ERG11 EXPRESSION IN AZOLE RESISTANT CANDIDA SPECIES ISOLATED FROM DIABETIC PATIENTS IN A TERTIARY CARE CENTRE

DISSERTATION SUBMITTED TO

In partial fulfillment of the requirement for the degree of

DOCTOR OF MEDICINE IN MICROBIOLOGY

(Branch IV) M. D. (MICROBIOLOGY)

of

THE TAMIL NADU DR. M. G. R MEDICAL UNIVERSITY

CHENNAI- 600032

DEPARTMENT OF MICROBIOLOGY

TIRUNELVELI MEDICAL COLLEGE

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BONAFIDE CERTIFICATE

This is to certify that the dissertation entitled **"ERG11 expression in azole resistant *Candida* species isolated from diabetic patients in a tertiary care centre"** submitted by **Dr. Gracia Paul L** to the Tamilnadu Dr. M.G.R Medical University, Chennai, in partial fulfillment of the requirement for the award of M.D. Degree Branch – IV (Microbiology) is a bonafide research work carried out by her under direct supervision & guidance.

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I solemnly declare that the dissertation titled **"ERG11 expression in azole resistant *Candida* species isolated from diabetic patients in a tertiary care centre"** is done by me at Tirunelveli Medical College hospital, Tirunelveli. I also declare that this bonafide work or a part of this work was not submitted by me or any others for any award, degree, or diploma to any other University, Board, either in or abroad.

The dissertation is submitted to The Tamilnadu Dr. M.G.R.Medical University towards the partial fulfilment of requirements for the award of M.D. Degree (Branch IV) in Microbiology.

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CERTIFICATE – II

This is certify that this dissertation work title **"ERG11 expression in azole resistant *Candida* species isolated from diabetic patients in a tertiary care centre"** of the candidate **Dr. Gracia Paul L** with registration Number **201614301** for the award of **M.D.** Degree in the branch of **MICROBIOLOGY(IV).** I personally verified the urkund.com website for the purpose of plagiarism check. I found that the uploaded thesis file contains from introduction to conclusion page and result shows **14 percentage** of plagiarism in the dissertation.

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CERTIFICATE OF REGISTRATION & APPROVAL OF THE TIREC REF NO:992/MICRO/2017

PROTOCOL TITLE: ERG11 EXPRESSION IN AZOLE RESISTANT CANDIDA SPECIES ISOLATED FROM DIABETIC PATIENTS IN A TERTIARY CARE CENTRE
PRINCIPAL INVESTIGATOR: Dr.GRACIA PAUL.L. MBBS.,
DESIGNATION OF PRINCIPAL INVESTIGATOR: PG STUDENT
DEPARTMENT & INSTITUTION: TIRUNELVELI MEDICAL COLLEGE, TIRUNELVELI

Dear, Dr.GRACIA PAUL.L MBBS., The Tirunelveli Medical College Institutional Ethics Committee (TIREC) reviewed and discussed your application during the IEC meeting held on 10.03.2017

THE FOLLOWING DOCUMENTS WERE REVIEWED AND APPROVED

1. TIREC Application Form ☐
2. Study Protocol ☐
3. Department Research Committee Approval ☐
4. Patient Information Document and Consent Form in English and Vernacular Language ☐
5. Investigator's Brochure ☐
6. Proposed Methods for Patient Accrual Proposed ☐
7. Curriculum Vitae of the Principal Investigator ☐
8. Insurance /Compensation Policy ☐
9. Investigator's Agreement with Sponsor ☐
10. Investigator's Undertaking ☐
11. DCGI/DGFT approval ☐
12. Clinical Trial Agreement (CTA) ☐
13. Memorandum of Understanding (MOU)/Material Transfer Agreement (MTA) ☐
14. Clinical Trials Registry-India (CTRI) Registration ☐

THE PROTOCOL IS APPROVED IN ITS PRESENTED FORM ON THE FOLLOWING CONDITIONS

1. The approval is valid for a period of 2 year/s or duration of project whichever is later
2. The date of commencement of study should be informed
3. A written request should be submitted 3weeks before for renewal / extension of the validity
4. An annual status report should be submitted.
5. The TIREC will monitor the study
6. At the time of PI's retirement/leaving the institute, the study responsibility should be transferred to a person cleared by HOD
7. The PI should report to TIREC within 7 days of the occurrence of the SAE. If the SAE is Death, the Bioethics Cell should receive the SAE reporting form within 24 hours of the occurrence.
8. In the events of any protocol amendments, TIREC must be informed and the amendments should be highlighted in clear terms as follows:
   a) The exact alteration/amendment should be specified and indicated where the amendment occurred in the original project. (Page no. Clause no. etc.)
   b) The PI must comment how proposed amendment will affect the ongoing trial. Alteration in the budgetary status, staff requirement should be clearly indicated and the revised budget form should be submitted.
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STANDS APPROVED UNDER SEAL

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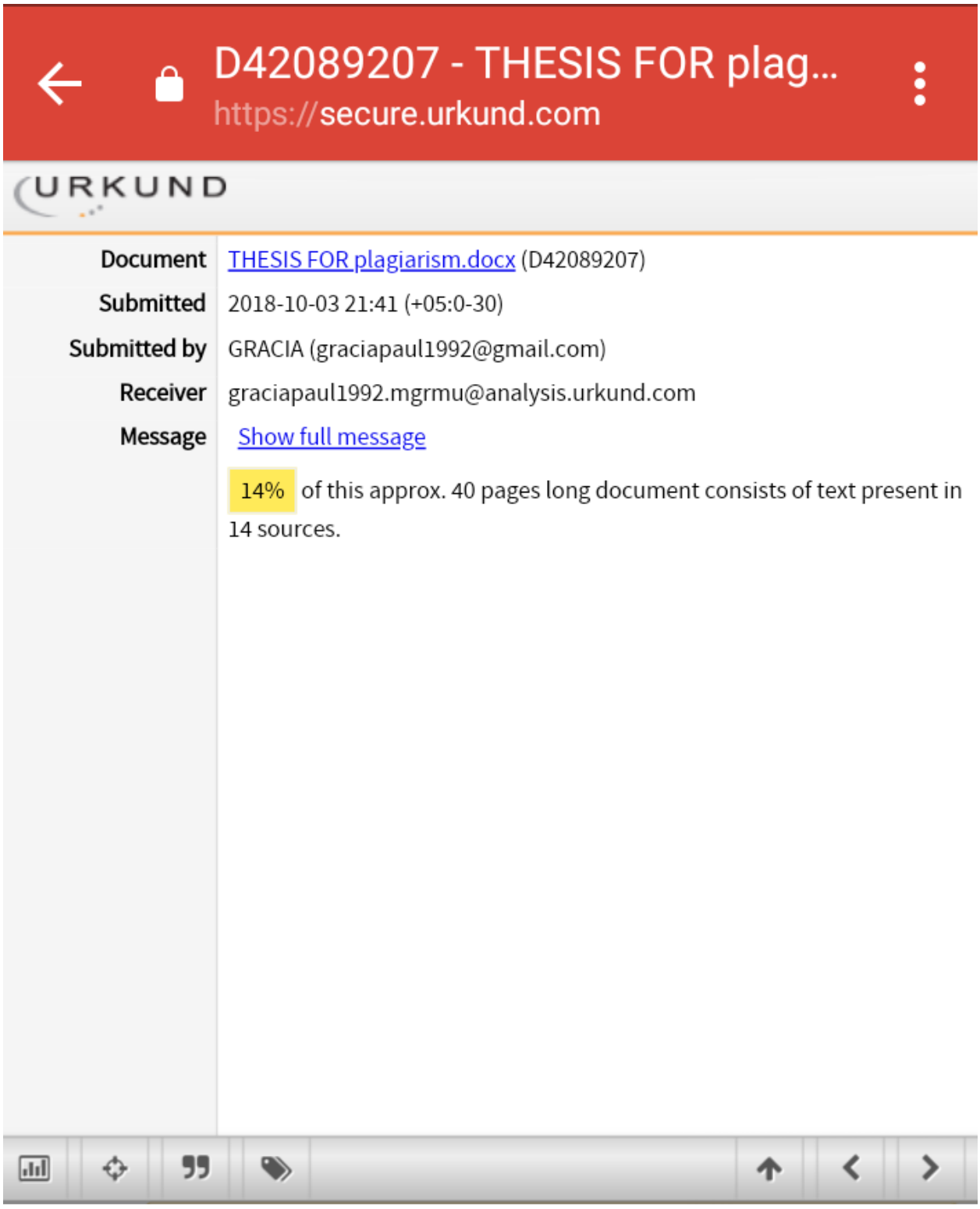
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1.INTRODUCTION

Candida species are commonly seen fungi that exist as an element of normal flora in the skin, gastrointestinal tract and reproductive tract of humans. Among *Candida* species, *Candida albicans* is the most common infectious agent but the non-*albicans Candida* species are emerging as pathogens and also colonize human mucocutaneous surfaces. Fungal infections are generally opportunistic so the pathogenesis and prognosis of candidial infections are affected by the host immune status and also differ greatly according to disease presentations.

Over the last decade, fungal infections and range of yeasts associated with human infections have increased, especially with *Candida*. Candidiasis has emerged as an alarming opportunistic infection with an increase in a number of patients who are immunocompromised, diabetics and the elderly. Extensive and prolonged use of antibacterial and cancer chemotherapeutic agents has further complicated the situation.

