Scholars LITERATURE

La Prensa Medica Argentina

Research Article

DOI: https://doi.org/10.47275/0032-745X-S2-004
S2-004

Detection of Human Follicular Fluid Microorganisms at the Time of Oocyte Retrieval and its Impact on *in-vitro* Fertilization Outcomes

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Abstract

The follicular fluid (FF) one of the important sources of microorganisms that may affect *in vitro* fertilization (IVF) outcomes. The present study investigated the microorganisms within 50 FF of women undergoing IVF procedure. All samples were identified according to the morphological features, biochemical, and molecular methods. Twelve microbial species belong to six genera were detected within 42% of FF samples and 58% did not show any microbial growth. The predominant species were *Streptococcus* spp. (34%), followed by *Corynebacterium* spp. (24%). Other microbes include *Bacillus* spp., *Enterococcus* spp., *Staphylococcus* spp., and Yeast. The FF microorganisms were close to statistically significant with IVF outcomes. Follicular fluid was not always sterile but contained a varied range of microorganisms that may effects the IVF outcomes and a larger sample of patients need to be studied to further examine our hypothesis.

Keywords: Follicular Fluid; *In-vitro* Fertilization; Oocyte Retrieval; Infertility

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Citation: Noor SO, Alosaimi EH, Najjar AA, et al. (2020) Detection of Human Follicular Fluid Microorganisms at the Time of Oocyte Retrieval and its Impact on *in-vitro* Fertilization Outcomes. Prensa Med Argent, S2:004. DOI: https://doi.org/10.47275/0032-745X-S2-004.

Received: June 20, 2020; **Accepted:** July 29, 2020; **Published:** August 03, 2020

Introduction

Infertility is a public health concern worldwide and considered a significant clinical problem today [1]. It is an inability of women to become pregnant for 12 months within unprotected intercourse according to WHO definition [2]. It can be either primary infertility, that is a delay for a couple to conceive after one year or more with no previous pregnancies or secondary infertility, that is a delay for a couple who have previously conceived children [3]. The prevalence of infertility estimated by a systematic analysis of 277 health surveys from 1990 to 2010 in 190 countries. They found that 48.5 million couples worldwide had this problem. This problem was the most prevalent in some regions such as South Asia, North Africa, the Middle East and Central Asia [4]. In 2012, the data of 457 infertility patients were analyzed in Alkhobar, Saudi Arabia and they reported that the prevalence of infertility was 18.93%, which was higher than the prevalence in developed countries 3.5% to 16.7% within one year [5]. There are many causes that can contribute to female infertility such as polycystic ovary syndrome, endometrioses, endocrine disorders, genetic factors, tubal factors and pelvic inflammatory disease [3]. In addition, many lifestyle factors such as age, nutrition, obesity, smoking, environmental pollutants, and others factors can influence overall health and contributing factor to infertility [6]. Infertility is now recognized as a big issue and a cause for concern in Saudi society. Therefore, many people choose to have an assisted reproductive technology (ART) to help women to get a child. One of these techniques is *in vitro* fertilization (IVF) procedure [7]. Prior to IVF cycles, couples are screened for the presence of sexually transmitted infections but the routines of microbiological tests are not performed for IVF procedure [8]. One of the most challenging situations when the embryologists discover that embryo contaminated after spending a lot of money and time. A second potential negative impact when the microorganisms transmitted from the contaminated embryo culture media into the female reproductive tissue that can lead to adverse pregnancy outcomes. Many cases of embryo contamination are due to microorganisms from a follicular fluid (FF) [9]. It is a liquid that surrounds the ovum and plays an important function in the communication between cells in the antral follicle while carrying nutrients to the oocyte. Also, it is a key part of the success of natural fertilization present in every stage of the conception procedure [10,11]. The FF can be colonized or contaminated by microorganisms [12].

Previous studies correlated between the FF microorganisms and decrease or increase of IVF outcomes [8,12-15]. To the best of our knowledge, in Saudi Arabia, no similar studies have been conducted on this topic yet and due to the high number of people with infertility who are going through the IVF cycle, this kind of study is important and will provide an important piece of information that will help doctors to improve the IVF procedure.

The aims of this study to identify the microorganisms in the FF of women undergoing IVF procedure at the time of oocyte retrieval and correlated these microorganisms with IVF outcomes.

We hypothesized that follicular fluid microorganisms may have an effect on the success rate of the *in vitro* fertilization procedure.

Methods

Specimen Collection

From October 2018 to April 2019, a cross-sectional study conducted for 50 FF specimens were collected from women undergoing IVF procedure at the time of oocyte retrieval. Ethics approval was obtained from the Biomedical Ethics Unit at King Abdulaziz University Hospital, Jeddah city, Saudi Arabia. All partners gave their written informed consent to participate in the study and permission for researchers to access their information, test results, and reproductive history.

