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Letter to the Editor

Genome sequence typing and antimicrobial susceptibility testing of infertility-associated *Enterococcus faecalis* reveals clonality of aminoglycoside-resistant strains

Editor Dr Teresa Coque

Sir,

Human infertility affects 9% to 12% of reproductive-aged couples. Both symptomatic and asymptomatic genital tract infections can contribute to infertility [1,2]. We have recently shown that the presence of *Enterococcus faecalis* in genital samples of asymptomatic infertile couples is associated with abnormal semen parameters and reduced levels of vaginal lactobacilli, and it is also predictive (together with *Mycoplasma hominis* and *Ureaplasma urealyticum*) of *in vitro* fertilization (IVF) failure [2]. *E. faecalis* is a member of the human gut microbiota, but it is also a pathogen responsible of urinary tract infections, sepsis, endocarditis, peritonitis, abdominal/pelvic and soft tissue infections. Beta-lactams are the preferred antimicrobials for most *E. faecalis* infections. Treatment of severe infections requires the combination of β-lactams or glycopeptides together with aminoglycosides, typically gentamicin. Acquisition of genes encoding aminoglycoside modifying enzymes (AMEs) and conferring resistance to high-level aminoglycosides is alarming as it eliminates the synergistic bactericidal effect. The present study aims at characterising antibiotic susceptibility and population structure of a collection of 41 *E. faecalis* strains isolated from infertile couples asymptomatic for genital infections.

The infertility-associated *E. faecalis* strains included 28 isolates from semen samples and 13 from vaginal swabs [2]. Antimicrobial susceptibility was tested using VITEK®2 with the AST-P658 card (Biomérieux), MIC with the Sensititre GPN3F plate (Thermo-Fisher) and disk diffusion assays covering the antibiotics recommended for enterococci by EUCAST, as previously described [3]. All the strains resulted susceptible to β-lactams, glycopeptides, tigecycline, linezolid and nitrofurantoin, whereas 8 of 41 (19.5%) isolates were resistant to at least one antimicrobial. All the eight strains were resistant to high-level aminoglycosides, of which seven (17%) were resistant to gentamicin (MIC≥1024 µg/mL), three to streptomycin (MIC≥2048 µg/mL), and one strain (4774) was resistant to gentamicin, streptomycin, ciprofloxacin and levofloxacin (MIC = 4 µg/mL). Resistance to high-level gentamicin in our population was lower than the European (26.6%) and Italian (35.2%) mean rate percentages in 2019 (https://www.ecdc.europa.eu/sites/default/files/documents/surveillance-antimicrobial-resistance-Europe-2019.pdf). Thus, four different phenotypic antimicrobial resistance patterns were defined: Gm^R (5/8 isolates), Sm^R (1/8), Gm^R Sm^R (1/8), Gm^R Sm^R Cip^R Lvx^R (1/8) (Fig. 1).

Complete genomes of all isolates were obtained by using both short-read (Illumina) and long-read (Oxford Nanopore) sequencing techniques followed by hybrid assembly. *E. faecalis* genomic DNA preparation, purification and sequencing were as reported [4].

Population analysis was conducted with the PopPUNK tool (https://github.com/johnlees/PopPUNK) using the: (i) complete genome sequences to estimate overall genetic diversity and relatedness of isolates, and (ii) core genome sequences to identify specific isolate clusters. The 41 strains were split into 9 partitioning clusters, of which the largest one (cluster 1) contained 8/41 isolates (Fig. 1). Six strains (4774, 5410, 5034, 5245, 2819, 4638) out of the seven high-level gentamicin-resistant isolates belonged to cluster 1, whereas strains 4153 (Gm^R) and 4953 (Sm^R) were found to be phylogenetically more distant and were placed under clusters 3 and 4 (Fig. 1). Of note, strains 4088 and 4089 isolated from the same infertile couple showed a Jaccard index of 1, indicating identical isolates and thus suggesting possible sexual transmission of *E. faecalis*. Core genomes were also used for multilocus sequence typing (MLST). Seventeen sequence types (STs) were assigned, of which ST40 (11/41 isolates), ST81 (7/41) and ST179 (5/41) were the most frequently found (Fig. 1). All STs were present in the *E. faecalis* database. Calculation of the Simpson's index of diversity of STs (D = 0.889; 95% confidence interval = 0.83–0.95; https://online.phyloviz.net/index) confirmed an elevated diversity of the infertility-associated *E. faecalis* population, except for the strains highly resistant to gentamicin. Among the six high-level gentamicin-resistant strains pertaining to cluster 1, four isolates belonged to ST179, one to ST16, and the sixth strain (4774) belonged to ST480, indicating clonality of the high-level gentamicin-resistant *E. faecalis* strains associated to infertility.

