Genomic-based characterization of Enterococcus spp.: an emerging pathogen isolated from human gut

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Abstract
Background Enterococci are ubiquitous microorganisms having diverse ecological niches but most prominently in gastrointestinal tract of humans and animals. Production of enterocins makes them a good probiotic candidate. However, their role as probiotics has become ambiguous in the last few years because of the presence of virulence factors and antibiotic resistance genes. These virulence traits are known to be transferred genetically, which makes them opportunistic pathogens in the gastrointestinal tract leading to serious concerns about their being used as probiotics. In the present study, Enterococcus spp. isolated from the human gut were subjected to Whole-Genome Sequencing (WGS) to determine the presence of resistance and virulence genes.

Methods and results Four human origins Enterococcus spp. including Enterococcus faecalis, Enterococcus casseliflavus, and two Enterococcus gallinarum were isolated from human fecal samples and further cultured on blood agar. Sanger sequencing was done using Applied Biosystems 3730xl DNA Analyzer. These strains were further subjected to WGS using oxford nanopore technology MinION. Raw data were analyzed using the free online tool epi2me. The Comprehensive Antibiotic Resistance Database (CARD) and RAST (Rapid Annotation using Subsystem Technology) software were used to look for the presence of antibiotic resistance genes in these strains. Resistance determinants for clinically important antibiotics (vancomycin) and functional virulence factor genes were detected. G-view server was used for comparative genomics of all strains.

Conclusion The genomic sequencing of Enterococcus suggested that E. faecalis, E. casseliflavus, and E. gallinarum strains are opportunistic pathogens, having antibiotic resistance genes. All isolates had vancomycin resistance genes, which were expressed phenotypically. Genes related to bacteriocin resistance were also present in E. casseliflavus and E. gallinarum.

Keywords Enterococcus spp. · Probiotics · Whole genome sequencing · Antimicrobial resistance · Vancomycin · MinION · Sanger sequencing

Introduction
Enterococcus genus is an important class of lactic acid bacteria (LAB) in the phylum Firmicutes that can survive in diverse ecological niches [1] including intestines of humans and animals and in food products. Most strains of enterococci are proven to have probiotic properties and are considered safe for hosts [2]. Probiotic bacteria are known for centuries mainly for their health benefits mostly in metabolic disorders. The Food and Agriculture Organization (FAO) and World Health Organization (WHO) have established some basic criteria before considering a bacterium as probiotic, e.g., tolerance level against gastrointestinal transit, production of antimicrobial peptides, susceptibility to antibiotics, and having immunomodulation activity [3].

Several genera of lactic acid bacteria (LAB) such as Aerococcus, Carnobacterium and Enterococcus have been studied due to their potential probiotic capability [4]. However, due to the presence sometimes of certain genes, their role is not as positive as thought to be previously. Hence, genomic analysis is useful in the identification and study of
such genes, as genes not only affect molecular and metabolic routes but also give specific properties to probiotics [5].

*Enterococcus* is the main genus that includes 50 species having probiotic properties. However, many strains of *Enterococcus* are known to cause disease in humans as opportunistic pathogens. There is now an alarming increase in multidrug resistance among enterococci, mainly vancomycin resistance. In addition, they also can transfer antibiotic resistance and virulence genes to other bacteria [6]. Whole-genome sequencing is now becoming a routine practice in many laboratories to characterize various genes related to antimicrobial resistance in Gram-negative bacteria but fewer studies are available on the antimicrobial resistance genes among Gram-positive bacteria [7]. Thus, it is important to do deep research on the virulence properties of *Enterococcus* before using them as probiotic strains [8]. Research is also needed to differentiate between pathogenic and nonpathogenic (safe) strains of *Enterococcus* so the latter can be used as an effective probiotic. We conducted this pilot study to determine the genome of *Enterococcus* spp. isolated from human stools. The main aim of this study was to analyze the virulence and antibiotic resistance factors in *Enterococcus* using WGS.

