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Identification of Single-nucleotide Polymorphisms (SNPs) of *Enterococcus faecalis* Isolated from HIV Seropositive Nigerian Patients with CD4+ Cells of<200 Cells/µl: A Possible Vaccine Target

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Abstract

Enterococci have gained significance as the cause of nosocomial infections. They occur as food contaminants and have also been linked to dental diseases. Currently, infective endocarditis (IE) caused by *Enterococcus faecalis* represents 10% of all IE and is marked by its difficult management and the frequency of relapses. Although the precise reasons for that remain to be elucidated, the evolution of the culprit strain based on single nucleotide polymorphism (SNP) could be, at least in part, involved. The cross sectional study randomly selected 40 consented (25 male and 15 female) HIV seropositive patients and body mass index of 16.7 ± 1.0 (Kg/m^2) with CD4$^+$ cells<200 cells/µl. Urine and feces samples were collected used for testing. Chrom agar was used for bacterial isolation. DNA isolations from the 24-hour growth cultures of possible *Enterococcus faecalis* were carried out using Zymo Research Bacterial DNA isolation kit. The twenty-nine (29) clinical isolates that showed black-colored colonies and were further subjected to polymerase chain reaction identification using *Enterococcus faecalis* gene specific primers. Only two (2) out of twenty-nine (29) suspected *Enterococcus spp* were PCR confirmed *Enterococcus faecalis*. We observed that about 85.36313% sites of the accessions are polymorphic among the two isolates. Considering the *Enterococcus faecalis* gene the polymorphic sites are 76.4% and 23.6% biallelic and triallelic respectively with a corresponding number of such sites as 447 and 331, respectively. The coding regions (CDs) for the *Enterococcus faecalis* genome displayed the majority of SNP loci at codon position C2 and C3 with 34.5% and 31.3% of their respective total SNP loci, respectively. The observed variability between the two sequences from Nigeria may be due to increased genetic diversity over time and could be a possible vaccine target in the prevention of infective endocarditis (IE) caused by *Enterococcus faecalis*.

Keywords: *Enterococcus faecalis* • Single-nucleotide polymorphisms • Vaccine target • Nigeria

Introduction

Approximately 10% of infective endocarditis (IE) is caused by *Enterococcus faecalis*, which makes such infections difficult to manage and frequently relapse for reasons not entirely elucidated [1]. The genetic evolution of this bacterial strain based on single nucleotide polymorphism could play an important role in determining the infection outcome. This hypothesis has been supported by the identification of small colony variants (SCV) in culture [2–4] and by the observation of phenotypic or genetic changes associated with *E. faecalis* IE relapses [5]. Also, in humans, numerous reports have demonstrated that natural variability to *infection* can be associated with single nucleotide polymorphisms [6].

Apart from nosocomial infections, *E. faecalis*, although not normally considered to be part of the healthy oral flora [7], has been found in common dental diseases, i.e., periodontitis, perimplantitis and caries [8,9]. *E. faecalis* has been found primarily in secondary endodontic infections with a prevalence of 24–70%, i.e., in previously filled root canals, where it can also form biofilms [10,11]. However, until now, the evaluation of genetic diversity based on SNPs of *E. faecalis* has not been described nor extensively characterized over the course of its infection. Such approaches were already proved relevant to understand the pathophysiology and to guide the therapy in other infectious diseases, such as osteoarticular infections or cystic fibrosis [12].

Methodology

The cross sectional study randomly selected 40 consented (25 male and 15 female) HIV seropositive patients of an average age of 48.3 ± 3.0 years and body mass index of 16.7 ± 1.0 (Kg/m^2) with CD4$^+$ cells < 200 cells/µl attending HIV clinic at the University of Benin Teaching Hospital, Benin City, Nigeria. Ethical approval was obtained from the Health research ethics committee of the university of Benin teaching hospital, Benin city, Nigeria. Urine samples were collected from 26 patients while stool samples were collected 16 patients respectively. For the isolation of suspected *E. faecalis* in urine and feces. Samples were inoculated with sterile swabs on chrom agar. Cultures were incubated 24-48 hours at 37°C under aerobic conditions. The twenty-nine (29) clinical isolates that showed black-colored colonies and were further subjected

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to polymerase chain reaction identification using *Enterococcus faecalis* gene specific primers.

DNA isolation from the suspected clinical isolates

The overnight culture from the suspected clinical isolated were harvest into 200 µl nuclease free water in micro centrifuge tube and centrifuged at 13000 rpm for 3 minutes. The pellet was re-suspended in 200 µl of nuclease free water and DNA was extracted using the ZR Fungi/Bacterial DNA MiniPrep (Zymo Research, Irvine, CA).