Genomes of the eight high-level aminoglycoside-resistant strains were searched for the presence of AME genes, which were found to be located either on the chromosome (6/8 strains) or on plasmids (2/8 strains) (Fig. 1). The seven strains highly resistant to gentamicin carried one copy of the *aac(6′)-aph(2′′)* gene encoding a bifunctional acetyltransferase-phosphotransferase enzyme, which mediates resistance to virtually all aminoglycosides, except for streptomycin. A single copy of the *ant(6)* gene, coding for a nucleotidyltransferase that confers streptomycin resistance, was found in the three high-level streptomycin-resistant strains. Strains 4774 and 5034 harboured both the *aac(6′)-aph(2′′)* and *ant(6)* genes. Interestingly, all the six strains with chromosomally located AME genes belonged to cluster 1, emphasizing clonality and arguing for the presence of a common genetic element mediating resistance to high-level aminoglycosides in the infertility-associated *E. faecalis* collection (data not shown). Finally, genome analysis of strain 4774 showed the presence of two point-mutations encoding amino acid substitutions in GyrA (Ser83Tyr) and ParC (Ser80Ile), known to be associated with fluoroquinolone resistance.

Infections of the genital tract, including asymptomatic and subclinical infections, can negatively impact on couple fertility [1,2,5].

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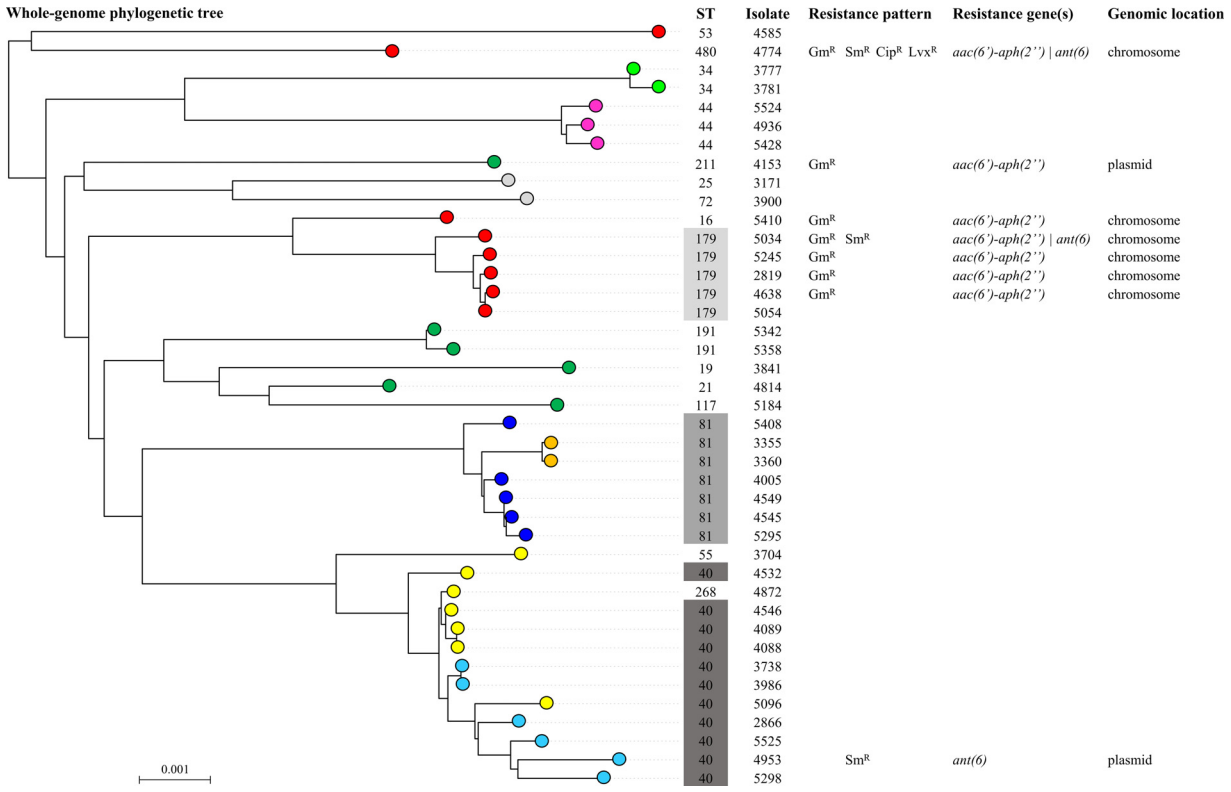

