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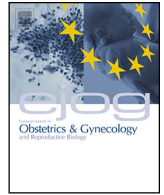

# Molecular detection of intrauterine microbial colonization in women with endometriosis

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ABSTRACT

*Objective:* Increased intrauterine microbial colonization by bacteria culture method and occurrence of endometritis have been reported in women with endometriosis. Here we investigated microbial colonization in intrauterine environment and cystic fluid of women with and without endometriosis by molecular approach.

*Study design:* This is a case-controlled biological study with a total of 32 women each with and without endometriosis. Among them, 16 each in these two groups of women received treatment with gonadotropin-releasing hormone agonist (GnRHa). Pattern of microbial colonization in endometrial swabs and endometrioma/non-endometrioma cystic fluid was examined using broad-range polymerase-chain reaction (PCR) amplification of bacteria targeting 16S rRNA gene (rDNA). After quantification of index PCR product, 16S rDNA metagenome sequence analysis was done by Illumina Miseq system.

*Results:* A wide proportion (0.01–97.8%) of multiple bacteria was detected in both endometrial swabs and cystic fluid collected from women with and without endometriosis. 16S metagenome assay indicated that proportion of *Lactobacillacae* was significantly decreased ($p < 0.01$) and of *Streptococcaceae*, *Staphylococaceae*, *Enterobacteriaceae* was significantly increased ($p < 0.05$ for each) in GnRHa-treated women with endometriosis than in GnRHa-untreated women. While bacteria culture method failed to detect a single colony, 16S metagenome assay could detect significantly higher percentage of *Streptococcaceae* ($p < 0.01$) and *Staphylococaceae* ($p < 0.05$) in the cystic fluid derived from women with ovarian endometrioma comparing to that in cystic fluid collected from non-endometrioma cysts.

*Conclusion:* These findings indicate the occurrence of sub-clinical infection in intrauterine environment and in the cystic fluid of ovarian endometrioma. Additional side effect of GnRHa treatment in promoting silent intrauterine and/or ovarian infection should be considered.

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## Introduction

Endometriosis is a multi-factorial disease mostly affecting women of reproductive age and is associated with chronic pelvic pain and infertility. Even after long three hundred years, most of the literatures [1–3] still claim that pathogenesis and/or pathophysiology of endometriosis is unclear. However, it is difficult to uniformly explain the pathogenesis of endometriosis by a single factor.

In an attempt to search additional factor, we established a new concept "bacterial contamination hypothesis" in endometriosis [4]. We found that menstrual blood of women with endometriosis was highly contaminated with *Escherichia coli* with consequent elevation in the concentrations of endotoxin in the menstrual fluid and peritoneal fluid [4]. As an initial inflammatory mediator, lipopolysaccharide (LPS) has been found to regulate Toll-like receptor 4 (TLR4)-mediated growth of endometriosis [4,5]. More recently, we demonstrated an increased intrauterine microbial colonization using endometrial samples in bacteria culture system and concurrent occurrence of endometritis in women with

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endometriosis that was further worsened after gonadotropin-releasing hormone agonist (GnRHa) treatment [6]. In clinical practice, GnRHa is commonly used for the alleviation of pain, decreasing the risk of intra-operative bleeding and/or for reducing the size of ovarian endometrioma.

The limitation of bacteria culture system is that only a fraction of the bacteria present in most microbial ecosystems are amenable to propagation in the laboratory [7]. Bacteria in complex microbial communities can be identified by characterizing their ribosomal RNA genes (rDNA), an approach that has the advantage of detecting fastidious or cultivation-resistant organisms [8]. Therefore, we plan to detect bacteria in endometrial samples derived from GnRHa-treated and -untreated women with and without endometriosis with the use of molecular methods. We also examined cystic fluid collected from women with ovarian endometrioma and non-endometrioma cysts in order to confirm their status of fluid sterility.

## Materials and methods

### *Study population*

During the period of November 2013 and June 2014, endometrial samples were collected from 32 women each with and without endometriosis during laparoscopy at Nagasaki University. Women with endometriosis aged between 21 and 47 years were recruited by either elective laparoscopy for infertility or diagnostic laparoscopy for dysmenorrhea and subsequently confirmed by histology. The control group, between 21 and 52 years old, consisted of fertile women without any evidence of endometriosis and were operated on for dermoid cyst/serous cyst adenoma/mucinous cyst adenoma or for uterine myoma. None of these control women had a prior history of endometriosis. The staging and the morphological distribution of peritoneal lesions were based on the revised classification of the American Society of Reproductive Medicine (r-ASRM) [9].

Sixteen control women with uterine myoma and 16 women in endometriosis group had gonadotropin-releasing hormone agonist (GnRHa) treatment for a variable period of 4–6 months. The GnRHa treatment with a dose of 1.88 mg IM per month was determined independently by a gynecologist (AF). The remaining study group and endometriosis-free group had no hormonal medication in the 3 months prior to the surgical procedure. All control women and women with endometriosis had regular menstrual cycles (28–32 days). The phases of the menstrual cycle was determined by histological dating of eutopic endometrial samples taken simultaneously with uterine swabs during laparoscopy.