**Materials and methods**

Stool samples collected from humans were stored at -80°C for isolation of common bacteria of gastrointestinal tract origin. The stools were inoculated in tetrathionate broth in glass bottles followed by incubation at 37°C. Broth cultures were then sub-cultured on blood agar plates. Identification of bacterial colonies was done using morphologic and biochemical tests (Gram staining and catalase and oxidase tests).

Kirby-Bauer disc diffusion test was used to determine antibiotic resistance in these strains [9]. Briefly, the strains were first precultured in TSB medium and then the microbial pellet was suspended in 2 mL of normal saline (0.9% NaCl) to achieve 0.5 M Mac Farland as turbidity standard. Antibiotic disks used were Vancomycin, Daptomycin, Gentamicin, Vancomycin, Tigecycline, Streptomycin, Nitrofurantoin, Linezolid, and Ampicillin (Oxoid and Liofilchem). After 24 h at 37°C, zones of inhibition were measured and recorded. Finally, colonies were stored at -80°C until DNA extraction. Stool DNA was isolated by using a commercial kit (Favrogen). Sanger sequencing was done to confirm the presence of *Enterococcus* spp. in all samples. Whole-genome sequencing of *Enterococcus* spp. was done using Oxford nanopore MinION technology using a protocol (Lambda-control-sqk-lsk109-CDE_9062_v109_revI).

Sequence quality was assessed by FASTQC [7, 10] followed by online software [https://epi2me.nanoporetech.com/workflow_instance]. FASTA files were uploaded at the RAST server for annotation, putative gene product identification [7, 11]. Moreover, to investigate the presence of antimicrobial resistance genes, the draft genome was uploaded at Metagenomic Rapid Annotations using Subsystems Technology; genomes uploaded here can easily be accessed and analyzed by everyone. To visualize and compare the genome with other published *Enterococcus* spp. genomes at the time of analysis, the G-view server ([https://server.gview.ca/](https://server.gview.ca/)) was also used [7].

**Results and discussion**

**Identification and physiochemical characterization**

* Enterococcus* spp. are commonly present in the human gut and are thought to be mostly safe and are used as probiotics. In the present study, *Enterococcus* spp. was isolated from the gastrointestinal tracts of humans. Stool samples on culturing produced small, pinpoint, cream, or yellowish colonies on agar. Gram staining revealed the presence of cocci, showing negative results for catalase and oxidase enzymes. API strips confirmed the presence of *Enterococcus* in our samples. Results from BLAST alignment also confirmed *Enterococcus*. To characterize the isolated strains of *Enterococcus*, WGS was performed. Analysis of WGS sequencing data showed the presence of *E. faecalis, E. casseliflavus*, and *E. gallinarum*. Sequences were submitted to NCBI with Bio project number PRJNA682015. The enterococci were also tested for their antibiotic resistances and variant genes. Identification of cultures stored at -80°C was confirmed by using Sanger sequencing via PCR amplification. Analysis of MinION data by EPITOME showed a difference in genome sizes of all strains suggesting that genetic variation is present within the *Enterococcus* strains.

**Genome size and features**

All four strains of *Enterococcus* showed different GC content ranging from 38 to 44%. The genome sizes also varied among strains (from 1,167,642–3,508,906 bp). A complete summary of the genome report is attached as S1 showing genome coverages, total read lengths in term of N50 and L50. Virulence and pathogenicity factors such as adhesins, invasions, pili, and hemolysin in *Enterococcus* make them potentially pathogenic for humans (Table 1). The circular map of *Enterococcus* spp. was generated using G view server (Fig. 1) and a comparative genomic study was performed between different *Enterococcus* spp.
Table 1  Presence of different antimicrobial genes and class of drug resistance and their mechanism according to CARD based upon WGS data