Mucocutaneous candidiasis can be divided into nongenital disease and genitourinary disease. Among nongenitourinary candidiasis, oropharyngeal manifestations are the most common and usually are diagnosed in immunocompromised patients, such as human immunodeficiency virus (HIV) infected persons . The most frequent manifestations of genitourinary candidiasis include Vulvovaginal candidiasis (VVC) in women, balanitis and balanoposthitis in men and Candiduria in both sexes. These diseases are remarkably common but occur in different populations, immunocompetent as well as immunocompromised. While VVC affects mostly healthy women, candiduria is commonly diagnosed in immunocompromised patients or neonates.

VVC is the second most common after bacterial vaginosis among the many causes of vaginitis and it is estimated that approximately 75% of all women suffer at least once in their lifetime from vulvovaginal candidiasis (VVC), with 40–50% experiencing at least one additional episode

of infection. A small percentage of women (5–8%) suffer from atleast four recurrent VVC per year. The prevalence of *Candida* infections accounts for about 47.5% among diabetic women.

In contrast to genital manifestations of candidiasis, candiduria is usually diagnosed in elderly hospitalized patients and *Candida* is the most frequently isolated pathogen in nosocomial urinary tract infections (UTIs). Candiduria most likely reflects colonization or infection of the lower urinary tract or the collecting systems of the kidneys but in some cases, it is a marker for hematogenous seeding in the kidney.

Candida colonization of the urinary tract is common in patients with Diabetes mellitus. In patients receiving broad-spectrum antibiotics or immunosuppressants or those with long-term urinary catheters, the clinical course of fungal urinary tract infection (UTI) vary from being an asymptomatic and self-limiting disorder to fungal septicemia, which can be fatal. *Candida albicans* has been the fungi most commonly isolated from urine, accounting for 50%–70% of isolates in various studies. *Candida glabrata* and *Candida tropicalis* are the next most common species found in cultures of urine.

Diabetes mellitus is a chronic, insidious disease that can affect any organ or system of the body and one of the major complications associated with it is infection. Although the prevalence of infection among diabetic and non-diabetic subjects is similar, the intensity of infection is being more severe and the response to therapy slow in diabetic patients. In patients suffering with Diabetes mellitus, excess glucose level in the bloodstream can cause several infectious diseases ranging from superficial candidiasis to deep seated mycoses. *Candida*rely on simple carbon compounds such as glucose, sucrose, maltose and lactose for their rapid growth and these simple sugars increase the fungal population density. Fungal load or count is an important parameter of antifungal susceptibility.

Candida albicans and related species in the recent times have developed resistance to anti‑fungal agents, in particular to the azole compounds. Hence, accurate species identification is important for the treatment of *Candida* infections, as the non-*albicans Candida* continue to be increasingly documented.

A number of antifungal agents especially azoles are used to treat candidiasis. Currently, Fluconazole is recommended in various guidelines as the first drug of choice because it is less toxic and can be taken as a single oral dose. Emergence of Fluconazole-resistant *Candida* species has been progressively reported in the last few years. Several major mechanisms leading to azole resistance have been elucidated. Declined effective drug concentrations can be achieved by overexpression of a drug's molecular target, which gives rise to drug resistance. Changes in sterol 14α-demethylase (*ERG11*), the target of azole antifungals, are associated with azole resistance in *C. albicans* . The azole activity is directed against lanosterol 14-α-demethylase (Erg11p), which is involved in the biosynthesis of ergosterol.

Erg11p is a cytochrome P450 enzyme from family 51 (CYP51) encoded by the *ERG11* gene . This enzyme converts lanosterol to ergosterol, which catalyses the oxidative removal of the 14α-methyl group from lanosterol. The sterol 14-α-demethylase contains a heme moiety in its active site. The unhindered nitrogen of the azoles binds to the heme iron of *Erg11p,* thus, inhibiting enzymatic reaction. In addition, second nitrogen in the azoles has the potential to interact directly with the apoprotein of lanosterol-demethylase. The inhibition of *Erg11p* leads to the accumulation of 14α-methylated sterols, thereby blocking the biosynthesis of ergosterol and leading to defects in membrane and cellular integrity as ergosterol is an essential component of the fungal plasma membrane.

Over-expression of *ERG11* gene results in production of a large amount of lanosterol 14α-demethylase and this favours continuous synthesis of ergosterol and maintenance of the integrity of the cell wall which enables *Candida* to resist Fluconazole . This type of resistance has been associated with widespread and continuous usage of Fluconazole as prophylaxis.

Therefore, the present study was carried out to determine the relative contribution of *Candida abicans* and non-*albicans* species in diabetic patients with suspected candidiasis , their antifungal susceptibility profile and role of ERG11 overexpression in azole resistance.

2. REVIEW OF LITERATURE

2.1 Diabetes mellitus:

Diabetes mellitus is a chronic disease caused by inherited and/or acquired deficiency in production of insulin by the pancreas or by the ineffectiveness of the insulin produced. Such a deficiency results in increased concentrations of glucose in the blood, which in turn damage many of the body's systems, in particular the blood vessels and nerves.

There are two principle forms of diabetes:

Type 1 diabetes is formerly known as insulin-dependent diabetes in which the pancreas fails to produce the insulin which is essential for survival. This form develops most frequently in children and adolescents, but is being increasingly noted later in life.

Type 2 diabetes is formerly named non-insulin-dependent diabetes in which results from the body's inability to respond properly to the action of insulin produced by the pancreas. Type 2 diabetes is much more common and accounts for around 90% of all diabetes cases worldwide. It occurs most frequently in adults.

Certain genetic markers have been shown to increase the risk of developing Type 1 diabetes. Type 2 diabetes is strongly familial, but it is only recently that some genes have been consistently associated with increased risk for Type 2 diabetes in certain populations. Both types of diabetes are complex diseases caused by mutations in more than one gene, as well as by environmental factors.

Symptoms:

The symptoms of diabetes may be pronounced, subdued, or even absent. Type 1 diabetes, the classic symptoms are excessive secretion of urine (polyuria), thirst (polydipsia), weight loss and tiredness.

These symptoms may be less marked in Type 2 diabetes. In this form, it can also happen that no early symptoms appear and the disease is only diagnosed several years after its onset, when complications are already present.

Prevalence:

Recently compiled data show that approximately 150 million people have diabetes mellitus worldwide and that this number may well double by the year 2025. Much of this increase will occur in developing countries and will be due to population growth, ageing, unhealthy diets, obesity and sedentary lifestyles.

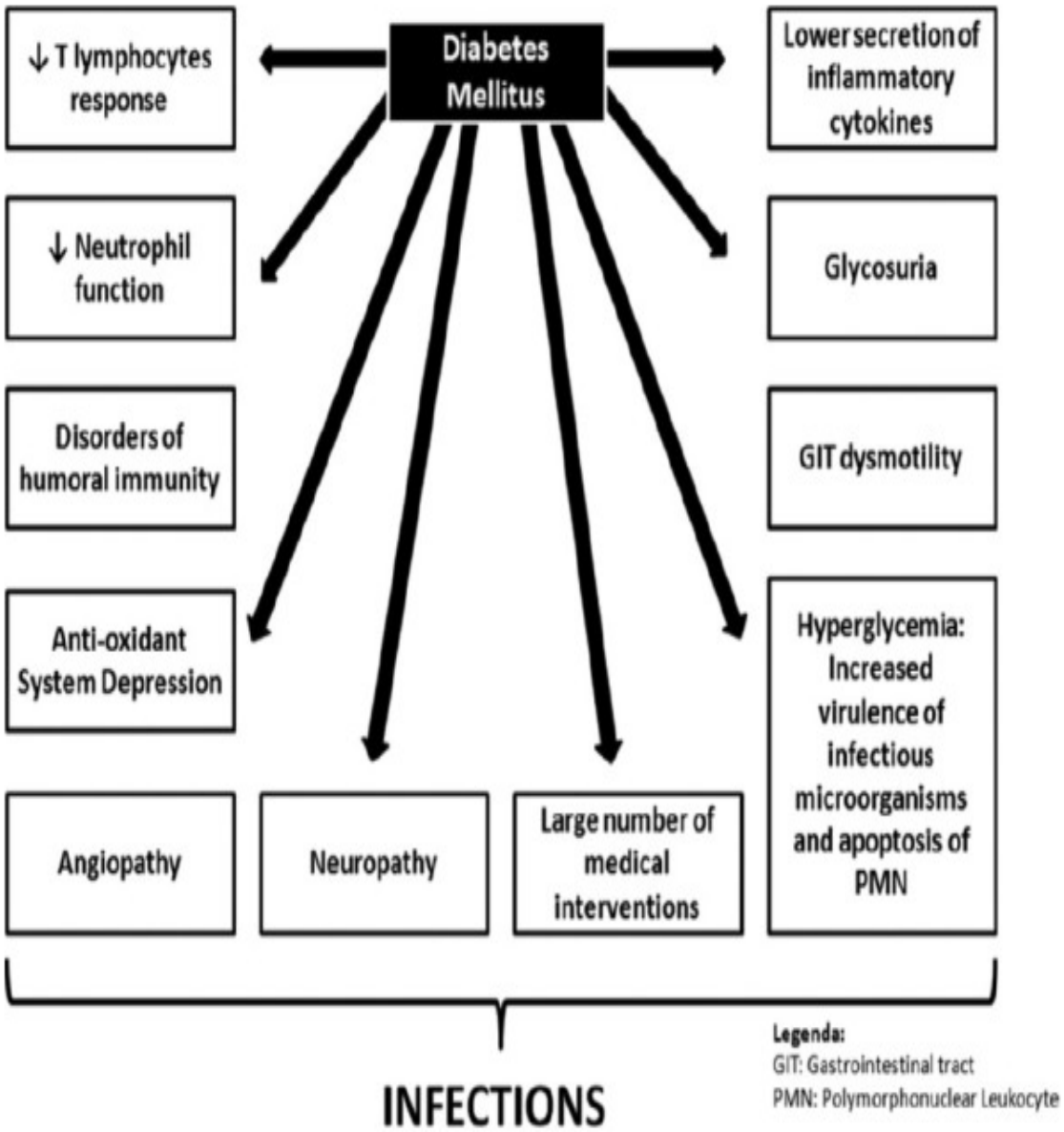
By 2025, while most people with diabetes in developed countries will be aged 65 years or more, in developing countries most will be in the 45-64 year age bracket and affected in their most productive years.

