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Establishment of a sensitive system for analysis of human vaginal microbiota on the basis of rRNA-targeted reverse transcription-quantitative PCR

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ABSTRACT

Ten specific primer sets, for *Lactobacillus gasseri*, *Lactobacillus crispatus*, *Atopobium vaginae*, *Gardnerella vaginalis*, *Mobiluncus curtisii*, *Chlamydia trachomatis*/*muridarum*, *Bifidobacterium longum* subsp. *longum*, *Bifidobacterium longum* subsp. *infantis*, *Bifidobacterium adolescentis*, and *Bifidobacterium angulatum*, were developed for quantitative analysis of vaginal microbiota. rRNA-targeted reverse transcription-quantitative PCR (RT-qPCR) analysis of the vaginal samples from 12 healthy Japanese volunteers using the new primer sets together with 25 existing primer sets revealed the diversity of their vaginal microbiota: Lactobacilli such as *L. crispatus*, *L. gasseri*, *Lactobacillus jensenii*, *Lactobacillus iners*, and *Lactobacillus vaginalis*, as the major populations at 10^7 cells/ml vaginal fluid, were followed by facultative anaerobes such as *Streptococcus* and strict anaerobes at lower population levels of 10^4 cells/ml or less. Certain bacterial vaginosis (BV)-related bacteria, such as *G. vaginalis*, *A. vaginae*, *M. curtisii*, and *Prevotella*, were also detected in some subjects. Especially in one subject, both *G. vaginalis* and *A. vaginae* were detected at high population levels of $10^{8.8}$ and $10^{8.9}$ cells/ml vaginal fluid, suggesting that she is an asymptomatic BV patient. These results suggest that the RT-qPCR system is effective for accurate analysis of major vaginal commensals and diagnosis of several vaginal infections.

1. Introduction

The vaginal microbiota of healthy women of reproductive age is dominated by *Lactobacillus* species, including major four species: *Lactobacillus crispatus*, *Lactobacillus jensenii*, *Lactobacillus gasseri* and *Lactobacillus iners*, followed by obligate anaerobes such as *Gardnerella vaginalis*, *Atopobium vaginae*, *Mobiluncus curtisii*, and *Prevotella* (Pavlova et al., 2002). Bacterial vaginosis (BV) results from the disturbance of the normal microbiota and is characterized by an overgrowth of these obligate anaerobes as well as the decreases in the population levels of lactobacilli (Fredricks et al., 2005; Meltzer et al., 2008; Menard et al., 2008; Verhelst et al., 2004). BV is associated with an increased risk of upper genital tract and sexually transmitted infections with organisms such as *Chlamydia*, and with the acquisition of HIV (Cohen et al., 1995; Plitt et al., 2005; Sewankambo et al., 1997).

Currently, microscopic evaluation by Gram staining using the Nugent score is the standard procedure for clinical examination in BV (Nugent et al., 1991). With this technique, diagnosis of BV is based on the semi-quantitative estimation of *Lactobacillus*, *G. vaginalis*, and *Mobiluncus* morphotypes. However, the use of this technique requires considerable skill, and several pathogens implicated in BV, such as *A. vaginae*, cannot be identified by Gram staining itself because of their variable morphology. Therefore, molecular-based methods such as PCR using specific primer sets have recently been introduced for better identification and quantification of such bacterial targets, and many studies have demonstrated the composition of the vaginal microbiota of both healthy subjects and BV patients (Biagi et al., 2009; De Backer et al., 2007; Menard et al., 2008; Yan et al., 2009). In most of these studies, however, only a few specific primer sets for *Lactobacillus* or BV-related bacteria such as *G. vaginalis* and *A. vaginae* have been applied, resulting in only partial identification of the vaginal microbiota.

YIF-SCAN® (Yakult Intestinal Flora-SCAN), a highly sensitive and rapid system that uses the reverse transcription-quantitative PCR (RT-qPCR), was initially developed for analyzing intestinal microbiota (Kubota et al., 2010; Kurakawa et al., 2013; Matsuda et al., 2007, 2009). The sensitivity of RT-qPCR has been shown to be 100- to 1,000-times higher than that of qPCR because the copy number of rRNA per

Abbreviations: BV, bacterial vaginosis; DAPI, 4′, 6′-diamidino-2-phenylindole; EB, elementary body; FISH, fluorescence in situ hybridization; IFU, inclusion forming unit; LOD, limit of detection; qPCR, quantitative polymerase chain reaction; RB, reticulate body; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; YIF-SCAN®, Yakult Intestinal Flora-SCAN.

