INVESTIGATION OF BACTERIAL DIVERSITY IN RELATION TO ASSISTED REPRODUCTIVE TECHNOLOGIES USING NEXT- GENERATION SEQUENCING

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ABSTRACT

Background: Infertility is an increasingly common problem around the world. There is a microbiome dependent host disease relationship. Infertility affects between 8% and 12% of couples of childbearing ages worldwide, However, in some regions of the world, infertility rates are much higher, reaching 30% in some populations. Microbiome analysis based on subunit 16S rRNA sequencing is a fast tool that can enable the identification of all the pathogenic microorganisms associated with ART. **Material & Methods:** This study comprised Infertile Couples (n=30) with idiopathic infertility Pacific IVF Center between July 2020 and July 2022. We investigated the impact of reproductive microbiome composition on reproductive outcomes within the context of infertility treatments, and its implications on assisted reproductive technology procedure outcome. **Results:** Of the 20 samples in 5 couples undergoing IVF treatment for infertility, all samples recovered for bacterial DNA in qPCR have been positive. Majority of bacteria were Lactobacillus crispatus, Lactobacillus delbrueckii, and Lactobacillus helveticus, compared to other bacteria in vaginal microbiota. On the other hand, in follicular fluid, endometrial sample and semen sample other bacteria like Staphylococcus aureus, Streptococcus pyogenes, Clostridiales bacterium KA00274, Prevotella amnii, Gammaproteobacteria bacterium 2W06, Bacillus cereus, Bacillus subtilis, Streptococcus pneumonia and Acinetobacter baumannii outnumbered Lactobacillus spp. These assessments were done using a 16S metagenomics approach with specific primers and probes. **Conclusion:** Presence of certain bacteria, regardless of pathogenicity, can alter fertilization, implantation, and subsequent embryonic development. This can result in unsuccessful reproductive treatments and a lower live birth rate (LBR).

Keywords: Infertility, Vaginal Microbes, Semen Microbes, Metagenomics.

INTRODUCTION

The world over, infertility is an increasingly common problem. The quantum of microbiome on the human body (3.8×10^{13}) outnumbers human cells (3×10^{13}). There is host disease relationship dependent on microbiome. In fact, this microbiota can be thought of as a second human genome influencing disease occurrence, severity and outcome. The microbiome related to the

reproductive tract constitutes 9% of human microbiota. This includes the flora in the vagina, endometrium andthe follicular fluid in the woman and the seminal fluid in the man. There have been reports of higher live birth rate (90-95%) after embryo transfer in presence of predominant lactobacillus as vaginal microflora while negatively influenced by pathogenic bacteria **(1)**. Presently we have culture-independent techniques which might help to explore bacterial signature in a small quantity of sample many folds compared to culture-dependent bacterial identification. These culture-independent techniques are called next-generation sequencing (NGS) of bacterial DNA. The vaginal microbiota of women of child bearing age consist of at least five different community state types. Four of these community state types are dominated by lactic-acid producing *Lactobacillus* spp. while the fifth is commonly composed of anaerobes and strict anaerobes **(2)**.

This community predominantly comprises *Lactobacilli* in healthy women, although other genera have been identified, namely, *Prevotella*, *Gardnerella*, *Atopobium*, *Sneathia*, *Bifidobacterium*, *Megasphaera*, and *Anaerococcus* **(3-5)**.The flora in the vagina consists of approximately 30 kinds of microbes, which are mainly distributed in the mucous membrane of the lateral wall in the vagina and a few of them in the cervix also **(6)**. *Lactobacillus* is the most common organism and plays a very important role in vaginal health **(7, 8)**. *Lactobacillus* can produce a large amount of lactic acid in the anaerobic respiration of glycogen to make the vaginal environment acidic, which helps to inhibit the propagation of pathogenic bacteria. *Lactobacillus* has strong absorbability to the mucous membrane of the vagina and thus can reduce the adsorption of other pathogenic bacteria to the vaginal mucosa and prevent the long-term existence of the pathogenic bacteria in the vagina. **(9, 10)** *Lactobacillus* plays a leading role in the distribution and balance of the vaginal flora. Vaginal microbiota and especially the presence of lactobacilli are important in maintaining the vaginal health and protecting the reproductive system from harmful organisms **(11)**. Some species found in the gut, such as *E. coli* and *Streptococcus, can* also be found in the vagina, indicating the proper receptors, nutrients, and oxygen tension are present for these organisms to grow. *Lactobacilli* also inhabit the mouth, gastrointestinal tract, and female genitourinary tract **(12)**.

