Therapeutic intervention based on analysis by 16S ribosomal RNA gene sequencing of intrauterine microbiome improves pregnancy outcomes of IVF patients: a prospective cohort study

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Research Article

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Abstract

Purpose: Lactobacillus -dominated microbiota (LDM, defined as >90% Lactobacillus species composition) in the endometrium was reported to be associated with favorable reproductive outcomes. We investigated in this study whether 16S ribosomal RNA (rRNA) gene sequencing technology analysis of the uterine microbiota improves pregnancy outcomes.

Methods: The prospective cohort study consisted of 195 women with recurrent implantation failure (RIF) (defined as at least three previous failed in vitro fertilization (IVF)-embryo transfer (ET) attempts) and was carried out between March 2019 and April 2021 at our fertility center. Analyses of endometrial microbiota by 16S rRNA gene sequencing were suggested to all patients who had failed three or more ETs. One hundred and thirty-one patients underwent microbial 16S rRNA biomarker analysis (study group) before additional transfers, while 64 patients with a history of RIF proceeded to ET without endometrium analysis (control group). The primary outcome was the cumulative clinical pregnancy rate after two additional ETs.

Main results: Abnormal endometrial microbiota was detected in 30 patients (22.9%). All but one of those 29 patients received antibiotics corresponding with the detected bacteria and probiotic treatment. As results, the cumulative clinical pregnancy rate (study group: 64.5% vs. control group: 33.3%, p=0.005) and the ongoing pregnancy rate (study group: 55.1% vs. control group: 32.8%, p=0.020) were both significantly higher in the study group compared to the control group.

Conclusion: Personalized treatment recommendations based on the 16S rRNA analysis of the uterine microbiota can improve IVF outcomes of patients who have been experiencing RIF.

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Introduction

The human microbiome project has revealed that approximately 9% of the total human microbiome is found in the female reproductive tract[1, 2]. In addition, a recent independent metagenomic study revealed differences in bacterial communities between the vaginal and uterine cavities of women of reproductive age[3–5]. These results challenge the conventional dogma that the human uterus is sterile[6] and support the existence of a low-biomass, active uterine microflora. The nature of the microbiota, whether it is transient or endogenous microorganisms that contribute to uterine homeostasis, is still a matter of debate[7]. However, accumulating evidence suggests that the microbiome of the female reproductive tract may play an important role in reproductive function. Observational clinical studies have shown that certain microbes identified by culture are associated with implantation failure and spontaneous

abortion[8–12]. Furthermore *Lactobacillus*-dominated microbiota (LDM, defined as > 90% *Lactobacillus* species composition) in the endometrium was reported to be associated with favorable reproductive outcomes, while non-LDM (< 90% *Lactobacillus* species composition) was found to decrease implantation, clinical pregnancy, and ongoing pregnancy [4].

Among the multiple factors that cause infertility, uterine factor infertility occurs in as much as one in five hundred reproductive-aged women[13]. Indeed, approximately 30% of cases of infertility are caused by uterine dysfunction[14]. This organ is therefore essential to reproductive success and continuity of the human species.

Using next-generation sequencing (NGS) technology to analyse by 16S rRNA gene sequences, the composition of the endometrial microbiome can be revealed in more detail, including culturable and unculturable bacteria [15]. The diagnostic tool used in this study, named Endometrial Microbiome Metagenomic Analysis (EMMA), is based on the analysis of microbial 16S rRNA by NGS technology reported by Moreno [15], and provides information on whether the uterine microbiota is LDM or non-LDM without bias in the natural abundances of the bacteria analyzed. However, the clinical efficacy of EMMA is still unknown. In the current study, we aimed to investigate whether the analysis of endometrial microbiota by 16S rRNA gene sequencing improves the endometrial microbial environment and impacts positively the pregnancy and miscarriage rates of patients with recurrent implantation failure (RIF).