The inclusion criteria are fertile women who have an infertile male partner (male factor), and women who have various causes of infertility include (ovulatory disorders, endometriosis, tubal factor, and unexplained infertility if the female and male underwent screening and not detected any abnormal results). The exclusion criteria are women who used antimicrobials (oral or topical) within the previous one month prior to sampling and women with chronic diseases such as cervical cancer and the human immunodeficiency virus (HIV).

The hospital informed us at the time of the IVF procedure for each patient. The vaginal wall was washed using sterile saline to remove excess mucous and cellular debris. The oocyte retrieval was conducted using a vaginal ultrasound probe covered with a disposable sheath attached to a sterile needle. The FF was aspirated directly into the sterile tube in the operation room. Then, the specimen was aseptically transferred into a sterile culture dish to remove an oocyte and the oocyte is implanted on embryo culture media containing gentamicin antibiotics. After that, the IVF scientist transferred the remaining of FF to a sterile collection tube and all specimens were sealed, placed in ice cooler until delivered to the laboratory and processed within two hours of collection [16].

Microbial Culture

Undiluted 50 FF samples were inoculated directly by 1 µL loops using a streak technique in triplicate on top of the (Nutrient agar; Oxoid, Adelaide, SA, Australia) for the bacterial growth. For the presence of fungi 100 µl of sample was spread using L-loops on top of Sabouraud Dextrose agarmedium (SDA) (Himedia, Mumbai, India) with 10% Lactic Acid to inhibits the bacterial growth [17]. All plates were incubated aerobically in 5% carbon dioxide incubator (Sanyo Com., Osaka, Japan) at 37℃ for 24-48 hrs. and SDA media were incubated aerobically at 37℃ for 2-5 days. All procedures of microbial isolates were performed under sterilized conditions inside the Biosafety cabinet (Daihanlabtech, Kyungki-Do, South Korea).

Morphological Structures Analysis

All microbial isolates were phenotypically identified from pure cultures. Colony characteristics including growth rate, colony color, texture, size, and shape were considered as important diagnostic criteria for identification. The microscopic appearance was observed in wet mounts prepared on microscopic slides and stained with Gram stain for bacteria and lactophenol cotton blue (LPCB) stain for fungi using the standard protocol [18,19].

Microbial Analysis

The total number of colonies was manually counted and the results were calculated as the mean of colony-forming units per milliliter (CFU/ml). The limit of detection was 10^3 CFU/ml as described by Pelzer [20].

Statistical Analyses

Data collected throughout history, basic clinical examination, and laboratory investigations, and IVF outcomes were analyzed using SPSS25.0to determine the significant difference among the data (Armonk, NY: IBM Corp). The data were tested using Kolmogorov-Smirnov test, Shapiro–Wilk tests, Chi-square test (χ^2), T-test, and ANOVA test. A p-value of ≤ 0.05 was considered statistically significant.

Results

Patient Demographics

Clinical specimens were collected from 50 women for microbiological analyses.

The mean (+SD) age of all women was 35.8 ± 5.47 years. The mean age of women with positive presence of microbes was (37.8 ± 4.7) compared with (34.4 ± 5.6) for negative presence of microbes (P=0.029). In addition, the mean weight with positive presence of microbes was (69.7±13.1) and (71.4±16.7) for negative presence of microbes (P=0.696). The mean number of oocyte retrieved with positive presence of microbes was (9.7±6.6) and (11.5±6.6) for negative presence of microbes (P=0.344) as shown in table 1. Furthermore, there was no statistically significant difference in the TSH hormone levels with the presence of microbes (P>0.05).

Overall cases, the ovulatory disorders consist most of infertility causes 44%, followed by male factor 32%, unexplained infertility, tubal factor, and endometriosis consist 12, 10 and 2% respectively as shown in figure 1. The positive IVF outcomes were 28% while 72% for negative IVF outcomes. The rates of microbial isolates from FF in women with ovulatory disorder, unexplained fertility, male factor, and tubal factor were 13/22 (59%), 3/6 (50%), 7/16 (44%) and 0/5 (0%) respectively with no statistically significant difference (P>0.05). The presence of microbes in the FF and endometriosis were found to be associated (P=0.002). However, the FF microorganisms was not associated with women fertility (P=0.291) as shown in figure 2. There was no statistically significant difference in the type of infertility and women participated in previous IVF procedure with the presence of microbes (P>0.05) and the IVF outcomes were close to statistically significant with the presence of microbes (P=0.062) as shown in table 2 and figure 3.

