A Method for Identification of Vaginal Epithelial Microflora by the Analysis of 16S Ribosomal RNA Gene

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Analysis of 16S ribosomal RNA gene was carried out in 2 DNA samples isolated from vaginal epithelial smears from women suffering from bacterial vaginosis. The composition of vaginal epithelial microbiocenosis in bacterial vaginosis was determined and its significant difference from normocenosis was shown. A laboratory protocol for identification of vaginal epithelial microflora was developed on the basis of 16S ribosomal RNA gene analysis.

Key Words: *vaginal microbiocenosis; 16S ribosomal RNA gene; bacterial vaginosis*

The prevalence of urogenital infections becomes a more and more pressing problem. Dysbiosis of vaginal microflora, or bacterial vaginosis (BV) is a highly incident diseases. This condition is detected in 24% women of reproductive age who consider themselves healthy and in 64% women complaining of pain [1]. Bacterial vaginosis is detected in 28-35% pregnant women. Miscarriages, preterm deliveries, preterm amniorrhea are several-fold more incident in patients with BV. The fetus can be infected by swallowing or aspirating the amniotic fluid. The status of vaginal microbiocenosis can be evaluated as normal in less than 50% pregnant women [1].

Several methods for identification of vaginal epithelial (VE) microbiocenosis composition are known; bacteriological and microscopic methods were the main methods used for this purpose up to recent time. However, not all microorganisms can be identified in this way. Approaches based on gene analysis are free from drawbacks of the majority of these methods, which are labor-consuming, their sensitivity and reproducibility are low, as is the reliability of results, *etc.* Of the new methods PCR diagnosis is used most often, but its application to studies of the entire spectrum of microorganisms is limited because no more than 3 microorganisms can be identified during one reaction. However, only complete and reliable information about VE microflora in health and disease can promote detection of the causes of disease (including BV) and selection of optimal therapy.

Other gene diagnosis methods, such as DNA-DNA biochip hybridization or determination of nucleotide sequences of evolutionally conservative genes (*e.g.*, 16S ribosomal RNA (rRNA) gene) in an isolated DNA sample are used for detection of rather many microorganisms. Studies of microbiocenosis on the basis of 16S rRNA gene are in progress. Microbiocenoses of the oral cavity, esophagus, stomach [2,7,8], and VE [3-6,9,10] were studied. No studies of this kind were carried out in Russia, though the problem is a pressing one.

The aim of our study was to develop a method for evaluating VE microbiocenosis composition by analysis of 16S rRNA gene.

MATERIALS AND METHODS

Specimens of DNA (M1 and M4) were isolated from VE smears collected from patients with decompensated BV (according to microscopic study). *Mycoplasma genitalium* was detected in these samples by PCR. The protocol of the study is presented in Fig. 1.

