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Molecular detection of microbial colonization in cervical mucus of women with and without endometriosis

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Abstract

Problem: Intrauterine microbial colonization and its association with the pathogenesis of endometriosis via an innate immune cascade have been reported. As a potential source of microbial transmission, information on microbial colonization in cervical mucus is unknown. We investigated pattern of microbiota in the cervical mucus collected from women with and without endometriosis using next-generation sequencing (NGS) technology.

Method of study: Cervical mucus samples were collected from women with (n = 30) and without (n = 39) endometriosis. The communities of microbiota in cervical mucus in the endometriosis group and the control group were examined by Gram staining and NGS targeting the V5-V6 region of 16S ribosomal RNA gene. Copy number of some target bacteria was detected by real-time PCR.

Results: We confirmed visual presence of bacteria in cervical mucus by Gram staining. NGS analysis showed that distribution of microbiota was similar in cervical mucus of women with and without endometriosis regardless of the phases of the menstrual cycle. In addition to predominant *Lactobacilli* spp., the populations of *Corynebacterium*, *Enterobacteriaceae*, *Flavobacterium*, *Pseudomonas*, and *Streptococcus* were increased in the endometriosis group. Of them, *Enterobacteriaceae* and *Streptococcus* were identified as the more significant candidates in the endometriosis group than in controls by real-time PCR ($P < 0.05$ for each).

Conclusion: Our NGS analysis of cervical mucus indicated that among a variable microbiota, two candidates (*Enterobacteriaceae* and *Streptococcus*) were more frequently detected in women with endometriosis. Further investigation is needed to elucidate a mechanistic link of these bacteria in the pathophysiology of endometriosis.

KEYWORDS
cervical mucus, endometriosis, metagenomic analysis, microbiota

1 | INTRODUCTION

Endometriosis, a chronic inflammatory condition characterized by the implantation of functional endometrium tissue outside the uterus, is estimated to affect approximately 6%-10% of reproductive aged women.[1] The main clinical symptoms, including chronic pelvic pain, dysmenorrhea, dyspareunia, and infertility, can seriously influence the quality of a patient's life suffering from endometriosis.[2] Numerous studies have clarified the regulatory factors supporting the development and the maintenance of endometriosis;[2,3] however,

the pathogenesis and/or pathophysiology of the disease is still unclear. Despite anatomical, genetic, endocrine, environmental, and inflammatory factors, recent evidence has showed that intrauterine microbial colonization and a cascade of innate immune system are involved in the growth promotion of endometriosis.[4] A national cohort study from Taiwan consisting of 79,512 patients showed that the incidence of lower genital tract infection remarkably increased the risk of endometriosis.[5] According to the report of Khan et al,[4] bacterial endotoxin (lipopolysaccharide, LPS), a component of Gram-negative bacteria, was elevated in the menstrual blood and peritoneal fluid of women with endometriosis and LPS could promote the growth of endometriosis through Toll-like receptor 4.[6,7] These findings provide a novel concept that bacterial contamination via the reflux of menstrual blood into the pelvis might play critical roles in the development of endometriosis.

Detection of pathogenic and non-pathogenic bacteria in uterine cavity is a consequence of ascending migration of microbiota from lower genital tract to upper genital tract bypassing an intermediate barrier, cervix and cervical mucus. Actually, it has been known that gut microbiota can serve as the source of genital tract infection,[8] and in recent years, when the use of NGS has increased, it has been suggested that genital tract microbiota is directly or indirectly associated with gut microbiota.[9,10] Cervix and cervical mucus play a fascinating role in first, preventing the ascent of pathogens from the vagina into the uterus, and second, allowing the ascent of sperm to the fallopian tube. Cervix/cervical mucus is also crucial for the maintenance of pregnancy until the onset of labor. If cervical mucus is contaminated with bacteria, it may have some detrimental effect on pregnancy outcome.

In addition, transmigration of bacteria from cervical mucus into the uterine cavity may switch the cascade as mentioned before[6,7] for the pathogenesis of endometriosis. Although accumulating evidences have focused on the bacterial detection of vagina and uterine cavity, there have been only a few studies to estimate the distribution of microbiota in cervical mucus.[11-13] One recent study demonstrated that microbiota in cervical mucus was more diverse than vagina accounting that a proportion of *Lactobacillus* in cervical mucus were lower than in vagina.[13] Although role of Gram-positive and Gram-negative bacteria in adverse reproductive outcome has been reported,[14,15] information on the distribution of microbiota in the cervical mucus of women with and without endometriosis remains unknown so far.