The most prevalent microbiota detected in colonized follicular fluids were Lactobacillus spp. (L. crispatus, L. gasseri, Actinomyces spp. and Propionibacterium spp.). Follicular fluid microbiome influences the outcome of IVF treatment, since the presence of Lactobacillus spp. is associated with better embryo quality leading to significantly higher rates of embryo transfer and pregnancy. Lactobacillus crispatus in follicular fluid on day of transfer predicts better live birth rate **(13)**.

Endometrium originally considered as sterile site too have shown presence of microbiota and *Lactobalillus spp* has been shown to improve implantation rate and lesser early abortions **(14)**

Male factors (either alone or in combination with female factors) leads to infertility in about 40%– 50% of infertile couples. Male infertility, unfortunately, is still considered ''idiopathic'' in a large proportion of cases **(15-17)**. In male infertility, it would be important to evaluate whether specific microbiological signatures correlate with the fertility status of the individual. In an initial study, **(18)** identified six microbiota clusters, none of which was specifically associated with infertility. The presence of *Anaerococcus* in semen, however, was negatively associated with its quality. A second study clustered bacterial content of semen into three groups, two of which, *Pseudomonas-* and *Prevotella*-predominant, were associated with abnormal semen parameters **(19)**.

The purpose of the present study was to determine, whether the microbiota obtained through metagenomics analysis present in the vaginal swab, follicular fluid, endometrial fluid, and semen are associated with poor treatment outcomes with assisted reproduction.

MATERIALS AND METHODS

From July 2020 to December 2022 infertile couples commencing fully stimulated IVF cycles at Pacific IVF Centre Udaipur India were enrolled this study. 30 couples having vaginal swab, follicular fluid, endometrial fluid, semen sample were included for metagenomics study. The exclusion criteria were couples who had a female partner older than 40 years, more than 3 failed embryo transfers, and abnormal uterocervical anatomy, known to have vaginal or systemic infections, pelvic inflammatory disease or infected semen. There was no exposure to antibiotics for at least one month before the collection of follicular fluid.

ETHICS STATEMENT

Ethical approval was obtained from the institutional Ethics committee of Pacific Medical University and hospital India, all patients provided informed consent for their vaginal swab, follicular fluid, endometrial fluid, semen sample to be used in this study.

PATIENT CHARACTERISTICS:

Characteristics of the study population are presented in the Table 1.

DNA EXTRACTION AND QUANTIFICATION AND 16S SEQUENCING

Bacterial DNA was extracted from samples using a commercially available DNeasypower soil Kit (Qiagen, Germany). 16S Amplicon-Seq hyper variable regions V1- V9 were amplified using primers, Forward 16S primer 5' - ATCGCCTACCGTGAC - barcode - AGAGTTTGATCMTGGCTCAG - 3'and Reverse 16S primer 5' ATCGCCTACCGTGAC - barcode - CGGTTACCTTGTTACGACTT - 3' MinION, Oxford Nanopore.

RESULTS

Out of 30 couples and 120 samples we thawed 20 samples of 5 consecutive couples with no biochemical pregnancy. All these were idiopathic infertility with no identifiable cause on standard routine workup.

