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The Detection of Ribosomal Mutations of *Enterococcuscasseliflavus* and *Enterococcusgallinarum* Isolated from Chronically Illness Patients

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Abstract

Background: The enterococci are Gram-positive lactic acid bacteria that inhabit different hosts' gastrointestinal tracts.*E. casseliflavus* and *E. gallinarum* are motile, low-level vancomycin resistant and opportunistic bacteria associated with infections in immunocompromised patients

Patients and methods: first case was febrile 44-year-old man, blood samples were collected and cultured to investigate infecting bloodstream organism. Second case was 65-year-old man chronically ill with prostate disorder with urinary tract infection, urine sample was collected and cultured to investigate the microbial cause.

Results: blood and urine culture were positive for bacterial growth which characterized by amplification of 16S rRNA gene.The sequence result of 520bp ribosomal replicon revealed that local samples belong to *Enterococcus casseliflavus* and *E.gallinarum*respectively.Sequencing results showed a total of seventeen mutations distributed in the investigated samples. Interestingly, both observed variations in both investigated samples were positioned in the same phylogenetic location in the currently generated comprehensive tree

Conclusion:The immunocompromised people are most vulnerable to infection with *E.casseliflavus* and *E.gallinarum*. additionally, the utilization of PCR-sequencing strategy in tow analyzed DNA samples of these local bacterial isolates have presented a confirmed identity of these strains within the Enterococuss sequences.

Keywords: E. casseliflavus , E. gallinarum, 16S rRNA

Introduction

Enterococci is known to inhabit the urinary and genital organs and gastrointestinal system in the human as a natural flora bacterium [1]. However, in patients with immunocompromisation and in those undergoi ng long term clinic treatment or earlier used concentrated doses of antibiotics, the propensity toward enterococcal infection is noted. [2].

Enterococci are bacterial cells exist as singles, doubles, or short chains, catalase is negative, use fermentation and oxidative metabolic pathway "facultative anaerobes", that develop in a medium containing 9.6 pH, 40 % bile salt. Enterococcus colonies are large, gray, rounded with zone of alpha or beta hemolysis other strain are not hemolysis in sheep blood agar. [3]
The genus Enterococcus was known to belong to "Lancefield group D Streptococcus "; however, studies of homology for DNA have shown that it is a separate genus. Up to now, over 40 species of Enterococcus have been identified and establish a diverse community of bacteria. [4]. In general, enterococci have been detected in the GTI of humans and animals, on plant surfaces, and in milk products[5]. Due to their capability to live under a severe circumstances, they may remain in the climate, such as drying, high temperatures, elevated osmolarity and the presence of disinfectants [6].

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unlike other enterococci, such as *E. faecium* and *E. faecalis*, which are the major harmful bacteria that a source of many hospital-acquired infections, *E. casseliflavus* is the gastrointestinal tract normal flora in human and animal. *E. casseliflavus* is not always isolated from clinical samples, the prevalence of isolation <1.3%, It is an opportunistic pathogen, which goals persons who are immunocompromised or persistently ill and sometimes, this pathogen is acquired as a result of admission to the hospital.[7-8] . While the infection of E. Caseliflavus is not common; if an infection occurs, it can be severely invasive. Lately, *E. casseliflavus* has been progressively involved in infections and epidemics in hospitals..A extensive range of hostile human infections, such as endocarditis, bacteremia, endophthalmitis and peritonitis, are associated with *E. Casseliflavus.* [9].

Vancomycin-resistant origins of enterococcal contagions have been one of the greatest significant causes of hospital acquired infections in recent years. There are two forms of resistance against vancomycin in enterococci. Inherent "Intrinsic " resistance is the first kind, the *E. casseliflavus /Flavescens* and *E. gallinarum* isolates have demonstrated inherent, low-level vancomycin resistance [7]. Acquired resistance is the second form of vancomycin resistance in enterococci. With the acquisition of genetic material from another microbe. This failure to response to antibiotics is, most generally, seen in "*E. Faecium, E. Faecalis*", but also was noted in. *E, raffinosus, E.avium, E.durans*[10].

