



Mycological Analysis for Seminal Fluid before *In Vitro* Fertilization Procedure

Azhar Abdullah Najjar^{1,2*}, Ameira Eid Albalawi¹, Ebtesam Hamoud Alosaimi¹, Hassan Salah Abduljabbar³, Howaida Abdulmonem Hashim³, Mohammed Abdulrahman Alem⁴, Sabah Mahmoud Hassan^{1,5}, Samah Omar Noor^{1,2}

¹ Department of Biological Sciences, Faculty of Science, King Abdulaziz University, Jeddah, Saudi Arabia,

² Microbiology Unit, King Fahd Medical Research Center, King Abdulaziz University, Jeddah, Saudi Arabia

³ Department of Obstetrics and Gynecology, Faculty of Medicine, King Abdulaziz University, Jeddah, Saudi Arabia.

⁴ Laboratory Directorate, Ministry of Health, Jeddah, Saudi Arabia.

⁵ Department of Genetics, Faculty of Agriculture, Ain Shams University, Cairo, Egypt.

ABSTRACT

Pathogenic fungi found in the seminal fluid has been shown to have a negative influence on sperm quality. **Aim:** The present study investigated the mycological analysis of semen before the IVF procedure. **Methodology:** All samples were identified according to the morphological, biochemical, molecular, and antimicrobial tests. **Results:** fungal species were detected within 28% of semen samples and 72% did not show any fungal growth. The prevalent fungal isolates were *Candida* spp., followed by *Candida guilliermondii*, *Candida parapsilosis*, and *Candida glabrata*. The use of mycological analysis to identify resistant fungal isolates is considered an effective method to reduce infertility in men by changing the contaminated sperm. **Conclusion:** Seminal fluid is not always sterile but it consists of diverse species of yeasts that are not related to the IVF outcomes and a larger sample is needed to further examine our hypothesis. Also, microbiological examination of semen in men who have undergone failed IVF cycles may provide a chance to instigate antimicrobial therapy before the next conception.

Key Words: Seminal fluid, *In vitro* fertilization, Semen, Fungi, and Infertility

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INTRODUCTION

Infertility is a disease of the reproductive system. It can be defined as the inability to achieve a clinical pregnancy after 12 months or more of regular unprotected sexual intercourse. *In vitro* fertilization (IVF) is a process of fertilization where an egg is combined with sperm outside the body, in a laboratory dish [1]. Microbial assessment of seminal fluid in the weeks preceding *in vitro* fertilization (IVF) procedure is requested to avoid low sperm quality. Contaminated semen, by microorganisms, is the most frequent diagnosis of unsuccessful IVF. The quantity of the pathogenic fungi in semen has been reported to be a serious medical threat as the low-quality sperm cannot fertilize eggs in a laboratory tube. Generally, the deficiency of seminal fluid accounts for 7% of Human infertility [2].

Although fungi isolated from this fluid account for a small proportion of the total microorganism number, they pose opportunistic behaviors that affect sperm quality, along with distortions (especially aberrant motility) loss of DNA integrity, and deficient mitochondrial function [3, 4]. The most frequent pathogenic fungi in the seminal fluid were *Candida* spp. [5]. Personal habits such as sexual promiscuity and poor hygiene, increase the fungi isolates number and change the basic characteristics of semen.

In a hospital, the laboratory protocol uses several antibiotics to treat fungal pathogenic flora existing in seminal fluid to increase sperm quality. The fungal antibiotics used are amphotericin B, fluconazole, nystatin, flucytosine, econazole, ketoconazole, and miconazole [6].

Corresponding author: Azhar Abdullah Najjar

Address: Department of Biological Sciences, Faculty of Science, King Abdulaziz University, Jeddah, Saudi Arabia.

E-mail: anajjar@kau.edu.sa

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The current study aims to isolate resistant fungi from seminal fluid and study their sensitivity to the antibiotics. It is hoped, by comparing the results obtained from client data, sperm quality will be improved along with increased rates of pregnancy using IVF at fertility clinics.

After obtaining ethical approval from the Biomedical Ethics Unit at King Abdulaziz University Hospital, we collected semen samples from sixty male subjects. The subjects were attending the *in vitro* fertilization clinics of King Abdulaziz University Hospital from October 2018 to October 2019. Proper hygiene was ensured in the collection of the samples which was done in one of the private rooms in the laboratory.