Amplification

Polymerase chain reaction amplification was done using *E. faecalis* gene specific forward and reverse primers (F: 5ACT TAT GTG ACT AAC TTA ACC 3'; R: 5' TAA TGG TGA ATC TTG GTT TGG 3). The PCR was performed in a 25 µl reaction mixture containing 12.5 µl oneTaq one-step reaction master mix (2×), 1.25 µl of each forward primer (20 µM), 1.25 µl of each reverse primer (20 µM), 5 µl of nuclease-free water and 5 µl of the DNA template was then added. The PCR was programmed as initial denaturation at 94°C for 1 min; denaturation at 94°C for 15 sec, annealing at 49°C for 30 sec; and extension at 72°C for 1 min, repetition of the denaturation step for 35 cycles; final extension at 72°C for 5 min and final holding at 4°C.

PCR product cleaning and purification

PCR product cleaning and purification The PCR products were cleaned using Exo/SAP. Exo/SAP Master mix was prepared by adding 50.0 µl Exonuclease I (NEB M0293) 20 U/ul and 200.0 µl Shrimp Alkaline Phosphatase (NEB M0371) 1U/ul in a 0.6 ml microcentrifuge tube. Exo/SAP Mix of 2.5 µl was then added to 10.0 µl of PCR Mixture, mixed well and was incubated at 37°C for 30 minutes after which the reaction was stopped by heating at 95°C for 5 minutes. Purification was done with the ABI V3.1 Big dye kit according to manufacturer's instructions. The labeled products were then cleaned with the Zymo Seq clean-up kit. Sequencing Binding Buffer of 240 µl was added to 20 µl sequencing reaction which was then transferred to a Zymo-Spin™ IB-96 plate mounted onto a collection plate. The mixture was centrifuge at 3,000 x g for 2 minutes. Sequencing Wash Buffer of 300 µl was added to each well of the plate and centrifuge at 3,000 x g for 5minutes. 15 µl of water was directly added to the column matrix of the filter plate. The ZymoSpin™ IB-96 plate was placed on top of the supplied 96-Well PCR plate and mount the assembly onto the Collection Plate which was then centrifuge at 3,000 x g for 2 minutes to elute the DNA.

Sequencing

The Ultra-pure DNA was sequenced with ABI3500XL analyzer with a 50 cm array, using POP7 at Inqaba Biotechnical Industries Ltd (Hatfield, South Africa). Sequences data generated was analyzed with genius version 9.0.5 and phylogenetic tree was constructed using neighbor joining method. Only two (2) out of twenty-nine (29) suspected Enterococcus spp were PCR confirmed *Enterococcus faecalis*.

Results and Discussion

Discriminant analysis of principal components (DAPC) for the PCR confirmed *Enterococcus faecalis* were carried out to detect the possible number of clusters among the 10 accessions within the accessions (Figure 1). The number of detected clusters was five, which coincided with the lowest BIC value obtained from the find. Clusters function (Figure 2A). First four PCs (98% of the variance conserved) of the PCA were retained (Figure 2B) and two discriminant eigenvalues were confirmed by the cross-validation analysis. The distribution of the accessions in the five populations was fully matched with the classification of variant in their respective location (Figure 2). Thus, each population was considered to have some genetic diversity which could be a possible target for vaccine production.

Clusters 1, 2, 3, 4 and 5 consisted of two accessions each (Figure 3). The distribution of the accessions in the five populations was fully matched with the classification of variant in their respective location (Figure 1). Thus, each population was considered for genetic diversity analysis. The fact that the accessions in the respective locations were grouped distinctly indicated that these accessions had the highest level of genetic variability. Our study clearly differentiated accessions from Nigeria, China, Turkey, India and Czech Republic into different clusters, which is in agreement with previous studies [13,14]. In order to assess the rate and nature of polymorphism within the *Enterococcus faecalis*, which we sequenced from the stool samples, we observed that about 85.36313% sites of the accessions are polymorphic. This is not entirely surprising, given that the segment of *Enterococcus faecalis* gene is known for its high mutation rate [15,16]. A previous study reported that the mutational rate of *Enterococcus faecalis* is found to be 6.4-fold higher than *Enterococcus faecium*, which accounts for the fact *Enterococcus faecalis* had a higher potential to develop linezolid resistance.