Methods

Study Design and Participants

This is a prospective cohort study carried out in our fertility center (Kamiya Ladies Clinic, Sapporo, Japan) from March 2019 to April 2021. Female patients under the age of 40 with RIF, defined as three or more failed embryo transfer (ET) attempts, who were in the process of *in vitro* fertilization (IVF)-ET were enrolled. Patients undergoing IVF treatment with scheduled frozen embryo transfers (FETs) at our institution were also included. Exclusion criteria englobed patients with (1) intrauterine lesions (surgically adapted uterine submucosal myomas and endometrial polyps, Asherman's syndrome, cesarean section scarring syndrome), (2) Hydrosalpinx without medical treatment, (3) allergy to antibiotics, or patients who cannot follow antibiotic treatment, (4) treatment with antibiotics within 3 months before sample collection, (5) current antibiotic treatment prescribed by other centers, (6) any illness or medical condition that is unstable or can present a risk to patient safety and compromise the compliance of the study. This study was performed following the Declaration of Helsinki medical research involving human subjects and Good Clinical Practice guidelines and was approved by the Ethics Committee of the Institutional Review Board of Kamiya Ladies Clinic. This trial is registered in the University Hospital Medical Information Network (UMIN) Clinical Trial Registry in Japan (UMIN000036050). Candidate participants were asked whether they would like analysis of their endometrial microbiota by 16S rRNA gene sequencing and written informed consent was obtained from all enrolled patients.

Procedures

Sample collection

Endometrial biopsies (EBs) were collected by a pipette cannula of Cornier Devices (Fuji medical, Tokyo, Japan). EB samples were decanted into a sterile tube (Cryotube, Biosigma S.p.A, Italy) containing RNA later solution (Sigma-Aldrich Co. LCC., MI). Tubes containing the samples were vigorously shaken, stored at 4ºC for at least 4 hours, and then sent to Igenomix Co., (Valencia. Spain).

Analysis of endometrial microbiota by 16S rRNA gene sequencing and intervention prior to frozen embryo transfer

This study was conducted using the EMMA test (https://www.igenomix.com/genetic-solutions/emma-clinics/) as a tool for assessing uterine microbiota using NGS analysis of microbial 16S rRNA. Detailed EMMA methods were described previously by Moreno [15, 16]. EMMA comprehensively detects a variety of bacteria present in the uterus and determines whether the uterine microbial environment is optimal for pregnancy. This molecular method is based on detecting and quantifying the amount of bacterial DNA present in EB samples. Briefly, total DNA was extracted using a QIAamp cador Pathogen Mini Kit (Qiagen Inc, Venlo, The Netherlands) and the resulting DNA was quantified using Nanodrop. Bacterial genomic DNA was analysed by high throughput sequencing, Ion Chef Instrument: Model 4247, Ion Torrent S5 XL Sequencer: Model 7728 (Thermo Fisher Scientific, Valencia, Spain). After samples were processed and analysed, patients received personalized treatments based on the results of analyses of endometrial microbiota by 16S rRNA gene sequencing. The different results of EMMA include: (1) “normal”, indicating LDM which means a *Lactobacillus* ratio of over 90% with no pathogens detected, (2) “abnormal”, indicating an endometrial microbiome dominated by non-*Lactobacillus* genera whose abundances account for more than 10% of the total bacterial composition, (3) “mild dysbiosis”, indicating that the endometrium is not *Lactobacillus*-dominant and pathogenic bacteria are not present in significant amounts, and (4) “ultralow” indicating very small amounts of bacteria, an almost sterile endometrium. Thus, treatments were determined based on these results.

In the case of a normal result, patients continued their FET cycles without any additional treatment; in the case of an abnormal result, patients followed the recommended therapy and underwent ET after confirmation of a normalized microbiome by repeating EB and the analysis by 16S rRNA sequencing. The recommended therapy for patients with an abnormal microbiota was one cycle of antibiotics treatment according to the pathogens detected in the test and clinical characteristics of each patient. Vaginal suppositories containing *Lactobacillus* strains available in Asia (Invag^{R}; Biomed, Krakow, Poland, or Lactoflora^{R}; STADA, Portugal) were recommended as probiotics after antibiotic treatment for 7-10 days. In patients with mild dysbiosis and ultralow biomass, vaginal probiotic treatment was recommended prior to their FET cycles. All patients in the control group received intravaginal probiotic treatment starting on day 5 of the menstrual cycle during the FET cycles for 7-10 days.

