research & Specialized Study, Sardar Patel Mahavidyalaya, Chandrapur (M.S), India

ARTICLE INFO

Received : 05 August 2023
Revised : 28 September 2023
Accepted : 15 October 2023

Available online: 10 January 2024

Key Words:
Antibiotics
Enterococcus
Fatty acid methyl esterase (FAME)
Gas chromatography

ABSTRACT

FAME Analysis is a simple and rapid technique based on Gas Chromatography analysis of bacterial cell membrane. Biochemical lipid profiling of bacterial cell membrane helps to identify bacterial isolates using MIDI Sherlock system. The main objective of present study is rapid and accurate identification of multidrug resistant *Enterococcal* isolates and to study their clinical profile for complicated nosocomial infection from the clinical samples collected at tertiary care center in Chandrapur region. It is essential to identify the causative organism for proper diagnosis and treatment of diseases as enterococcus is fastly emerging pathogen responsible for life threating nosocomial infection and other health hazards.

Introduction

Enterococcus species are a part of the normal flora of humans, but in recent years, they have been identified as opportunistic pathogens that can cause serious health problems. The most common *causes of enterococcal* infection include hospital-acquired infection (Upadhyaya *et al.,* 2009), fecal contamination of drinking water, consumption of unhygienic food, environmental contamination, cross infection, etc. Thus, from a medical standpoint, identifying enterococcal species, which leads to various infections, and determining the prevalence rate of infection caused by bacterial isolates are essential (Fisher *et al.,* 2009). Currently, *Enterococcus species* are characterized by natural resistance to various broad-spectrum antibiotics and by easily acquired resistance to antibiotics. Enterococcus species have rapidly emerged and are of great concern due to the development of antimicrobial resistance (Bekhit *et al.,* 2012). Infections caused by multiantibiotic-resistant strains of *Enterococcus* are more difficult to treat and can be recurrent, highly infectious, chronic and sometimes fatal. In the present study, surveillance was performed at the Tertiary Care Hospital in the Chandrapur District among patients visiting for various diagnostic purposes and treatments; most patient clinical samples were screened for the presence of multidrug-resistant enterococcal isolates for proper diagnosis and treatment of the disease (Arias *et al.,* 2010). The identification of causative organisms is highly essential. Thus, a rapid and reliable method for identifying microorganisms is used to isolate and identify *enterococcal* isolates.

FAME analysis is a technique developed to identify bacterial species more quickly than differential biochemical and media culture testing. In FAME analysis, gas chromatography-accumite ultraviolet spectroscopy was used to determine the importance of bacterial fatty acid methyl esters in identifying and discriminating bacteria based on the fatty acid profile of the bacterial cell wall. The unique configuration of the Sherlock system was designed for the automatic analysis of fatty acid methyl esters via Sherlock pattern recognition software (Morey *et al.,* 2013). A study is therefore essential to identify *enterococcal* isolates that cause various infections

Corresponding author E-mail: *ashwinimuttawar07@gmail.com*
Doi:https://doi.org/10.36953/ECJ.26612639

within a short period of time and to determine the associations of these isolates with potential virulence factors and antimicrobial resistance patterns for appropriate management, diagnostic treatment and treatment.

Materials and Methods

For the present study, urine samples were collected aseptically from the Pathology and Microbiology Department of Government Hospital, Chandrapur. The samples were labeled, sealed properly and transported to the Department of Microbiology, Sardar Patel Mahavidyalaya, Chandrapur, for further processing and isolation. In the Department of Microbiology, Sardar Patel Mahavidyalaya and Chandrapur, the presence of *an enterococcal* isolate from urine samples was detected using microscopic, biochemical and growth methods on differential culture media. Chromogenic UTI media is a differential agar media used to identify the pathogens causing UTI infection. Solid UTI media was inoculated with inoculum from urine samples under aseptic conditions. It was then incubated at 37°C for 24 hr to obtain blue colonies with good growth. A thin smear of the bacterial preparation was mounted on a clean glass slide and subjected to Gram staining. The stained slides were then observed under an oil immersion objective to identify gram-positive cocci that occurred in pairs. Bile Esculine agar is both a differential and selective medium for the isolation of members of the genus *Enterococcus.* The bacterial inoculum was inoculated on sterile slants of bile esculine agar under aseptic conditions and incubated at 37°C for 24 hr. Enterococcus isolates hydrolyze esculine in the presence of bile acid, which turns more than half of the medium to a dark brown color, indicating a positive test. Sheep blood agar is used to grow fastidious microbes that require nutrient-rich environments. It helps to distinguish nonhemolytic, alpha-hemolytic and beta-hemolytic organisms. The bacterial inoculum was inoculated on blood agar, where nonhemolytic, circular, convex colonies with entire margins were observed after 24 hours of incubation.