This study was undertaken based on serial experiments from our group[4,6,7] on the intrauterine microbial colonization in women with and without endometriosis and its association with LPS/TLR4 engagement of innate immune system in the pathogenesis of endometriosis. All of the previous studies were carried out with intrauterine samples. We plan to clarify the hypothesis if cervical mucus is contaminated with bacteria in women with endometriosis, this local microbial colonization at the entry of uterine canal could transmigrate into intrauterine cavity. If this is true, then we can make a link with our previous studies in the pathogenesis of endometriosis. Therefore, we aim to investigate the bacterial population in cervical mucus collected from women with and without endometriosis. We already know that cervical mucus harbors bacteria, and this can be found in healthy women and in women with gynecological diseases. We attempted to re-confirm this information by visual existence of bacteria in cervical mucus and their molecular analysis by NGS. In addition, residual pathogens in cervical mucus may adversely affect pregnancy outcome in these two groups of women. With these concepts in mind, first of all, we investigated visible existence of bacteria in cervical mucus by Gram staining. Secondly, a comprehensive analysis based on next-generation sequencing (NGS) technology was done targeting 16S ribosomal RNA (rRNA) gene. Finally, real-time polymerase chain reaction (PCR) analysis was performed to quantify the amount of various bacteria in cervical mucus.

2 | MATERIALS AND METHODS

2.1 | Patients and samples

Cervical mucus samples were obtained from 30 women with and 39 women without endometriosis who were of reproductive age (range, 20-44 years). All samples were taken before laparoscopic surgery. Endometriosis was diagnosed by laparoscopy and confirmed by pathology, and the stages were classified according to the revised American Society for Reproductive Medicine (r-ASRM) scoring system.[16] In this study, we enrolled the cases of r-ASRM stage III-IV endometriosis to highlight the difference from control group. Women without endometriosis (control group) comprised of fibroids or benign ovarian tumor other than endometriosis. All samples, collecting from patients with a normal-appearance cervix, were negative for vaginal culturing test. Women with gynecological malignancies, pelvic inflammatory disease, bacterial vaginosis, and endocrine disorders including thyroid diseases and diabetes mellitus were excluded. No patients received endocrine therapy or antibiotics at least six months before sampling cervical mucus. All women had a regular menstrual cycle, and each phase of the menstrual cycle was determined from the recorded files based on last menstrual period (LMP). Informed consent was obtained from all patients in accordance with a protocol approved by the Institution Review Board of Kyoto Prefectural University of Medicine (RBMR-C-1181-3).

2.2 | Preparation of cervical mucus

Cervical mucus was aspirated from the cervical canal using a sterile 1-mL syringe after prudently wiping with a sterile swab to prevent contamination of vaginal bacteria. After the treatment with 500 μL Sputazyme (Kyokuto Pharmaceutical Industrial Co.) to dissolve mucins, samples were then diluted with 2 mL of Dulbecco's phosphate-buffered saline and stored at −20°C for subsequent analysis.

2.3 | Gram staining

To remove host cells, 100 μL of cervical mucus sample was centrifuged at 100 x g for 5 minutes at 4°C and the supernatant was recentrifuged

at 800 x *g* for 5 minutes at 4°C. After discarding the supernatant, the pellets were resuspended with distilled water. Gram staining was performed using 0.2% Victoria Blue solution, 2% picric acid solution, and 0.04% Fuchsine solution (Nissui Pharmaceutical Co.).

2.4 | Bacterial DNA extraction and PCR amplification of 16S rRNA gene

The samples were centrifuged at 3500 x *g* for 5 minutes at 4°C, and then, the supernatant was discarded. Bacterial DNA was extracted from the pellets using NucleoSpin Microbial DNA (Macherey-Nagel). All steps of DNA extraction were performed according to the manufacturer's protocol. Bacterial DNA was subjected to PCR to amplify the 16S rRNA gene V5 - V6 region using a pair of the NGS.784F (5'-CCATCTCATCCCTGCGTG TCTCCGACTCAG|barcode sequence| AGGATTAGATACCCTGGTA–3') and NGS.1061R (5'- CCACTACGCCTCCGCTTTCCTCTCTATGGGCAG TCGGTGATCRRCACGAGCTGACGAC -3') primers[17] that contained the adaptor and barcode sequences for NGS. PCR products such as amplicon were size-selected and purified.

2.5 | Amplicon sequencing using NGS

Library preparation, including the determination of DNA quantities, dilution of each samples, emulsion PCR using Ion One Touch (Thermo Fisher Scientific), and purification of amplified DNA using Ion OneTouch ES system (Thermo Fisher Scientific), was performed according to the method proposed by Nishioka et al.[18] NGS was performed using the Ion Torrent Personal Genome Machine.