Endometrial Preparation and Frozen Embryo Transfer

Endometrial preparation was performed similarly in both groups. Endometrial preparation and FET were performed in either a natural or a hormone replacement therapy (HRT) cycle. Natural cycle was used for women with regular menstrual cycles (25–35 days in duration). The ET time of the natural cycle was adjusted to the LH surge of each patient. For patients with luteal insufficiency or thin endometria during the natural cycle, hormone treatment was implemented. Patients started oral administration of estradiol valerate (2.00 mg per a day; Progynova®; Bayer, NSW, Australia) and oral progestin (Duphaston®; 20 mg of dydrogesterone) after ovulation. The protocol for HRT was performed as follows: patients used dermal patches of 1.44 mg estradiol every other day (Estrana®; Hisamitsu Pharmaceutical Co., Ltd., Tokyo, Japan) and took estradiol valerate (2.00 mg per a day; Progynova®; Bayer, NSW, Australia) orally starting from day 2 or 3 of the menstrual cycle. Once endometrial thickness reached >7 mm, women started with daily administration of progestin capsules (500 mg of progesterone vaginal suppositories) and oral progestin (Duphaston®; 20 mg of dydrogesterone). The transfer of day-3 embryos or blastocysts was scheduled based on embryo and endometrium synchronization. Once pregnancy was achieved, exogenous estrogen and progestin supplementation were continued until week 10 of gestation.

Outcome Measurements

The main objective of this study was to determine whether the analysis of endometrial microbiota by 16S rRNA gene sequencing and its recommended treatments have positive impacts on the pregnancy outcomes of patients with RIF. The primary outcome was to compare the cumulative clinical pregnancy rate of two additional ETs between the study group and the control group after the suggested test. The secondary outcomes were the morbidity rates of intrauterine microbiome disturbance in patients with RIF, the rate of cumulative ongoing pregnancy rate and birth rate after the suggested test, early miscarriage rate, and adverse events. The definition of clinical pregnancy adopted in this report was the presence of gestational sacs assessed by ultrasound up to 7 weeks of gestation. Ongoing pregnancy was defined as fetal heartbeat detected until 12 weeks of gestation. Early miscarriage rate was defined as the proportion of pregnancies arrested before 12 weeks of gestation.

Sample size and Statistical analysis

A previous report has shown that the clinical pregnancy rate differs by 30% between LDMand non-LDM, and that, approximately, half of infertile patients undergoing IVF have a less-than-90% abundance of *Lactobacillus* species in their endometria [4]. To verify these data, a minimum sample size of 112 patients in total and 56 patients in each study group would be required with an alpha level of 0.05 and a power of 80%. Considering a dropout rate of approximately 10% due to possible lack of compliance in treatment, follow-up or monitoring, the total number of patients required per group would be 61.

Data were presented as the mean ± SD. Statistical analysis was performed using StatFlex version 7.0 (Artech Co., Ltd., Osaka, Japan). The results were compared between the two study groups using the chi-squared test, unpaired Student's *t*-test, or Mann–Whitney nonparametric *U* test. $P < 0.05$ was considered statistically significant.

Results

Patients' characteristics

A total of 195 patients were available for analysis, two patients were excluded due to lack of test data, and three patients dropped out of this study for personal reasons (Fig. 1). One hundred and thirty-one patients underwent analysis of endometrial microbiota by 16S rRNA gene sequencing (study group) before additional embryo transfers, and 64 patients with a history of RIF were unwilling to undergo that analysis and chose to continue ET (control group). Patients in the control group were negative about the possibility of delaying ET due to testing and results-based treatment interventions. Table 1 shows the patients' characteristics. There were no differences in age, body mass index (BMI), ovarian reserve, number of previous egg retrievals, history of miscarriage, or history of delivery between the two groups.

Table 1
Patients' characteristics

	Study group (EMMA tested)	Control group (not tested by EMMA)	P value
Number of patients	131	64	N/A
Age (y)	36.17 ± 3.45	35.50 ± 3.56	0.210
BMI (kg/m^2)	21.92 ± 2.79	21.95 ± 2.84	0.942
AMH (ng/mL)	3.55 ± 3.12	3.43 ± 3.00	0.791
Basal FSH (mIU/mL)	8.08 ± 3.84	8.31 ± 3.35	0.692
Duration of infertility (m)	40.31 ± 30.80	40.51 ± 27.60	0.948
History of delivery (n)	29 (22.1%)	17 (26.6%)	0.587
History of miscarriage (n)	62 (47.3%)	23 (35.9%)	0.239
Mean number of previous ET cycles	4.31 ± 2.40	3.97 ± 1.94	0.438
Number of patients affected by tubal factors	20 (15.3%)	15 (23.4%)	0.163
Number of patients affected by male factor infertility	39 (29.8%)	19 (29.7%)	0.990
Number of patients affected by endometriosis factors	20 (15.3%)	12 (18.8%)	0.538