Table 1: Sample processing for FAME analysis

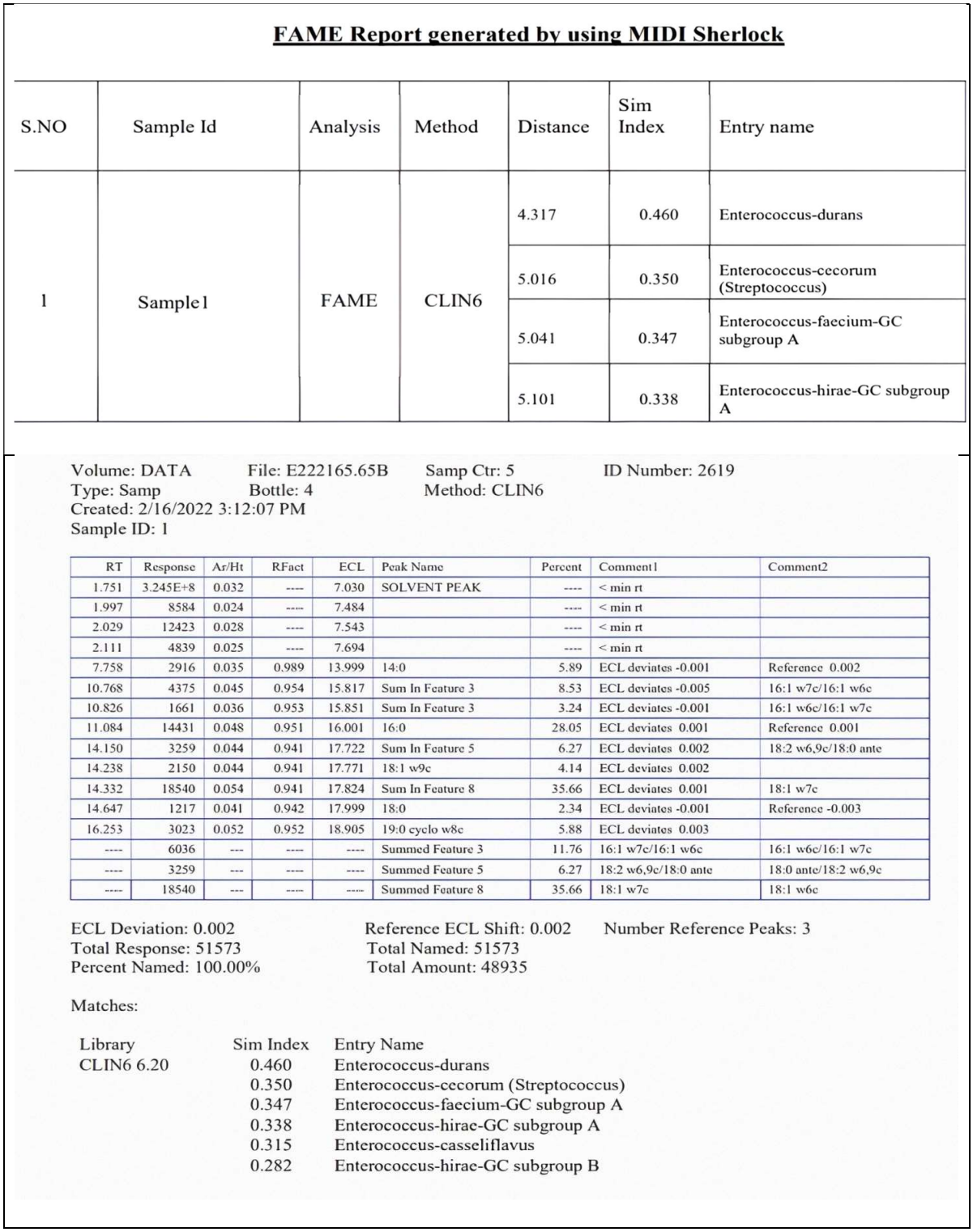
SN	STEPS	PURPOSE	REAGENTS	PROCEDURE
1	Harvesting	Removal of cells from cultural media for bacterial identification.	-	A 4 mm loop is used to harvest approximately 40 mg of bacterial cells from III quadrant of culture plate in 13 * 100 culture tube
2	Saponification	Lysis of cells to liberate fatty acids from the cellular lipids.	REAGENT 1 NAOH - 45 gms METHANOL - 150 gms DIST WATER -150 ml	Add 1.0 ml Reagent to culture tube and vortex for 5 – 10 sec and keep it in water bath at 100 degree C for 5 min, again vortex for 5 – 10 sec and again keep in water bath for 5 min and then let it cool for 25 min.
3	Methylation	Formation of Fatty Acid Methyl Ester (FAMEs) which is poorly soluble in aqueous phase.	REAGENT 2 6.00 N HCL - 325 ml METHANOL - 275 ml	Add 2.0 ml of Reagent 2 and vortex for 5 – 10 sec, now keep it in water bath at 80 degree C for 10 min and cool it rapidly.
4	Extraction	Transfer of FAMEs from aqueous phase to organic phase for use with Gas Chromatography	REAGENT 3 HEXAN -200 ml METHYL TERT-BUTYL ETHER -200 ml	Add 1.25 ml of Reagent 3 and stir well for 10 min, remove bottom aqueous phase and save top organic phase
5	Base wash	Aqueous wash of organic extract prior to chromatography which reduce contamination of injection port line, the column and detector.	REAGENT 3 NAOH -10.8 gms DEIONIZED DIST WATER -900 ml	Add 3.0 ml of Reagent 4 to organic phase and stir for 5 min and allowed it to rest, Remove 2/3 of top organic phase and transfer it into GC vial, capped it and introduce to GC analyzer.