2.1.1 Diabetes Mellitus and Infections:

The incidence of infections is increased in patients with diabetes mellitus. Some of these infections are also more likely to have a complicated course in diabetics than in nondiabetic patients. Diabetic ketoacidosis is precipitated or complicated by an infection in 75% of the cases. The mortality rate of patients with an infection and ketoacidosis is 43%. The question then arises as to which pathogenic mechanisms are responsible for this high infection rate in patients with DM. Possible causes include defects in immunity, an increased adherence of microorganisms to diabetic cells, the presence of micro- and macroangiopathy or neuropathy and the high number of medical interventions in this group of patients.

The immune system can be divided into the innate and adaptive-humoral or cellular immune systems. Concerning the humoral adaptive immunity, serum antibody concentrations in patients with DM are normal. Concerning the adaptive cellular immunity, inhibition of the

proliferative response to different stimuli has been observed in the lymphocytes of diabetics with poorly controlled disease. Differences in innate immunity between diabetic and non-diabetic patients and in adherence of microorganisms to diabetic and non-diabetic cells are more important in the pathogenesis of the increased prevalence of infections in these patients.



2.1.2 Defects in Innate Immunity

Complement Function:

Vergani et al found that 26% of DM type 1 patients had a serum complement factor 4 concentration (C4) below the normal range. The low C4 values did not appear to be the result of consumption. Since nondiabetic identical twins also had a C4 concentration below normal, and the genes encoding C4 are linked with the antigens DR3 and DR4 (which are expressed in 95% of the Caucasian diabetic patients in contrast to 40% of the general population), it suggests that this reduced C4 may be an inherited phenomenon. However an isolated C4 deficiency is not a known risk factor for infections in nondiabetic patients and therefore seems not to play an important role in the increased risk of infections in patients with DM.

Cytokines:

Geerlings et al and Peleg et al studies showed that mononuclear cells and monocytes of persons with DM secrete less interleukin-1 (IL-1) and IL-6 in response to stimulation by lipopolysaccharides. It appears that the low production of interleukins is a consequence of an intrinsic defect in the cells of individuals with DM. However, some studies reported that the increased glycation could inhibit the production of IL-10 by myeloid cells, as well as that of interferon gamma (IFN-γ) and tumor necrosis factor (TNF)-α by T cells. Glycation would also reduce the expression of class I major histocompatibility complex (MHC) on the surface of myeloid cells, impairing cell immunity (Price et al)

Hyperglycemia/Glucosuria:

Hostetter et al studied the enhancing effect of hyperglycemic environment on the virulence of certain microorganisms. An example is *Candida albicans*, which expresses a surface protein that has great homology with the receptor for complement factor 3b (CR3). Normally, opsonization

of microorganisms takes place by attachment of complement factor 3b (C3b). Receptors on phagocytizing cells recognize this bound C3b and attach, thereby initiating ingestion and killing. In a hyperglycemic environment, the expression of the receptor-like protein of *C. albicans* is increased, which results in competitive binding and inhibition of the complement-mediated phagocytosis

Glucosuria found in poorly regulated patients enhances growth of different *Escherichia coli* strains, which probably plays a role in the increased incidence of urinary tract infections in diabetic patients. So an optimal diabetes regulation can decrease the virulence of some pathogenic microorganisms (Geerlings et al).

2.1.3 Cellular Immunity

Chemotaxis:

Mowat et al study showed a significantly lower chemotaxis in PMNs of diabetic patients (type 1 and type 2) than in those of controls. Study by Delamaire et al showed that the chemotactic responses of the PMNs did not alter after the incubation of either glucose or insulin, but returned to normal values after the incubation with glucose and insulin together. Since most PMN functions are energy-dependent processes, an adequate energy production is necessary for an optimal PMN function. Glucose needs insulin to enter the PMNs to generate this energy, which may explain the improvement of the chemotactic response after the addition of these two substances.

Phagocytosis:

Tater et al and Delamaire et al studies demonstrated that the PMNs of diabetic patients have same and a lower phagocytotic capacity compared to PMNs of controls respectively. A study by Balasoiu et al showed that the mean HbA1c concentration was lower in patients without impaired phagocytosis than in those with impaired phagocytosis. Marhoffer et al showed an inverse relationship between the HbA1c levels and the phagocytotic rate. Another study by Gin et al showed that the decreased phagocytosis improved, but did not become normal after 36 h of normoglycemia. Therefore, it seems that impairment of phagocytosis is found in PMNs isolated from poorly regulated patients and that better regulation of the DM leads to an improved phagocytotic function

Oxidative Burst:

Shah et al used Chemiluminescece (CL) to evaluate the oxidative potential of PMNs, a process during which free radicals are synthesized early in the phagocytotic process. CL correlates well with antimicrobial activity and may be used as a measure of phagocytotic capacity. Compared to controls, CL at baseline was the same in PMNs of diabetic patients. Studies showed after stimulation, the CL of diabetic PMNs was lower than that of control PMNs.

Killing by PMNs:

The killing capacity of diabetic PMNs is lower than that of control PMNs. An impaired killing function of diabetic PMNs was found in studies by Tater et al and Tan et al with *Staphylococcus aureus* as the microorganism, but not in the stugy by Balasoiu et al in which the killing of *Candida albicans* was used as the measure. No correlation was found with glycemic level, although some studies have shown that bactericidal activity improved, but did not normalize after achieving normoglycemia.(Gin et al)

Monocytes And Macrophages:

Both impaired chemotaxis and phagocytosis of the monocytes of diabetic patients have been described by Deresinski et al and Katz et al. In combination with the earlier mentioned decreased production of pro-inflammatory cytokines after LPS stimulation in DM type 1 patients, it shows that monocyte/macrophage functions are impaired in DM type 1 patients.

Adherence

Adherence of a microorganism to mucosal or epithelial cells is an important step in the pathogenesis of infections. Host-related factors may influence this adherence. Schaeffer et al demonstrated that women with recurrent urinary tract infections have a greater adherence *of E.coli* to their vaginal and buccal cells compared to controls

C. albicans infection is frequently found in diabetic patients. Since infection mostly is preceded by colonization Aly et al. investigated which risk factors increased the risk of *Candida* carriage in diabetic patients. Risk factors for oral *Candida* carriage in patients with DM type 1 were a lower age and a higher HbA1c level (poor regulation of DM). Continuous wearing of dentures and the presence of glucosuria (also an indication of a poor DM regulation) increased the risk of *Candida* carriage in DM type 2 patients, the mean number of cigarettes smoked per day was correlated with *Candida* carriage in DM type 1 and type 2 grouped together.

Cameron et al study showed that the carbohydrate composition of receptors probably plays an important role in the susceptibility to infections. These receptor changes possibly lead to an increased adherence of microorganisms and play a role in the high prevalence of Gram-negative bacterial colonization in the respiratory tract of these patients. This mechanism of increased adherence, due to an altered receptor carbohydrate composition, is possibly also present in diabetic patients.

2.1.4 Major Infections Associated With Diabetes Mellitus

Bacterial Infections:

1. Respiratory infections
2. Tuberculosis
3. Urinary tract infections
4. Bacterial pyelonephritis
5. Emphysematous pyelonephritis
6. Emphysematous cystitis
7. Perinephric abscess
8. Emphysematous cholecystits
9. Skin and soft tissue infection – Furunculosis, Folliculitis and Subcutaneous abscess
10. Foot infections
11. Necrotizing fasciitis
12. Fournier gangrene
13. Invasive external otitis
14. Periodontits

Fungal Infections

- Fungal cystitis
- Mucocutaneous candidiasis
- Rhinocerebral mucormycosis

2.2 *Candida*

2.2.1 Discovery

The perception of *Candida* has evolved from the presence of an exudate in the human host to a known infectious agent. 200 years of medical history was recorded before the etiological agent of oral thrush, the first form of candidiasis described, was correctly identified as a fungal pathogen. “Thrush” appears as whitish plagues within the oropharynx or the buccal mucosa or tongue.

One of the main points of contention when defining thrush was whether it originated from the host or was an infectious agent, or a combination of the two. The earliest reports of thrush predated the concept of a microbial pathogen. In “Of the Epidemics,” Hippocrates described oral candidiasis (around 400 B.C.) as “mouths affected with aphthous ulcerations”. In 1665, Pepys Diary reported “a patient hath a fever, a thrush and a hiccup” perpetuating the idea that oral thrush originates from the host.

In 1771, Rosen von Rosenstein defined an invasive form of thrush. In 1839, Langenbeck was credited with first recognizing a fungus in a patient with typhoid fever. Oropharyngeal and esophageal thrush with pseudomembranes were found at autopsy. “Under the microscope magnified, the pseudomembranes consisted of an immense number of fungi” .He described in detail what is now referred to as septate hyphae, branched pseudohyphae and blastoconidia However, he ascribed the entity to the typhoid bacterium rather than the fungus. In 1844, J.H. Bennett observed a similar fungus in the sputum and the lungs of a patient with a pneumothorax and criticized the conclusion by Lagenbach. The morphologic description of Bennett was essentially that as described by Langenbeck. Two years later, Berg explicitly concluded that thrush was caused by a fungus and found that spread could occur from communal feeding

bottles. Most importantly, he also stated “descriptions of the disease unsupported by demonstration of the fungus could not substantiate the diagnosis”. He was able to reproduce the infection in healthy children and thereby confirmed his hypothesis that the fungus caused the infection . After this discovery, other infections would be ascribed to this dimorphic fungus including vaginitis and gastrointestinal candidiasis.

