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Characterization of vaginal *Lactobacillus* species by *rplK* -based multiplex qPCR in Russian women

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ABSTRACT

We describe a multiplex qPCR assay for identification and quantitative assessment of a set of vaginal *Lactobacillus* species, including *L. acidophilus*, *L. crispatus*, *L. gasseri*, *L. helveticus*, *L. iners*, and *L. jensenii*. The assay extends the previously developed qPCR method for *Lactobacillus* detection and total quantification based on targeting the *rplK* gene. Both assays use only single pair of primers and a set of probes combined in three reactions, comprising a vaginal *Lactobacillus* diagnostic assay panel. The utility of the diagnostic panel was evaluated by analyzing of vaginal swab specimens from 145 patients with different status of vaginal health. Most frequently, only one *Lactobacillus* species was dominant (68,9%), mostly *L. crispatus* (18,6%) or *L. iners* (33,1%), but two or three *Lactobacillus* species were also being simultaneously detected (24,9%). The diagnostic panel will facilitate investigations of the role of *Lactobacillus* species in the health of the female reproductive system and promote studies of variability of the vaginal microbiota.

1. Introduction

Lactobacilli are a major component of the vaginal microbiota. These bacteria are proposed to support healthy vaginal conditions by using several mechanisms, which include competitive elimination of pathogenic microorganisms, modulation of the host immune response, and production of various antibacterial compounds, such as lactic acid, hydrogen peroxide, and bacteriocins. Alteration of the microbial ecosystem colonizing the vagina of healthy women frequently results in a deficiency of lactobacilli and is usually associated with an overgrowth of anaerobes [1–3]. The basic ecology of the genitourinary microbiota, namely, the composition, relative abundance, and temporal fluctuations of vaginal bacterial species, including lactobacilli, is poorly understood.

Application of genomic technologies to the vaginal ecosystem has enabled identification of several distinctive microbial communities, the specific proportions of which are shaped by a woman's race/ethnicity, geographic location, personal hygiene habits, infectious diseases, and others. However, in general, the vaginal microbiota of healthy women appears to be dominated by one of four major *Lactobacillus* species, i.e., *L. crispatus*, *L. gasseri*, *L. jensenii*, and *L. iners* [4–8]. Other *Lactobacillus* species, such as *L. acidophilus*, *L. coleohominis*, *L. helveticus*, *L. plantarum*, *L. rhamnosus*, *L. salivarius*, and *L. vaginalis* have also been occasionally reported [5,8–12]. The individual role of different species of lactobacilli in maintaining vaginal health, and particularity their prevalence, remains unclear and is a subject of interest. It has been observed that *L. iners* is present both in apparently healthy women and those with vaginal dysbiosis, while *L. crispatus* is typically isolated from healthy women. In addition, the major vaginal *Lactobacillus* species respond differently to environmental and physiological challenges, such as menses or antibiotic therapy [13–15]. Thus, it can be anticipated that the detection and quantification of lactobacilli to a species level could prove valuable in clinical applications, facilitating the use of lactobacilli as a vaginal health biomarker in the clinical setting.

Identification of lactic acid bacteria to the species level is difficult because of their variety, their complex and undetermined classification, the uncertainty of taxonomic criteria [16,17], and the genetic variability of closely related species [18,19]. Quantitative polymerase chain reaction (qPCR) is a well-established technique for the detection and quantification of many microorganisms. With regard to vaginal lactobacilli, a number of qPCR assays have been developed, targeting mainly the *16S rRNA* gene and designed as a

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mono-specific assay [13,14,20–25]. Besides *16S rRNA*, the *tuf* gene was also reported as a suitable amplification target for vaginal lactobacilli, and a multiplex assay for detection and quantification of four major vaginal *Lactobacillus* species targeting this gene has been developed [26]. Recently, we reported that the gene *rplK*, encoding L11 ribosomal protein, could be a suitable target for PCR-based detection and quantification of *Lactobacillus* content specific to vaginal microbiota [27]. This assay allowed accurate quantification of lactobacilli in the vaginal fluid without speciation giving the total value. The aim of the present study was using the same *rplK* amplification platform to develop a qPCR assay allowing identification and quantification of the detectable lactobacilli to the species level. In result, species-specific hydrolysis (TaqMan) probes for *L. acidophilus*, *L. crispatus*, *L. gasseri*, *L. helveticus*, *L. iners*, and *L. jensenii* were designed for the same amplification target, *rplK*, and a multiplex qPCR assay was developed. The assay was arranged into a two-tube format, detecting three *Lactobacillus* species in each tube and reaction. The assay was used to characterize the diversity and prevalence of *Lactobacillus* species in vaginal microbiocenosis in Russian women.

2. Materials and methods

2.1. Primers and probes

Lactobacillus group-specific primers LacI2 and LacANr were described previously [27]. Pan-bacterial primers E782F (5′-ACAGGATTAGACACCCTGGTAG-3′) and 1061Rm1 (5′-GTCGACACGAGCTGACGAC-3′) were modified from Ref. [28]. The primers were ordered from Evrogene, Ltd. (Moscow, Russia).