FAME analysis

FAME analysis is a technique developed to identify bacterial species more rapidly and easily using the fatty acid profile of the bacterial cell wall. Based on these findings, further work was carried out at Royal Life Science Pvt Ltd., Secanderabad, and Telengana. The sample was aseptically transported and processed for fatty acid profiling and identification of *the enterococcal* isolates. The process of FAME analysis involved harvesting bacterial cells from quadrant III of the culture plate in the late log phase for processing in the culture tube. Various reagents were added to the culture tube containing harvested cells, and proper mixing of the reagents was performed via a vortex machine. In the first reaction, the sodium salt of fatty acids is produced, which then undergoes methylation to form fatty acid methyl esters. A boiling water bath was used to maintain and control the temperature throughout the process. Vigorous mixing via a vortex machine results in the separation of the aqueous and organic phases. The organic phase was then extracted using a base wash. Approximately 2/3 of the organic phase from the culture tube was then transferred to a clean GC sample vial, and the cap was tightly sealed and loaded in an automatic sampler of the MIDI Sherlock system. The Sherlock system is a unique configuration designed for the analysis of fatty acid methyl esters by gas chromatography. The results were compared with those from the stored database of the library using Sherlock pattern recognition software, which helps in the identification of bacterial isolates based on the fatty acid profile (Table 1).