2.6 | Sequence data analysis

Quality control was performed using the open source pipeline Quantitative Insights Into Microbial Ecology (QIIME) version 1.8.0,[19] and fasta files containing more than 250 base pair in length and Phred score of more than 20 were obtained. Using SeqKit, 20 000 reads for each sample were picked up randomly from the obtained fasta file.[20] Operational taxonomic units (OTUs) picking process at 97% sequence identity against the Greengenes database (13_8),

followed by alpha and beta diversity analysis (by Shannon Index and weighted UniFrac), were performed using QIIME. The Shannon index, one of the popular alpha diversity indexes, was used to estimate the microbial diversity in each sample. It is calculated by the following equation:

$$H = -\sum_{i=1}^{s} p_i \ln (p_i)$$

where p_i means the rate of ith genus in the population.[21] Beta diversity served as an indicator of microbiota similarity. In this study, it was displayed graphically using principle coordinates analysis. After construction of a phylogenetic tree from NGS results, UniFrac distance was calculated by using the following equation[22]:

Sum of unshared branch lengths/Sum of total branch lengths

2.7 | Quantitative real-time PCR

Real-time PCR was performed using PowerUp SYBR Green Master Mix (Thermo Fisher Scientific) and 0.5 μmol/L each primer for detection of each genus (Table 1)[23-26] in the StepOnePlus Real-Time PCR System (Applied Biosystems) using the following parameters: initial denaturation at 50°C for 2 minutes and at 95°C for 2 minutes, followed by 40 cycles of 95°C for 3 seconds, and at 60°C (16S rRNA gene) and 62°C (*Enterobacteriaceae*, *Streptococcus*, *Pseudomonas*, *Corynebacterium*) for 30 seconds. The copy number of 16S rRNA gene was estimated as an indicator of total bacterial amount. The normalized value of the specific bacterial family and genus was calculated from 16S rRNA gene copy number.

2.8 | Statistical analysis

Data were expressed as the mean ± SD and SEM. A non-parametric Mann-Whitney *U* test and chi-squared test were used to compare the difference between groups. *P* values < 0.05 were considered statistically significant. All statistical analyses were performed using the Statcel 4 software (OMS Publishing Inc).

TABLE 1 Primer sets used in this study

Target	Primer	Sequence	Product size	References
Bacteria 16S rRNA	16S-784F	5'-AGGATTAGATACCCTGGTA-3'	280 bp	[17]
	16S-1061R	5'-CRRCACGAGCTGACGAC-3'		
Enterobacteriaceae 16S rRNA	Eco1457F	5'-CATTGACGTTACCCGCAGAAGAAGC-3'	195 bp	[23]
	Eco1652R	5'-CTCTACGAGACTCAAGCTTGC-3'		
Pseudomonas spp.16S rRNA	PSD7F	5'-CAAAACTACTGAGCTAGAGTACG-3'	215 bp	[24]
	PSD7R	5'-TAAGATCTCAAGGATCCCAACGGCT-3'		
Corynebacterium spp.rpoB	C-rpoB-F	5'-CAAACACCAAGCCGAACC-3'	196 bp	[25]
	C-ropB-R	5'-TGCTGCGTAATCATGGAGT-3'		
Streptococcus spp.23S rRNA	g-Str-F	5'-AGCTTAGAAGCAGCTATTCATTC-3'	309 bp	[26]
	g-Str-R	5'-GGATACACCTTTCGGTCTCTC-3'		

3 | RESULTS

The clinical characteristics of patients with and without endometriosis are shown in Table 2. There was no significant difference in age, BMI, parity, phases of menstrual cycle, and incidence of smoking/alcohol intake between these two groups of women. All patients with endometriosis belonged to r-ASRM stages III and IV. Complaint of dysmenorrhea was significantly higher in women with endometriosis than in control women (73.3% vs 43.6%, $P = 0.01$).

3.1 | Visual presence of bacteria in cervical mucus by Gram staining

Gram staining was done to verify the existence of microbiota in cervical mucus derived from women with and without endometriosis. The representative images of Gram staining of cervical mucus are shown in Figure 1A, B. A heterogeneous distribution of bacteria was observed in cervical mucus such as Gram-positive cocci, rods, and Gram-negative rods (Figure 1). Gram-positive rod-like bacilli including *Lactobacilli* were seen in large amount. A similar distribution of Gram-stained rods and cocci was found in the cervical mucus of endometriosis and non-endometriosis women. These findings indicate that individual variability of bacteria might be present regardless of the presence or absence of endometriosis.