	Couple 1	Couple 2	Couple 3	Couple 4	Couple 5
Age	F30/M37	F32/M34	F26/M32	F28/M38	F25/M28
Indication					
Duration of infertility	14	5 yrs	8 yrs	12 yrs	6 yrs
Stimulation protocol	Antag	Antag	Antag	Antag	Antag
Sperm parameter	22 Million	70 Million	15 Million	20 Million	22 Million
No of oocytes retrieved	10	13	16	11	14
No of embryos formed	6	8	7	6	9
Biochemical pregnancy	Negative	Negative	Negative	Negative	Negative

results

Table: 2 PRESENCE OF COMMON BACTERIA

	Semen Samples	Vaginal Swab	Follicular Fluid	Endometrial
Common Bacteria Found in Meta-genomic analysis of all samples	*Bacillus cereus* *Bacillus subtilis* *Lactobacillus iners* *Lactobacillus gasseri*	*Lactobacillus crispatus* *Lactobacillus delbrueckii* *Lactobacillus johnsonii* *Staphylococcus aureus* *Lactobacillus iners*	*Bacillus cereus* *Bacillus sp. UFRGS-B20* *Streptococcus pneumonia* *Staphylococcus aureus*	*Acinetobacter baumannii* *Lactobacillus crispatus*

Relative bacterial genera identified in vaginal swab are *Lactobacillus crispatus (45%), lactobacillus delbrueckii (24%), lactobacillus johnsonii (12%), staphylococcus aureus (9%), lactobacillus inners (6%)*

On further analysis predominant bacteria present in semen were *Bacillus cereus* (35 %), *Bacillus subtilis* (25%), *Lactobacillus iners (24%), Lactobacillus gasseri (11%).*

On follicular fluid analysis predominant bacteria present are *Bacillus cereus* (44%), staphylococcus aureus (24%), streptococcus pneumonia (16%), *Bacillus sp. UFRGS-B20* (4%).

Endometrial sample analysis revealed predominant bacteria like *Lactobacillus crispatus* (54%), Acinetobacter baumannii (28%).

AT GENUS LEVEL

Almost 267 bacteria were identified from all these 20 samples. We may further classify them in three groups first Lactobacillus spp predominantly present (87%) in vaginal sample while second non-lactobacillus pathogenic low or high virulence bacteria were predominantly present in follicular fluid, endometrial sample and semen. Third group presence can be nonpathogenic commensal, or contaminant as per literature.

DISCUSSION

This study was undertaken to look for additional etiology for failed pregnancy after ruling out all possible genetic, hormonal, overt infection and any other organic cause of infertility. These all subjects were apparently physically and mentally fit to conceive. We wanted to explore presence of normal healthy and low grade or nonvirulent pathogenic bacteria in reproductive tract especially in supposedly relatively sterile sites like follicular fluid and endometrium in absence of any evidence of frank sepsis **(20).**

Foetal development right from fertilization onward is not sterile process. We found plethora of bacteria, normal and abnormal flora in our population of five patients. This study was too small to make correlations to the fertility treatment outcomes. All were patients were failed pregnancy after IVF treatment despite no apparent identified cause ascribed to it. Culture yield is very poor in reproductive tract samples so new generation sequencing, a culture independent technique will identify and quantify bacterial DNA signature by many fold. Presence of non lactobacillus spp like bacillus, staphylococcus, streptococcus, Acinetobacter, enterococci, prevotell, sneathia, gardenella in our patient population at a certain titre individually or together exceeding a certain threshold may obviate the positive influence of lactobacillus spp on pregnancy in otherwise normal internal milieu, thereby diminishing live baby rate by various mechanism. **(21)**

Biofilm properties like enterococci, bacillus spp., gardenella vaginalis, Stenotrophomonas maltophilia, prevotella amnis, and sneathia present in our samples help them to lie dormant in reproductive tract,escaping antibiotics like metronidazole and trigerring biofilm dispersion on specific trigger like stress and produce inflammation in local milieu. A bacterial biofilm is a structured community of bacteria that is adherent to an inert surface or biological tissue. The biofilm is enclosed in a mucous substance: a self-produced matrix of extracellular polymeric substances (EPS). This inflammation may affects gametogenesis, fertilization and implantation, further embryo development.