Results and Discussion

The microbial culture obtained from clinical samples (urine) collected at the Tertiary Care Center in the Chandrapur region revealed gram-positive Diplococci, which grow well on UTI Media to produce Blue colonies. The microbial isolates were differentiated by growing them on bile esculine agar, where half of the media turned dark brown in color, and growth on blood agar showed nonhaemolytic, circular, colonies with entire margins. The interpretation of the results for FAME analysis is a visual representation of the result of the library search given after listing the best possible matches and corresponding similarity indices, which are as follows:It is generally found that routine identification of bacterial isolates using conventional methods is common and time consuming. For rapid and accurate identification of bacterial strains, a convenient and precise mechanism is needed. FAME analysis is a standard, sensitive, rapid and rapid method for identifying bacteria. In this technique, screening of bacterial isolates for morphological, physiological, biological and cultural reasons is not needed. The most stable and reproducible cellular fatty acid profile is achieved by regulating growth conditions. A specific temperature and differential media are essential for determining the fatty acid composition of specific bacteria. Most aerobic bacteria grow on TSBA agar, whereas clinical isolates utilize blood agar. Thus, a separate database with standards is established depending on specialized differential media used by specific organisms (Hoffmann *et al.,* 2010). The Sherlock system is based on the similarity index. The fatty acid composition of unknown organisms is numerically related to the fatty acid composition of organisms present in the database library of the Sherlock system (Kunitsky *et al.,* 2006). A sample with a similarity index of 0.500 or greater and a separation of 0.100 is considered to be a close match and highly related. A similarity index between 0.300 and 0.500 and a separation greater than 0.100 were considered good matches but with an atypical strain. A value less than 0.300 indicates that there is no match in the database (Figure 1). A visual representation of the results is given after listing the best possible matches and similarity indices in the form of comparison charts for easy understanding. For each fatty acid, the bar represents a +/- 2 standard deviation window around the entry mean for fatty acid identification with a vertical line (Figure 2). A sample chromatogram is a visual plot that traces the electronic signal generated by the flame ionization indicator as it burns fatty acids eluting from the column (Figure 3).Histogram used for graphical summarization and display of the distribution of process data. A dendrogram provides information about pair matching based on fatty acid composition to show the relatedness of the isolates. (Figure 4). The NJ tree shows the relationship between the organisms, whereas the NJ Rooted Tree



FAME Report generated by using MIDI Sherlock

S.NO	Sample Id	Analysis	Method	Distance	Sim Index	Entry name
1	Sample1	FAME	CLIN6	4.317	0.460	Enterococcus-durans
				5.016	0.350	Enterococcus-cecorum (Streptococcus)
				5.041	0.347	Enterococcus-faecium-GC subgroup A
				5.101	0.338	Enterococcus-hirae-GC subgroup A

Volume: DATA File: E222165.65B Samp Ctr: 5 ID Number: 2619
Type: Samp Bottle: 4 Method: CLIN6
Created: 2/16/2022 3:12:07 PM
Sample ID: 1

RT	Response	Ar/Ht	RFact	ECL	Peak Name	Percent	Comment1	Comment2
1.751	3.245E+8	0.032	----	7.030	SOLVENT PEAK	----	< min rt	
1.997	8584	0.024	----	7.484		----	< min rt	
2.029	12423	0.028	----	7.543		----	< min rt	
2.111	4839	0.025	----	7.694		----	< min rt	
7.758	2916	0.035	0.989	13.999	14:0	5.89	ECL deviates -0.001	Reference 0.002
10.768	4375	0.045	0.954	15.817	Sum In Feature 3	8.53	ECL deviates -0.005	16:1 w7c/16:1 w6c
10.826	1661	0.036	0.953	15.851	Sum In Feature 3	3.24	ECL deviates -0.001	16:1 w6c/16:1 w7c
11.084	14431	0.048	0.951	16.001	16:0	28.05	ECL deviates 0.001	Reference 0.001
14.150	3259	0.044	0.941	17.722	Sum In Feature 5	6.27	ECL deviates 0.002	18:2 w6,9c/18:0 ante
14.238	2150	0.044	0.941	17.771	18:1 w9c	4.14	ECL deviates 0.002	
14.332	18540	0.054	0.941	17.824	Sum In Feature 8	35.66	ECL deviates 0.001	18:1 w7c
14.647	1217	0.041	0.942	17.999	18:0	2.34	ECL deviates -0.001	Reference -0.003
16.253	3023	0.052	0.952	18.905	19:0 cyclo w8c	5.88	ECL deviates 0.003	
----	6036	---	----	----	Summed Feature 3	11.76	16:1 w7c/16:1 w6c	16:1 w6c/16:1 w7c
----	3259	---	----	----	Summed Feature 5	6.27	18:2 w6,9c/18:0 ante	18:0 ante/18:2 w6,9c
----	18540	---	----	----	Summed Feature 8	35.66	18:1 w7c	18:1 w6c