Bacteremia of enterococcus, including both primary bacteremia, possibly of GTI origin, and bacteremia secondary to UTI and intra-abdominal infection or intravascular system use. [11]

In a 2005-2014 Japanese surveying study [12], 9% and 2.7% of enterococcal bloodstream infections (BSI) were caused by *E. casseliflavus / flavescens and E.gallinarum*, respectively, Therefore, enterococcal BSI is the third etiological cause and accounts for the totality of vancomycin-resistant isolates. Although the abundance of some reports, the dispersal of infections with *E. gallinarum* and *E. casseliflavus/flavescens* partially unidentified remains, but its occurrence can increase worldwide, according to current trends. [13]

Currently accordingly to their "16S rRNA reverse transcriptase sequence analysis", E. gallinarum group encompasses *E. gallinarum*and *E. casseliflavus/flavescens* [14].

The present study aims to shed light on the extent of *E. casseliflavus* and *E.gallinarum* involvement in infections for hospitalized and under treatment patients

Patient and methods

First case report

An contagious diseases specialists was demanded for a 44-year-old man due to fever exceeded 96 hours after post –operative of remove gallbladder who admitted to Al-SadiqHilla hospital during the period November 2019. Patients WBC value was 13000/mm3 and CRP level was 98mg/l as well as blood culture was achieved to investigate the microbial cause of bloodstream infection.

Blood specimen was collected for culturing, two blood specimes (5 ml) obtained in sequence by separate venipunctures each of them inoculated into screw cup bottles contains 25ml BHI broth incubated aerobically at 37C for 48 hrs., thancultured on blood agar incubated overnight at 37 C0 for isolation the bacterial bloodstream pathogen.

Second case report

A 65-year-old man patient with a prostate disorder. With symptoms of urinary tract infection like burning feeling when urination concomitant with fever. The attending physician suggested a procedure general urine examination and urine culture by clean-catch method for identification microbial cause of UTI. The urine pus cell was 50 Hpf and urine culture was positive for bacterial growth.

Genotyping assay

Bacterial Genomic DNA of *E.casseliflavus*and*E.gallinarum* were separated according to the procedure supplied by the of the scientific manufactured institutes (Promega, USA).Primers dissolved and prepared according to manufactured company (Alpha-USA). which was designed to amplify one ribosomal locus (520bp) within the bacterial genomic sequences. The forward primer sequence ,357F (5-CTACGGGGGGCAGCAG-3) and the revers primer sequence, 806 R (GGACTACCGGGGTATCT) [15].The PCR reaction was made using AccuPower PCR premix (Bioneer, Daejeon, South Korea). Each 20µl of PCR premix was contained 1 U of Taq DNA polymerase, 250 µM of dNTPs, 10 mM of Tris-HCl (pH 9.0), 30 mM of KCl, 1.5 mM of $MgCl_2$. The reaction mixture was completed with 10 pmol of each primer and 50 ng of genomic DNA.

The following program was done in PCR thermo cycler. The initial denaturation was the first step for amplification at 94°C for 5 min, after that 30 cycles of denaturation at 94˚C, annealing (61 ˚C for 1 min), and elongation at 72˚C, and was completed with a final extension at 72°C for 10 min. Amplification was tested by using electrophoresis technique of 1.5% (w/v) agarose gel, pre-stained with ethidium bromide (0.5 µg /ml). As a molecular size marker, selecting a 100-bp ladder (Bioneer, South Korea) All PCR fixed bands were checked to be precise and consist of only one pure and distinct band in order to be successfully subjected to sequencing.

DNA Sequencing of PCR amplicons

According to guidance notes from the sequencing agency (Macrogen Inc. Geumchen, Seoul, South Korea), the clarified PCR amplicons were commercially sequenced from termini, forward, and reverse. Only transparent chromatographs from A B I sequence archives have been further analyzed to ensure that the annotation and differences are not attributable to PCR or artifact sequencing. By comparing the observed nucleic acid sequences of samples with the saved reference sequences of the bacterial database .The virtual locations and other specifics of the PCR pieces obtained were established.

Interpretation of sequencing data

"BioEdit Sequence Alignment Editor Software Version 7.1 (DNASTAR, Madison, WI, USA)" has modified, synchronized, and analyzed the sequencing outcomes of the P C R products of various samples providing the respective sequences are in the reference file. The variations observed were numbered in PCR amplicons in each sequenced sample as well as in their corresponding location within the referring genome..