Conventionally follicular fluid, endometrial fluid and , semen samples are considered relatively sterile in conventional culture technique but metagenomics analysis have revealed presence of diverse bacterial population. These may contribute to infertility, and failed fertility treatments.

The vaginal microbiota of reproductive-aged women is largely made up of at least five different community state types (22). Four of these community state types (CSTs) are dominated by lactic-acid producing Lactobacillus spp. while the fifth is commonly composed of anaerobes and strict anaerobes and is sometimes associated with vaginal symptoms. CST-IV, does not contain a significant number of Lactobacillus but is composed of a polymicrobial mixture of strict and facultative anaerobes including species of the genera Gardnerella, Atopobium, Mobiluncus, Prevotella and other taxa in the order Clostridiales (23-26).More than 90% of vaginal microbiota consist of Lactobacillus spp and even higher up in endometrium and follicular fluid it makes predominant microbiota in people with higher live birth rate. When this balance is disturbed it may result in infertility as shown in this study.

Semen samples and Follicular fluid showed presence of bacillus spp in abundance in our patient pool. Interestingly microorganisms such as Bacillus subtilis, Pseudomonas aeruginosa, and Escherichia coli isolated from cervices of infertile human females inhibited motility and agglutinated human sperm by virtue of their elastase positive activity (27).

This study found that human follicular fluid contains microor- ganisms. The presence of some microorganisms' bacillus spp, Propionibacterium spp., Streptococcus spp. Actinomyces spp., Staphylococcus spp. and Bifidobacterium spp correlated with adverse IVF outcomes. Our data demonstrates that not only is follicular fluid not sterile but that bacterial species in follicular fluid can have both positive and negative effects on IVF outcomes (13).

The "normal" flora of the reproductive tract includes a variety of Lactobacillus species, which promote a healthy, supportive environment for the embryo in the pre- and periconceptual period. Not only by their presence but also by production of lactic acid hydrogen peroxide (H_2O_2) ,bacteriocins, anti- biotic toxic hydroxyl radicals, and probiotics, do lactobacilli promote a supportive environment for implantation (28,29,30) . In our study lactobacillus was predominant in vaginal microbiota but other bacterial species dominated the follicular fluid, endometrium and semen samples thereby signifying their role in infertility through direct and indirect mechanisms.

In recent years, much of the focus regarding intrauterine inflammation has been on the cytokine network in presence of bacterial infection. This signaling system is thought to play an important role from conception until implantation (31). Expression of cytokine receptors by the embryo allows cytokines and growth factors secreted from the maternal oviduct and uterine epithelial cells to influence proper development of the embryo and adaptation to its microenviroment (31). Growth factors such as granulocyte-macrophage colony-stimulating factor (GM-CSF), colony-stimulating factor (CSF-1), leukemia inhibitory factor (LIF), heparin-binding EGF-like growth factor (HB- EGF), insulin-like growth factor (IGF-1, IGF-2) and cytokines IL-4, IL-10, IL-11 are crucial for normal blastocyst development, whereas other growth factors such as tumor necrosis factor alpha (TNF-α), interferon gamma (IFN-γ), and cytokine IL-2 inhibit implantation (32).

Staphylococcus species is most of which are commensal of skin without causing infections, The infection of bacteria such as *Staphylococcus* sp. has been detected at the male reproductive tract. Staphylococcal infection in male reproductive organs and accessory glands may exert detrimental effect on sperm activity **(33).** *S. aureus* infection significantly interferes with semen quality and activity. It deteriorates the volume of semen and the concentration of sperm as well as the motility, morphology, and vitality of sperm. Therefore, a causative relationship may exist between staphylococcal infection and male infertility. A previous study reported a 20.6% infection of *S. aureus* in the semen samples from males with fertility problems. More importantly, *S. aureus* infection was found to be closely related to poor semen quality and reduced sperm motility **(34).** S. pneumoniae is a major respiratory pathogen. However, intestinal pneumococci may colonize the perineum and introitus vaginae and probably give rise to infection of Bartholin's glands and - via colonization of the vagina and cervix infection **(35).**