ECL Deviation: 0.002 Reference ECL Shift: 0.002 Number Reference Peaks: 3
Total Response: 51573 Total Named: 51573
Percent Named: 100.00% Total Amount: 48935

Matches:

Library	Sim Index	Entry Name
CLIN6 6.20	0.460	Enterococcus-durans
	0.350	Enterococcus-cecorum (Streptococcus)
	0.347	Enterococcus-faecium-GC subgroup A
	0.338	Enterococcus-hirae-GC subgroup A
	0.315	Enterococcus-casseliflavus
	0.282	Enterococcus-hirae-GC subgroup B

Figure 1: Sample profile information generated by the MIDI Sherlock tool

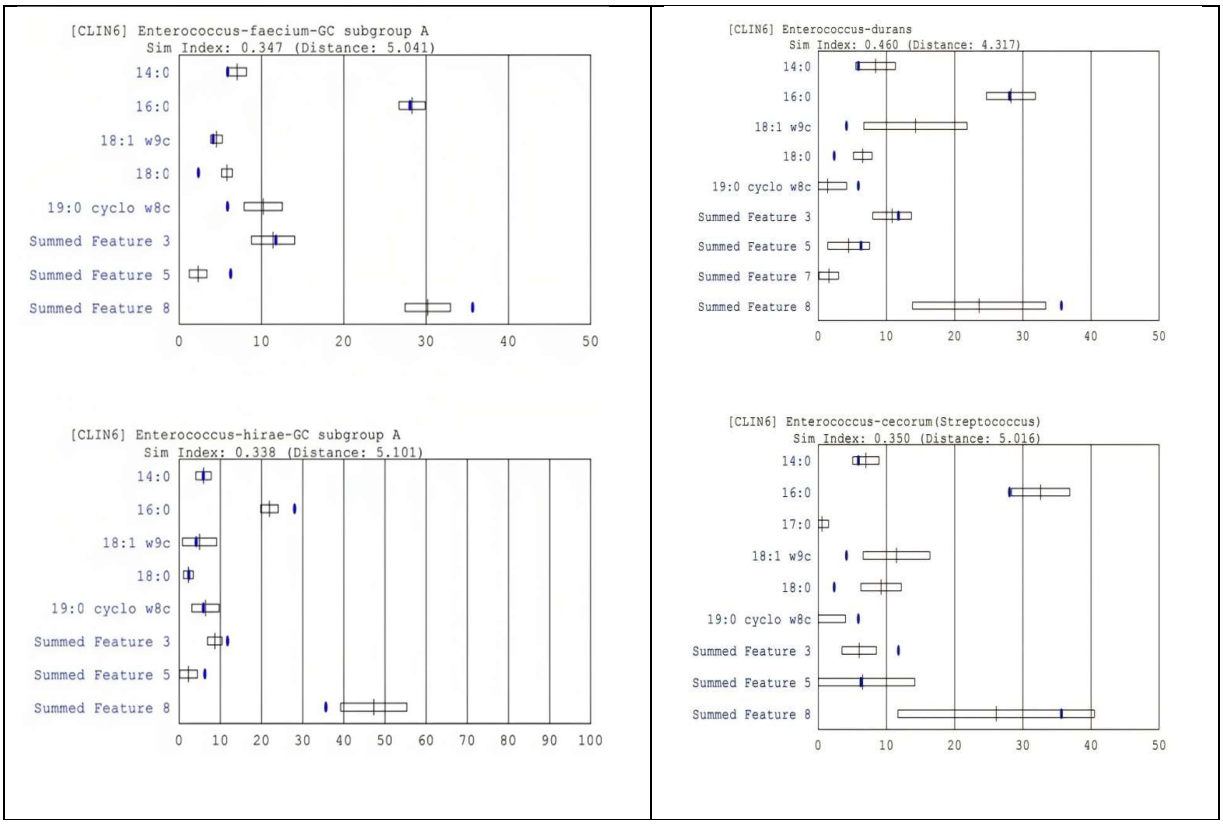


Figure 2: Sim index–comparison chart for bacterial isolates