3.2 | The distribution of microbiota in cervical mucus by NGS

To further investigate the distribution of microbiota in cervical mucus, a comprehensive microbiome analysis using NGS was performed. Consistent with the findings from Gram staining, the microbiota in cervical mucus indicated highly variations in individual as shown in Figure 2A. Various bacteria were detected in the samples of cervical mucus in both groups, although *Lactobacillus*

TABLE 2 Characteristics of Patients with or without endometriosis

	Control (n = 39)	Endometriosis (n = 30)	P value
Age (mean ± SD)	32.5 ± 6.0	33.9 ± 5.7	0.41
Range of age (y)	20 - 41	20-44	
BMI (mean ± SD)	20.58 ± 2.89	21.33 ± 3.27	0.33
Parity (mean ± SD)	0.37 ± 0.75	0.40 ± 0.82	0.88
Range of parity (n)	0-3	0-3	
Menstrual cycle (n) P(proliferative)/ S(secretory)	17/22	16/14	0.42
Dysmenorrhea (n)	17/39 (43.6%)	22/30 (73.3%)	0.01
Smoking (n)	10/28 (35.7%)	9/24 (37.5%)	0.89
Alcohol intake (n)	16/26 (61.5%)	11/23 (47.8%)	0.34

Note: The results were expressed as the mean ± SD. Statistics were analyzed by a non-parametric Mann-Whitney *U* test and chi-squared test.

was the most frequently seen (Figure 2A). There was no difference in the distribution of microbial community in cervical mucus between the endometriosis group and the controls (Figure 2B). When we compared the pattern of microbial community in the cervical mucus between the proliferative phase (from day 5 to 14 of menstrual cycle) and the secretory phase of the menstrual cycle (from day 15 to the last day of menstrual cycle), beta diversity analysis using weighted UniFrac indicated no significant difference in microbial community between the phases of the menstrual cycle, and this was similarly observed for control women (Figure 2C) and women with endometriosis (Figure 2D). Comparisons in beta diversity between the above groups further confirmed that microbiota in cervical mucus could depend on the individuals regardless of the menstrual cycle.

3.3 | Identification of bacteria in cervical mucus by NGS

We were curious to know whether different bacterial components would harbor in cervical mucus. To investigate this possibility, alpha diversity (the diversity of bacterial community within one sample) was analyzed using the Shannon index. As shown in Figure 3A, alpha diversity in the endometriosis group was significantly higher than in the control group ($P < 0.05$), indicating a more diverse distribution of microbiota in the cervical mucus of the endometriosis group. To identify the bacteria more frequently detected in cervical mucus in endometriosis group than the control, the data obtained from NGS were analyzed. The candidate bacteria were selected according to the following criteria: (a) bacteria that accounted for more than 1% of microbiota in the endometriosis group and (b) bacteria significantly higher in prevalence than the control. *Enterobacteriaceae*, *Corynebacterium*, *Pseudomonas*, *Flavobacterium*, and *Streptococcus* were selected as candidates (Figure 3B). Although *Streptococcus* was too small in amount in the control group to show the significant difference between the two groups, the population of *Streptococcus* was seen by far more than 1% of microbiota in the endometriosis group. On the other hand, while the prevalence rate of *Lactobacillus* (main bacteria among normal vaginal microbiota) ranged from 40%-60%, there were no significant differences in *Lactobacillus* population between endometriosis and control group (Figure 3C). The prevalence rate of *Prevotella*, and *Gardnerella* (two common pathogens of bacterial vaginosis) displayed no difference between control and endometriosis group (Figure 3C).