METHODS

Study population

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Specimen Collection

Semen specimens were collected by masturbation after 3–7 days of abstinence. Before the sample collection, instructions were provided to the participants on procedures to be followed to avoid sample contamination. The subject's hands were washed using soap more than two times. The penis, especially the glans and the coronal sulcus, was washed with warm soapy water and swabbed after with 75% alcohol more than two times. Without any contact with the interior of the sterile wall of the container, the semen was then ejaculated into a sterile glass receptacle. The freshly provided seminal fluid was used for routine semen clinical examination, Gram staining, microscopy, and molecular technique. Within 2 hours of collection, the remainder of the semen samples were transferred to sterile Eppendorf tubes and stored at 80°C. The exclusion criteria are men using antibiotics for less than three months before sampling and patients with chronic diseases such as prostate cancer and HIV. We will be informed about the time of the IVF procedure of each client.

Cultures

The following fungi culture media plates were used: Sabouraud dextrose agar (SDA) and yeast peptone dextrose agar (YPD). All seminal fluid samples were inoculated directly using 100 µl of the sample was spread by L-loops in triplicate on the previous media and incubated for 5 days at 25°C and 37°C. The growth was examined for size, shape, elevation, and other cultural characteristics. Lactophenolcotton blue cotton blue has stained the isolates

and pure growth was subjected to sensitivity to a range of antibiotics testing according to standard [7].

Microbial analysis

The total number of colonies was done by manually counting of colonies on plates and the results were calculated as the mean of colony-forming units per milliliter (CFU/ml). The limit of detection was 10³ CFU/ml as described by Pelzer [8].

Morphological structures analysis

All 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 and recorded as macro-morphological structures. The micro-morphological structure was observed in wet mounts prepared on microscopic slides and stained with LPCB using the standard protocol [9].

Molecular identification of microbial isolates

All samples were mixed with 70% glycerol in Eppendorf tubes and store in 80°C. Then the samples were sent to Macrogen Company, Seoul, South Korea for purification and sequencing.

DNA Sequencing

Sequence identities were characterized using BLAST GenBank general databases from the National Center for Biotechnology Information (NCBI) database (<http://www.ncbi.nlm.nih.gov>) [10]. The sequence alignment of the constructions of neighbor analysis was done by theBLAST and MEGA-X program (version 10.0.5). The result reports were received from the Macrogen molecular analysis laboratory and the sequencing data were submitted to GenBank and the obtained accession number was recorded for each isolate.

Antimicrobial susceptibility test

The susceptibility to five antifungal agents was determined using the VITEK 2 antifungal susceptibility testing system (bioMerieux, Durham, USA) (card number AST-YS08) to determine the antifungal susceptibility of yeasts compared with the Clinical and Laboratory Standards Institute (CLSI M100-S25, 2015) reference standards within 24 hours. The antifungal used included Caspofungin (CAS) (0.25-4 µg/ml), Fluconazole (FLU) (1-64µg/ml), Flucytosine (FCT) (1-64µg/ml), Micafungin (MCF) (0.06-4 µg/ml), and Voriconazole (VRC) (012-8µg/ml).The cassettes were loaded and placed into the VITEK-2 instrument, and the suspensions were diluted by the instrument. Afterward, the cards were filled, incubated, and read automatically. The complete data for each fungal isolate were expressed as minimal inhibitory concentrations (MICs) in micrograms per milliliter (µg/ml).

Statistical Analyses

Data collected were analyzed using SPSS to determine the significant difference among the data (Armonk, NY: IBM Corp). A P-value of < 0.05 was considered statistically significant.

RESULTS

Microbial isolation

The result of microbial isolation of semen specimen was collected from the client would go for the IVF procedure. Overall, 17 (28%) of the 60 semen specimens were positive for fungal growth, while no fungal colonies were observed on 43 (72%) specimens. All fungal colonies were counting with counting forming unit (CFU) table (1). The fungal colonies grew on the two different media with two different temperatures 25°C and 37°C but with the different growth rates. The total CFU/ml of all fungal colonies isolated from different media at 25°C were 872.1×10^3 CFU/ml whereas 727.2×10^3 CFU/ml at 37°C. In the SDA medium, the total CFU/ml of fungal colonies grown at 25°C were 331.1×10^3

CFU/ml while the total CFU/ml grown at 37°C were 541×10^3 CFU/ml. Furthermore, the total CFU/ml of yeast colonies grown on YPD medium at 25°C were 64.2×10^3 CFU/ml, and 333.3×10^3 CFU/ml at 37°C.