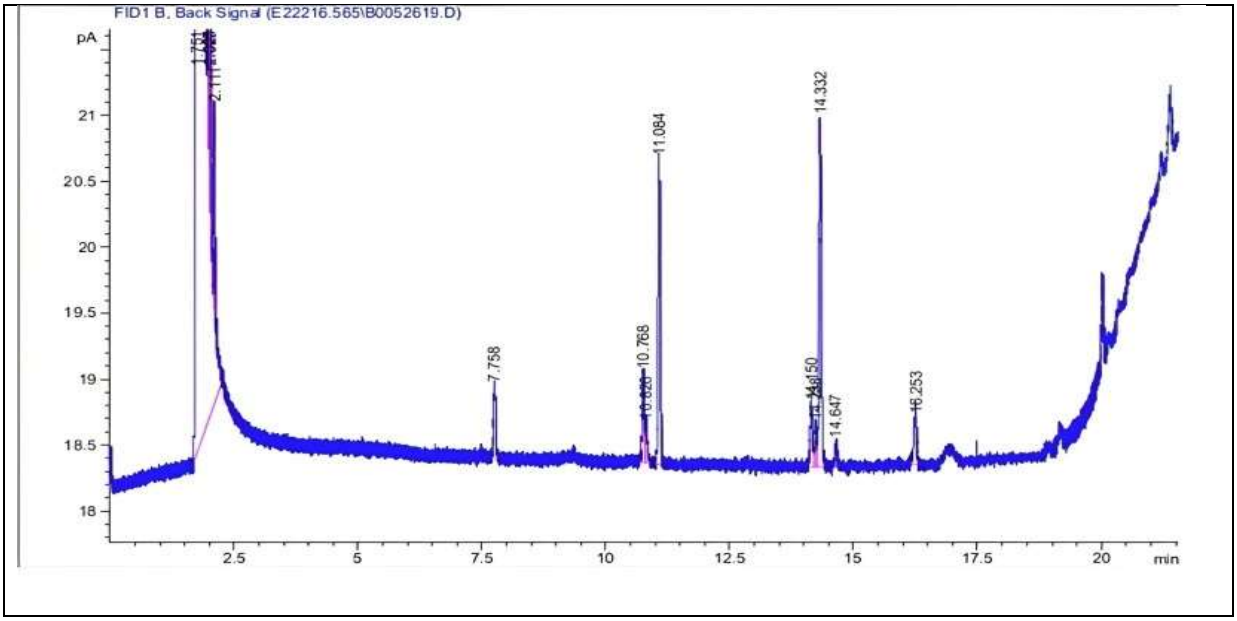
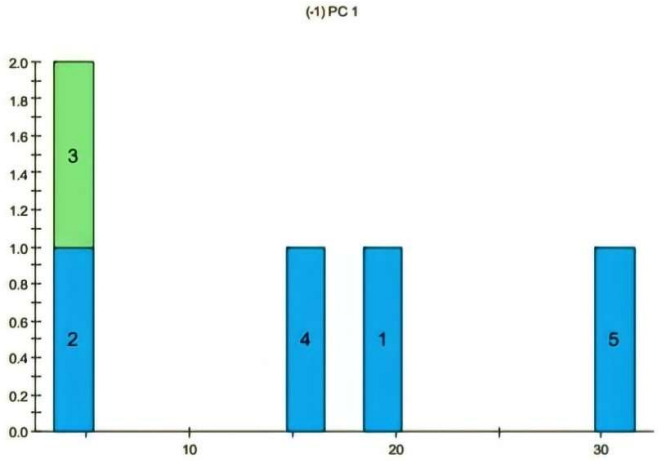


Figure 3: Chtomatogram

HISTOGRAM: Used to graphically summarize and display the distribution of a process data set.

Index	Sel	Volume:Filename#Cntr	Bottle	ID Num	Sample ID
1	Y	DATA:E222165.65B#5	4	2619	1
2	Y	CLIN6 # 173		173	Enterococcus-cecorum(Streptococcus)
3	Y	CLIN6 # 176		176	Enterococcus-durans
4	Y	CLIN6 # 179		179	Enterococcus-faecium-GC subgroup A
5	Y	CLIN6 # 183		183	Enterococcus-hirae-GC subgroup A



DENDROGRAM: Used to produce pair matching based on fatty acids composition.