Rifampicin resistance was associated with mutations in the rpoB gene. Rifampicin MICs for the *Enterococcus faecalis* mutant were 2048 mg/l, but rifampicin MICs for *Enterococcus faecium* mutants ranged from 64 to 1024 mg/l [17]. Considering the *Enterococcus faecalis* gene the polymorphic sites are 76.4% and 23.6% biallelic and triallelic respectively with a corresponding number of such sites as 447 and 331, respectively (**Figure 4**). Quigley (2020) showed that most of the SNPs associated with human disease have been described as biallelic, which confirms increased pathogenicity associated with *Enterococcus faecalis.* At regulatory sites, SNP rates are better and

Figure 1. Discriminant analysis of principal components (DAPC) for 10 *Enterococcus faecalis* accessions using the identified single nucleotide polymorphisms (SNPs). Four first principal components (PCs) and two discriminant eigenvalues were retained during the analyses to describe the relationship between the clusters.

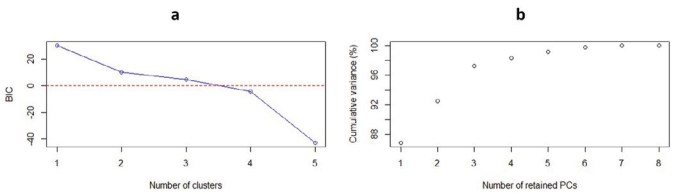

Figure 2. (a) Value of BIC versus number of cluster and **(b)** Variance explained by PCA.

Figure 3. Clusters of *Enterococcus faecalis* accessions based on their genetic distance.

conservation is higher [15]. Single-nucleotide polymorphisms (SNPs) are considered to be the most common genetic changes that result from alterations in a single nucleotide [16]. The densities of polymorphic sites of genomes or individual coding sequences (CDSs) were plotted against their nucleotide position to visualize the distribution of SNPs across the length of the genome (Figure 5). The genome-wide SNP distribution appeared random in the graph.

The Monte Carlo simulation was performed to analyze whether SNPs are clustered or randomly distributed in the genome. Based on 999 replicates with a simulated p-value of 0.007, the values for standard observation, expectation value and variance were found to be -2.7560099, 3.7117117 and 0.3857445, respectively, indicating that the alternative hypothesis is not true and the distribution of SNPs in the complete genomes of *Enterococcus faecalis* genome is random. The CDS for *Enterococcus faecalis* exhibited SNP at several loci, with the 5 highest densities clustered together from 475th to 482nd nucleotide position (Figure 5). The SNP loci of various CDS were also analyzed for the presence of SNP at nucleotide positions C1, C2 and C3 in the codons. The CDSs for the *Enterococcus faecalis* genome displayed the majority of SNP loci at codon position C2 and C3 with 34.5% and 31.3% of their respective total SNP loci, respectively (Figure 6). Single-nucleotide polymorphisms (SNPs) are considered to be the most common genetic changes that result from alterations in a single nucleotide [17]. Among SNPs, nonsynonymous SNPs (nsSNP) are associated with single amino acid substitution in the coding regions of a gene that may have the drastic effect on its structural and functional properties [18]. The second-codon position is the most functionally constrained; any change to the second codon position causes a nonsynonymous change in the coding sequence [19].

To discover any potential pattern in SNP data of *Enterococcus faecalis* genome, the genind object of the entire genomes was subjected to the principal component analysis (PCA). The missing data (NAs) in the genind object were replaced by the mean allele frequency. The eigenvalues, which indicate the amount of variance represented by each principal component, were also calculated and a scatter plot was generated. The PCA report suggests that except *Enterococcus faecalis* genome identified from the patients stool samples in Nigeria, accessions of *Enterococcus faecalis* sequences from China, Turkey, Czech Republic and India are closely related (Figure 7A and 7B). The noticed variability between the two sequences in Nigeria may be due to increased genetic diversity over time. This is further confirmed with a wide genetic distance observed between the accessions (OL307982.1 and OL307984.1) representing *Enterococcus faecalis* isolate from Nigeria based on the phylogenetic relationships among the 10 accessions (Figure 8). A large number of loci are nearly fixed (frequencies close to 0 or 1) in *Enterococcus faecalis* accessions (OL307982.1 and OL307984.1) (**Figure 9**) with an increased susceptibility to be associated with certain phenotypic traits.

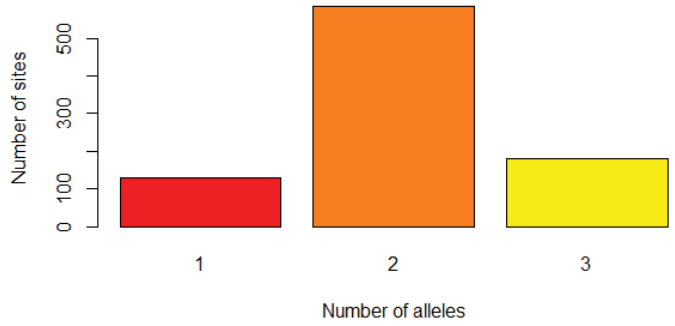

Figure 4. SNP in the 16s RNA accessions of *Enterococcus faecalis* genome isolated in Nigeria. The numbers of sites (y-axis) were plotted against the number of alleles (x-axis).

