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RESEARCH ARTICLE

# Laboratory Model of Molecular-Genetic Interactions of the Protose Blastocystis Spp. With Bacteria Enterococcus Faecalis

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## ABSTRACT

Changes in the pathogenicity of opportunistic enterobacteriaceae are currently acquiring more and more medico-biological significance. The rates and scale of transformations of symbiont bacteria do not fit into the norm of the phenotype reaction to changes in environmental conditions. All this determines the intensification of studies of the phenomenon of pathogenicity at the molecular genetic level. This paper presents the results of a molecular genetic analysis of the interactions of E. faecalis in association with Blastocystis spp. in vitro. It was found that the nucleotide sequences of genes that determine the synthesis of pathogenicity factors are found in the studied enterococci in different ratios, and the frequency of detection of fragments of the desired genes changes after their co-cultivation with blastocysts isolated from the feces of gastroenterological patients. The simplest Blastocystis spp. caused an increase in the frequency of occurrence of the gene gelE (gelatinase) in enterococci after their joint cultivation, which is evidence of their influence on the ability to realize the pathogenic potential of associative symbionts.

KEYWORDS:
Enterococcus faecalis,
Blastocystis spp., virulence, PCR

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## INTRODUCTION

Molecular genetic analysis in the study of associative symbiotic interactions helps to identify the main directions of mutual adaptation of microorganisms in the changing conditions of symbiocenosis [5, 6, 8]. Changes in the pathogenicity of opportunistic enterobacteriaceae are currently acquiring more and more medico-biological significance. Relatively recently, "islands" and "islets" of pathogenicity were found in the traditionally opportunistic bacteria E. faecalis [3, 4, 5]. However, the contribution of these mechanisms to the formation of new phenotypic variants of microorganisms remains unexplored. In recent years, data have appeared on an important role in the formation of intestinal pathobiocenoses, in addition to bacteria and fungi, such protozoa as Entamoeba hystolytica, Giardia lamblia, and Criptosporidium [7, 11]. Less well known is protozoal disease caused by parasitism mainly in the colon of protozoa Blastocystis hominis [12, 14]. The causative agent of blastocyst invasion is a microorganism, whose aggression, on the one hand, can be increased by factors that reduce the defenses of the macroorganism, and, on the other hand, by microorganisms that are part of the biocenosis. The works of V.I. Pushkareva et al. [10] showed that bacterial cells, being in protozoa, avoid death, and their hosts - protozoa - their number is virulence. Research on the characteristics of the associative symbiosis of protozoa with other microorganisms is limited to the study of survival mechanisms and their interaction outside the macroorganism, which is also a habitat for a huge number of microbial species [8, 11, 12].

To assess the importance of opportunistic microflora for the human body, it was of interest to carry out a molecular genetic analysis of the interactions of enterococci in association with blastocysts in vitro. In this regard, the aim of this work was to

study the occurrence of genetic determinants of pathogenicity of the E. faecalis microsymbiont in association with blastocysts in vitro.

## MATERIALS AND RESEARCH METHODS

In the work was used used 132 E. faecalis strains isolated from associations with Blastocystis spp. varying degrees of virulence before and after their co-cultivation, as well as 67 strains of enterococci isolated from microbial consortia, where blastocysts were not members of the microbial community. The study of the intestinal microflora in patients and individuals of the control group was carried out according to the order of the Ministry of Health of Russia dated 09.06.2003, № 231 “On the approval of the industry standard “Patient Management Protocol. Intestinal dysbiosis” (AUS 91500.11.0004-2003). The presence of blastocysts was detected by microscopy of native or stained preparations prepared from the feces of patients. Cultivation of protozoa B. hominis was carried out using Suresh CEM medium [13].

To isolate pure enterococcal cultures, enterococcus agar (NPC, Obolensk) was used, followed by accumulation on Columbia agar (BioRad, France) with blood and identification on Diskinson Oxoid medium (Himedia, India).

When performing PCR in order to identify the genetic determinants of the pathogenicity of enterococci, we used the Enterococcus faecalis strain № 111, obtained from the Museum of Cultures of the Institute of Cellular and Intracellular Symbiosis of the Ural Branch of the Russian Academy of Sciences (Orenburg).

To obtain a pure culture of blastocysts, fecal samples were poured in an equal volume (1: 1) of saline, suspended, and filtered. The filtrate in a volume of 0.5-1.0 ml was introduced into a test tube with Suresh culture medium.

The virulence of blastocysts was determined by intraperitoneal injection of white mice (weighing 23.1 ± 2.2 g) with 0.5 ml of a suspension of a protozoan culture grown on K. Suresh medium. One day later, the LD50 value was determined for each strain. In accordance with the obtained indicators, strains with LD50 from 101 to 103 CFU/ml were classified as highly virulent, strains with LD50 from 103 to 106 CFU/ml were classified as moderately virulent, and strains with LD50 over 106 CFU/ml were considered weakly virulent (Kostyukova et al., 1996).