Index	Sel	Volume:Filename#Cntr	Bottle	ID Num	Sample ID
1	Y	DATA:E222165.65B#5	4	2619	1
2	Y	CLIN6 # 173		173	Enterococcus-cecorum(Streptococcus)
3	Y	CLIN6 # 176		176	Enterococcus-durans
4	Y	CLIN6 # 179		179	Enterococcus-faecium-GC subgroup A
5	Y	CLIN6 # 183		183	Enterococcus-hirae-GC subgroup A

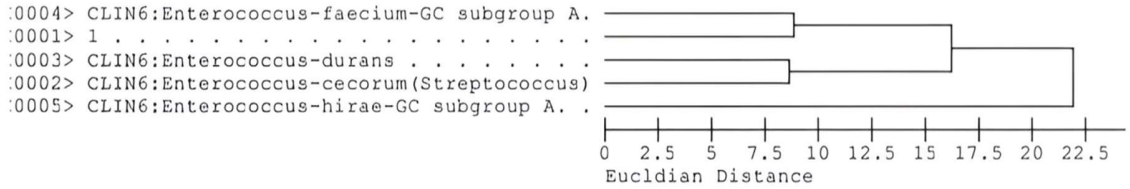


Figure 4: Graphical representation and pair matching of related organisms

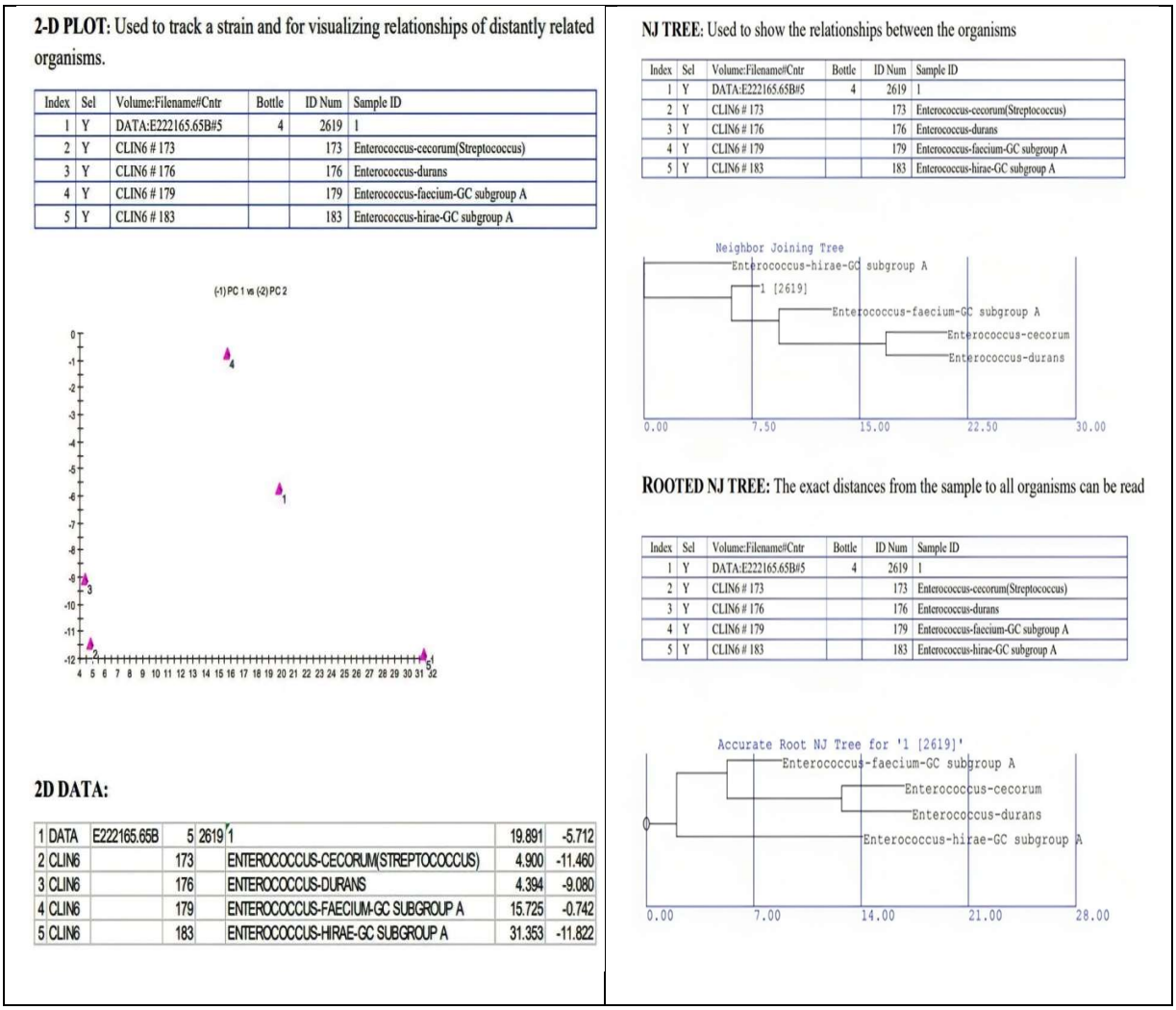


Figure 5: Relationships among distantly related organisms

gives the exact distance of the identified sample from other isolates. A 2D plot was generated to visualize the relationships between distantly related organisms (Figure 5).

Conclusion

The results of the above study showed that the microbial culture obtained from clinical samples collected at the Tertiary Care Center in the Chandrapur region was an enterococcal isolate, which was detected by FAME analysis very rapidly and accurately via the database library of the MIDI Sherlock system. For novel studies, microbiologists should implement this technique for accurate identification of bacterial isolates. There is also a need to upgrade the MIDI Sherlock system by establishing new FAME libraries with chromatographic profiles of additional microorganisms. Based on the fatty acid profile, enterococcal isolates were identified by comparison with TSBA6 and the CLIN6 database library of the MIDI Sherlock system, as microscopic examination revealed gram-positive cocci that grew well on specific media chosen for clinical isolation. If the infective microbial agents are identified accurately and diagnosed properly in a short duration along with the sensitivity pattern for a specific antibiotic, this will help medical practitioners prescribe specific

sensitive antibiotics against the causative bacterial isolate. This approach helps in preventing the frequent use of broad-spectrum antibiotics for longer periods, which may cause multiple antibiotic drug resistance in the future.