Comprehensive phylogenetic tree construction

According to the neighbor-joining protocol defined by [16], a particular comprehensive tree was constructed in this study. The observed variants were compared using the NCBI-BLASTn server with their neighbor's homologous reference sequences [17]. Then, by the neighbor-joining process, a complete inclusive tree was constructed, including the observed version, and visualized as a circular tree using iTOL suit [18]. The sequences in the complete tree of each classified phylogenetic species group were colored..

Resultsand discussion:

***E. casseliflavus* and *E.gallinarum* isolation** .

The two blood specimens were positive for bacterial culture of a same bacterial species. Also urine culture was positive for bacterial growth .Both bacterial isolates were identified as Gram positive, cocci ,catalase test was negative . the molecular characterization by using PCR amplification of 16 S rRNA of these bacterial strains showed that it belong to Enterococcus *casseliflavus* and E.gallinarum respectively.

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Concerning the isolation of bacterial pathogen from clinical human specimens, rare results have been found for "*E.casseliflavus / flavescens and E.gallinarum*". However, it has been suggested that incomplete characterization of the clinical influence may be responsible for limiting the detection and recognition of these species. [19].
High mortality rates are associated with enterococcal bacteremia; the death rates vary from 12% to 68%, and due to sepsis with enterococci is 4%-50% of patients. The role of enterococci as a cause of infection has become ever more significant just not because of its known pathogenic potential, but also because of increased antimicrobial resistance in some strains (especially glycopeptide resistance). [11].
Recovered from routine cultures in a sample of 302 consecutive enterococcal isolates by Ruoff et al. [20]they noted that *"E. gallinarum.*.and *E. Casseliflavus"* accounted for just 1% of the isolates in each, a finding that has been established by other studies [21-22]. These enterococcal organisms have also been shown to colonize both hospitalized and non-hospitalized people's intestinal tracts, with average colonization rates varying from 5.7 % to 12.1 %. [23].

Sequencing
Within this locus, the local one sample included, which showed about 520 bp amplicons length. Before sending these ribosomal amplicon to sequencing, it was made sure that all the amplified amplicon had shown sharp, specific, and clean bands
The sequencing reactions had indicated the confirmed identity of the amplified products by performing NCBI blastn. Concerning the 520 bp PCR amplicons of the ribosomal gene, the NCBI BLASTn engine showed entire sequences of similarities between the sequenced samples and *Enterococcus* sp. sequences. NCBI BLASTn engine has indicated the presence of about 97% of homology with the expected target that covered a portion of the 16-S ribosomal RNA (rRNA) region. The exact locations and other specifics of the retrieved PCR fragments were established when comparing the detected D N A sequences of these test samples with the retrieved DNA sequences (Gen Bank acc. KR001878.1). (Fig. 1).

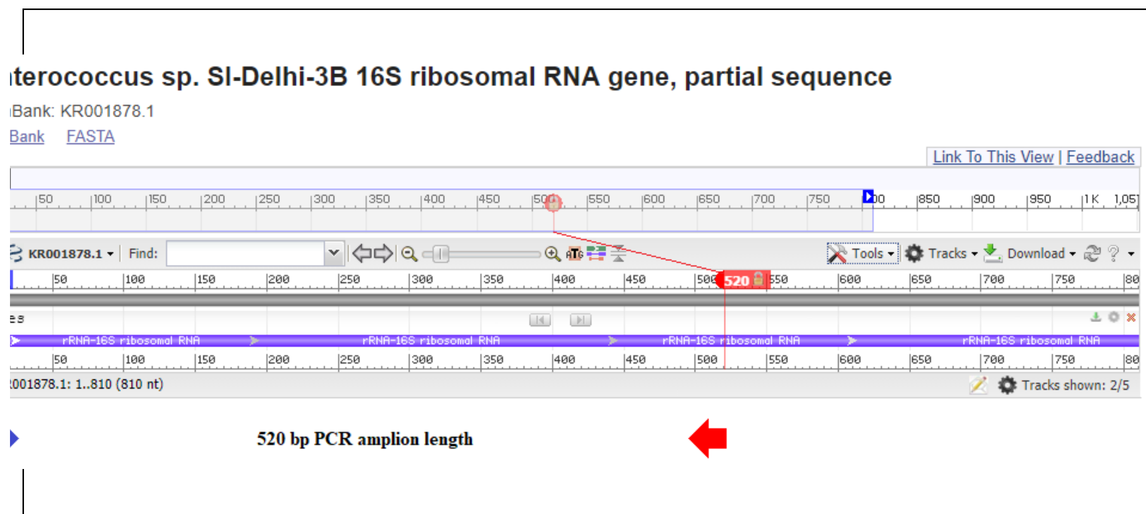

Fig. 1. The strict position of the retrieved 520 bpamplicon that partially covered the 16-S rRNA region within Enterococcus genomic sequences (Gen Bank acc. no. KR001878.1). The blue arrow refers to the starting point of this amplicon while the red arrow refers to its endpoint.