Total bacterial DNA was isolated from a 24-hour agar culture, 1 ml of cell suspension was besieged at 12000 rpm in a centrifuge. The pellet was resuspended and diluted in a buffer containing 50 mM KCl, 10 mM Tris-HCl (pH 8.4), 2.5 mM MgCl2, 0.01% gelatin, to a final concentration of 108 CFU/ml. Lysis was performed with lysozyme (Germany) at a concentration of 1 mg/ml for 15 min at room temperature (22–25°C), followed by the addition of proteinase K to a final concentration of 200 μg / ml and incubated for 30 min at 60°C. To detect genes encoding pathogenicity factors of E. faecalis, polymerase chain reaction (PCR) was used. For this, we used sets of NPF "Litekh", Moscow. Storage of samples before use was carried out at 4°C.

In this work, the selection of primers was made to identify the gene for the pathogenicity of enterococci, as well as the optimization of the conditions and mode of real-time PCR, which allows simultaneous combination of amplification and detection. For this, a primer was used for the E. faecalis gene, which determines its pathogenicity: gelE (gelatinase). Statistical data processing was carried out using the "Statistica for Windows" program [1].

## RESEARCH RESULTS AND THEIR DISCUSSION

In our own studies, we studied the distribution of the genetic determinant gelE (gelatinase) in E. faecalis isolated from individuals with diseases of the gastrointestinal tract. To carry out this series of experiments, E. faecalis strains (n = 132) isolated from the examined subjects were selected, in the associative symbiosis of which blastocysts with varying degrees of virulence (the first group) participated. In addition, strains of enterococci (second group) isolated from consortia where protozoa were not members of the microbial community were studied (n = 67).

In our experiment, we assumed that one gene is one bacterium; therefore, the number of detected desired DNA fragments - the gelE gene – corresponds to the number of bacteria. Characteristics of the primer to the E. faecalis gelE gene region, which determines its pathogenicity, is presented in table 1.

Further, the work was carried out to identify the nucleotide sequences of genes that control the synthesis of gelatinase (gelE). Testing of E. faecalis strains for the presence of the gelE gene isolated from association with avirulent, moderately and highly virulent blastocysts showed (Table 2) that fragments of specific DNA corresponding to the gelE gene region before co-cultivation were found in 3.72 ± 0.3; 15.25 ± 1.6 and 62.55 ± 4.6% of strains, after co-cultivation these indicators increased to 5.43 ± 0.5%, 24.64 ± 2.8% * and 98.51 ± 7.3 % *, respectively ($p < 0.05$). In subsequent periods of the study, the frequency of occurrence of the studied fragments did not change. ($p > 0.05$).

During co-cultivation of E. faecalis with virulent blastocysts caused an increase in the number of virulent strains of bacteria with the presence of the gelatinase gene, it was especially significant after co-cultivation with highly virulent blastocysts (Fig. 1).

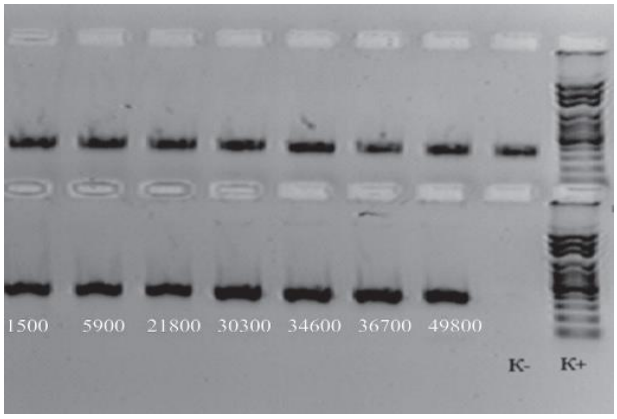
**Table 1:** Characterization of the primer to the gelE gene region

| Parameter | Characteristic |
|---|---|
| Gene gelE | |
| Direct primer (f) 5'-3' | TCAAGCGCCATCACTAGCAA |
| Reverse primer (r) 5'-3' | AAACCGGCAGTATGTTCCGT |
| Calculated Melting Point of Forward Primer | + 60,0°C |
| Calculated melting point of the reverse primer | + 60,0°C |
| Theoretical specificity | All E. faecalis strains available for study |
| Amplified length (bp) | 297 |

**Table 2:** Frequency of occurrence of gelE gene fragments in E. faecalis cultures in the total biomass before and after co-cultivation with Blastocystis spp.

| E. faecalis in association with blastocysts: | Co-cultivation | Frequency of occurrence of the gelE gene fragment (%) |
|---|---|---|
| Weakly virulent (n=24) | before co-cultivation | 3.72 ± 0.3 |
| | after 3 days of co-cultivation | 5.43 ± 0.5 |
| Moderately virulent (n=71) | before co-cultivation | 15.25 ± 1.6 |
| | after 3 days of co-cultivation | 24.64 ± 2.8* |
| Highly virulent (n=37) | before co-cultivation | 62.55 ± 4.6 |
| | after 3 days of co-cultivation | 98.51 ± 7.3* |
| E. faecalis isolated as monoculture (n = 67) | before co-cultivation | 3.22 ± 0.2 |
| | after 3 days of co-cultivation | 4.34 ± 0.6 |

Note: * – an indicator of the significance of the difference between the frequency of occurrence of the gelE gene fragment in E. faecalis cultures in the total biomass before and after their co-cultivation with Blastocystis spp. ($p < 0.05$).