3.4 | Quantification of candidate bacteria in cervical mucus by qRT-PCR

In microbiome analysis using NGS, only a part of 16S rRNA gene sequences was recognized and automatically assigned to each OTU. Hence, the copy number of respective candidate for bacteria was analyzed by quantitative real-time PCR using respective primer for detection of each genus (Table 1). The average copy numbers of 16S rRNA gene in the control and the endometriosis group were 9.6×10^5 copies/mL and 6.8×10^5 copies/mL, respectively (not significant,
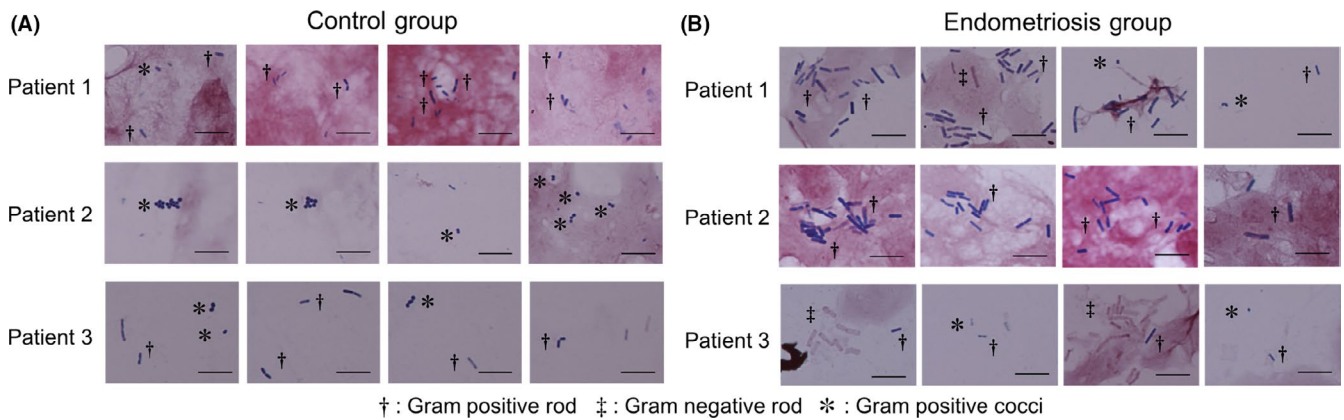
FIGURE 1 The existence of bacteria in cervical mucus by Gram staining. The representative images of Gram staining of cervical mucus samples from the control group (A) and the endometriosis group (B) are shown. Gram-positive cocci, rods, and Gram-negative rods were observed although Gram-positive rods were seen in large amount. The microbiota varied in individual and was similarly seen in both groups. Each marker indicates the following bacteria: †: Gram-positive rod, ‡Gram-negative rod, *Gram-positive cocci. Scale bar, 10 μm

FIGURE 2 The composition of microbiota in cervical mucus by NGS. (A) Cumulative bar chart of the main operational taxonomic units (OTUs) at genus level presents microbiota in cervical mucus (control group n = 39, endometriosis group n = 30). The microbiota indicated highly variations in individual, although *Lactobacillus* was the most frequently seen. (B) Beta diversity analysis was done to show the difference in microbial community composition between the control group (red) and the endometriosis group (blue), between the proliferative phase (red, from day 5 to 14 of menstrual cycle) and the secretory phase (blue, from day 15 to the last day of menstrual cycle) of women in (C) the control group and (D) the endometriosis group. The analysis was performed by weighted UniFrac, and the principal component (PC) analysis plot is shown. Each dot represents one sample. The percentage on the axes indicates the contribution rate of each principal component
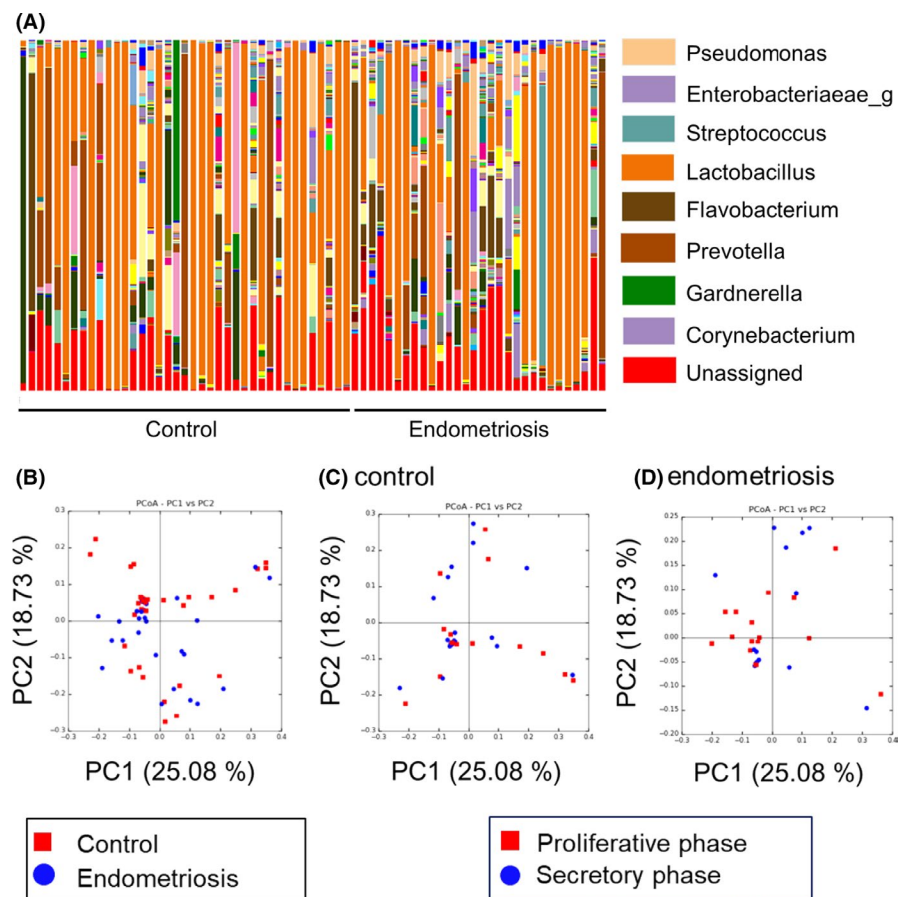