AMH: Anti-Müllerian hormone, BMI: body mass index, EMMA: endometrial microbiome metagenomic analysis, ET: embryo transfer, FSH: Follicle-stimulating hormone

The Results Of The Analysis Of Endometrial Microbiota By 16s Rrna Gene Sequencing

The results for the 131 patients in the study group are shown in Table 2. The analysis revealed that 67 patients (51.1%) had an LDM, 64 patients (49.9%) had non-LDM uterine microbiota. Twenty-three patients (17.6%) had mild dysbiosis, and 11 patients (8.4%) had an ultralow biomass. Probiotic treatment was recommended for them. Abnormal microbiota was detected in 30 patients (22.9%), and, among those patients, Fig. 2 shows the frequency of bacteria other than *Lactobacillus* spp. detected. A wide variety of bacteria other than *Lactobacillus* spp. was identified, but the most frequently detected pathogens were *Streptococcus*, *Gardnerella*, *Atopobium*, and *Bifidobacterium*. *Chlamydia* and *Ureaplasma*, previously suggested to cause chronic endometritis (CE) [17], were not detected in this cohort of patients. The percentage of bacteria detected in individual patients with abnormal microbiota was shown in the supplemental data (supplemental Table 1). The most frequently detected bacteria in patients with a non-LDM were *Gardnerella* and *Bifidobacterium*. Metronidazole (500mg twice a day, respectively) was recommended as the first-choice antibiotics in cases where *Gardnerella* was widely detected as the cause of abnormal intrauterine flora. For patients whose microbiota presented more than 10% of *Streptococcus*, which has been reported to be associated with CE [17], a combination of amoxicillin and clavulanic acid (500mg-125mg per 8 hours, respectively) was recommended as the first-line treatment. The single case where, even though the initial result was abnormal microbiota, no antibiotic treatment was recommended was case 30 in Fig. 2, in which the patient presented a *Bifidobacterium* abundance of 97.3%. Thus, antibiotic therapy was recommended for 97% of patients who presented an abnormal microbiota in the initial test (Table 3). Patients who were recommended to receive antibiotics were treated for 7–8 days, followed by probiotic therapy starting on day 5 of the menstrual cycle and underwent a 2nd biopsy. Among them, 23 patients (75.0%) improved to less than 10% pathogens present in their uterus after the initial treatment. The remaining 7 patients (25.0%) who remained with more than 10% pathogens required additional antibiotic treatment and proceeded to the 3rd biopsy. For these 7 patients, the recommended antibiotics were clindamycin (300mg twice a day, respectively) (4 patients), amoxycillin/clavulanic acid (2 patients), and metronidazole (1 patient) (Table 3). All the patients improved their microbiota to less than 10% pathogens after the additional treatment.

Table 2
Detailed data for each EMMA initial result

Initial results of EMMA	Abnormal hysteroscopic findings with a suspicion of CE *	Ongoing pregnancies after EMMA/ALICE and its recommended treatment
Normal 67 cases (51.1%[$])	12 cases (17.9%[#])	31 cases (46.2%[#])
Abnormal 30 cases (22.9%[$])	6 cases (20.0%[#])	12 cases (40.0%[#])
Mild dysbiosis/negative 23 cases (17.6%[$])	3 cases (13.0%[#])	18 cases (78.2%[#])
Ultralow/negative 11 cases (8.4%[$])	2 cases (18.1%[#])	5 cases (45.4%[#])
Total 131 cases	23 cases (17.5%[#])	66 cases (50.3%[#])
*: Hysteroscopy performed within 3 months prior to endometrial microbiota 16S RNA gene sequencing		
$: percentage found out of a total of 131 cases		
#: prevalence in each endometrial microbiota 16S RNA gene sequencing group		
CE: chronic endometritis, EMMA: endometrial microbiome metagenomic analysis		

Table 3
Recommended antibiotic treatments for patients with Abnormal results in EMMA