Isolation and Identification of Microbial Isolates

Microorganisms were detected within 21 (42%) FF samples and 29 (58%) did not show microbial growth.

In the microscopic specimens, 67 bacterial isolates were Gram-

Table 1: The relation between patient demographics and the presence of microbes.

Patient demographics	Groups		P- value
	Presence of microbes	Absence of microbes	
	21/50 (42%)	29/50 (58%)	
Age	37.8±4.7	34.4±5.6	0.029*
Weight	69.7±13.1	71.4±16.7	0.696
Number of oocytes	9.7±6.6	11.5±6.6	0.344

*P-value < 0.05 is significant.

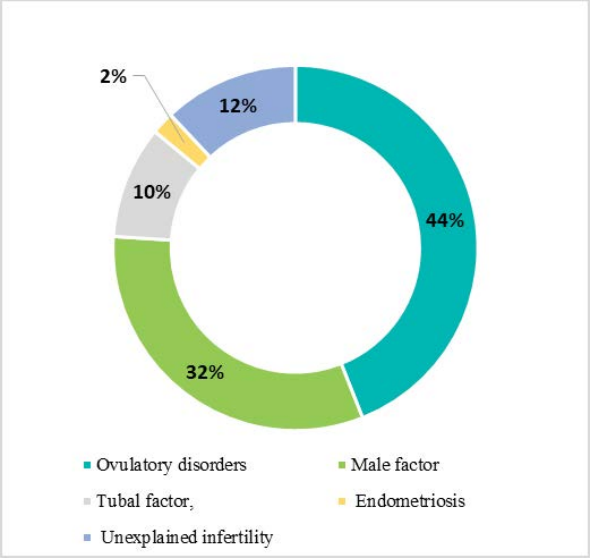

Figure 1: The percentage of the etiology of infertility, graph showing that the predominant case was ovulatory disorders with 44%, followed by male factor 32%, unexplained infertility, tubal factor, and endometriosis 12%, 10% and 2% respectively.

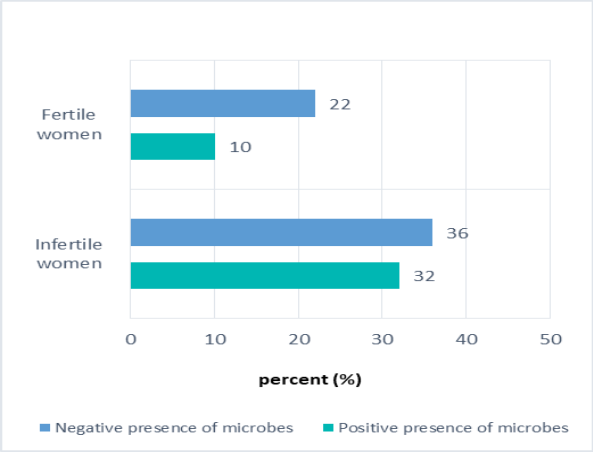

Figure 2: The relation between FF microorganisms with women fertility.

Table 2: A comparison between IVF outcomes, type of infertility and women participated in previous IVF procedure with the presence of microbes.

Microbe Detection		Presence	Absence	P- value
		21/50 (42%)	29/50 (58%)	
IVF Outcome	Non-pregnant	18	18	0.062
	Pregnant	3	11	
Type of infertility	Primary	10	13	0.536
	Secondary	11	16	
Participation in previous IVF procedure	Yes	9	12	0.573
	No	12	17	

positive reaction and five isolates showed Yeast cells with (LPCB) stain. The morphological features were identified up to the genus level according to the morphological reference published [19,21].

The effect of microbial load was also considered in this study. The quantitative method assessed the prevalence of microorganisms within the FF samples. The microbial species were isolated at concentrations ranging from 10^3 CFU/ml to $>10^5$ CFU/ml in FF samples. There appeared to be no correlations between the microbial load in FF and the etiology of infertility.

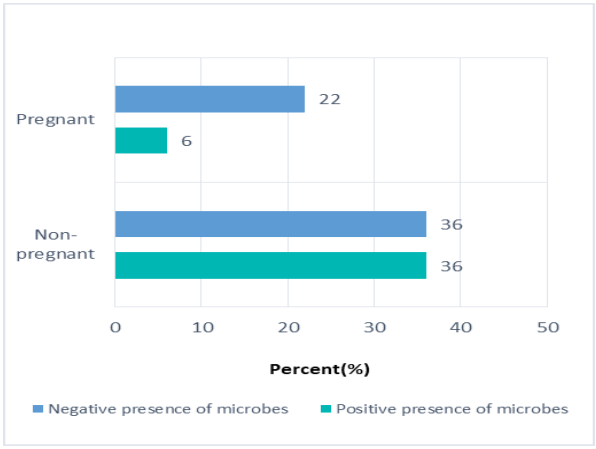

Figure 3: The relation between FF microorganisms with IVF outcomes.