Figure 4A). The copy number of candidate bacteria, *Enterobacteriaceae*, *Streptococcus*, *Pseudomonas*, and *Corynebacterium*, was also confirmed by real-time PCR using each primer pairs. Consistent with the NGS results, the amount of *Enterobacteriaceae* and *Streptococcus* was significantly higher in the cervical mucus of endometriosis group than that in the control group (Figure 4B). On the other hand, even a relatively higher amount was found in the endometriosis group, there were no significant differences in copy numbers of *Pseudomonas* and *Corynebacterium* between the two groups (Figure 4B).

4 | DISCUSSION

In this study, we for the first time conducted a comprehensive analysis using NGS technologies to clarify the microbiota in cervical mucus of women with and without endometriosis. Our findings by Gram staining and NGS analysis revealed the existence of various microbiota in cervical mucus of both women with and without endometriosis. Individual variability was observed in the microbiota irrespective of the phases of menstrual cycle. Furthermore, *Enterobacteriaceae*
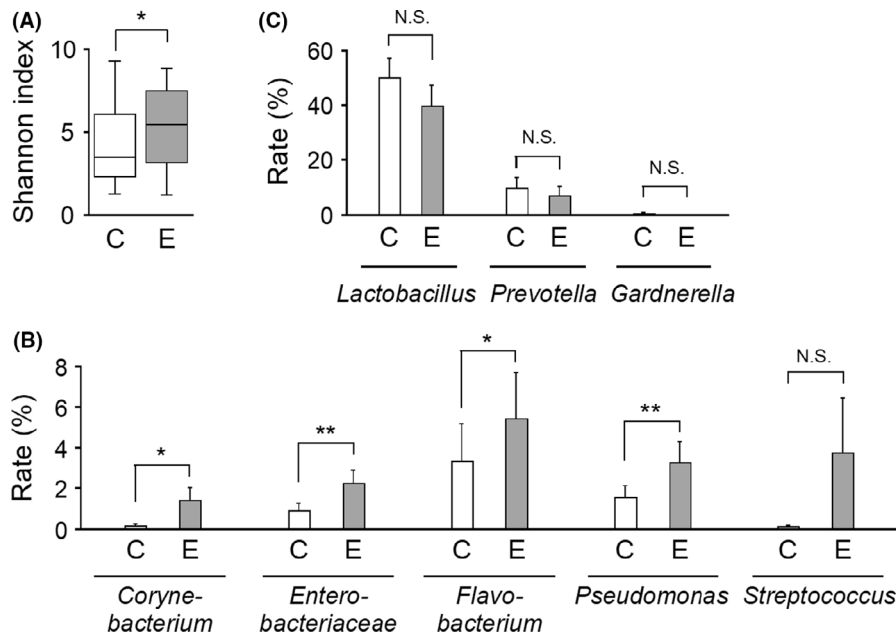
FIGURE 3 Identification of candidate bacteria in cervical mucus by NGS. (A) Shannon index, one of the indexes indicating the alpha diversity community of bacteria within one sample, was calculated. The boxes denote the interquartile range between the 25th and 75th percentiles (*$P < 0.05$). (B) The prevalence rate of candidate bacteria in the cervical mucus collected from women with endometriosis and control women as detected by NGS analysis. (C) The prevalence rate of common bacteria related to bacterial vaginosis is shown between the two groups. Data are expressed as the mean ± SEM (*$P < 0.05$, **$P < 0.01$, NS = not significant). E: endometriosis group, C: control group
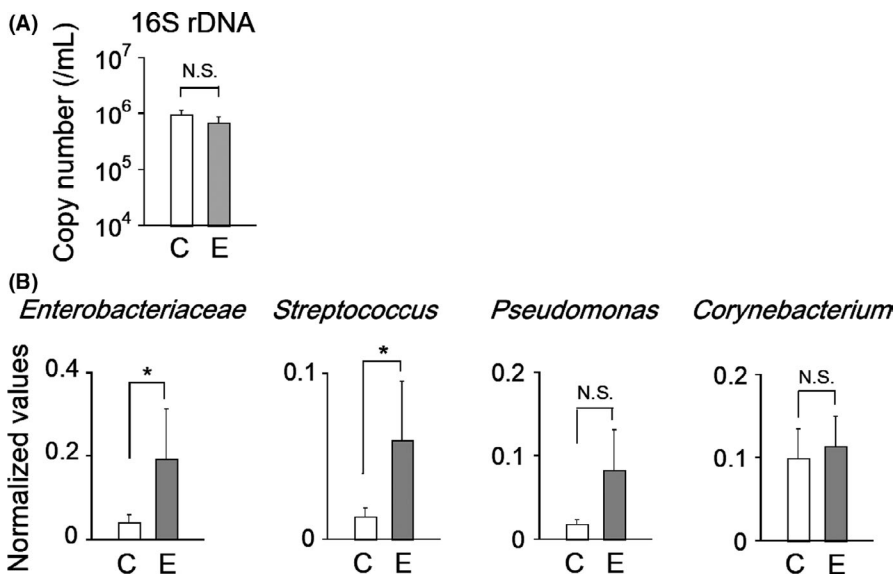