Acknowledgment

The authors sincerely acknowledge the Department of Microbiology, Sardar Patel Mahavidyalaya, Chandrapur, for the laboratory facilities and various reviewers for improving the research quality and Royal Life Science Pvt. Ltd. Secanderabad (Telengana) for providing the Life Science Laboratory and is the most rapid, accurate and cost effective method of microbial identification.

Conflict of interest

The authors declare that they have no conflicts of interest.

References

Arias, C. A., Contreras, G. A., & Murray, B. E. (2010). Management of multidrug-resistant enterococcal infections. *Clinical microbiology and infection*, *16*(6), 555-562.

Fisher, K., & Phillips, C. (2009). The ecology, epidemiology and virulence of Enterococcus. *Microbiology*, *155*(6), 1749-1757.

Hoffmann, M., Fischer, M., & Whittaker, P. (2010). Evaluating the use of fatty acid profiles to identify deep-sea Vibrio isolates. *Food chemistry*, *122*(4), 943-950.

Kunitsky, C., Osterhout, G., & Sasser, M. (2006). Identification of microorganisms using fatty acid methyl ester (FAME) analysis and the MIDI Sherlock Microbial Identification System. *Encyclopedia of rapid microbiological methods*, *3*, 1-18.

Morey, A., Oliveira, A. C., & Himelbloom, B. H. (2013). Identification of Seafood bacteria from cellular fatty acid analysis via the Sherlock® microbial identification system. *J Biol life sci*, *4*(2), 139-151.

Salem-Bekhit, M. M., Moussa, I. M. I., Muharram, M. M., Alanazy, F. K., & Hefni, H. M. (2012). Prevalence and antimicrobial resistance pattern of multidrug-resistant enterococci isolated from clinical specimens. *Indian journal of medical microbiology*, *30*(1), 44-51.

Upadhyaya, P. G., Ravikumar, K. L., & Umapathy, B. L. (2009). Review of virulence factors of enterococcus: an emerging nosocomial pathogen. *Indian journal of medical microbiology*, *27*(4), 301-305.