Fig. 1. Characterisation of infertility-associated *Enterococcus faecalis* clinical isolates. Whole genome sequence and antibiotic susceptibility were determined in 41 *E. faecalis* strains isolated from the genital tract of infertile couples. Genetic relatedness was evaluated with the PopPUNK tool using the '–fit-model lineage' parameter for data fitting (https://github.com/johnlees/PopPUNK). PopPUNK exploits the Jaccard index (J) to establish the similarity between *k*-mer data sets (oligonucleotide sequences of *k* length) of two genome sequences (0<J<1, with J=1 describing two genome sequences sharing the same *k*-mers). The phylogenetic tree was generated based on whole genome sequences with branch lengths indicating the number of nucleotide substitutions per site (scale bar), whereas nine population clusters (coloured dots) were obtained from core genome sequences. Major cluster 1 (red dots) contains 6 out the 8 high-level aminoglycoside resistant strains. J between strains 4088 and 4089 is 1. MLST was carried out by searching the *E. faecalis* database (https://pubmlst.org/organisms/enterococcus-faecalis/) with the MLST tool (https://github.com/tseemann/mlst) and assigned 17 different STs. The most predominant STs (ST40, ST81 and ST179) are highlighted as grey-gradient boxes. The presence of resistance genes was investigated by searching the ARG-ANNOT, CARD, MEGARes and ResFinder databases with the ABRicate tool (https://github.com/tseemann/abricate). The antimicrobial resistance pattern, resistance gene(s) and genomic location of AME genes are shown. The AME genes *aac(6′)-aph(2″)* and *ant(6)* are responsible for resistance to gentamicin (Gm^R) and streptomycin (Sm^R), respectively. Resistance to ciprofloxacin (Cip^R) and levofloxacin (Lvx^R) was mediated by two point-mutations in *gyrA* and *parC*.

The presence of *E. faecalis* in genital samples of infertile couples has been associated to reduced sperm quality [2,5,6], altered vaginal microbiota [2,7,8] and IVF negative outcomes [2,8]. Our collection of infertility-associated *E. faecalis* was tested for population structure and antibiotic susceptibility. Two different typing approaches confirmed a high level of diversity in this *E. faecalis* population, indicating that association with infertility is not a feature of a specific cluster of *E. faecalis* strains. As far as antibiotic susceptibility is concerned, all isolates were susceptible to most clinically relevant antibiotics, except for 8/41 strains (19.5%), which were resistant to high-level aminoglycosides and clonally related, suggesting that these clonal isolates derive from an ancestral clone that acquired a mobile genetic element carrying high-level gentamicin-resistance genes.

Declaration of Competing Interest

None declared.

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Ethical approval

Not required.

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