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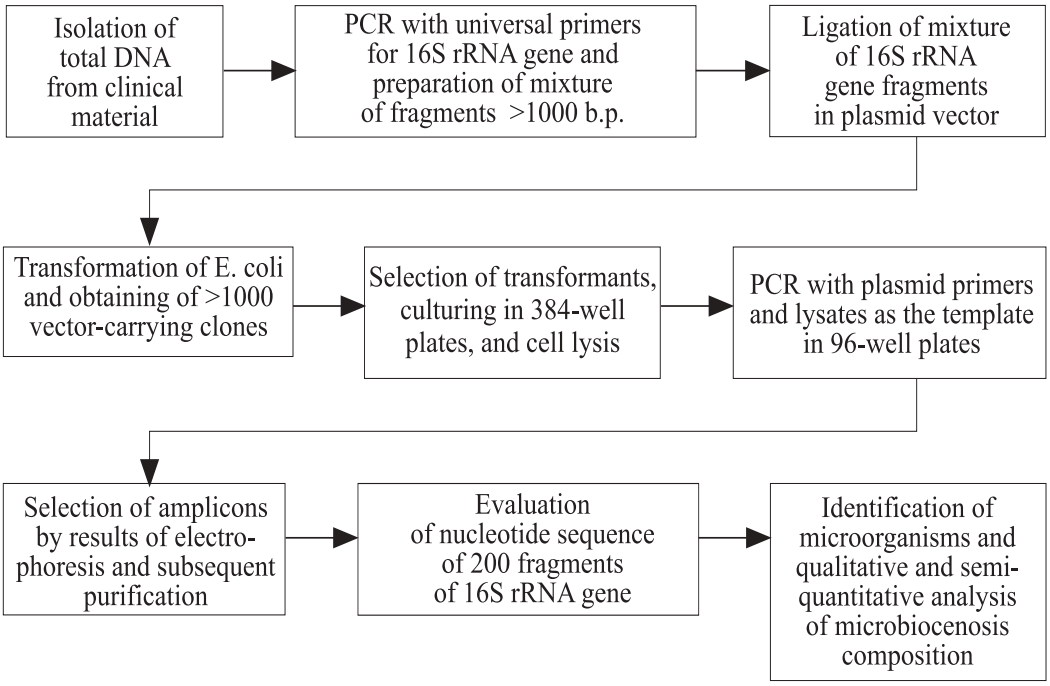

Fig. 1. Identification of VE microbiocenosis composition by analysis of 16S rRNA gene.

Universal primers UP450 (5'-ACTCCTACGG GAGGCAGCAGTGG-3') and LOW1205 (5'-TCT CACGACACGAGCTGACGACAAC-3') were used for amplification of the set of 16S rRNA gene fragments. The primers limit ~750 b.p. sequence in the middle of the gene (338-1081 for *E. coli*). Amplification program included 25 cycles: 30 sec at 94°C, 30 sec at 55°C, and 90 sec at 72°C.

The PCR products were purified by Wizard PCR Preps DNA Purification System (Promega) according to the instruction.

Strain DH5α *E. coli* cells were transformed by thermal shock, inoculated in large (22×22 cm) Petri dishes in Luriya—Bertani solid nutrient medium (LB agar) containing ampicillin, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside, and isopropyl-thiogalactoside and incubated at 37°C overnight. Transformation was considered effective, if more than 1000 colonies formed in a dish. Colonies containing the plasmid with insert were selected automatically on a QPix2 device (Genetix). The cells were transferred into 384-well plates with liquid nutrient LB medium with ampicillin and incubated overnight at 37°C. The cells were lyzed in Tris-EDTA buffer. Cell lysates were stored at -20°C.

Fragments of 16S rRNA gene present in plasmids were amplified from universal plasmid primers pUC/M13 Forward and Reverse using cell lysates as the templates. PCR was carried out in 96-well plates according to a similar program of 30 cycles on a PTC-225 DNA Engine Tetrad thermocycler (MJ Research). Amplification products containing the only fragment ~1000 b.p. (insert+vector region) were selected by the results of electrophoresis and purified in 96-well MultiScreen-PCR plates (Millipore) according to the instruction. The degree of purification and concentration of DNA were evaluated by electrophoresis.

Nucleotide sequences of the resultant DNA were determined on an ABI Prism Genetic Analyzer 3100 automated sequencer (Applied Biosystem).

Primary analysis of DNA sequences was carried out using Sequence Scanner 1.0 and Vector NTI 9.0 software. The resultant sequences of 16S rRNA fragments were compared using AlignX Vector NTI 9.0 multiple alignement software. The appurtenance of each group of fragments to this or that microorganism was identified using NCBI Blast software. More than 97% coincidence of the nucleotide sequence of a fragment with the 16S rRNA sequence presented in NCBI was considered as a reliable evidence of appurtenance of the analyzed 16S rRNA fragment to a certain microorganism.