The fungal morphological features were identified up to the genus level according to the morphological reference published [11]. The color of all the isolates is creamy to yellowish, and depending on the species, the colony has a glistening, smooth, or dry texture. Under microscopic analysis with LPCB stain, all fungal isolates showed yeast cells with blastoconidia produce singly or in small clusters as shown in Figures (1, 2, and 3) and followed by *Candida guilliermondii* (60%), *Candida parapsilosis* (23%), *Candida glabrata* (17%).

The effect of the microbial load was also considered in this study. The quantitative method estimated the diffusion of yeast within the semen samples. The yeast species were isolated at concentrations ranging from 10^2 CFU/ml to $>10^3$ CFU/ml in semen samples. There manifest to be no correlations between the microbial load in semen and the etiology of infertility



Fig. 1: The isolate of *C. guilliermondii*(a) colonies represents flat, moist, smooth, and cream to yellow on SDA. (b) Cellular morphology of yeast cells stained with LPCB and observed blastoconidia produce in clusters in size $5\mu\text{m}$ under 100x objective by light microscopy.

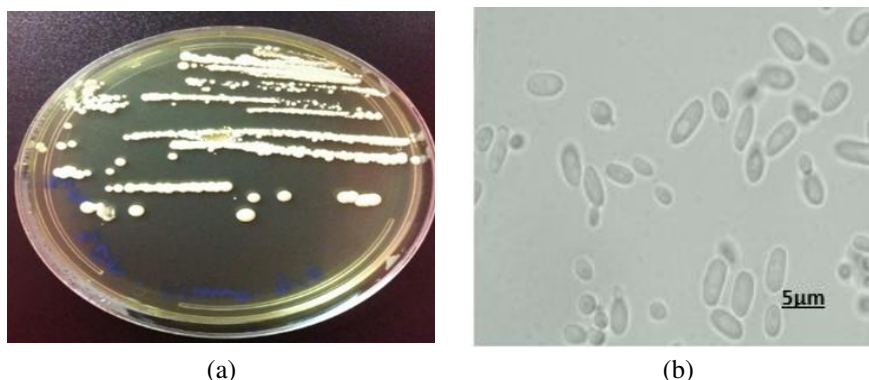


Fig. 2: The isolate of *C. parapsilosis*(a) colonies represents white, creamy, and shiny colonies on SDA (b) Cellular morphology of yeast cells stained with LPCB and observed small blastoconidia produce singly in size $5\mu\text{m}$ under 100x objective by light microscopy.

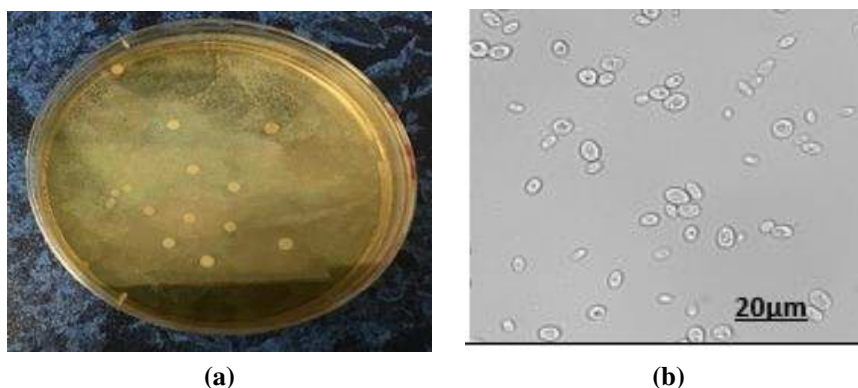


Fig. 3: The isolate of *C. glabrata* (a) colonies represents white, smooth, and glistening colonies on SDA. (b) Cellular morphology of yeast cells stained with LPCB and observed small blastoconidia produce in clusters in size 20µm under 100x objective by light microscopy.