 S. pyogenes isolated from invasive infections in women after delivery. Furthermore, this study sought to verify whether S. pyogenes strains show special phenotypic and genotypic (sla, spy1325) characteristics that may play a decisive role in adherence to the genital tract epithelium. *Prevotella amnii*, prevotella buccalis, *Sneathia*,amnii ,sneathia sanguinegens ,significantly associated with genital inflammation and bacterial vaginosis and decreased frequencies of *Lactobacillus reuteri*, *Lactobacillus crispatus*, *Lactobacillus jensenii*, and *Lactobacillus iners*. Aerobic vaginitis (AV) is mainly differentiated from bacterial vaginosis by the presence of an inflammatory response predominately associated with aerobes, such as group Streptococcus, Staphylococcus aureus ,Escherichia coli, and Enterococcus faecium**.(36,37)** The inflammatory response manifests symptomatically as itching or burning, molecularly as increased IL-6and IL-1β, and cellularly as the presence of leukocytes in a microscopic wet mount **(38).** Routine standard work up does not warrant use of NGS to look for bacterial DNA, which may play

role in so called idiopathic infertility. These bacterial florae though relatively innocuous but at increasingly crossing certain threshold on one hand and diminishing population of lactobacillus on other hand may lead to infertility. We need larger study to derive more precise information on this and possible use of probiotics or antibiotics in infertility management armamentarium.

CONCLUSIONS

This is the first NGS based study of whole reproductive microbiota showing the presence of diverse population of bacteria in our population and possible impact on fertility potential and outcome of ART treatment. Certain bacteria may affect fertility outcome more than others, making it avenue for further research.

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REFERENCES

1. Moreno I, Simon C. Deciphering the effect of reproductive tract microbiota on human reproduction. Reprod Med Biol. 2019 Jan;18(1):40-50. doi: 10.1002/rmb2.12249, PMID 30655720.

2. Smith SB, Ravel J. The vaginal microbiota, host defence and reproductive physiology. J Physiol. 2017 Jan 15;595(2):451-63. doi: 10.1113/JP271694. PMID 27373840, PMCID PMC5233653.

3. Human Microbiome Project. Consortium. Structure, function and diversity of the healthy human microbiome. Nature. 2012;486(7402):207-14.

4. González A, Vazquez-Baeza Y. Knight R. SnapShot: the human microbiome. Cell. 2014;158(3):690-690.e1.

5. Sirota I, Zarek SM, Segars JH. Potential influence of the microbiome on infertility and assisted reproductive technology. Semin Reprod Med. 2014;32(1):35-42. doi: 10.1055/s-0033-1361821, PMID 24390919.

6. Song JH, Zheng JJ. Identification and analysis of vaginal lactobacillus in patients with bacterial vaginosis and healthy women in nationality of Pastoral area. Chin J Obstet Gynecol. 2011;46(1):41-4.

7. Xiao BB, Liao QP. Analysis on diversity of vaginal microbiota in healthy Chinese women by using DNA fingerprinting. Beijing Da Xue Xue Bao Yi Xue Ban. 2012;44(2):281-7. PMID 22517004.

8. Zhang D. A study of vaginal flora. J Pract Obstet Gynecol. 2010;26(2):89-90.

9. Xiao BB, Zhang D, Liao QP, Liu ZH. Microecological evaluation of vaginal flora in gestation period. Chin J Clin Obstet Gynecol. 2007;8(6):412-49.

10. Xiao CY, Zhang HW. Analysis on the composition of 2066 cases with vagina microecological imbalance. Int J Obstet Gynecol. 2012;39(2):191-3.