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cell (approximately 10^4 copies per actively growing cell) is higher than that of rRNA genes (approximately 10 copies in a genome) (Kubota et al., 2010; Kurakawa et al., 2013; Matsuda et al., 2007, 2009). The high sensitivity of RT-qPCR has also been shown to improve the clinical diagnosis in several clinical settings such as bacteremia in the patients with febrile neutropenia and bacterial translocation during surgical operation (Fujimori et al., 2010; Mizuno et al., 2010; Sakaguchi et al., 2010). Here, we constructed 10 new primer sets specific for *Chlamydia trachomatis/muridarum* and other vaginal bacteria, and the standard curves for the new primer sets were prepared for RT-qPCR by using the corresponding standard strains. We then analyzed the vaginal flora of 12 Japanese healthy volunteers by using these primer sets together with 25 other primer sets that have been applied for microbiota analysis by RT-qPCR.

2. Materials and methods

2.1. Strains and culture conditions

Bacterial strains listed in Table 1 were obtained from the American Type Culture Collection (ATCC), the Culture Collection; University of Goteborg (CCUG), the German Collection of Microorganisms and Cell Cultures (DSMZ), the Institute for Fermentaion, Culture Collection of Microorganisms (IFO), the Japan Collection of Microorganisms (JCM), the National Collection of Dairy Organisms (NCFB), Gifu University Culture Collection (GIFU), the National Collection of Industrial, Food and Marine Bacteria (NCIMB), the NODAI Research Institute Culture Collection (NRIC) and the Anaerobe Laboratory, Virginia Polytechnic Institute and State University (VPI). All the strains of *Lactobacillus* species, as well as *Enterococcus faecalis* and *Streptococcus bovis*, were cultured anaerobically in Lactobacilli MRS broth (Becton Dickinson Co., Sparks, MD) at 37 °C for 24 h. *Bifidobacterium* species and *A. vaginae*, *Atopobium rimae*, *Atopobium pervulum*, *Collinsella aerofaciens*, *Collinsella intestinalis*, *Collinsella stercoris*, *Eggerthella lenta*, *Slackia exigua*, *G. vaginalis*, *M. curtisii*, *Mobiluncus mulieris*, *Alloscardovia omnicolens*, *Metascardovia criceti*, *Aeriscardovia aeriphila*, *Peptostreptococcus anaerobius*, *Peptostreptococcus magnus*, *Anaerococcus tetradius*, *Blautia producta*, *Ruminococcus obeum*, *Flavonifractor plautii*, and *Prevotella melaninogenica* were cultured anaerobically at 37 °C for 24 h in modified GAM broth (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) containing 1.0% (wt/vol) glucose. *Escherichia coli*, *Enterobacter cloacae*, *Klebsiella pneumoniae*, *Serratia marcescens*, *Proteus vulgaris*, *Pseudomonas aeruginosa*, *Streptococcus agalactiae*, *Streptococcus intermedius*, *Streptococcus mitis*, and *Streptococcus anginosus* were cultured aerobically in Brain Heart Infusion broth (Becton Dickinson Co.) at 37 °C for 16 h. *Neisseria gonorrhoeae* was cultured at 37 °C for 24 h in modified GAM broth containing 1.0% (wt/vol) glucose under the microaerophilic conditions generated in the CampyPak Plus microaerophilic system (Becton Dickinson Co.).

Obligate intracellular bacteria, *C. trachomatis* and *C. muridarum* were propagated in a McCoy cell line, whereas *Chlamydia suis* and *Chlamydophila pneumoniae* were propagated in an HEp-2 cell line. Each cell line was cultured in Dulbecco's minimal essential medium (DMEM) (Sigma-Aldrich, St. Louis, MO) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 100 μg/ml kanamycin and incubated at 37 °C under 5% CO_2. Samples of Chlamydiae were added to McCoy or HEp-2 confluent cell cultures in 24-well plates, and the plates were centrifuged at 1,000 *g* for 60 min at 25 °C. After removal of the supernatant, the infected cells were incubated in DMEM supplemented with 10% FBS and 1 μg/ml cycloheximide (Sigma-Aldrich). They were then incubated at 37 °C under 5% CO_2. After 72 h of incubation (in the case of *C. trachomatis*, *C. suis*, and *C. pneumoniae*) or 16 h of incubation (for *C. muridarum*), infected cells peeled from the bottoms of the plates were collected in microtubes. They were centrifuged at 15,000 *g* at 25 °C for 60 min, and the supernatant was removed. The pellets were homogenized with SPG buffer (7.5% sucrose, 0.072% L-glutamic acid, 0.6148% $Na_2HPO_4 \cdot 12H_2O$, 0.0247% KH_2PO_4; pH 7.0). The homogenate was sonicated 10 times with Ultrasonic Multi Cleaner W-113 (Honda, Aichi, Japan) at 28 kHz for 5 s. Cellular debris was removed by centrifugation at 1,000 *g* at 4 °C for 5 min. The resultant supernatant was used for RNA extraction, cell counting by culture, or FISH.

