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Incidence of virulence determinants in clinical *Enterococcus faecalis* and *Enterococcus faecium* isolates collected in Bulgaria

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ABSTRACT

Objectives: To evaluate the prevalence of some virulence genes among 510 clinical *Enterococcus* spp. isolates and to assess the association of those genes with the species, infection site, and patient group (inpatients/outpatients).

Methods: Adhesins genes (aggregation substances *agg* and *asa1* of *Enterococcus faecalis* and *Enterococcus faecium*, respectively), enterococcal surface protein (*esp*), endocarditis-specific antigen A (*efaA*), collagen-binding proteins (*ace/acm*)); invasins (hyaluronidase (*hyl*) and gelatinase (*gelE*)); cytotoxines (activation of cytolysin (*cylA*) in *E. faecalis*); and modulators of the host immunity and inflammation (enhanced expression pheromone (*eep*) in *E. faecalis*) were detected by polymerase chain reaction.

Results: The overall prevalence was: *esp* – 44.3%, *agg/asa1* – 38.4%, *ace/acm* – 64.3%, *efaA* – 85.9%, *eep* – 69.4%, *gelE* – 64.3%, *hyl* – 25.1%, and *cylA* – 47.1%. *E. faecalis* isolates had significantly higher frequency of adhesin genes (*esp* and *agg/asa1*) and gelatinase in comparison to *E. faecium*. Multiple virulence genes in *E. faecalis* were significantly more prevalent than in *E. faecium* isolates. Domination of *E. faecium* with or without only one gene compared to the isolates of *E. faecalis* were found. *Enterococcus* spp. isolates obtained from outpatients compared to inpatients isolates had significantly higher frequency of *agg/asa1*, *eep*, *gelE* and *cylA*. Some adhesins genes (*esp*, *agg/asa1* and *efaA*) had higher prevalence among the non-invasive *Enterococcus* spp. isolates compared to those causing invasive bacteremia, while *ace/acm* revealed higher dissemination in isolates causing invasive infections compared to non-invasive isolates.

Conclusion: Most *E. faecalis* attaches to abiotic surfaces in hospital environment, which correlates with higher prevalence of gene encoding for virulence factors involved in biofilm

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formation, such as enterococcal surface protein, aggregation substance, and gelatinase. The intestinal tract is an important reservoir for opportunistic enterococcal pathogens and allows them to access infectious sites through different virulence factors, demonstrated in outpatient isolates in this study.

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Introduction

Enterococci (mostly *Enterococcus faecalis* and *Enterococcus faecium*) are natural inhabitants of the gastrointestinal tract of humans and animals,[1] but are also found in other anatomical sites, including the vagina and oral cavity,[2] and in the soil, water, plants, and food.[3–5] Several reports have documented that the two most important species are among the leading causes of opportunistic human infections,[6] including urinary tract infections,[7] infections of the surgical site and burn wound infections,[8,9] bacteremia and sepsis,[10] endocarditis,[11] cholecystitis,[12] peritonitis,[13] neonatal meningitis,[14] and others.

The severe infections caused by *Enterococcus* spp. are difficult to treat due to the organism's capacity to survive in the hospital environment, their intrinsic resistance to many antimicrobials, their remarkable ability to acquire further resistance mechanisms toward strategic antibiotics during the treatment period and a variety of virulence factors.[15]

Virulence factors contribute to the pathogenesis of enterococcal infections through mediation of adhesion, colonization and invasion into the host tissues, modulation of the host immunity, and extracellular production of enzymes and toxins, which enhance the severity of the infection.[16] Many virulence factors, such as the enterococcal surface protein (Esp), aggregation substance, capsule formation and gelatinase, are involved in bacterial adherence to host cells and/or in biofilm formation on abiotic surfaces in hospital environment.[17–21] Biofilm production has an important role in the pathogenesis of enterococcal infections and also favors disease sustenance because of restricted penetration of antimicrobials.[19] Invasion is usually facilitated by damage to host tissues and presence of virulence factors, including hyaluronidase and gelatinase, which assist in the advancement and further survival in newly infected places.[21]

Currently, our knowledge about the possible relationship between the presence of virulence factors and their implication in human enterococcal infections is still limited.

The aim of the present study was to examine the prevalence of genes encoding cell-associated and extracellular virulence factors in clinically relevant isolates of *Enterococcus* spp. and to assess the association of those genes with species, infection site, and patient population (inpatients or outpatients).

Materials and methods

Bacterial isolates

A collection of 510 non-duplicate clinical strains of *Enterococcus* spp., causing symptomatic infections, was investigated. All urinary tract infections were associated with significant bacteriuria ($\geq 10^5$ CFU/mL). The isolates were recovered between June 2013 and June 2015 from 398 inpatients and 112 outpatients in seven large Bulgarian hospitals. They were obtained from urine ($n = 256$), surgical wound or abscesses ($n = 138$), genital tract samples ($n = 74$), blood ($n = 26$), peritoneal fluid ($n = 6$), lower respiratory tract samples ($n = 8$), and bile ($n = 2$). *E. faecalis* ATCC 29212 and *E. faecium* ATCC 19434 were used as control strains for species identification with biochemical and molecular genetic techniques.

Culture media

HiCrome *E. faecium* Agar Base (Himedia Labs) was applied to isolate *Enterococcus* spp. strains. *E. faecalis* forms blue colonies, while *E. faecium* gives green colonies, surrounded by yellowish coloring of the ambient.

Biochemical identification with commercial kits and systems

Species identification was done using API Rapid ID 32 Strep (bioMérieux), BBL Crystal Gram-positive ID kit (Becton Dickinson) and the automated system VITEK 2 (bioMérieux).

DNA isolation

Total DNA from all used strains was extracted with GenElute™ Bacterial Genomic DNA Kit (Sigma–Aldrich), according to the manufacturer's instructions, from 3 mL overnight cultures inoculated with a single colony.

Molecular genetic genus and species identification

Polymerase chain reaction (PCR) amplification of the 16S rRNA gene of *Enterococcus* spp. was used for genus identification of all isolates included in this study. The *sodA* genes encoding the enzyme manganese-dependent superoxide dismutase in the most common enterococci (*E. faecalis*, *E. faecium* and *Enterococcus durans*) were detected with multiplex PCR. *E. faecalis* identification was confirmed by PCR for the *eda1* gene (encoding 2-keto-3-deoxy-6-phosphogluconate aldolase, which is an enzyme involved in the Entner–Doudoroff pathway and is species-specific for *E. faecalis*). Oligonucleotides used as primers and amplification conditions were previously described.[22]

PCR assay for detection of virulence genes

PCR amplification was performed in order to confirm the presence of genes coding for different virulence factors: adhesins

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