RESULTS

Nucleotide sequences of 186 from 1131 fragments of 16S rRNA gene were identified for M1 specimen and 288 from 1137 for M4. *Gardnerella vaginalis* and *Veillonella spp.* predominated (75% of all microorganisms) in M1 specimen, *Veillonella* constituting almost 50%. The content of *Ureaplasma urealyticum*, *Gemella spp.*, and *Streptococcus spp.* was much lower (4-8%). Solitary fragments belonging

TABLE 1. Presence of Microorganisms (in %) in Normocenosis (According to [5]) and BV (M1 and M4 specimens)

Microorganisms	Hyman R.W. *et al.* [5]	M1	M4
Aerococcus	1	2	3
Atopobium	1	0	0
Bifidobacterium	6	0	0
Comamonas	0	1	0
Corynebacterium	1	2	0
Gardnerella vaginalis	10	27	24
Gemella	2	5	2
Lactobacillus	63	0	7
Leptotrichia	0	0	22
Mycoplasma genitalium	0	1	2
Mycoplasma hominis	0	0	1
Peptoniphilus	0	0	1
Prevotella	9	0	11
Staphylococcus	0	1	0
Streptococcus	8	8	5
Ureaplasma urealyticum	0	4	6
Veillonella	0	48	15

to *M. genitalium* and some other microorganisms were identified (Table 1).

Sample M4 in addition to *G. vaginalis* and *Veillonella spp.*, contained fragments of 16S rRNA gene from *Leptotrichia spp.* and *Prevotella spp.* (altogether 75% of all microorganisms). The content of *U. urealyticum*, *Streptococcus spp.*, and *Lactobacillus spp.* amounted to 5-7% each. *M. genitalium* and some other bacteria were presented by solitary copies of 16S rRNA gene fragment (Table 1).

We compared our findings with the results of a previous report [5] in which normal VE microbiocenosis was characterized by using analysis of 16S rRNA gene. The authors studied VE microflora in 20 healthy women by analyzing 1000 fragments of 16S rRNA gene ~1400 b.p. long for each sample. On the whole, the sequences of more than 30 million nucleotides were determined.

No appreciable differences from the parameters of normocenosis were detected for *Aerococcus spp.*, *Comamonas spp. Corynebacterium spp.*, *Gemella spp.*, *Peptoniphilus spp.*, *Prevotella spp.*, and *Streptococcus spp.* (Table 1). The content of *Lactobacillus spp.* was very low in comparison with normocenosis (in sample M4) or zero (in sample M1). The content of *G. vaginalis* 2-fold surpassed the normal [5]. However, in 2 samples studied previously [5] the content of this bacterium differed little from our results (about 25%).

A significant difference of our data is increased content of *Veillonella spp.*: more than 100-fold in comparison with normocenosis in both samples (*Veillonella spp.* was the predominant microorganism in M1) and of *Leptotrichia spp.* (more than 50-fold in M4 sample) (Table 1). These microorganisms are opportunistic. In addition, opportunistic microorganisms, not occurring in normal microflora [5], were detected: *M. genitalium* and *U. urealyticum* (in both samples) and *M. hominis* (in sample M4). However, the content of these microorganisms was low, and presumably, clinical manifestations of BV in the patients were largely due to the presence of *Veillonella spp.* and *Leptotrichia spp.*

Hence, changes in the microflora of the studied samples detected in our study correspond to the modern concepts of BV: a significant reduction or complete absence of *Lactobacillus spp.*, increased content of *Gardnerella spp.* and other microorganisms normally present in small quantities. On the whole, the microbiocenosis in samples M1 and M4 can be characterized as dysbacteriosis.

Introduction of this approach to research and clinical practice will appreciably reduce the duration of analysis (to just 3 days) in comparison with bacteriological methods, when the duration of culturing in special selective media takes up to 15 days. In addition, the spectrum of identified bacteria is wider than in microscopic analysis. In contrast to identification of bacteria on DNA chips, when the spectrum of identified microorganisms is known beforehand, direct identification of the nucleotide sequence of the whole cloned 16S rRNA library allows detection of new microorganism species.

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