FIGURE 4 Quantification of candidate bacteria in cervical mucus by real-time PCR. (A) The average copy number of 16S rRNA gene of total bacteria in the cervical mucus collected from control women and women with endometriosis. (B) The amount of candidate bacteria was estimated, and normalized values were calculated against 16S rRNA copy number in each sample. Data are expressed as the mean ± SEM (*$P < 0.05$, NS = not significant). E: endometriosis group, C: control group

and *Streptococcus* in cervical mucus were more frequently detected in women with endometriosis than the control, which was also confirmed by real-time PCR analysis. NGS analysis revealed that even the prevalence rate of *Lactobacilli* in the cervical mucus was 20-40 times higher than other detected bacteria, a substantial proportion of *Enterobacteriaceae*, *Corynebacterium*, *Pseudomonas*, and *Flavobacterium* still exist in the cervical mucus of women with endometriosis. Cervical mucosal colonization of these bacteria in women with endometriosis was significantly higher than in control women. This might be due to impaired innate immune system of the female reproductive tract or by overcoming the antimicrobial host defense

capacity of *Lactobacilli*, thereby allowing these selected pathogens to persist in the alkaline environment of cervical mucus.[3,27,28]

Cervical mucus pug is a large, complex structure within cervical canal that is shed shortly before menstruation or during labor. Cervical mucus plug functions as a critical "gatekeeper" that prevents ascending infection from lower genital tract into uterine cavity. It has been reported that viscoelastic properties of the cervical mucus are determined by mucins (large glycoproteins) that can inhibit viral replication and exclude bacteria and other large molecules by preventing their diffusion through cervical mucus.[29] Considering that individual distribution of microbial diversity was similar in control

women and in women with endometriosis regardless of the phases of menstrual cycle, the question is how can these colonized bacteria in cervical mucus enter into uterine cavity.

There are some possible explanations that may address this question: (a) Under the influence of the corpus luteum, the ovary begins to secrete progesterone and the amount of cervical mucus is reduced and diluted,[30] (b) serial collection of cervical mucus on different days of menstrual cycle indicated that maximum inhibitory effect of cervical mucus on common microbial agents including *Escherichia coli* and *Streptococci* was apparent on day 14 and declined toward the end of the menstrual cycle,[31] (c) a number of antimicrobial peptides (beta-defensin/secretory leukocyte protease inhibitor, SLPI) are reported to be expressed in cell linings of urogenital tract, and the expression of all these antimicrobial peptides is regulated by cyclic estrogen.[32] Immunoexpression of human beta-defensin and SLPI was the highest during the proliferative phase, intermediate during the secretory phase, and the lowest during the menstrual phase.[33] In addition, several studies demonstrated that the materials in the cervix were transferred into the uterine cavity.[34,35] These reports suggested that there is a period of menstrual cycle when transfer of the bacteria in the cervix is likely to occur. Furthermore, there was no difference in the distribution of microbiota between women with and without endometriosis and between proliferative and secretory phase of the menstrual cycle as we found by beta diversity of NGS analysis. We hypothesized that population of bacteria in cervical mucus was not susceptible to change across the menstrual cycle. Therefore, we can at least postulate that bacteria that exist in cervix, including candidate bacteria in this study, may enter into the intrauterine cavity together with materials in cervix.