**Rice. 1:** Electropherogram of the amplification products of the E. faecalis gelE gene region after co-cultivation with blastocysts

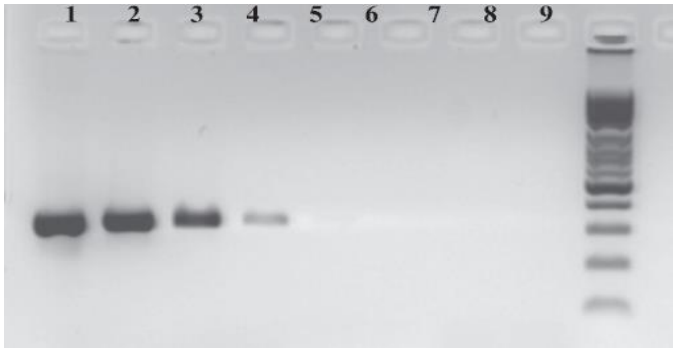
Concentration of E. faecalis of gelE gene copies is presented in protocol № 1.

**Protocol № 1:** Determination of the absolute concentration of the E. faecalis gelE gene after co-cultivation with Blastocystis spp.

| Hole number | Tube ID | Cp, Fam | Cp, Hex | Concentration copies/ml |
|---|---|---|---|---|
| B1 | Sample 1(CPS Ent f) | 17.4 | | 1500 |
| B2 | Sample 2(CPS Ent f) | 17.6 | | 5900 |
| B3 | Sample 3(CPS Ent f) | 17.5 | | 21800 |
| B4 | Sample 4(CPS Ent f) | 12.4 | | 30300 |
| B5 | Sample 5(CPS Ent f) | 12.8 | | 34600 |
| B6 | Sample 6(CPS Ent f) | 12.7 | | 36700 |
| B7 | Sample 7(CPS Ent f) | 13.6 | | 49800 |
| B8 | K-(CPS Ent f) | | | |

An important characteristic of any PCR system is its sensitivity. Using variations in sensitivity, test systems are currently being created to determine, for example, conditionally pathogenic flora in a clinically significant amount. Thus, if the sample contains representatives of conditionally pathogenic flora in an amount less than a certain threshold, the result of the PCR study will be negative. In practice, this avoids overdiagnosis.

The task of this work was also to determine the sensitivity of PCR with all primer systems used for the indication of E. faecalis. Thus, serial 10-fold dilutions were prepared from the initial bacterial suspension of E. faecalis containing $5.0 \cdot 109$ cells/ml. The baseline concentration was determined from the turbidity standard and CFU counts. The results of determining the sensitivity are shown in Fig. 2.




**Rice. 2:** Electropherogram for determining the sensitivity of PCR with primers to the gelE gene region of the E. faecalis genome. 1–5.5·106 bacterial cells/ml; 2–5.5·105 bacterial cells/ml; 3–5.5·104 bacterial cells/ml; 4–5.5·103 bacterial cells/ml; 5–5.5·102 bacterial cells/ml; 6–5.5·101 bacterial cells/ml; 7–5.5·100 bacterial cells/ml; 8 - negative control; 9 - molecular weight marker

As a result, the sensitivity of the PCR study with the primers used in this work was: for the gelE gene region of the E. faecalis genome - 5.0·103 bacterial cells/ml. Thus, in the course of the work done, the dependence of the increase in the number of positive signals with primers specific to the gelE gene on the virulence of associates ($r = 0.75$) both before and after co-cultivation ($p < 0.001$) was revealed. Subsequently, the frequency of occurrence of the gelE gene in E. faecalis cultures isolated from intestinal microbial communities, in which blastocysts were absent, was studied. It was found that in the group of enterococci isolated without protozoa, the frequency of occurrence of the genetic determinants of gelE before and after co-cultivation with blastocysts did not change significantly ($p < 0.005$).

## CONCLUSION

Thus, the selection of primers was made to identify the gene for the pathogenicity of enterococci, as well as the optimization of the conditions and mode of carrying out PCR in "real time", which allows simultaneous combination of amplification and detection. For this, a primer for the E. faecalis gene, which determines its pathogenicity, gelE (gelatinase), was used. It was found that the nucleotide sequences of the gelE gene, which determines the synthesis of pathogenicity factors, are found in the studied enterococci in different ratios, and the frequency of detection of fragments of the desired gene changes after co-cultivation of enterococci with blastocysts isolated from gastrointestinal diseases. The simplest Blastocystis spp. caused an increase in the frequency of occurrence of the gelE gene in enterococci after their joint cultivation, which is evidence of their influence on the ability to realize the pathogenic potential of associative symbionts. Therefore, it can be assumed that the mechanisms of mutual adaptation of E. faecalis strains under conditions of a macroorganism and on artificial nutrient media in a consortium with blastocysts of different virulence include an increase in the number of individuals in the genotype of which the gelE gene is localized.

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