2.2.2 Nomenclature

Once the etiology was conclusively demonstrated by mycologists, the next point of contention was the identity of the pathogen. While Langenbeck (1839) first documented the fungus associated with thrush, he failed to make the direct connection. In 1847, the distinguished French mycologist, Charles Philippe Robin, classified the fungus as *Oidium albicans* using albicans (“to whiten”) to name the fungus causing thrush. Hill and later Martin and Jones misclassified *Candida* into the genus Monilia, a genus containing fungi that commonly grow in plants. Subsequently, clinicians erroneously referred to the etiology of thrush as “Monilias” despite the fact mycologists had already elucidated the morphological differences between the fungus associated with thrush and the fungus in the genus Monilia.

Christine Berkhout and others noted these differences, particularly the ability of this fungus to infect humans. Berkhout reclassified it under the current genus *Candida* (1923). *Candida* is derived from Latin where toga candida was a white robe worn by Roman Senators. However, it was not until 1954 that the Eighth Botanical Congress officially endorsed the binomial *Candida albicans* as the nomen conservandum formally ending the 200 year long uncertainty over the etiology and taxonomy of Candida. Currently, there are some 200 organism species within the genus *Candida.*

2.2.3 Morphology

These yeast-like cells are anamorphic (sexual imperfect) fungi belonging to the class Blastomycetes. They are characterized by their polymorphic nature and ability to produce budding yeast cells (blastoconodia), mycelia, pseudomycelia and blastospores . Of the nearly 200 species, only few species are considered to be significant pathogens associated with various infections in human - *Candida albicans , Candida glabrata ,Candida tropicalis, Candida parapsilosis, Candida krusei ,Candida auris, Candida kefyr, Candida dubliniensis, Candida guilliermondii, Candida rugosa, Candida haemulonii, Candida viswanathii* and *Candida lusitaniae.*

Out of these, six species- *C. albicans, C. glabrata, C. tropicalis, C. parapsilosis Candida dubliniensis* and *Candida krusei* are the most commonly associated with human infection.

Candida is a yeast like fungus that primarily exists and propagates through blastospore phenotype (also called blastoconidia). Blastospores are characterized by their oval-shapes, mono-nucleated cells and propagation through cellular budding. Upon perception of environmental signals, *C. albicans* is able to transform into one of two filamentous forms: psuedohyphae and hyphae. Elongated, ellipsoidal cells that are attached to one another are referred to as psuedohyphae, while cells that are considered to be true hyphae are characterized by a cylindrical cellular morphology and are separated by perpendicular septal walls. These hyphal forms are comprised of conjoined cells that are divided by septal walls and are not syncytial in composition.

C. albicans has evolved into a commensal organism as well as an opportunistic pathogen, implying that it is routinely present in what is considered to be a healthy mucosal microflora while retaining the ability to establish an infection in its host if the factors are favourable.

2.2.4 Clinical Classification Of Candidiasis:

Infectious diseases:

- Mucocutaneous manifestations
    - Oral : thrush, stomatitis, glossitis, chelitits
    - Alimentary : esophagitis, gastritis
    - Vulvovaginitis, balanitis, balanoposthitis
    - Chronic mucocutaneous candidiasis
    - Ocular candidiasis
- Cutaneous manifestations
    - Paronychia ,Onychomycosis, Diaper dermatitis ,Candidal granuloma
- Systemic manifestations
    - Urinary tract infection, Endocarditis, Pulmonary candidiasis, Meningitis, Arthritis Osteomyelitis, Endophthalmitis, Candidemia, Disseminated candidiasis

Allergic diseases:

- Candidids, Eczema, Asthma, Gastritis

2.3 Genitourinary Candidiasis:

2.3.1 Definition of Genitourinary Candidiasis :

Vulvovaginal Candidiasis:

The presence of *Candida* in the vagina, in the absence of immunosuppression or damaged mucosa is usually not associated with any signs of disease and is thus referred to as colonization. In contrast to asymptomatic colonization, VVC is defined as signs and symptoms of inflammation in the presence of *Candida* species and in the absence of other infectious etiology. Over a decade ago, VVC was classified into uncomplicated and complicated cases, a classification that has been internationally accepted and adapted (Pappas et al and Sobel et al). Uncomplicated VVC is characterized by sporadic or infrequent occurrence of mild to moderate disease caused by *C. albicans* in immunocompetent women.

Complicated VVC includes cases of

- Severe VVC
- VVC caused by non-*C. albicans* species
- VVC associated with pregnancy or other concurrent conditions such as uncontrolled diabetes or immunosuppression
- Recurrent VVC (RVVC) in immunocompetent women.

RVVC is defined as at least four episodes of VVC during 1 year. RVVC can be further broken down into two subgroups: primary and secondary RVVC. Primary infections are idiopathic and do not correlate to any known predisposing factors identified with acute VVC (Sobel et al, 1998). Secondary infections are defined as frequent episodes of acute VVC brought on by unavoidable predisposing factors such as diabetes mellitus or hormone replacement therapy.

Long-term suppressive antifungal therapy is commonly required to control RVVC and recurrence rates of up to 40% to 50% occur after discontinuation of suppressive therapy (Sobel et al, 2004). Compared to the case for women with other chronic vaginal symptoms, symptoms of women with RVVC are reported to have the greatest negative impact on work and social life

Candiduria:

This presentation is relatively rare, manifesting as cystitis (more commonly caused by *Candida glabrata*) and pyelonephritis, either ascending from a bladder infection or from hematogenous spread from a distant primary site of infection. The CFU criteria to diagnose candiduria range from 10^3 to 10^5 CFU/ml of urine. Chabasse et al study showed an significant correlation between heavy candiduria (>10^4 CFU/ml urine) and a high Pittet *Candida* colonization index (>0.5) has been established

2.3.2 Epidemiology of Genitourinary Candidiasis:

VVC:

Studies of Bauters et al and Beigi et al showed that Candida species mostly *C. albicans*, can be isolated in the vaginal tracts of 20 to 30% of healthy asymptomatic non pregnant women at any single point in time and in up to 70% if followed longitudinally over a 1-year period. If the balance between colonization and the host is temporarily disturbed, *Candida* can cause disease such as VVC which is associated with clinical signs of inflammation. Such episodes can happen sporadically or often can be attributed to the presence of a known risk factor like the disturbance of local microbiologic flora by antibiotic use.

VVC is not a reportable disease and is often diagnosed without confirmatory tests and treated with over-the-counter medications, and thus the exact incidence is unknown. It is estimated that

around 75% of all women experience at least one episode of VVC during their childbearing years, of which about half have at least one recurrence (Sobel et al, 1998).

Candiduria:

Incidence numbers given for candiduria are dependent on the setting and the populations studied and have to be carefully compared because of the discrepancies with definitions of candiduria. Schaberg et al and Kauffman et al studies shows that candiduria is very common in hospitalized patients . There is evidence that the incidence is linked to antibiotic usage (Weinberger et al). In general, most estimates of incidence based on culture results are likely underestimated because standard urine culture is not very sensitive.

Depending on the population examined, *Candida* is reported in up to 44% of urine samples sent for cultur . Colodner et al and De Francesco et al report lower rates (0.14 to 0.77% and 0 to 1.4%) in urine cultures from both hospitalized patients and outpatients. The incidence of candiduria also varies with hospital setting, being most common in intensive care units (ICUs) (Schaberg et al) and among those in burn units (Bougnoux et al). Studies by Richard et al and Lundstorm et al reported that 11 to 30% of nosocomial urinary tract infections (UTIs) are caused by *Candida*.

2.3.3 Predisposing Risk Factors For Genitourinary Candidiasis:

Risk Factors for VVC:

Although many healthy women develop VVC sporadically, several behavioral and host-related risk factors have been associated with VVC and recurrent episodes. These episodes are caused by *Candida* overgrowth from the gastrointestinal or the vaginal tract or through sexual transmission (Reed et al). Studies by Cetin et al and Foxman et al showed that Behavioral risk

factors like frequent sexual intercourse and receptive oral sex, as well as the use of high-estrogen oral contraceptives, condoms, and spermicides have been significantly associated with a higher incidence of VVC. Host-related risk factors that have been significantly associated with VVC and RVVC include antibiotic use, uncontrolled diabetes, conditions with high reproductive hormone levels and genetic predispositions (Goswami et al).

After antibiotic use, the increase in vaginal colonization with *Candida* species mostly *Candida albicans* is estimated to range from 10 to 30% and VVC occurs in 28 to 33% of cases by Sobel in 2007as antibiotics alter the bacterial microflora of the vaginal and gastrointestinal tracts and thus allow for overgrowth of *Candida* species. It is commonly hypothesized that the reduction of lactobacilli in the vaginal tract predisposes women to VVC. Lactobacilli play a key role in the vaginal flora through the production of hydrogen peroxide, bacteriocins, and lactic acid which protect against invasion or overgrowth of pathogenic species (Eschenbach et al). However, studies by Vitali et al and Zhou et al have failed to provide evidence that an altered or abnormal vaginal bacterial flora predisposes women to recurrent episodes of VVC in the absence of antibiotic intake.

Episodes of VVC occur mostly during childbearing years and are rare in premenarchal and postmenopausal women. An increased frequency of VVC has been reported during the premenstrual week (Eckert et al) and during pregnancy (Cotch et al).