Molecular identification

Initially inoculated a single colony of culture from YPD agar into 5 ml of YPD broth and incubated for 48 h at room temperature in a shaking incubator (SI-100, Human lab, Gyeonggi-do, South Korea). The cells were harvested from 1 ml of the culture broth in an Eppendorf tube mixed with 70% glycerol then store at -80°C. Then the samples were sent to Macrogen Company, Seoul, South Korea for DNA purification and sequencing.

The PCR genetic sequences alignment of yeast isolates was determined as known species based on the 99% similarity of their sequences with that of the known species already published in NCBI databases. All of the yeast species isolates could be characterized by *Candida* with three different species as *C. guilliermondii* for SF1, *C. glabrata* for SF2, and *C. parapsilosis* SF3. New accession numbers were recorded of the mentioned fungal strains in the GenBank database as shown in the table. *C. guilliermondii* identified similarities percentages 99% for the SF1. SF2 was showed 99% similarity with the type strain *C. parapsilosis* and *C. glabrata* reported a similarity amount of 99% for the SF3 as shown in Table (1).

Table 1: List of yeast strains GenBank accession number isolated from semen and % identity with closely related strains found in the NCBI website.

Isolate code	Closest related species	Gen Bank numbers	Similarity (%)
SF1	<i>C. guilliermondii</i>	EU236703.1	99%
SF2	<i>C. parapsilosis</i>	GQ280301.1	99%
SF3	<i>C. glabrata</i>	KP675205.1	99%

Antimicrobial test

According to CLSI documents, the *Candida* isolates were indicated different antifungal sensitivity to five antifungal agents including CAS, FLU, FCT, MCF, and VRC unless *C. parapsilosis* that not advanced any MIC values against fluconazole with an analysis message (this antibiotic is not claimed). Micafungin showed the highest susceptibility to

isolates of *C. parapsilosis* ($\leq 0.06 \mu\text{g/mL}$) and VRC showed the highest susceptibility to the other two isolates of *C. glabrata* and *C. guilliermondii* ($\leq 0.12 \mu\text{g/mL}$) as shown in Table (2).

Table 2: scale azole MICs (in mm) of antifungal agents for the *Candida* species isolated from seminal fluid.

<i>Candida</i> species	CAS	FLU	FCT	MCF	VRC
	MIC ($\mu\text{g/mL}$)	MIC ($\mu\text{g/mL}$)	MIC ($\mu\text{g/mL}$)	MIC ($\mu\text{g/mL}$)	MIC ($\mu\text{g/mL}$)
<i>C. guilliermondii</i>	0.5	2	≤ 1	0.5	≤ 0.12
	(S)	(S)	(S)	(S)	(S)
<i>C. parapsilosis</i>	0.12	-	≤ 1	≤ 0.06	≤ 0.12
	(S)	(-)	(S)	(S)	(S)
<i>C. glabrata</i>	0.25	2	≤ 1	0.5	≤ 0.12
	(S)	(S)	(S)	(S)	(S)

Scale azole of the minimum inhibitory concentration (MICs) was measured using the VITEK-2 system. (S) Susceptible; (R) Resistant. CAS, caspofungin; FLU, fluconazole; FCT, flucytosine; MCF, micafungin; VRC, voriconazole; NS, MIC value not showed with analysis message (this antibiotic is not claimed).

DISCUSSION

In the present study, three *Candida* species were identified from 17 semen specimens. These yeasts were present in 28% of semen while there were 72% of semen did not show any fungal growth. However, no complete agreement on the detrimental role of the presence of fungi in the semen [12]. The most common pathogenic fungi isolated from semen were *Candida albicans* and these fungal infections may adversely affect reproduction and may consequently influence ejaculate production [13]. Opportunistic microorganisms that can usually cause some classical infections of the urogenital tract as prostatitis and epididymitis and subclinical reproductive tract infections in many cases. Some possible

pathophysiological mechanisms that could cause the development of infertility is associated with the presence of microorganisms in the ejaculation [14]. The seminal fluid may be contaminated by the hematogenous invasion of different microbes spread via the oral mucosa and skin. It may also be colonized with collecting semen samples procedure [15]. In our study, *Candida* spp. is a common commensal yeast of the male reproductive tract and investigated as opportunistic pathogens that may sometimes cause diseases and it can affect fertility in several ways including damage of spermatozoa, hampering their motility. Mostly cases were diagnosed amount (80% to 90%) infected with *Candida albicans*, while infection with other species such as *C. glabrata* and *C. parapsilosis* occurs less frequently [16]. The presence of *C. Albicans*, *C. glabrata* affects seminal parameters including motility, viability, and sperm morphology [17].