Once positioning the 520 bp amplicons' sequences within the ribosomal sequences, the details of these sequences were highlighted within the amplified 16-S rRNAsequences(Table 1)

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Table 1. The position and length of the 520 bp PCR amplicons that used to amplify a portion of the 16-S rRNA within Enterococcus genomic DNA sequence.

Amplicon	Reference locus sequences (5′ - 3′)	Length
16S rRNA sequences	GGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAA TCTTCGGCAATGGACGAAAGTCTGACCGAGCAACGCCGCGTGAGTGA AGAAGGTTTTCGGATCGTAAAACTCTGTTGGTAGAGAAGAACAAGGA TGAGAGTAAAATGTTCATCCCTTGACGGTATCTAACCAAAAAGCCCCG GCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTT GTCCGGATTTATTGGGCGTAAAGCGAGCGCAGGCGGTTTCTTAAGTC TGATGTGAAAGCCCCCGGCTCAACCGGGGAGGGTCATTGGAAACTG GGAGACTAGAGTGCAGAAGAGGAGAGTGGAATTCCATGTGTAGCGG TGAAATGCGTAGATATATGGAGGAACACCAGTGGCGAAGGCGGCTC TCTGGTCTGTAACTGACGCTGAGGCTCGAAAGCGTGGGGAGCGAAC AGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAG TGTTGGA	520 bp

The amplified sequences were extended from 1 to 520 of the NCBI reference sequence (GenBank acc. no. KR001878.1).
The alignment results of the 520 bp samples revealed the detection of seventeen nucleic acid variations with the corresponding *Enterococcus* referring sequences (Fig. 2). These sequences were prepared by aligning our investigated samples with the most relative sequences deposited in the NCBI database (GenBank acc. KR001878.1).

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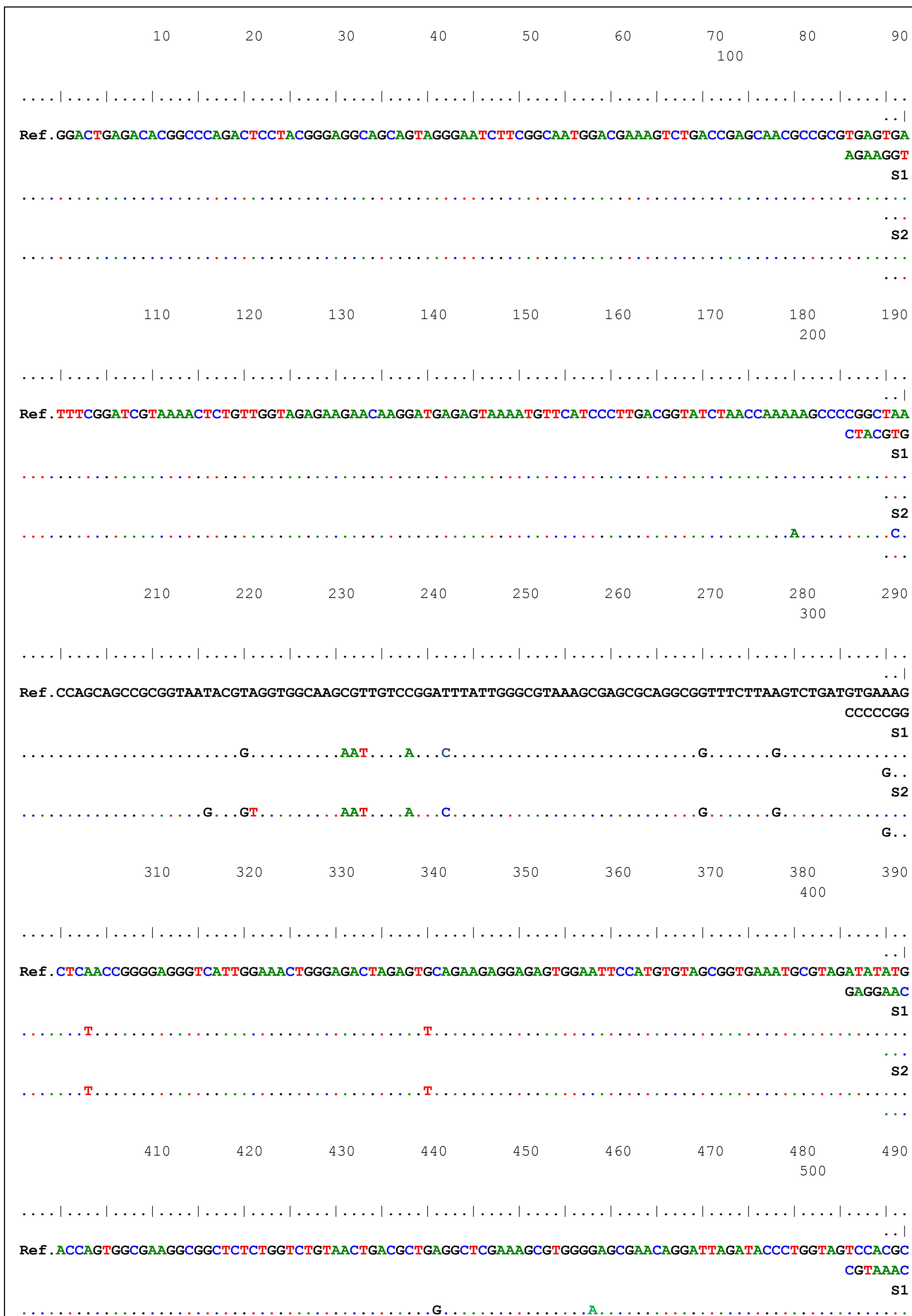