11. Van de Wijgert JH, Borgdorff H, Verhelst R, Crucitti T, Francis S, Verstraelen H et al. The vaginal microbiota: what have we learned after a decade ofmolecularcharacterization?. PLoS One. 2014;9(8):e105998. doi: 10.1371/journal.pone.0105998, PMID 25148517.

12. Borriello SP, Hammes WP, Holzapfel W, Marteau P, Schrezenmeir J, Vaara M, et al. Safety of probiotics that contain lactobacilli or bifidobacteria. Clin Infect Dis. 2003;36(6):775-80. doi: 10.1086/368080, PMID 12627362.

13. Pelzer ES, Allan JA, Waterhouse MA, Ross T, Beagley KW, Knox CL. Microorganisms within human follicular fluid: effects on IVF. PLOS ONE. 2013;8(3):e59062. doi: 10.1371/journal.pone.0059062, PMID 23554970.

14. Moreno I, Codoñer FM, Vilella F, Valbuena D, Martinez-Blanch JF, Jimenez-Almazán J et al. Evidence that the endometrial microbiota has an effect on implantation success or failure. Am J Obstet Gynecol. 2016;215(6):684-703. doi: 10.1016/j.ajog.2016.09.075, PMID 27717732.

15. Kamischke A, Nieschlag E. Analysis of medical treatment of male infertility. Hum Reprod. 1999;14;Suppl 1:1-23. doi: 10.1093/humrep/14.suppl_1.1, PMID 10573021.

16. Carr BR. Optimal diagnosis and medical treatment of male infertility. Semin Reprod Med. 2013;31:231-2.

17. Rittenberg V, El-Toukhy T. Medical treatment of male infertility. Hum Fertil (Camb). 2010;13(4):208-16. doi: 10.3109/14647273.2010.534833, PMID 21117930.

18. Hou D, Zhou X, Zhong X, Settles ML, Herring J, Wang L, et al. Microbiota of the seminal fluid from healthy and infertile men. Fertil Steril. 2013;100(5):1261-9. doi: 10.1016/j.fertnstert.2013.07.1991, PMID 23993888.

19. Weng SL, Chiu CM, Lin FM, Huang WC, Liang C, Yang T, et al. Bacterial communities in semen from men of infertile couples: metagenomic sequencing reveals relationships of seminal microbiota to semen quality. PLOS ONE. 2014;9(10):e110152. doi: 10.1371/journal.pone.0110152, PMID 25340531.

20. Einenkel R, Zygmunt M, Muzzio DO. Microorganisms in the healthy upper reproductive tract: from denial to beneficial assignments for reproductive biology. Reprod Biol. 2019 Apr 22;19(2):113-8. doi: 10.1016/j.repbio.2019.04.001, PMID 31023521.

21. Nuriel-Ohayon M, Neuman H, Koren O. Microbial changes during pregnancy, birth, and infancy. Front Microbiol. 2016 Jul 14;7:1031. doi: 10.3389/fmicb.2016.01031, PMID 27471494, PMCID PMC4943946.

22. Zhou X, Brown CJ, Abdo Z, Davis CC, Hansmann MA, Joyce P et al. Differences in the composition of vaginal microbial communities found in healthy Caucasian and black women. ISME J. 2007;1(2):121-33. doi: 10.1038/ismej.2007.12, PMID 18043622.

23. Fredricks DN, Fiedler TL, Marrazzo JM. Molecular identification of bacteria associated with bacterial vaginosis. N Engl J Med. 2005;353(18):1899-911. doi: 10.1056/NEJMoa043802, PMID 16267321.

24. Campos ACC, Freitas-Junior R, Ribeiro LFJ, Paulinelli RR, Reis C. Prevalence of vulvovaginitis and bacterial vaginosis in patients with koilocytosis. Sao Paulo Med J. 2008;126(6):333-6. doi: 10.1590/s1516-31802008000600008, PMID 19274321.