On the other hand, several researchers reported yeast species based on a molecular technique to confirm species determination [10]. It is a big challenge to categorize between different yeasts because they have very comparable morphological structures. The molecular identification for the microbial strains exposed that the identification of yeast isolates using the morphological features was practically giving genus scientific name, thus submission of the correct identification for all yeast isolates tested were required to distinguish species name. Many studies have viewed the effectiveness of microbial semen contamination in male fertility; however, the putative detrimental effect of fungi on sperm quality is still controversial [18]. Fungi can impact on the male reproductive ability, causing the agglutination of motile sperm, decreasing the causing alterations in cell morphology and indirectly, ability of acrosome reaction, and through the production of reactive oxygen species generated by the inflammatory response to the infection [19].

The relationship between pathogenic fungal growth and male infertility, as well as some quantitative and qualitative features of the sperm, has been studied. Researchers noted that the most common fungal isolate was *C. spp.* Therefore, the presence of fungi in the seminal fluid may strongly affect the survival of sperm [20], these findings have confirmed in the present study. The role of infectious yeast such as *Uropathogenic sp.* and *Candida tropicalis* in the seminal fluid decreased sperm density, motility, and morphology, resulting in increased male infertility [21]. On the other hand, the presence of pathogenic fungi in seminal fluid failed to show the cause-and-effect relationship between fungi infections in seminal fluid and male infertility. To further complicate the problem, the presence of fungi in sperm samples of infertile men has a prevalence similar to that seen in fertile

men. Therefore, the clinical significance of fungi in seminal fluid is still unclear [22].

Investigators have demonstrated that antibiotic treatments reduced the number of fungal isolates and increased ejaculate volume, spermatozoa, number of motile spermatozoa, sperm concentration, and normal morphology [13]. The *Candida* species have been detected in semen samples and have been shown to harm the IVF success. After antibiotic treatments, the reduction of *Candida* growth has been shown to correlate with increased IVF pregnancy rates. [23]. Maciejewska *et al.*, (2005) supports our finding with antibiotic treatment for the genitourinary tract infection (GTI) resulting in a significant improvement in sperm volume, movement, sperm concentration, morphology, and vitality in multiple samples resulting in increased IVF success. Moving on from this research, Bielanski attempted a preventive protocol with an embryo culture media were mixed with fungal antibiotics to reduce pathogenic microorganisms' development [24].

The fresh semen may contain different kinds of fungi. Yeasts like *Candida parapsilosis* are skin commensals. we can reduce them by rigorous hygiene of the collection and processing rooms. The yeasts' concentration in semen samples is quite constant indicating an internal source of contamination [25]. The literature concerning the fungal contamination of male semen is relatively poor and it could be a justification for the opportunity of our study. The embryo culture media is mixed with fungal antibiotics such as amphotericin B, fluconazole, nystatin, flucytosine, econazole, ketoconazole, and miconazole [6]. Also, all *C. spp.* in this study were sensitive with five common antifungal agents, unless *C. parapsilosis* that not advanced any MIC values against fluconazole.

The finding of this study showed that 28 % of semen culture was found to be fungal isolates. *C. spp.* was found the commonest organism followed by *C. guilliermondii*, *C. parapsilosis*, and *C. glabrata*. The regular screening of fungal pathogens in infertile man seems necessary because it affects infertility in several ways.

CONCLUSION

This research investigated the fungal isolates within the seminal fluid of males before the IVF procedure. The semen was not always sterile but contained a diverse range species of Yeasts that may reduce the IVF outcomes. Furthermore, the Identification of fungus within the semen in males with repeated unsuccessful IVF cycles may extend a chance to initiate antifungal treatment before the next conception.

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