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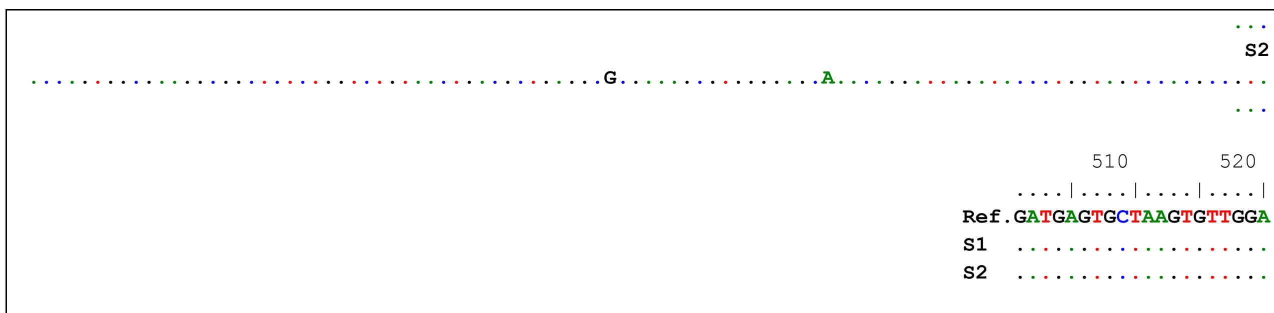

Fig. 2. Nucleic acid sequences alignment of two local samples with their consistent reference sequences of the 16-S rRNA within the Enterococcus genomic DNA sequences. The symbol "ref" refers to the NCBI reference sequences, while "S No.#" refers to sample number

Highly interesting differences were observed in the currently observed nucleic acid substitution as detected in the analyzed samples. However, the sequencing chromatograms of the identified variation region, as well as its detailed annotations, were verified and documented, and the chromatograms these sequences were shown according to their positions in the PCR amplicons (Fig 3).

Figure 3. The pattern of the detected mutations within the DNA chromatogram of the targeted 520 bp amplicons of the 16-S rRNA sequences. The identified substitution mutations are highlighted according to their positions in the PCR amplicons. The symbol ">" refers to "substitution" mutation.

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To summarize all the results obtained from the sequenced 520 bp fragments, the exact positions and annotations of the observed nucleic acid substitution mutations were described in the NCBI reference sequences as shown in (Table 2).