VVC and Diabetes:

A study by Faraji et al showed that the prevalence of candiduria among diabetic women was 20%. The species of *Candida* isolated in this study were *C. albicans* (62.5%), *C. glabrata* (18.7%), *Candida tropicalis* (9.4%) and *Candida parapsilosis* (9.4%) with *C. albicans* as the

most predominant isolate. Another study by Emeribe et al showed that the prevalence was about 14%.

Risk Factors for Candiduria:

Studies by Kauffman et al and Lundstorm et al have identified similar risk factors associated with candiduria in adults. They include anatomic urinary tract abnormalities, comorbidities, indwelling urinary drainage devices, abdominal surgery, ICU admission, broad-spectrum antibiotics, diabetes mellitus, increased age, and female sex. The patients who present with candiduria from the community are younger, more commonly female, pregnant and more likely to have dysuria.

Candiduria and diabetes:

A study by Janifer et al in South India showed that 13% of UTI in diabetic patients was caused by *Candida.* Among the specimens containing *Candida*,57 (80.3%) were other *Candida species* and 14 (19.7%) were *Candida albicans*. Poor glycemic control was significantly associated with UTI in both sexes. Age, duration of diabetes, and glycemic control did not show any significant differences between men and women.

Yismav G et al showed that significant candiduria was detected in 29 of 387 (7.5%) and 6 of 35 (17.1%) asymptomatic and symptomatic diabetic patients, respectively. The overall prevalence of significant candiduria was 35 of 422 (8.3%). Of the 38 *Candida* species isolated, *C. albicans* accounted for 42% of the cases, followed by *C. glabrata* (34.2%),*C tropicalis* (15.8%), *C.famata* (5.3%) and *C.kefyr* (2.6%).

2.3.4 Virulence Factors Of Candida Pertaining To Genitourinary Candidiasis:

- Bud-Hypha Formation
- Aspartic Proteinases and Phospholipases
- Adhesion Proteins
- Biofilm Formation
- Phenotypic Switching

2.4 Laboratory Diagnosis of Genitourinary Candidiasis:

2.4.1 Direct Examination:

The preferred method for the direct microscopic examination of genitourinary candidiasis incudes

(i) Wet mount

(ii) KOH mount

Processing of the specimen does not generally require treatment with keratinolytic substance.

Wet mounts can be unstained, prepared in saline or stained with lactophenol cotton blue or calcofluor white. About 50% of patients have positive microscopy of a wet mount or saline preparation, where yeast cells and hyphal elements can be seen. A 10% potassium hydroxide (KOH) preparation is more sensitive than a saline preparation in identifying yeast cells or hyphae Microscopic examination of specimens from candidiasis will demonstrate the presence of budding yeast cells, pseudohyphae and/ or hyphae. Demonstration of hyphal elements in direct microscopic examination is important as *Candida* species normally colonises the oral mucosa and the presence of the hyphal elements in addition to the yeasts, is an indicator of infection. However *C.glabrata*, a significant non-*albicans Candida* species does not produce hyphae or pseudo hyphae in clinical specimens

2.4.2 Culture:

Vaginal culture is the most accurate method for the diagnosis of VVC and is indicated if microscopy is negative but VVC is suspected or in cases of high risk for non-*C. albicans* VVC.

Candida species grow on almost all common laboratory media particularly Blood agar and Sabouraud's dextrose agar with antibacterial antibiotics for primary isolation.

Isolation of Candida:

Sabourauds Dextrose Agar with antibiotics:

The routine medium used for isolation of fungi in culture from mucocutaneous infections is Sabouraud's dextrose agar supplemented with antibiotics like Gentamicin , Chloramphenicol or Tetracycline to prevent bacterial overgrowth. The addition of cycloheximide to inhibit fungal contaminants permits the growth of *Candida albicans* but inhibits most strains of *C.tropicalis, C.krusei* and *C.parapsilosis*. Use of two SDA containing chloramphenicol supplemented with or without cycloheximide is recommended.

Cultures can be incubated at 28^0 C or / and at 37^0 C.*Candida* colonies appears in two to three days and more than three days for some *Candida species* like *C. guillermondii* and *C.glabrata*.

In case of isolation of candida from urine, $>10^5$ colony forming units/ml can be considered significant.

Colony Characteristics and Microscopic Morphology of Candida on SDA:

- *Candida albicans*

(i) Macroscopically – Colonies are smooth , creamy , pasty , glistening

(ii) Microscopically – globose or short ovoid cells(5 - 7 μm)

- *Candida tropicalis*

(i) Macroscopically – white to cream coloured colonies with peripheral fringe

(ii) Microscopically – globose – ovoid or short ovoid cells (4 -8 μm X 5-11 μm)

- ·*Candida krusei*

(i) Macroscopically - Colonies are flat ,dry becoming dull, smooth or wrinkled with the dense of mycelium extending as lateral fringe around the colony

(ii) Microscopically - Cylindrical , few ovoid cells / elongated cells (3-5 μm x 6-20μm)

- *Candida parapsilosis*

(i) Macroscopically – Soft, smooth, white sometimes lacy

(ii) Microscopically – Short ovoid to long ovoid cells (2.5 - 4μm x 2.5 -9 μm)

- ·*Candida guilliermondii*

(i) Macroscopically – Thin , flat, glossy , cream to pinkish colonies

(ii) Microscopically - short ovoid / ovoid cells(2-5 μm x 3-7 μm)

- *Candida glabrata*

(i) Macroscopically-Cream coloured, soft, glossy ,smooth colony

(ii) Microscopically-small, round yeasts (2.5 – 4.5 μm x 4-6 μm)

- *Candida kefyr*

(i) Macroscopically- Smooth , creamy appearance

(ii) Microscopically- short, ovoid with a few elongate cells(2.5 -5 μm x 5-10 μm)

2.4.3 Speciation of Candida:

Germ Tube Test (Reynolds Braude Phenomenon):

A germ tube is defined as a filamentous extension from a yeast cell that is about half the width and three to four times the length of the cell. The principle of the test is the ability of *Candida albicans or Candida dubliniensis* to produce germ tube when incubated in serum at 37^0 C for two hours.

It helps in the presumptive identification of *Candida albicans* or *Candida dubliniensis*. A true germ tube has no constriction at the point of origin. Early pseudohyphae of *Candida tropicalis* may be confused but characteristically show a point of constriction adjacent to the mother cell.

C.albicans and its variants are able to produce germ tube when incubated with various substances like human or sheep serum, rabbit plasma, egg albumin, thioglycollate broth and various peptone medium at 37^0C for 2 hours.

CHROM agar:

A new differential culture that allows selective isolation of yeasts and presumptive identification of most commonly isolated *Candida* species especially *Candida albicans*, *Candida tropicalis* and *Candida krusei*. Identification of yeast pathogen by traditional methods requires several days and specific mycological media. Chromogenic media contain chromogenic substrates which react with enzymes secreted by the target microorganisms to yield colonies of varying colours after incubation at 37^0 C for 48 to 72 hours.

Cornmeal Agar:

The commonly used differential medium both for genus identification as well as speciation is the Corn meal Agar plate (Dalmau plate) supplemented with Tween 80 (Polysorbate 80) or Rice starch agar

Corn meal agar is a general purpose medium used for the isolation of fungi. In 1960, Walker and Huppert modified the basic formulation by adding polysorbate 80 which stimulated rapid and abundant chlamydospore formation. Dextrose is added to Corn meal agar to enhance fungal growth and pigment production.

Subcultures made by furrowing the Corn meal agar plates with coverslips applied on the streak line and incubated at 28^0C for 2-5 days. Cover slips applied provides a microaerophilic

environment which enhances the formation of hyphae and blastoconidia and the polysorbate 80 reduces the surface tension. After 2-5 days of incubation, plates are examined directly under the microscope for the presence of pseudo hyphae/ true hyphae, chlamydospores , arthroconidia and blastoconidia.

Chlamydospore formation are seen in the isolates of *C.albicans* and *C.dubliniensis. C.dubliniensis* often produces chlamydospores abundantly in clusters.

Microscopic Morphology of different species on Corn Meal Agar:

- *C.albicans* –Hyphae and pseudohyphae formed with clusters of blastospores at internodes and large thick walled terminal chlamydospores
- *C.tropicalis*- Abundant long, branching pseudohyphae .Ovoid / elongate pseudohyphae found anywhere along the pseudohyphae .True hyphae may be present.
- *C.krusei* – Pseudohyphae with elongated blastoconidia in tree like arrangements / Crossed match stick appearance
- *C.parapsilosis*- Fine pseudomycelium with single or small clusters of blastoconidia. Thick pseudo mycelium and giant cells also found.
- *C.glabrata* –No pseudohyphae. Small, spherical and tightly compacted blastoconidia
- *C.guilliermondi* –Pseudohyphae with blastospores in small chains or in clusters
- *C.kefyr* –Abundant pseudomycelium of elongate cells that lie parallel giving log in stream appearance. Blastoconidia are infrequent.

2.4.4 Biochemical Characterisation:

Biochemical speciation of *Candida* is based on

1) Assimilation tests

2) Fermentation tests.

Candida species can utilize carbohydrates both oxidatively (assimilation) & anaerobically (fermentation) .Yeasts possessing the ability to ferment a given carbohydrate do also assimilate that substance but not necessarily vice versa.

Sugar assimilation tests:

Sugar assimilation determines the ability of particular yeast to utilize a particular carbohydrate as the sole source of carbon in the presence of oxygen. Carbohydrate utilization patterns are the most commonly used conventional methods for the definitive identification of yeast recovered in a clinical laboratory.