| S. no. | E. gallinarium QAU17 | E. gallinarum QAU16 | E. casseliflavus QAU15 |
|--------|----------------------|---------------------|------------------------|
| RGI criteria | Perfect VanRC | Strict VanXY | Strict VanC | Strict ACC(6)-li | Strict VanXY |
| ARO term SNP | | | | | |
| Detection criteria | Protein homolog model | Protein homolog model | Protein homolog model | Protein homolog model | |
| AMR gene family | glycopeptide resistance gene cluster, vanR | glycopeptide resistance gene cluster, vanR | glycopeptide resistance gene cluster, vanR | AAC(6’) | glycopeptide resistance gene cluster, vanXY |
| Drug class | glycopeptide antibiotic | glycopeptide antibiotic | glycopeptide antibiotic | aminoglycoside antibiotic | glycopeptide antibiotic |
| Resistance mechanism | Antibiotic target alteration | Antibiotic target alteration | Antibiotic target alteration | Antibiotic inactivation | Antibiotic target alteration |
| % identity | 100 | 99.4 | 98.83 | 99.4 | 79.8 |
| % length of reference sequence | 100 | 88 | 100 | 100 | 100 |

Metabolic network

The metabolic pathway/genome database (PGDB) was created computationally with KEGG metabolic pathways in RAST annotation server version 2020 [12]. The genome size of all strains was between 1 M-4 M, showing size diversity among different strains. GC content also varied among strains ranging from 38%-44%. These sizes are in agreement with Enterococcus spp. genome present on NCBI. We performed a different type of analysis on the genomic data to evaluate Enterococcus strain as a potential probiotic. Thirteen genes related to bacteriocin production were found in E. casseliflavus QAU15. Eight genes co-occur together in a cluster-based subsystem. One of these genes is responsible for producing colicin V (a bacteriocin produced by some strains of Enterococcus). Nine genes were present in two strains of E. gallinarium for bacteriocin production. Production of colicin V in these bacteria suggests their role in the progression of gastrointestinal infection. Many recent studies are now developing a relationship between pathogenicity and colicinogeny in different bacterial strains [13].

However, the presence of bacteriocins only is not enough to declare Enterococcus as a non-suitable candidate for GRAS (generally regarded as safe). Hence, we did antibiotic resistance gene analysis of our strains; phenotypic data showed vancomycin resistance among a few strains of Enterococcus. Using RAST software, we found the presence of different antibiotic resistance genes but the most common was vanXY. This is of clinical significance as it is also expressed phenotypically in E. faecalis QAU14, E. casseliflavus QAU15, and E. gallinarum QAU16.

Evaluation of antibiotic resistance

The comprehensive antibiotic resistance database was used to determine the presence of antibiotic resistance genes. Enterococcus spp. were then tested against commonly used antibiotics, e.g., Daptomycin, Gentamicin Vancomycin, Tigecycline, Streptomycin, Nitrofurantoin, Linezolid, and Ampicillin (Table S2). Results show that E. gallinarium QAU17, E. faecalis QAU14, E. casseliflavus QAU15, and E. gallinarum QAU16 were resistant against vancomycin. E. faecalis and E. gallinarium were also resistant to linezolid while E. casseliflavus was resistant against ampicillin and vancomycin. We then used CARD software [13] to find presence of antibiotic resistance genes so we can see the presence of antibiotic resistance genes in these isolates. VanXYC glycopeptide resistance gene cluster was observed in E. gallinarium suggesting a strong antimicrobial resistance pattern here.

E. gallinarium has AAC-6 (aminoglycoside acetyltransferase-li having role against aminoglycoside antibiotics) protein homology model belonging to the aminoglycoside class of antibiotics that work by antibiotics inactivation which is somehow expressed phenotypically that is its resistance towards vancomycin and linezolid. Three types of genes related to vancomycin resistance were found in E. gallinarium isolates i.e. vanRC, vanXYC and vanC, which are protein homologs and act against glycopeptide antibiotics. These results were verified by in vitro analysis of drug susceptibility testing; most strains were only resistance to vancomycin, suggesting vancomycin resistance as an intrinsic property of Enterococcus genome.