We cannot ignore the possibility of bidirectional pathway in the bacterial contamination of cervical mucus. This study could be an interesting piece of evidence that if cervical mucus is colonized with bacteria, it could transmigrate to the uterine cavity (ascending pathway). In this case, the causative bacteria may have migrated from the gut microbiota. It is also true that transmigration could be from bacterial species or genus being shed from the uterus and contaminate cervical mucus during passing down through the cervical opening (descending pathway). In addition, disruption of the immune system, including the changes caused by gut microbiota,[36] may have affected the microbiota of the upper genital tract. Further studies are warranted to clarify this bidirectional pathway related to bacterial colonization in cervical mucus.

Another critical question remains to be addressed how bacterial population in cervical mucus and its subsequent entry into uterine cavity may be involved in the pathophysiology of endometriosis. Our qRT-PCR data of cervical mucus indicated that amount of *Enterobacteriaceae* and *Streptococci* was significantly higher in women with endometriosis than in control women. *Escherichia coli*, a Gram-negative bacteria and a candidate among *Enterobacteriaceae* retains LPS as a cell wall extract and its entry into pelvis during retrograde menstruation may trigger TLR4 signaling in inducing pro-inflammatory response in pelvis and growth promotion of endometriosis. This

phenomenon has already been described by a series of study by Khan et al[4,6] Similarly, lipopeptide, a ligand of *Streptococci*, may be involved in the growth of endometriosis through homodimeric or heterodimeric binding with TLR2/TLR6.[37] Several studies have shown that the microorganisms in female genital tract could be involved in endometriosis. The initial study of Khan et al[6,7] was based on the analysis of menstrual blood by bacteria culture method. Recently, NGS analysis was conducted using samples from uterine cavity and cystic fluid of ovarian endometrioma, and *Streptococcaceae* and *Moraxellaceae* in the endometriosis group were significantly more frequently detected than the control.[38] Our comprehensive study of cervical mucus showed that *Enterobacteriaceae* and *Streptococcus* were most frequently seen in women with endometriosis, and this finding was in agreement with candidate microorganisms previously reported.[6,38]

In microbiome analysis using NGS, only a part of 16S rRNA gene sequences was recognized and automatically assigned to each operational taxonomic unit (OTU). Unlike previous studies, the new information of our study is that we used cervical mucus as the study sample and we performed qRT-PCR to quantify some target bacteria in cervical mucus derived from women with and without endometriosis. There have been major concerns that collection of samples via cervical canal might increase the technical bacterial contamination; therefore, samples from uterine cavity were usually considered to be taken during surgery. Surgical samples might not be appropriate for the analysis of microbiota since the composition of microbiota in living body rapidly changes after the resection of the tissues.[39] Thus, the collection of cervical mucus samples, readily accessed and less contaminated, was conducted in the present study.

There are some limitations in our current study: (a) The sample size was relatively small. (b) In the current NGS analysis, we analyzed cervical mucus bacteria at the genus level, since it was not necessarily reliable to analyze bacteria at the species level. Further study focusing on bacteria at the species level in our detected bacteria may bring to precisely clarify the link between microbiota in cervical mucus and endometriosis. (c) We targeted moderate-to-severe endometriosis to highlight the difference from control group. Further study including minimal and mild stages may be useful to clarify the pathophysiology of endometriosis.

Our current findings may have some biological and clinical significance. Higher accumulation of bacteria in cervical mucus and its consequent entry into the uterine cavity may be involved in the pathogenesis or pathophysiology of endometriosis via LPS/TLR4 or lipopeptide/TLR2 or TLR6 engagement of innate immune system. Bacterial colonization of cervical mucus may have some detrimental effect on the adverse pregnancy outcome by interrupting sperm-mucus interaction. Sperms are at risk of phagocytosis and apoptosis by leukocytes, antibodies, and complements including microbiota in the female genital tract.[40] In fact, the role of microbial pathogens in adverse reproductive outcome has already been reported.[14,15] A tissue inflammatory reaction in response to bacteria in the endometrium may stimulate occurrence of acute endometritis or chronic endometritis by releasing IL-8 or lymphocyte stimulating factor by the infiltrating

immune cells.[41-44] This tissue inflammatory response in the cervix and/or endometrium may adversely affect pregnancy outcome.[45-49]

In conclusion, our 16S rRNA gene sequencing analysis of cervical mucus indicated that among a variable microbiota, two predominant candidates (*Enterobacteriaceae* and *Streptococcus*) were more frequently detected in women with endometriosis. These two microbial candidates may narrow the range of whole microbiome at the genus level. The identification of these bacteria was consistent with the microorganisms detected by several previous studies suggesting that our analysis of cervical mucus sample using NGS was useful and valid. Further investigation is needed to elucidate a mechanistic link of these bacteria in the pathophysiology of endometriosis.

CONFLICT OF INTEREST

The authors have nothing to disclose.

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