Table 2. The pattern of the observed SNPs in the 520 bp amplicons of the 16-S rRNA sequences of the Enterococcus in comparison with the NCBI referring sequences (GenBank acc. no. KR001878.1).

Sample No.	wild	mutant	Position in the PCR fragment	Position in the reference genome	SNP summary
S2	C	A	185	185	KR001878.1;g.185C>A
S2	A	C	196	196	KR001878.1;g.196A>C
S2	T	G	221	221	KR001878.1;g.221T>G
S1, S2	T	G	225	225	KR001878.1;g.225T>G
S2	G	T	226	226	KR001878.1;g.226G>T
S1, S2	G	A	236	236	KR001878.1;g.236G>A
S1, S2	T	A	237	237	KR001878.1;g.237T>A
S1	C	T	238	238	KR001878.1;g.238C>T
S1, S2	T	A	243	243	KR001878.1;g.243T>A
S1, S2	T	C	247	247	KR001878.1;g.247T>C
S1, S2	C	G	275	275	KR001878.1;g.275C>G
S1, S2	T	G	283	283	KR001878.1;g.283T>G
S1, S2	C	G	298	298	KR001878.1;g.298T>G
S1, S2	G	T	308	308	KR001878.1;g.308G>T
S1, S2	A	T	345	345	KR001878.1;g.345A>T
S1, S2	T	G	446	446	KR001878.1;g.446T>G
S1, S2	G	A	463	463	KR001878.1;g.463G>A

The symbol "S" followed by a number referred to the investigated sample numbers

A comprehensive phylogenetic tree was generated, which was based on the observed nucleic acid sequences detected in the investigated bacterial samples. Along with the other deposited D NA sequences, this phylogenetic tree contained both currently investigated samples (S1 and S2). In this complete tree, the total number of alignednucleic acid sequences was 85 sequences . This comprehensive tree indicated the presence of only one genus, *Enterococcus*, which represents the only incorporated nucleic acid sequences within the tree. Based on the currently analyzed ribosomal sequences, *Enterococcus* sequences were clustered into four adjacent clades. The first one was represented by the incorporation of our investigated samples alongside with *Enterococcus* species and *E. casseliflavus* sequences. this positioning provided more confirmation about the proximity between our samples and the genus of Enterococcus. As well, the pattern of the positioning of both *Enterococcus* and *E.casseliflavus*implied extremely high homology of this species with our investigated isolates. Another confirmation of this proximity was come from the adjacent clade, in which *E.casseliflavus*was positioned. This clade was made of eight strains of *E.casseliflavus*adjacent to each other. Clade-3 was also made of the positioning of both Enterococcus species and E.*casseliflavus*, giving more validation concerning the high similarity of our investigated samples with this species. Whereas it was found that clade-4 was made of the positioning of both *Enterococcus* and *E. gallinarum*. This close positioning indicated another high similarity with this

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species. therefore, our investigated samples of both S1 and S2 exerted close homology to *Enterococcus*, *E.casseliflavus*, and *E. gallinarum* respectively. Furthermore, the presence of mixing phylogenetic positioning showed the extreme similarity between the *Enterococcus*, the main genus in which all sequences were incorporated, with both *E.casseliflavus*and *E.gallinarum*species. However, the observed pattern of positioning indicated that our investigated samples tended to exhibit a closer positioning with *E.casseliflavus*than *E.gallinarum*sequences, respectively.

These observations indicated that these three different species of *Enterococcus* had not exhibited any obvious differences in their 16-S ribosomal sequences. though, the total number of the observed variations in the currently investigated isolates were seventeen nucleic acid substitutions. However, there was no deviation from *Enterococcus* as these observed variations were only minor variations within the sequences of these bacterial sequences (Fig. 3). Thus, the distinctive role of the generated phylogenetic tree in the accurate detection of the currently analyzed samples could not be ignored. Accordingly, this notion provided a further indication of the bacterial identity and accurate genotyping of these local studied samples. This 16-S ribosomal -based comprehensive tree has provided an extremely inclusive tool about the high ability of such sequences to efficiently identify *Enterococcus* samples using these genetic fragments.