All endometrial samples and cystic fluid were collected in accordance with the guidelines of the Declaration of Helsinki and were approved by the Institutional Review Board (Ethics No. 14011) of Nagasaki University. An informed consent was obtained from all women.

### *Collection of endometrial samples and cystic fluid*

With strict aseptic measure, endometrial samples were collected from control women [GnRHa (−), $n = 16$, GnRHa (+), $n = 16$] and women with endometriosis [GnRHa (−), $n = 16$, GnRHa (+), $n = 16$] using Seed Swab (No. γ3, E-MS64, Eiken Chemical Co. Ltd., Tochigi, Japan). Cystic fluid was collected during laparoscopy from 8 GnRHa-untreated women with r-ASRM stage IV endometriosis (ovarian endometrioma, OE) and 8 women with serous/mucinous cyst adenoma (non-endometrioma, NE). To minimize the risk that endometrial cultures could be contaminated in the vagina after placing a vaginal speculum, the Seed Swab was inserted under visual control into the uterine cavity taking care to avoid any contact with vaginal walls. After collecting samples, all Seed Swabs/cystic fluids were transferred to bacteriological laboratory and stored at −80 °C.

In the bacteriological laboratory, cystic fluids derived from 16 each of GnRHa-untreated women with and without endometriosis was plated into appropriate agar medium for bacteria culture as described previously [6]. All colonies were counted for each plate, all experiments were performed in triplicates, and data were expressed as colony forming unit/ml (CFU/ml).

### *DNA extraction*

Endometrial and cystic fluid samples were placed in 15-ml conical vials with 2 ml saline and endometrial samples were vortex mixed for 1 min to dislodge cells from Seed Swab. The sample solution was centrifuged at 10,000 rpm for 10 min, and the pellet was resuspended in 100 μl supernatant. DNA was extracted from the total amount of endometrial cell pellets and cystic fluid using the Ultra Clean PowerSoil® DNA isolation Kit (MoBio Laboratories Inc., Carlsbad, CA) according to the manufacturer's instruction. Since concentration of DNA for each sample was diverse, therefore we performed PCR cycles to gain sufficient amount of PCR products and adjusted final concentration to be employed for later analysis. The similar PowerSoil kit was previously used in the extraction of DNA from human samples [10].

### *Bacterium-specific PCR assays*

A flow-chart of polymerase-chain reaction (PCR) amplification of bacteria targeting 16S rDNA and sequence analysis is shown in Fig. 1. Bacterium-specific PCR assays were developed based on detection of species- specific regions of the 16S rRNA gene (rDNA). The PCR primers were designed to combine 16S rRNA gene region sequence with adapter overhang nucleotide sequence to attach the index sequence for deep sequencing [11]. The primer sequences are as follows: the adapter overhang sequences are underlined and 16S rRNA gene locus specific sequences are indicated by bold letters.

(1) forward primer, 5′TCGTCGGCAGCGTCAGATGTGTATAA-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG**CTACG GGGGGCAG-CAG**-3′ (Tm: 77 °C), (2) reverse primer, 5′GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG**GGACTACCGGGGTATCT**-3′ (Tm: 74 °C)

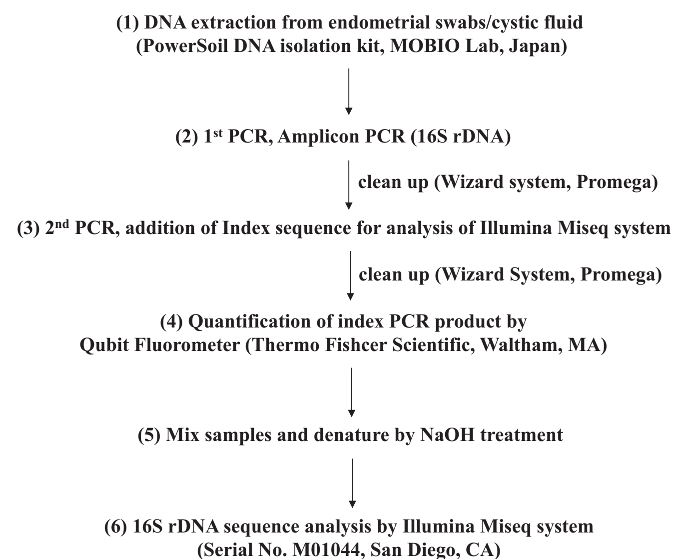


**Fig. 1.** A flow chart of methods in the 16S rDNA sequence analysis by Illumina Miseq system that we used for current study. A sequential steps of DNA extraction from endometrial swabs/cystic fluids, amplicon PCR, index PCR, quantification of PCR products for subsequent sequence analysis are shown in this figure.

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