- Auxanographic methods
- Commercial Kits

Sugar fermentation test:

The classic tests involved liquid media supplemented with different carbohydrates, a colour indicator to assess pH changes to measure acid formation and the Durham's tube to assess gas production .There are several modifications for assessment of gas production such as use of semisolid media or a wax layer on top of liquid medium. Production of gas and not the change of colour in the fermentation fluid is considered as the indicator of positive fermentation

2.4.5 Growth in Sabourauds Dextrose broth:

This method serves as an important differentiating method for various *Candida species*. Ring around the surface of the tube at the broth interface indicates *Candida tropicalis*, a thick pellicle creeping along the sides indicates *C.krusei* while growth occurring at the bottom indicates other *Candida species.*

2.4.6 Automated methods:

The species of *Candida* can be identified within a day by using automated/semiautomated systems such as Vitek 2 or ID32C yeast identification systems which are based on phenotypic identification

2.4.7 MALDI- TOF:

Matrix-Assisted Laser Desorption/Ionization-Time Of Flight Mass Spectrometry (MALDI-TOF MS), based on protein fingerprints, has been used for the identification of clinical *Candida* spp. isolates. Even closely related species/species complexes have been identified by MALDI-TOF MS within a few minutes.

2.4.8 Molecular Methods:

i) Use of specific DNA probes like those encoding for actin gene, part of the 18s RNA gene complex, chitin synthetase gene, mitochondrial DNA or Candida DNA Repititive Elements

ii) Electrophoretic patterns of DNA

iii) RNA profiling

iv Restriction enzyme analysis

v Amplification techniques like Polymerase chain reaction

2.5 Treatment of Genitourinary Candidiasis:

2.5.1 Treatment of VVC:

Treatment recommendations for VVC are separated into treatment of uncomplicated VVC caused by *C. albicans* and of complicated VVC, which includes RVVC, severe VVC, VVC caused by non-*C. albicans* species, and VVC in immunocompromised hosts (Workowski et al). In cases of complicated VVC, contributing factors such as diabetes or behavioral factors should

be controlled or avoided. Treatment should not differ on the basis of HIV infection (Pappas et al).

2.5.2 Treatment of uncomplicated VVC:

Successful treatment of uncomplicated VVC is achieved with single-dose or short-course therapy in over 90% of cases. Several topical and oral drugs are available, without evidence for superiority of any agent or route of administration, although among the topically applied drugs, azoles are more effective than Nystatin. As an oral agent, Fluconazole at 150 mg as a one-time dose is recommended for uncomplicated VVC. Because oral and topical antimycotics have shown to achieve equivalent results for the treatment of VVC (Watson et al), both Fluconazole given orally and topical agents have received the same recommendation in the IDSA guidelines (A-1) and no preference is given to either treatment (Pappas et al).

2.5.3 Treatment of complicated VVC and RVVC:

Complicated VVC with azole-susceptible strains requires topical therapy administered intravaginally daily for at least 7 days or multiple doses of oral Fluconazole (150 mg every 72 h for three doses) (Sobel, 2001). In cases of RVVC, 10–14 days of induction therapy with a topical agent or oral Fluconazole, followed by Fluconazole, 150 mg weekly for 6 months is recommended. Long-term suppressive therapy with oral Fluconazole is the most convenient and well-tolerated regimen among other options and was shown to be effective in over 90% of patients with RVVC. Against expectations, study by Sobel in 2004 have shown little evidence of developing Fluconazole resistance in *C. albicans* isolates or superinfection with non - *C.albicans* species in patients on suppressive therapy with Fluconazole . However, species

identification and MIC testing should be performed in women experiencing breakthrough or refractory infection. Other oral treatment options that have been shown to be effective for RVVC with azole-susceptible strains include suppressive therapy with Ketoconazole (100 mg daily) (Sobel, 1985) and with Itraconazole (200 mg twice daily for one day each month) (Witt et al). However because of liver toxicity associated with oral Ketoconazole , other regimens are now preferred as maintenance therapy.

Non-*Candida albicans*-related disease is less likely to respond to azole therapy (Nyirjesy et al). For *C.glabrata* vulvovaginitis that is unresponsive to oral azoles, topical intravaginal boric acid, administered in a gelatin capsule, 600 mg daily, for 14 days is an alternative.Another alternative agent for *C. glabrata* infection is Nystatin intravaginal suppositories, 100 000 units daily for 14 days .A third option for *C. glabrata* infection is topical 17% Flucytosine cream alone or in combination with 3% AmB cream administered daily for 14 days (Pappas et al).

2.5.4 Treatment of Candiduria:

The presence of yeast in the urine, whether microscopically visualized or grown in culture, must be evaluated in the context of the clinical setting to determine its relevance and make an appropriate decision about the need for antifungal therapy. Similar to the case for asymptomatic bacteriuria, there has been a revolving debate on whether and how to treat patients with candiduria (Nicolle et al).

2.5.5 Treatment of asymptomatic Candiduria:

The current guidelines recommend observation of asymptomatic patients and elimination of predisposing factors if feasible. Specifically, removal of an indwelling catheter may be sufficient

to eliminate candiduria without antifungal therapy. Treatment with antifungal agents is not recommended unless the patient belongs to a group at high risk for dissemination; high-risk patients include neutropenic patients, very low-birth-weight infants and patients who will undergo urologic manipulation. Neutropenic patients and very low–birth-weight infants should be treated as recommended for candidemia with prolonged high-dose antifungal intravenous therapy. Patients undergoing urologic procedures should be treated with oral Fluconazole 400 mg (6 mg/kg) daily or AmB deoxycholate 0.3–0.6 mg/kg daily, for several days before and after the procedure

2.5.6 Treatment of symptomatic Candiduria:

For Fluconazole-susceptible organisms, oral Fluconazole 200 mg (3 mg/kg) daily for 2 weeks is recommended. For Fluconazole-resistant *C. glabrata,* AmB deoxycholate 0.3–0.6 mg/kg daily for 1–7 days or oral Flucytosine 25 mg/ kg 4 times daily for 7–10 days is recommended. For *C.krusei,* AmB deoxycholate 0.3–0.6 mg/kg daily, for 1–7 days is recommended. Removal of an indwelling bladder catheter if feasible is strongly recommended. AmB deoxycholate bladder irrigation 50 mg/L sterile water daily for 5 days may be useful for treatment of cystitis due to Fluconazole-resistant *C. glabrata* and *C. krusei* species.

2.6 Anti Fungal Drug Resistance:

Microbiological resistance refers to non-susceptibility of a fungus to an antifungal agent by in vitro susceptibility testing, in which the MIC of the drug exceeds the susceptibility breakpoint for that organism. Microbiological resistance can be primary (intrinsic) or secondary (acquired).

Primary resistance is found naturally among certain fungi without prior exposure to the drug and emphasizes the importance of identification of fungal species from clinical specimens. Resistance of *Candida krusei* to Fluconazole is an example.

Secondary resistance develops among previously susceptible strains after exposure to the antifungal agent and is usually dependent on altered gene expression. The development of Fluconazole resistance among *Candida albicans* strains illustrates this type of resistance.

Clinical resistance is defined as the failure to eradicate a fungal infection despite the administration of an antifungal agent with in vitro activity against the organism. Such failures can be attributed to a combination of factors related to the host, the antifungal agent or the pathogen. Although clinical resistance cannot always be predicted, it highlights the importance of individualizing treatment strategies on the basis of the clinical situation.

2.6.1 Mechanism of Action of Azoles:

Azoles exert their action by inhibiting the lanosterol 14-α-demethylase (Erg11p), which is involved in the biosynthesis of ergosterol. *Erg11p* is a cytochrome P450 enzyme from family 51 (CYP51) encoded by the *ERG11* gene. This enzyme converts lanosterol to ergosterol, which catalyses the oxidative removal of the 14α-methyl group from lanosterol. The sterol 14-α-demethylase contains a heme moiety in its active site. The unhindered nitrogen of the azoles binds to the heme iron of *Erg11p,* thus inhibiting enzymatic reaction. In addition, second nitrogen in the azoles has the potential to interact directly with the apoprotein of lanosterol-demethylase.

2.6.2 Epidemiology of Azole Resistance:

Widespread use of Itraconazole and Fluconazole is thought to have been the major driver of azole resistance. Up to one-third of patients with advanced AIDS in one study harbored

Fluconazole-resistant *C. albicans* in their oral cavities (Law et al). Azole-resistant *C. albicans* is less common among patients with other diseases, such as vaginal candidiasis and candidemia (Sanglard et al). In general, the rates of azole resistance among the most commonly encountered invasive *Candida* species remain low, with reported rates of 1.0%–2.1% in *Candida albicans*, 0.4%–4.2% in *Candida parapsilosis*, and 1.4%–6.6% in *Candida tropicalis* [Pfaller et al,2005]. A clear exception is *C.glabrata*, which is second to *C.albicans* in causing systemic fungal infection. According to data from the ARTEMIS Global Antifungal Surveillance Program, the incidence of Fluconazole resistance in *C.glabrata* increased from 7% in 2001 to 12% in 2004. In addition to the changing trends in antifungal susceptibility, there has been a recent shift towards more infections in the immunocompromised host being caused by Candida species other than *Candida albicans* as shown in the study by Hajjeh et al. Studies by Abi-Said et al and Price et al have initially incriminated the environmental pressure imposed by exposure to Fluconazole. Other factors such as exposure to antibacterial agents, immunosuppressive therapy, and the underlying medical condition of the host might also prove to be better predictors of the distribution of *Candida* species than Fluconazole.

2.6.3 Azole resistance in diabetics:

The genus of *Candida* is commensal to the human gastrointestinal and genitourinary tract. In patients suffering with DM, an extra glucose level in the bloodstream can cause several infectious diseases ranging from superficial candidiasis to deep seated mycoses. As a major component of the culture medium, carbon source plays an important role in the growth of microorganisms including *Candida* species. Carbon compounds generally range from sugars to organic acids, alcohols, polysaccharides whereas microbes prefer simple sugars such as glucose,

sucrose, maltose and lactose for their rapid growth and these simple sugars increases the fungal population density. Fungal load or count is an important parameter of antifungal susceptibility.