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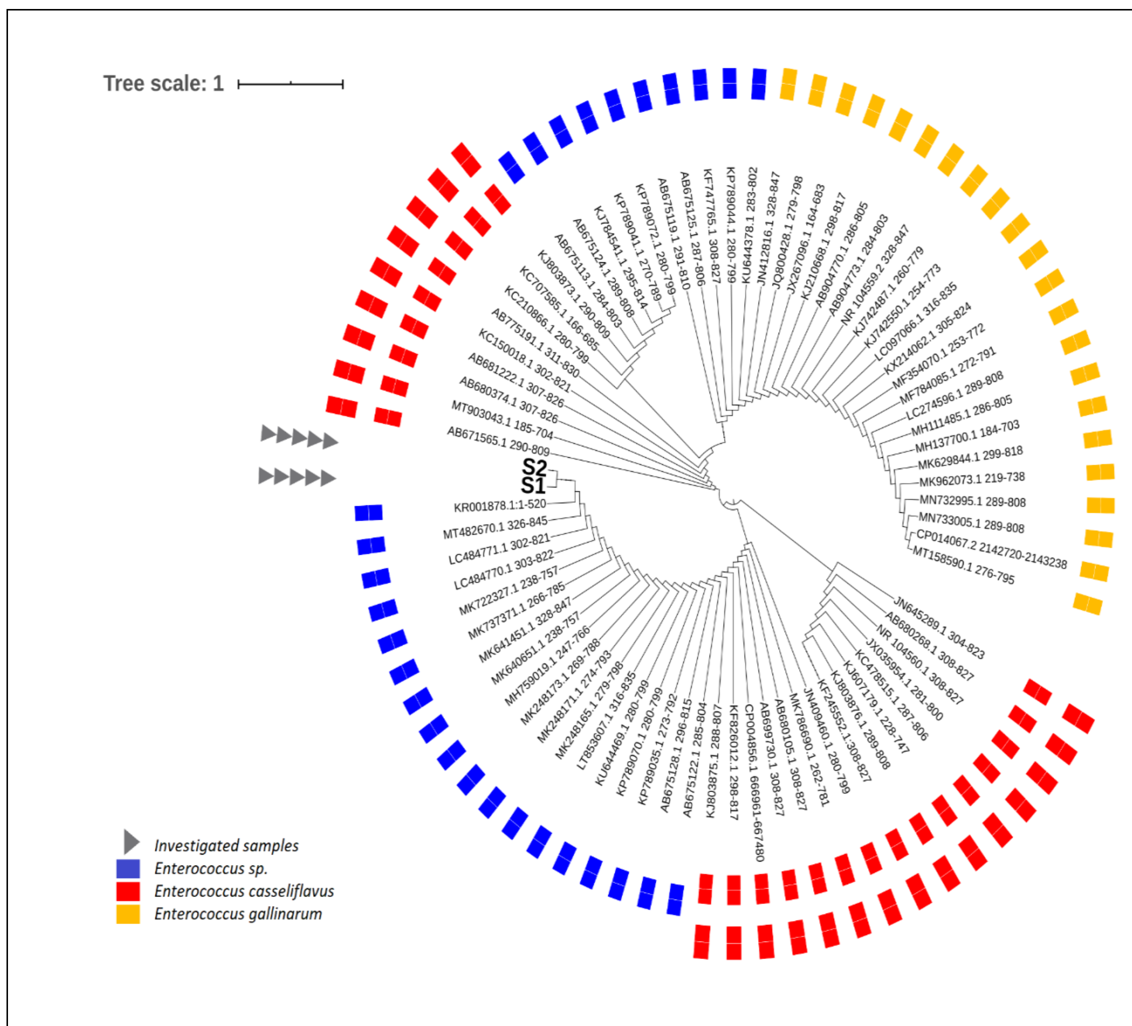

Fig. 3. The comprehensive phylogenetic tree of the local isolates of the 520 bp amplicons that partially covered the 16S ribosomal region within Enterococcus genomic sequences. The variable colors refer to the variable grouping of the analyzed variants, within their Genbank deposited sequences. The number "1" at the top left portion of the tree refers to the degree of scale range among the comprehensive tree categorized organisms. The symbols S1 and S2 refer to the code of the investigated samples.

Conclusion

The immunocompromised people are most vulnerable to infection with E.*casseliflavus* and *E.gallinarum*. additionally, the utilization of PCR-sequencing strategy in tow analyzed DNA samples of these local bacterial isolates have presented a confirmed identity of these strains within the *Enterococcus* sequences

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