Recommended first-line antibiotic treatment for patients with Abnormal results (n = 30)	
Amoxicillin/ Clavulanic acid (500mg-125mg per 8 hours for 8days)	13 (43.3%)
Metronidazole (500mg twice a day for 7days)	16 (53.3%)
None	1 (3.3%)
Recommended **second-line** antibiotic treatment for patients with Abnormal results (n = 7)	
Clindamycin (300mg twice a day for 7days)	4 (57.1%)
Amoxicillin/ Clavulanic acid (500mg-125mg per 8 hours for 8days)	2 (28.6%)
Metronidazole (500mg twice a day for 7days)	1 (14.3%)
EMMA: endometrial microbiome metagenomic analysis	

Importantly, the prevalence of hysteroscopic abnormalities suggesting CE [18, 19] such as moderate micro-polyps, hyperemia, and edema was comparable between normal (12 out of 67 cases, 17.9%) and

abnormal (6 out of 30 cases, 20.0%) results in the group of patients who underwent analysis of the uterine microbiota (p = 0.807, Table 2).

In terms of adverse events observed in the study, there were no patients who developed serious complications such as uterine perforation. Although there were 3 patients who experienced vagal reflex and hypotension due to pain, the symptoms improved after 5 minutes of rest. There were no cases of serious complications such as anaphylactic shock due to the administration of antibiotic agents.

Pregnancy Outcomes

Table 4 shows the pregnancy outcomes with the FETs of patients between the two groups after the suggested analysis of microbial 16S rRNA gene sequencing. Patients in the study group restarted their FET cycles after completing the recommended treatment suggested in the report of the microbial 16S rRNA biomarker analysis results. When comparing pregnancy outcomes, there was no difference in the number of transferred embryos between the two groups. The study group had significantly more favorable results than the control group in regard to clinical pregnancy rate (42.0% and 12.5%, $p < 0.001$), ongoing pregnancy rate (33.6% and 12.5%, $p < 0.001$), and delivery rate (33.6% and 9.4%, $p < 0.001$) after the first ET. In contrast, there were no significant differences in early miscarriage rates (20.0% and 25.0%, $p = 0.665$) between the two groups. Comparing the cumulative pregnancy rates within two FET cycles after the suggested test, the study group had significantly higher implantation (48.3% and 33.7%, $p = 0.025$), clinical pregnancy (64.5% and 33.3%, $p = 0.005$), ongoing pregnancy (55.1% and 32.8%, $p = 0.020$), and delivery (48.9% and 31.2%, $p = 0.020$) rates. These results show that approximately 55% of patients who underwent endometrial microbiota 16S rRNA gene sequencing achieved ongoing pregnancies over a short study period.

Table 4
Pregnancy outcomes of frozen embryo transfer

	Study Group (EMMA tested)	Control Group (not tested by EMMA)	P value
Transferred embryos (n)	1.36 ± 0.54	1.34 ± 0.48	0.844
Results after the first FET following the proposed tests			
hCG positive rate	62.6% (82/131)	14.1% (9/64)	< 0.001
Clinical pregnancy rate	42.0% (55/131)	12.5% (8/64)	< 0.001
Ongoing pregnancy rate	33.6% (44/131)	9.4% (6/64)	< 0.001
Early miscarriage rate	20.0% (11/55)	25.0% (2/8)	0.665
Delivery rate	33.6% (44/131)	9.4% (6/64)	< 0.001
Results after 2 cumulative FET cycles			
Implantation rate	48.3% (87/180)	33.7% (29/86)	0.025
Clinical pregnancy rate	64.5% (79/131)	33.3% (25/64)	0.005
Ongoing pregnant rate	55.1% (64/131)	32.8% (21/64)	0.020
Multiple pregnancy rate	9.4% (6/64)	5.0% (1/20)	0.680
Biochemical pregnancy rate	19.8% (26/131)	10.9% (7/64)	0.119
Early miscarriage rate	13.0% (15/79)	11.8% (4/25)	0.498
Stillbirth rate	0% (0/64)	4.8% (1/21)	0.247
Delivery rate	48.9% (64/131)	31.2% (20/64)	0.020

ALICE: analysis of infectious chronic endometritis, EMMA: endometrial microbiome metagenomic analysis, FET: frozen embryo transfer, hCG: human chorionic gonadotropin