2.2. Inclusion-forming-unit (IFU) counting

Cell lines were precultured at 37 °C under 5% CO_2 for 24 h on poly-L-lysine-coated cover glass (Matsunami Glass Ind., Ltd., Osaka, Japan) in DMEM supplemented with 10% FBS in 24-well plates. The appropriate dilutions of the Chlamydiae samples were added to the corresponding cells, and the cells were centrifuged at 1,000 *g* at 25 °C for 60 min. After removal of the supernatant, the infected cells were incubated at 37 °C under 5% CO_2 for 24 h in DMEM supplemented with 10% FBS and 1 μg/ml cycloheximide. The poly-L-lysine coated cover glass was washed with PBS (−) and fixed with ethanol and acetone (95:5 [v/v]) for 15 min at room temperature. The cover glass was completely air dried, and 50 μl of FITC-labeled anti-Chlamydiae LPS monoclonal antibody (Denka Seiken, Tokyo, Japan) was put on top of the fixed cells to detect Chlamydiae inclusion bodies. Observation and acquisition of the fluorescent images were performed with a Leica imaging system (an automatic fluorescent microscope [Leica DM6000], image-acquisition software [QFluoro], and a cooled black-and-white charge-coupled-display camera [Leica DFC3500FX]) (Leica Microsystems GmbH, Wetzlar, Germany). The fluorescent images obtained at a magnification of 200 times were analyzed by using image analysis software (Image-Pro Plus v. 4.5; Media Cybernetics, Inc., Bethesda, MD) to count the IFUs in each sample. Microscopic counts were determined for 10 images per sample.

2.3. Design and validation of rRNA-targeted primers

By using 16S and 23S rRNA sequences obtained from the DDBJ/GenBank/EMBL databases, multiple alignment of *Lactobacillus*, the *Atopobium* cluster, the Bifidobacteriaceae, *Mobiluncus*, Chlamydiae species, and the reference organisms were constructed with the program Clustal X. After comparison of the sequences, potential target sites for specific detection were manually identified, and the *L. crispatus* (s-Lcri-F, s-Lcri-R), *L. gasseri* (s-Lgas-F, LgassR (De Backer et al., 2007)), *G. vaginalis* (s-Gvag-F, s-Gvag-R), *A. vaginae* (s-Avag-F, s-Avag-R), *M. curtisii* (s-Mcur-F, s-Mcur-R), *C. trachomatis/muridarum* (s-Ctra-F, s-Ctra-R), *Bifidobacterium longum* subsp. *longum* (BLON-23S-F, BLON-23S-R), *Bifidobacterium longum* subsp. *infantis* (BINF-23S-F, BINF-23S-R), *Bifidobacterium adolescentis* (BADO-23S-F, BADO-23S-R), and *Bifidobacterium angulatum* (BANG-F, BANG-R) primers (16–26 bp, GC contents: 30–70%, Tm: 51–61) were newly constructed (Table 2). Specificity and sensitivity in each primer set were checked as shown below. The other primers we used have been reported previously (Table 2).

2.4. Samples

Vaginal samples were collected from 12 healthy Japanese volunteers (IDs 1 to 12, 36 ± 10 years old); each subject collected the sample herself by inserting a flocked swab R80 (Copan Italia S.P.A., Brescia, Italy) into the vagina. Immediately after sampling, the swab was suspended in 2 ml of RNA*later*, RNA-stabilizing agent (Ambion, Inc., Austin, TX), in a Cryovial (Simport, Beloeil, Canada), and the vials maintained at a chilled condition were sent to Yakult Central Institute. The weight of the vaginal fluid was calculated by measuring the weight of the vial before and after sampling. All the volunteers were confirmed by questionnaire not to have taken any antibiotics in the 7 days before testing. In accordance with the Declaration of Helsinki, all subjects were adequately informed of the study, and written informed consent for participation was obtained from all subjects. Ethics committee in Yakult Central Institute approved the study.