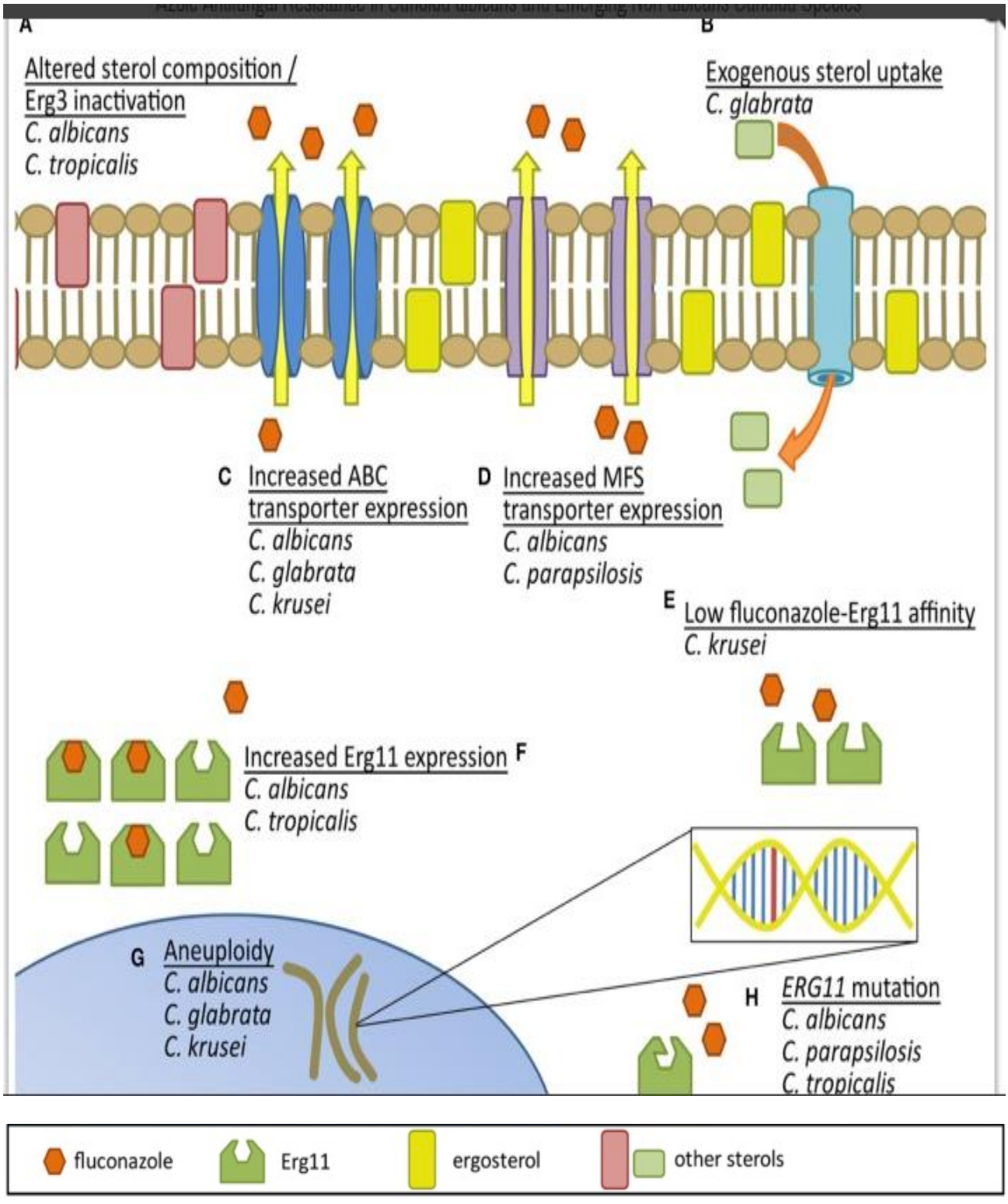
Mandal et al investigated the antifungal drug susceptibility in culture medium containing different antifungal agents in combination with fixed concentrations of glucose in each set of experiments and demonstrated the failure of azole (Voriconazole) followed by polyene (AmpB) antifungal agent in the presence of high glucose in medium. Antifungal agents showed a decreased rate of susceptibility when the glucose concentration in culture medium was increased.

Rodaki *et al.* conducted a study based upon the global impact of glucose on *C.albicans* transcriptome for the modulation of carbon assimilatory pathways during pathogenesis.The study revealed that glucose concentrations in the bloodstream have a significant impact upon *C. albicans* gene regulation which reflects on the elevated resistance to oxidative, cationic stresses and resistance to an azole antifungal agent, Miconazole. In this study, it was observed that no significant susceptibility level was observed for Anidulafungin whereas Voriconazole becomes the most resistant followed by Amphotericin B.

The in vitro antifungal susceptibility testing by Al-Attas et al revealed that the yeast isolated from the diabetic patients had different rates of resistance to the tested antifungal drugs except amophotericin B and nystatin, against which they had no resistance . In contrast, in the healthy controls, none of the isolated yeast showed any resistance to the tested antifungal agents. When patients with different types of DM were compared, there was statistically no significant difference in the antifungal susceptibility.

2.6.4 Mechanisms of Azole Resistance:

Many mechanisms of resistance to azoles have been described in Candida species. More than one mechanism can be functioning in any given fungal strain with additive effects.


A
Altered sterol composition / Erg3 inactivation
C. albicans
C. tropicalis
B
Exogenous sterol uptake
C. glabrata
C Increased ABC transporter expression
C. albicans
C. glabrata
C. krusei
D Increased MFS transporter expression
C. albicans
C. parapsilosis
E Low fluconazole-Erg11 affinity
C. krusei
Increased Erg11 expression F
C. albicans
C. tropicalis
G Aneuploidy
C. albicans
C. glabrata
C. krusei
H ERG11 mutation
C. albicans
C. parapsilosis
C. tropicalis
fluconazole
Erg11
ergosterol
other sterols

Reduced intracellular accumulation of azoles:

Interactions between sterols and phospholipids in the cytoplasmic membrane affect membrane fluidity and asymmetry and consequently influence the transport of materials across the membranes.

A decrease in the amount of azoles taken up by the fungal cell may result from changes in the sterol and/or the phospholipid composition of the fungal cell membrane. Intracellular accumulation of azoles can hence be reduced by the lack of drug penetration because of low ergosterol levels or possibly decreased ratio between phosphatidyl-choline and phosphatidyl-ethanolamine in the plasma membrane, which may change the membrane barrier function [Loeffler e al].

However, an important cause of reduced intracellular accumulation of the drug is active efflux of the drug out of the cell. In *C.albicans,* two families of drug efflux pumps are described: ATP-binding cassette (ABC) transporters and major facilitator (MF) proteins. These efflux pumps differ in their energy source for transport: ABC transporters use adenosine triphosphate (ATP) as energy source for transport; MFs use the proton gradient across the membrane as the driving force for transport. In *C.albicans,* ABC transporters are encoded by CDR (Candida drug resistance) genes, whereas the MF transporters are encoded by the MDR1 (multidrug resistance) gene. Several clinical azole-resistant isolates of *Candida* species are unable to accumulate sufficient concentrations of intracellular azoles to inhibit growth due to overexpression of genes encoding drug efflux pumps. Direct evidence for the involvement of efflux pumps in azole resistance was presented by Niimi . In this study, overexpression of CDR1 in a *C. albicans* cdr1 deletion mutant conferred resistance to Fluconazole. These molecular mechanisms were evaluated by Sanguinetti et al in 29 clinical *C.glabrata* isolates of which 20 isolates were

Fluconazole resistant and 9 were susceptible dose dependent to Fluconazole . It became apparent that the upregulation of the CgCDR1-, CgCDR2- and CgSNQ2- encoded ABC transporter proteins might explain the azole resistance in these isolates.

Alteration of membrane sterol:

Some of the earliest studies on azole resistance pointed to alterations in sterol biosynthesis other than those caused by the ERG11 gene product. Growth inhibition by azoles in *Candida* is caused by ergosterol depletion and its replacement by the toxic 14α-methyl-3, 6-diol. This growth arrest can be circumvented if 14α methylfecosterol accumulates instead. This change in sterol accumulation and azole resistance is achieved if cells are deficient in sterol Δ5,6-desaturase, encoded by the ERG3 gene . The altered sterol composition of ERG3 deletion mutants allows growth during azole treatment and the production of functional membranes Recent research by Kelly et al using 7 Itraconazole-resistant *C.dubliniensis* isolates demonstrated that mutation of their ERG3 gene and the consequent loss-of-function of Erg3p was an important mechanism of in vitro Itraconazole resistance in six out of the seven *C dubliniensis* derivatives.

Altered cell wall composition:

In Fluconazole-resistant *C.albicans* strains, a change in GAP profile in the cell wall was observed by Angiolella et al as compared to the GAP profile of susceptible *C.albicans* strains. In Fluconazole-resistant strains, the GAP proteins enolase and phosphoglyceromutase disappeared, whereas larger amounts of β-1,3-glucanase and of polydisperse, high-molecular, highly-glycosylated material appeared. Hence, this overall change from a prevalently “glycotic” one (rich in the GAP proteins enolase and phosphoglyceromutase) to a prevalently “glucanase” one may be seen as a general reaction to an inhibitor, a reaction that may be stably incorporated into

a resistant strain as a sort of anti-stress response because it offers the cell some selective advantages .