Discussion

To the best of our knowledge, this prospective cohort study is the first report to demonstrate the advantageous effects on pregnancy outcomes of optimizing the endometrial microbiome using endometrial microbiota 16S rRNA gene sequencing in patients with RIF. Our results showed that only one in four of the patients tested (study group) needed antibiotics to improve their uterine microbiome. The first ET after endometrial microbiota 16S rRNA gene sequencing presented significantly improved implantation, ongoing pregnancy, and birth rates in the study group compared to the control group. That is, those with a good uterine environment (LDM) performed ETs while in good condition, and only those requiring intervention were given appropriate treatment, and ETs were performed after treatment, resulting in significantly better pregnancy outcomes in the study group. Retrospective studies conducted by

Cicinelli and collaborators have shown that patients with hysteroscopic CE findings have better pregnancy outcomes after antibiotic treatment [20] and, in several studies, the recommended antibiotic was the broad-spectrum antibiotic doxycycline [21, 22]. In the present study, 90% of the recommended antibiotics were not broad-spectrum, and the percentage of endometrial *Lactobacillus* spp. improved after 6–8 days of antibiotic treatment. Prolonged administration of broad-spectrum antibiotics is discouraged because of the increased risks of developing multidrug-resistant bacteria and decreasing the abundance of beneficial bacteria like *Lactobacillus* spp.. For this reason, patients should receive reliable tests with high reproducibility and appropriate antibiotics.

Classic diagnostic techniques for CE are histology, hysteroscopy, and microbial culture [19, 23, 24]. However, histology and hysteroscopy are highly subjective, unspecific, and rely on individual observations of pathologists or endoscopic surgeons [25]. Because of contamination, low abundances and certain unculturable endometrial bacteria, it has been difficult to diagnose CE using microbial culture. In this regard, as Moreno et al. reported, sequencing of the 16S rRNA gene, a specific marker to identify bacteria at the species level, allows for the detection of both culturable and unculturable bacteria without bias in their endometrial compositions [4]. The EMMA, the diagnostic tool we used in this study, also addresses the risk of contamination [16]. In our study, when hysteroscopic findings were compared among the patients who underwent endometrial microbiota 16S rRNA gene sequencing, the incidence of abnormal findings by hysteroscopy was similar in both the patients with LDM and patients with abnormal microbiota. The percentage of non-LDM in the current study was approximately 50%, but antibiotics were recommended for the purpose of achieving a LDM in only 23% of patients tested. By examining uterine microbiota and administering antibiotics only when required, based on NGS technology results, we were able to avoid the use of broad-spectrum antibiotics.

Moreno et al. reported that the implantation and ongoing pregnancy rates were higher when *Lactobacillus* species represented more than 90% of endometrial microbiota [4]. It has been suggested that, if bacteria other than *Lactobacillus* spp. predominate in the endometrium and bacterial infection continues, CE may set in and lead to implantation failure and premature miscarriage due to infection. This study, as well as their report [4], suggests that the interventions to promote LDM of the uterine microbiota may be the key to achieve an early pregnancy. Recently, several mechanisms have been proposed as potential causes of reproductive failure in CE pathophysiological models where an altered endometrial microbiota produces endometrial inflammation and a series of secondary effects, including abnormal cytokine and leukocyte expression, abnormal uterine contractility, impaired immune tolerance to the embryo, altered vascular permeability, defective decidualization and trophoblast invasion [26, 27].

In a healthy human vagina, the bacterial flora is composed mainly of lactic acid bacteria of the *Lactobacillus* genus. According to previous reports, the bacterial concordance between vaginal and endometrial cultures is low, ranging from 32.6–50.2%, and the bacterial flora of these two sites does not necessarily coincide [19, 28]. In cases where the intrauterine environment cannot be assessed by vaginal microbial culture alone, the detection of abnormal microbiota by NGS is the most effective and reliable tool.

Limitations

Firstly, the lack of randomization is a limitation of the present study, although it has a prospective design. This could not completely control for possible interfering factors. Therefore, randomized controlled study should be added to properly prove the validity of endometrial microbiota 16S rRNA gene sequencing. Secondly, preimplantation genetic testing for aneuploidy (PGT-A) was not performed in the study because this test is currently restricted by the Society of Obstetrics & Gynecology of Japan. The pregnancy rate is expected to improve if good-quality embryos are selected by PGT-A and transferred once the intrauterine environment is optimized by endometrial microbiota 16S rRNA gene sequencing. Further prospective studies are needed to confirm the pregnancy rates following ETs with PGT-A and with or without endometrial microbiota 16S rRNA gene sequencing.