All microbial isolates belong to six genera which were identified as *Bacillus* spp. (10%), *Corynebacterium* spp. (24%),Yeast (16%), *Enterococcus* spp. (3%), *Streptococcus* spp.(34%), and *Staphylococcus* spp.(10%).

Discussion

Infertility is a public health concern worldwide and considered a significant clinical problem today, therefore, many people choose to have IVF procedure to help women to get a child [1]. The presence of FF microorganisms was associated with negative or positive IVF outcomes [8,12-15].

This study investigated the presence of microorganisms within fifty FF specimens collected from women undergoing IVF procedure with different causes of infertility and the effect of microbial species isolated on IVF outcomes. The mean age of women was statistically significant higher with positive presence of microbes. Another study were consistent with our finding, they have reported that the age of women associated with FF colonization[13]. In addition, there were no statistically significant difference in the mean of weight, TSH hormone levels, number of oocyte retrieved, type of infertility and women who participated in previous IVF procedure with the presence of microbes. Lan, Huang [22] demonstrated significantly increase in the number of oocytes retrieved from the right ovary compared to those from the left ovary. Hamad TA, et al. (2013) [13] reported that secondary infertility was associated with FF colonization and there was no correlation between women who participated in previous IVF procedure and FF colonization.

In the present study, sex genera were identified from 21 FF specimens. The microbial isolates were present in 42% of FF while there were 58% of FF did not show any microbial growth. The ovulatory disorders consist most of infertility causes 44%, followed by male factor 32%, unexplained infertility, tubal factor, and endometriosis consist 12, 10 and 2% respectively. We concluded that the presence of microorganisms *Bacillus* spp., *Corynebacterium* spp., *Enterococcus* spp., *Streptococcus* spp., *Staphylococcus* spp. and Yeast were close to statistically significant with adverse IVF outcomes. Previous studies have investigated the microorganisms within FF samples and *Lactobacillus* spp., *Staphylococcus* spp., *Streptococcus* spp., *Escherichia coli, E. faecalis* have been recovered with no significant adverse impacts on IVF outcomes [13,15]. Other studies reported that the presence of FF microorganisms was correlated with negative IVF outcomes [8,12,14]. Furthermore, another study concluded that the FF microorganisms

may result in poor quality oocytes that leading to reduced IVF outcomes [23]. In one retrospective study, the IVF cultures for 5/729 patients contaminated by yeast species and the embryo quality was not compromised and all five patients conceived [9].

The FF may colonize by hematogenous invasion of different microorganisms spread via the oral mucosa and respiratory tract [12]. It is may also be contaminated by vaginal microorganisms when the clinical embryologist starts to collect the sample by a large needle passed through the vagina into the ovary. In our study, *Staphylococcus* spp., *Enterococcus* spp., *Corynebacterium* spp., *Streptococcus* spp. and Yeast isolated from FF samples appear to be opportunistic pathogens in the vagina according to the work published by others [24-26]. *S. agalactiae*is one of the pathogenic organisms that can be an association with the complications of pregnancy and it is a main infectious reason for neonatal disease and death [27,28]. Other studies isolated *S. aureus,* and *B. subtilis* from cervical swabs samples among women with bacterial vaginosis [29]. We have also shown that FF microorganisms were not associated with endometriosis. This is consistent with the findings of others who have reported that there is a correlation between FF colonization by microorganisms and endometriosis [12].

The microbial isolated from FF samples recovered in this study at concentrations ranging from 10^3 CFU/ml to $>10^5$ CFU/ml and there was no correlations between the microbial load and the etiology of infertility. Another study showed that most microbial species were isolated at concentrations ranging from 10^3 CFU/mL to $>10^6$ CFU/mL in both colonized and contaminated FF and the microbial load was not associated with the etiology of infertility or the IVF outcomes [20].

Conclusion

This study investigated the microorganisms within FF of women undergoing IVF procedure. The FF was not always sterile but contained a varied range of microorganisms that may effects the IVF outcomes and a larger sample of patients need to be studied to further examine the hypothesis that FF microorganisms may adversely affect IVF outcomes. Furthermore, the Identification of microbes within the FF in women with repeated failed IVF cycles may provide an opportunity to initiate antimicrobial treatment prior to the next conception.

Acknowledgment

Authors are appreciated by Dr. Mona GAlharbi, Department Head of Biological Science at King Abdulaziz University for supporting and kind gaudiness.

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