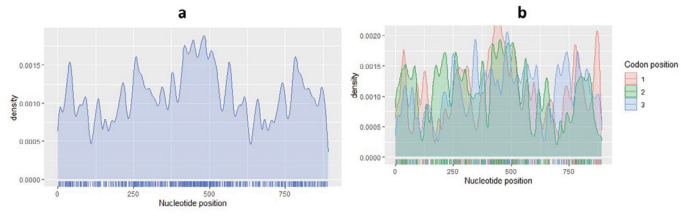

Figure 5. (a) Distribution of polymorphic sites on *Enterococcus faecalis* genome nucleotide position only and **(b)** Nucleotide with codon position.

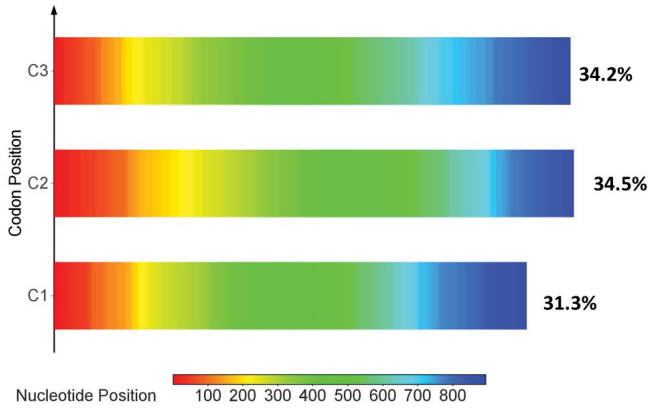

Figure 6. Codon positions of SNP loci.

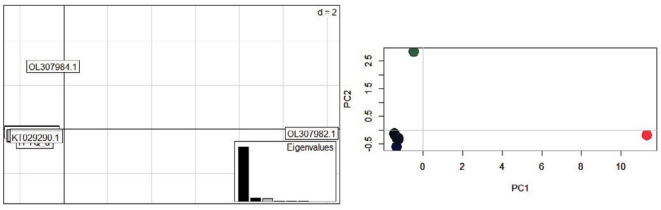

Figure 7. Principal component analysis (PCA) of the SNP data extracted from *Enterococcus faecalis* genome.

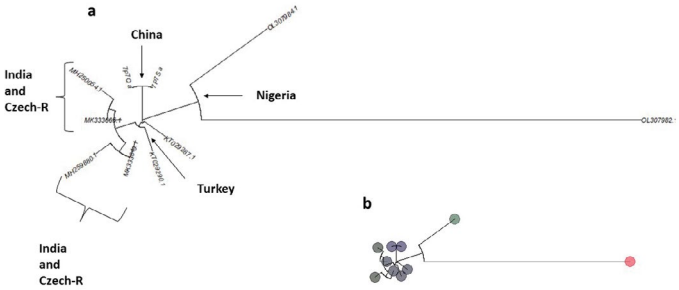

Figure 8. (a) Phylogenetic relationship between accessions of *Enterococcus faecalis* and **(b)** *16s* rRNA based on different geographical locations.

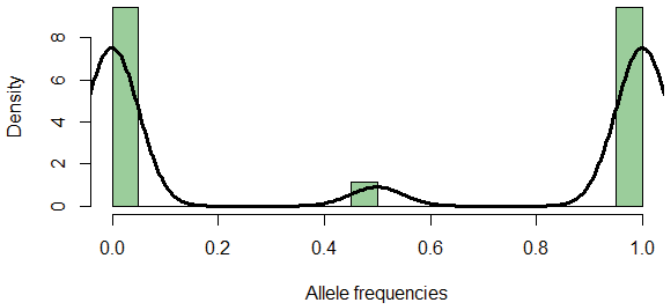

Figure 9. Distribution of alleles frequencies of *Enterococcus faecalis* accessions. (OL307982.1 and OL307984.1).

Conclusion

The observed variability between the two sequences from Nigeria may be due to increased genetic diversity over time and could be a possible vaccine target in the prevention of infective endocarditis (IE) caused by *Enterococcus faecalis*.

Competing interests

The authors declare that they have no competing interests.

Author's contributions

This work was carried out in collaboration between all authors. Author EFA, EIJ, RCC, OCC and MCS designed the study and performed the statistical analysis. Authors EFA, EIJ, OEC, OMTO, UCB, OCM, URN, OON, MPO, IAC and EPI conducted and managed the Laboratory analysis. All authors read and approved the final manuscript.

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