Conclusion

Our study suggests that an appropriate uterine microbiota is essential to improve pregnancy outcomes in patients with RIF, even if the patients were not diagnosed with CE. Personalized treatment recommendations based on endometrial microbiota 16S rRNA gene sequencing can help achieve an optimal intrauterine environment and improve IVF outcomes for RIF. Moreover, by using analysis of endometrial microbiota 16S rRNA gene sequencing, broad-spectrum antibiotic treatment can be avoided and the physical and economic burdens on patients can be reduced.

Declarations

Data availability:

The results of EMMA and datasets analyzed during the current study are available from the corresponding author on reasonable request.

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Compliance with ethical standards

Conflicts of Interest: None of the authors have any conflicts of interest to declare regarding the manuscript.

Ethical approval: All procedures performed in this study were in accordance with the ethical standards of the Kamiya Ladies Clinic and with the 1964 Helsinki declaration and its later amendments or similar ethical standards.

Informed consent: Informed consent was obtained from all individual participants included in the study.

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Figures

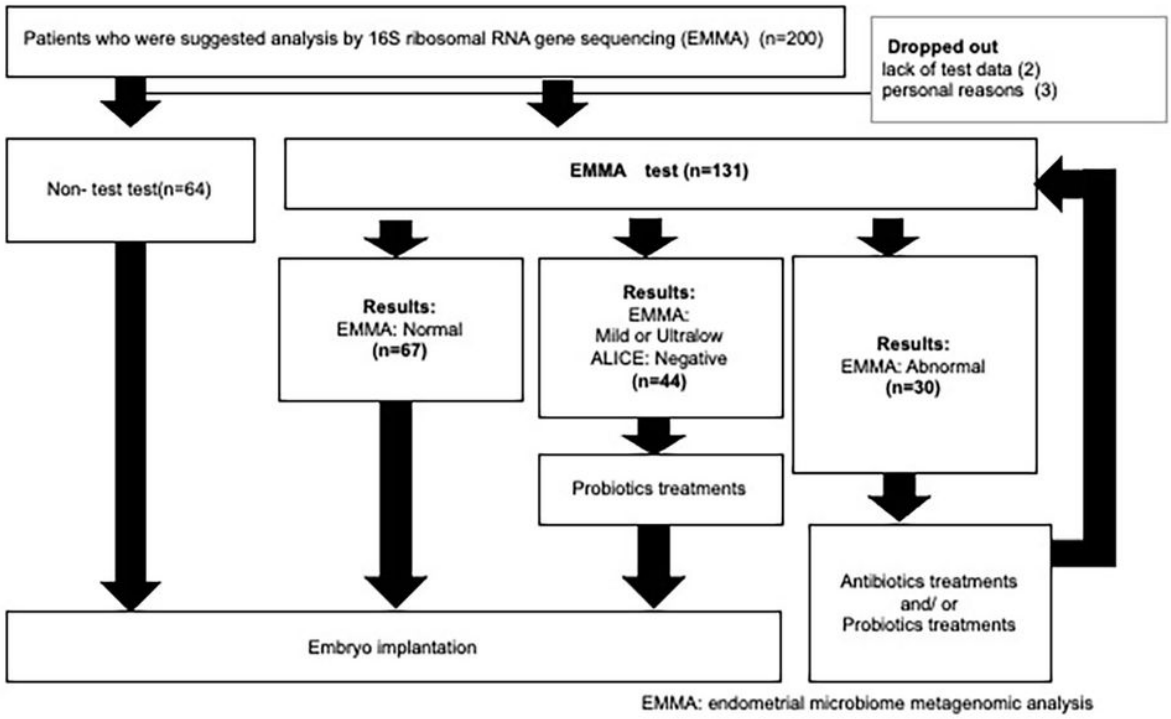

Figure 1

The flowchart of research participants.

Study flowchart of the participants. A total of 195 women with recurrent implantation failure (defined as at least three previous failed *in vitro* fertilization-embryo transfer (ET) attempts) were analyzed from July

2018 to December 2020. One hundred and thirty-one patients requested endometrial microbiota 16S rRNA gene sequencing prior to additional ETs, and the results of those tests are shown in the figure. The remaining 64 patients did not wish to have endometrial microbiota 16S rRNA gene sequencing and continued with additional ET attempts.