25. Ravel J, Gajer P, Abdo Z, Schneider GM, Koenig SSK, McCulle SL et al. Vaginal microbiome of reproductive-age women. Proc Natl Acad Sci U S A. 2011;108;Suppl 1:4680-7. doi: 10.1073/pnas.1002611107, PMID 20534435.

26. Gajer P, Brotman RM, Bai G, Sakamoto J, Schütte UME, Zhong X et al. Temporal dynamics of the human vaginal microbiota. Sci Transl Med. 2012;4(132):132ra52. doi: 10.1126/scitranslmed.3003605, PMID 22553250.

27. Kaur M, Tripathi KK, Bansal MR, Jain PK, Gupta KG. Bacteriology of the cervix in cases of infertility: effect on human and animal spermatozoa and role of elastase. Am J Reprod Immunol Microbiol. 1988 May;17(1):14-7. doi: 10.1111/j.1600-0897.1988.tb00195.x, PMID 3189646.

28. Hawes SE, Hillier SL, Benedetti J, Stevens CE, Koutsky LA, Wolner-Hanssen P, et al. Hydrogen peroxide-producing lactobacilli and acquisition of vaginal infections. J Infect Dis. 1996;174(5):1058-63. doi: 10.1093/infdis/174.5.1058, PMID 8896509.

29. Aroutcheva AA, Simoes JA, Behbakht K, Faro S. Gardnerella vaginalis isolated from patients with bacterial vaginosis and from patients with healthy vaginal ecosystems. Clin Infect Dis. 2001;33(7):1022-7. doi: 10.1086/323030, PMID 11528575.

30. Ng SC, Hart AL, Kamm MA, Stagg AJ, Knight SC. Mechanisms of action of probiotics: recent advances. Inflam Bowel Dis. 2009;15(2):300-10. doi: 10.1002/ibd.20602, PMID 18626975.

31. Hillier SL, Krohn MA, Rabe LK, Klebanoff SJ, Eschenbach DA. The normal vaginal flora, H2O2-producing lactobacilli, and bacterial vaginosis in pregnant women. Clin Infect Dis. 1993;16;Suppl 4:S273-81. doi: 10.1093/clinids/16.supplement_4.s273, PMID 8324131.

32. obertson, SA CPY, Glynn DJ, Thompson JG. Peri-conceptual cytokines—setting the trajectory for embryo implantation, pregnancy and beyond. Contraceptive vaccines: success, status, and future perspective. Am J Reprod Immunol. 2011;66(1);Suppl 1:2-4. doi: 10.1111/j.1600-0897.2011.00999.x, PMID 21645164.

33. Shi L, Wang H, Lu Z. Staphylococcal infection and infertility. doi: 10.5772/62663.

34. Onemu SO, Ibeh IN. Studies on the significance of positive bacterial semen cultures in male fertility in Nigeria. Int J Fertil Womens Med. 2001;46(4):210-4. PMID 11563831.

35. Westh H, Skibsted L, Korner B. Streptococcus pneumoniae Infections of the Female Ge nital RACT and in the newborn Child REVIEWS OF INFECTIOUS DISEASES *. Vol. 12(3) * MAY-JUNE 1990.

36. Donders GGG, Vereecken A, Bosmans E, Dekeersmaecker A, Salembier G, Spitz B. Definition of a type ofabnormal vaginal flora that is distinct from bacterialvaginosis: aerobic vaginitis. BJOG. 2002;109(1):34-43. doi: 10.1111/j.1471-0528.2002.00432.x, PMID 11845812.

37. Donders GGG. Definition and classification of abnormal vaginal flora. Best Pract Res Clin Obstet Gynaecol. 2007;21(3):355-73. doi: 10.1016/j.bpobgyn.2007.01.002, PMID 17434799.

38. Han C, Wu W, Fan A, Wang Y, Zhang H, Chu Z et al. Diagnostic and therapeutic advancements foraerobicvaginitis. Arch Gynecol Obstet. 2014;291:251-7.