Virulence factors play an important role in enhancing the ability of these organisms to cause illness. Virulence factors
in the case of Enterococcus include extracellular proteins, bacteriocins, cell adhesion proteins, invasions, and intracellular proteins, and metabolic proteins. In general, although virulence-related genes are absent from our strains, some genes related to the above-mentioned factors are present in these strains; detail of each factor along with the number of genes are mentioned in Table 2. Virulence factor alone is not enough to explain the pathogenicity of Enterococcus as antimicrobial genes also have a major role in its pathogenicity.

The presence of genes related to transposons and mobile elements also contributes to increasing in pathogenicity of Enterococcus, as these mobile genetic elements transfer AR (antimicrobial genes) to nonpathogenic strains. This trans-conjugation mechanism, by which they acquire AR resistance and even transfer of virulence factors, raises serious concern of using them as probiotics. Other genes related to antibiotics and toxic compounds were also seen in these isolates detail of which is incorporated in S3. Some important genes mentioned in S3 are related to resistance from fluoroquinolones shows presence of acquired gene resistance as described previously in many studies [14, 15].

Additional analysis of plasmid-associated genes was also calculated using plasmid finder by RAST software. It did not show the presence of genes associated with plasmids.
indicating that in these strains plasmid are not much involved in antibiotics resistance transfer genes, as plasmid have a central role in the transfer of resistance genes, but in place of plasmids mobile genetic elements are there which help in transfer of AR genes in strains.

Conclusions

Vancomycin and linezolid are the most used drugs against Enterococcus in hospitals. Vancomycin-resistant organism (VRE) is known to cause serious infections that cannot be treated with common antibiotics. Treatment of VRE is a real challenge to clinicians, as vancomycin is usually the drug of last choice in treatment of enterococcal infections. Vancomycin is used as a replacement for penicillin, ampicillin, and aminoglycosides in patients with allergies [16]. In our isolates, we found resistance genes against vancomycin only in E. gallinarum QAU17 and E. casseliflavus QAU15. E. faecalis QAU14 and E. gallinarum QAU16 were also resistant against Linezolid, which concludes the presence of 23S rRNA mutations and horizontally acquired resistance genes cfr and optrA. Three chromosomally located clustered genes vanC1, vanXYC, and vanRC were detected in these strains except E. faecalis but always there is a chance that these resistance genes can be transmissible to other bacteria. Therefore, it is suggested that more research is needed to study vanC1, vanXYC, and vanRC in Enterococcus strains as they are the reservoir for antimicrobial resistance genes. The WGS analyses proved that E. faecalis, E. casseliflavus, E. gallinarum isolates are not promising candidates for probiotics as they have antibiotic resistance and virulence genes. More comprehensive studies on metabolic pathways are needed to further evaluate the probiotic role of Enterococcus. Moreover, we used Oxford Nanopore technology MinION, which is proven to be less time-consuming and is cost-effective for screening normal gut flora of humans.

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Authors’ contributions All authors contributed significantly in this study. Ms. Zumara: did all laboratory work and drafted the manuscript. Prof. Goyal: Supervised the WGS. Dr. Vikash: worked on WGS data analysis. Prof. Aamer: supervised the clinical aspects especially sampling. Dr. Imran: supervised the experiment design and did data analysis.

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Data availability Sequences data was submitted to NCBI with Bio project number PRJNA682015.

Code availability Not applicable.

Declarations

Conflict of interest Authors have no conflict of interest or competing interest in this study.

Ethical approval The work was approved by the Biological Ethical Committee of Quaid-i-Azam University, Islamabad, Pakistan.
Consent to participate Written informed consent was obtained from all participants of this study.

Consent for publication All authors have agreed to publish this work in the current form.

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