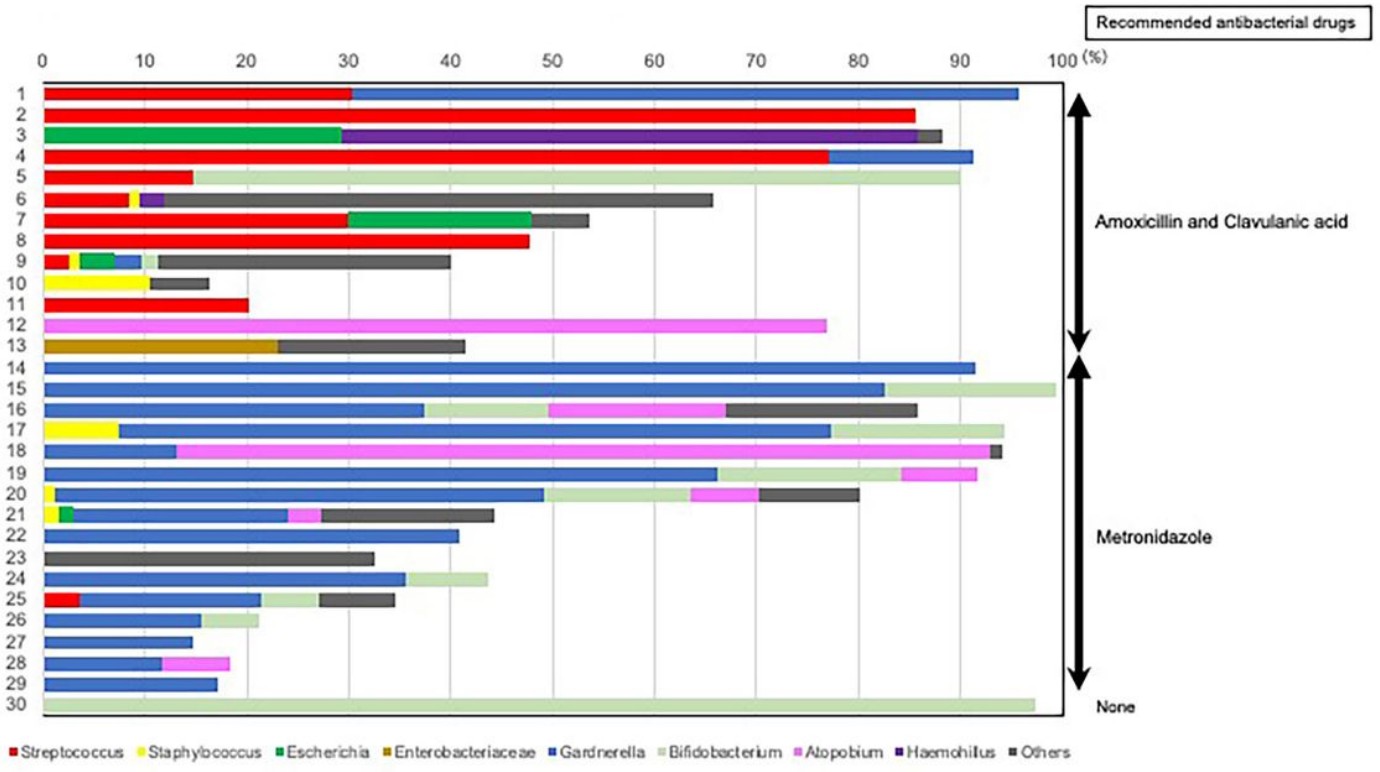

Figure 2

Intrauterine bacteria detected in patients with an initial abnormal result for the microbiota detected by 16S rRNA sequencing and their recommended treatments.

The most frequently detected bacteria other than *Lactobacillus* spp. are shown in 30 patients with abnormal microbiota. “Others” includes *Corynebacterium*, *Prevotella, Flavobacterium, Propionibacterium, Enterococcus, Microbacterium, Rothia, Klebsiella, Micrococcus, Enterobacter, Granulicatella, Actinomyces, Fusobacterium, Gemmata, Aerococcus, Sneathia, Megasphaera, Parvimonas, Finegoldia, Morganella*, and *Serratia*. Based on the results of the analysis of uterine microbiota, amoxicillin and clavulanic acid were recommended in 13 patients and metronidazole was recommended in 16 patients. The remaining patient had probiotic treatment. Please see supplementary data 1 for a detailed list of the bacteria detected in each patient.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementalData1.docx