The probes used in this study are listed in Table 1 and were ordered from DNA Synthesis, Ltd. (Moscow, Russia). LNA nucleotides were incorporated into the probes, exept PbLin, to increase their melting temperature and specificity. The probes were labeled with a reporter dye at 5'- terminus and with a quencher at the 3'-terminus (Table 1). *In silico* probe specificity was tested using the Primer-BLAST web service (http://www.ncbi.nlm.nih.gov/tools/primer-blast). The melting temperatures of the probes were calculated using Exiqon LNA™ Oligo Tools (https://www.exiqon.com/oligo-tools).

2.2. DNA templates

Templates for *L. acidophilus* and *L. gasseri* were genomic DNAs from *L. acidophilus* FSI4 and *L. gasseri* DSM 20243, respectively [27]. For *L iners*, *L. jensenii*, *L. crispatus*, and *L. helveticus*, artificial synthetic DNA templates were constructed to mimic the amplifiable fragments.

Artificial templates, mimicking the corresponding amplifiable sequences, were designed as follows. Due to the large size of the potential amplicons (345 bp), template reconstruction was carried out using smaller fragments. For this, four overlapping sequences covering the targeted amplicon were designed for each *Lactobacillus* species and chemically synthesized as single-stranded polynucleotides (Evrogene, Ltd.; Moscow, Russia). Two 5′-polynucleotides were synthesized in the direct orientation, and two 3′-polynucleotides were synthesized in the complementary orientation. The full-size templates were then assembled in two steps. The assembly of the central part was carried out by annealing and extending two central polynucleotides. The reaction was performed in a 30 μL reaction volume containing PCR buffer (10 mM Tris HCl, pH 8.3, 8% sucrose, 50 mM KCl, 0.5% Tween 20, 3% formamide, 3.6 mM $MgCl_2$, and 180 μM each dNTP), 2 U Taq polymerase (Evrogene, Ltd.; Moscow, Russia), and 25 pM each polynucleotide. The thermal cycling conditions for the first step reaction were as follows: 3 min at 95 °C, followed by decreasing the temperature to 50 °C in 1 °C/10 s increments, repeated for two cycles, with a final extension at 72 °C for 3 min. For the second step reaction, an aliquot of the first step product was used as template in a PCR reaction with two external polynucleotides as primers, to amplify the full-length sequence. Amplification was performed as follows: 95 °C for 3 min, followed by 40 cycles of 95 °C for 10 s and 60 °C for 30 s. The polynucleotides used for preparation of the *Lactobacillus* templates are given in Supplementary Table S1. The quality of the resulting products was confirmed by melting curve analysis with SYBR Green and by electrophoresis on 1.5% agarose gels stained with ethidium bromide. Template concentration was determined using microvolume spectrophotometer Agilent 8453 (Agilent Technologies, Germany).

2.3. PCR

Multiplex and uniplex qPCRs were performed in 30 μL reaction volumes using a BioRad CFX 960 machine. The amplification program was as follows: 95 °C for 3 min, followed by 40 cycles of 95 °C for 10 s and 60 °C for 30 s. DNA (5 μL) was added to 25 μL of PCR mix containing the PCR buffer, 5 pM each primer, 5 pM each labeled probe, 1 × SYBR Green reagent, and 1 U Taq DNA polymerase (Evrogene, Ltd.; Moscow, Russia). In the multiplex reaction format, one reaction mixture contained probes PbLin, PbLje, and PbLjoga, while the other reaction contained probes PbLcr2b, PbLhe3a, and PbLaci1b. Negative controls were included in each reaction plate.

By following the MIQE guidelines [29], qPCR efficiency (E), coefficient of determination (R^2), and the limit of detection (LOD) of

Table 1
Probes.

Name	Sequence	Specificity	Reference
PbLacAAr2	Vic-TTCATTACTAAGACTCCACC-BHQ2	A group of lactobacilli, including *L. acidophilus*, *L. crispatus*, *L. helveticus*, *L. iners*, *L. jensenii*, *L. gasseri*, *L. johnsonii* and others	[27]
PbLaci	Cy5-TGAAGCCTTATCGATCTTAGCAGC-BHQ3	*L. acidophilus*	This work
PbLcr	Vic-AGCCTTTTGGATCTTAGCAG-BHQ2	*L. crispatus*	This work
PbLhe	Rox-AGCCTTCTTGATCTTAGCAGC-BHQ2	*L. helveticus*, *L. acetotolerans*, *L. acidophilus L. amylovorus*, *L. gallinarum*, and *L. kefiranofaciens*	This work
PbLin	Vic-AGGATTTCAATGCACGTACCGCAGATC-BHQ2	*L. iners*	This work
PbLje	Rox -ACTTCAATGCAAGAACTGCAGACC-BHQ2	*L. jensenii*	This work
PbLjoga	Cy5-CACGTACTGCTGATCAAAAGGGT-BHQ3	*L. gasseri*, *L. johnsonii*	This work

Abbreviations: Cy5, Rox, Vic, fluorescent dyes.
BHQ2, BHQ3, fluorescent dye quenchers.