Since the glycolytic enzymes are highly antigenic, thus their decreased levels of expression in the cell wall may render the cell less antigenic, therefore helping the cell to evade the host's immune responses.(Chaffin et al)

Overproduction of the target enzyme of azoles:

Upregulation of the ERG11 gene, encoding the major target enzyme of the azoles lanosterol 14α-demethylase, has been observed in azole-resistant *C.albicans* and *C.glabrata* isolates by Chau et al. However, studies by Sanguinetti et al reported no significant change in expression levels of the ERG11 gene in azole-resistant clinical isolates of C. glabrata.

Alterations in the target enzyme of the azoles:

A successful approach to demonstrate the involvement of ERG11 mutations in Fluconazole resistance has been the heterologous overexpression of different ERG11 alleles and comparison of the susceptibility of the resulting strains to Fluconazole [Sangard e al]. The amino acid changes G129A, Y132H, S405F, G464S and R467K were shown to cause Fluconazole resistance. Direct evidence for certain mutations resulting in decreased affinity to the drug were provided by biochemical analysis of heterologously expressed enzymes. The affinity of Fluconazole for lanosterol 14α-demethylase containing the mutations Y132H, G464S or R467K was reduced as compared with the wild-type enzyme, confirming that these naturally occurring mutations indeed caused drug resistance in clinical *C.albicans* isolates [Lamb et al].

Multiple mechanisms contribute to a stepwise development of azole resistance :

Each of the mechanisms described above can cause resistance of Candida species to azoles. Several studies have shown that multiple mechanisms may be combined to result in a stepwise

development of azole resistance. Widespread and prolonged use of azoles has led to the rapid development of the phenomenon of multiple drug resistance (MDR), which poses a major hurdle in antifungal therapy.

2.7 Antifungal Susceptibility Testing:

The rising prevalence of serious fungal infections and antifungal drug resistance has created an increased demand for reliable methods of in vitro testing of antifungal agents that can assist in their clinical use.

Invitro susceptibility tests are mainly used for

1. Epidemiological surveys for determination of susceptibility profiles and resistance rates of the infecting strains against commonly used antifungal drugs at a particular center.
2. Determination of the degree of antifungal activity of the newly developed compounds
3. Prediction of clinical outcome and optimization of antifungal therapy in routine mycology laboratory practice.

Methods used for in vitro antifungal susceptibility testing of Yeasts are

2.7.1 Disk Diffusion method:

Agar disk diffusion is a simple, flexible and cost effective alternative to broth dilution testing. CLSI subcommittee has proposed a standard disk diffusion method for susceptibility testing of *Candida species* to the Fluconazole and Voriconazole. The subcommittee has established zone interpretative criteria for Fluconazole and Voriconazole .CLSI recommends MHA medium supplemented with 2% glucose and 0.5µg/ml of methylene blue over RPMI agar because of less intrazonal growth. It also recommends the inoculum size of 0.5 McFarland standard and

incubation temperature of 35^0 C for 20 to 24 hr but some strains may require 48 hr incubation. Addition of a low concentration of methylene blue (0.5μg/ml) makes the zones of inhibition clearer and easier to measure precisely.

2.7.2 Broth microdilution method.

The Clinical and Laboratory Standards Institute developed and published an approved reference method for the broth microdilution testing (CLSI document M27-A3) of *Candida* species. The standard powders of Fluconazole , Amphotericin B , Voriconazole And Caspofungin are used as antifungals with Distilled water as a solvent for Fluconazole and Caspofungin and DMSO (dimethylsulphoxide) as a solvent for water-insoluble Amphotericin B and Voriconazole. The stock solutions can be prepared at the rate of 1280 μg/mL for Fluconazole, 1600 μg/mL for Amphotericin B, 1600 μg/mL for Voriconazole, and 1600 μg/mL for Caspofungin. For the susceptibility test, RPMI 1640 (with glutamine, bicarbonate-free, and containing phenol red as the pH indicator) is used as a medium. The final concentrations should be in the range 64–0.125 μg/mL for Fluconazole, 16–0.03 μg/mL for Amphotericin B and Voriconazole, and 8–0.015 μg/mL for Caspofungin.. The results should be evaluated 24 hours later for Caspofungin and 48 hours later for Fluconazole, Amphotericin B, and Voriconazole. For Amphotericin B, the MIC endpoint is defined as the lowest drug concentration that resulted in a reduction in growth by 90% or more compared with that of a drug-free growth control well. For Fluconazole, the MIC endpoint is defined as a 50% reduction in optical density. For Caspofungin, the endpoint is given as the concentration of the drug in the assay at which 50% of growth control was observed.

2.7.3 Epsilometer test (E- test)

E-test uses a non-porous plastic strip immobilised with a predefined gradient of a given antimicrobial agent on one side and printed with an MIC on the other side. The medium that provides the best performance for E-test MICs is solidified RPMI supplemented with 2% dextrose. When the strip is placed on an inoculated agar plate, a continuous, stable and exponential antimicrobial gradient is established along the side of the strip. After incubation, the MIC value can be read directly from the MIC scale printed on the strip

2.7.4 Colorimetric and spectrophotometric methods:

A novel alternative to the standard method of visual grading of turbidity is the use of colorimetric methods for the determination of MIC endpoints. The colorimetric method using 2,3-Diphenyl-5-thienyl-(2)-tetrazolium chloride (STC) which is an oxidation-reduction indicator that, in the presence of growing organisms, changes from colorless to red is identical to the broth microdilution method with two exceptions: STC was added to RPMI 1640-morpholinepropanesulfonic acid medium with antifungal agents at a concentration of 100 μg/ml (final STC concentration, 50 μg/ml) and the solubilizing agents were added at 48hr of incubation and plates were incubated for 2 hrs. The MICs were determined by three methods: visual reading before the addition of solubilizing agents, visual reading after the addition of solubilizing agents and spectrophotometer determination after solubilization at 540 nm.

2.7.5 Flow cytometric methods:

Flow cytometric methods have been used for antifungal susceptibility testing by introducing DNA- binding vital dyes into the culture to detect fungal cell damage after exposure to an

antifungal agent. Inspite of faster results, the need for flow cytometer preclude their use in small laboratories

2.7.6 Automated system:

Vitek 2 system provides a very promising alternative to reference methods for antifungal susceptibility testing of isolates belonging to the most clinically relevant *Candida* species, thus providing fast and reliable means for detecting azole resistance. Susceptibility testing with the Vitek 2 system can be performed by preparing a standardized 2.0 McFarland inoculum suspension and then placing it in a Vitek 2 cassette along with a sterile polystyrene test tube and a Vitek 2 card containing serial twofold dilutions of Fluconazole (range, 1 to 64 μg/ml) and Voriconazole (range, 0.125 to 8 μg/ml). After the loaded cassettes are placed in the Vitek 2 instrument, the cards are filled with the appropriately diluted yeast suspensions, incubated (for a maximum of 24 h) and read automatically. The MIC results are expressed in μg/ml.

2.7.7 Candifast

The Candifast kit allows the identification of *Candida* species and testing of their susceptibility to various antifungal agents. It is a 20 well tray with two rows, one for identification and the other for susceptibility testing. The determination of the resistance of yeasts to antifungal agents is based on growth or absence of growth of the yeasts in the presence of various antifungal agents. An isolated colony of *Candida* should be inoculated into reagent-1 (R1) bottle and mixed well. The turbidity of the suspension should be then compared with the turbidity control. 100 μl of inoculated R1 was added to R2. 100 μl of R2 was then added to each of the wells, 2 drops of paraffin oil was added to each well and the test tray was sealed and incubated at 37°C for 24 hours. Reading was taken once the yeast grew in the control well. The indicator used is phenol red. A yellow or orange-yellow color in the susceptibility test row, due to glucose fermentation,

indicated that the yeast was able to grow in the presence of the antifungal agent and hence was resistant to that drug. If the color in the well was red or pink, the isolate was inhibited by the drug in that well and so was sensitive to that drug. The susceptibility testing by Candifast kit can be done for the following anti-fungal drugs: Amphotericin B (4 µg/ml), Fluconazole (16 µg/ml), Ketoconazole (16 µg/ml), Nystatin (200 Units/Ml), Flucytosine (35 µg/ml), Econazole (16 µg/ml) and Miconazole (16 µg/ml).

2.7.8 Molecular methods for detection of ERG11 mediated resistance:

Detection of ERG11 mutations by DNA sequencing:

ERG11 genes obtained from the genomic DNAs of all *C. albicans* isolates are amplified by PCR with high-fidelity *Pwo* DNA polymerase. Fragments of the expected length (1.6 kb) obtained are sequenced in order to identify the point mutations present in the *ERG11* genes of the resistant isolates. The sequences are compared with the published sequence of *ERG11* for any variation. Globally, 11 amino acid substitutions were found to be associated with a resistance phenotype: D116E, G450E, G307S, Y132F, D446N, G464S, F126L, K143R, S405F, F449S, and T229A. On the other hand, two amino acid substitutions, K128T and V437I, were confirmed to not participate in azole resistance (Perea et al).

Indirect detection of ERG11 expression by PCR:

11 amino acid substitutions were found to be associated with a resistance phenotype: D116E, G450E, G307S, Y132F, D446N, G464S, F126L, K143R, S405F, F449S, and T229A.

ERG11 expression can be evaluated by detecting point mutations which results in aminoacid substitutions using specific primers. Specific primers can be used to detect point mutation within the *ERG11* gene that results in ERG11 overexpression as done by Kelly et al and Pam et al.

ERG 11 expreesion studies by Reverse transcriptase PCR:

Total RNA was isolated using the RNeasy Mini Kit in accordance with the manufacturer's instructions. RNA quality and quantity were verified both electrophoretically and using a NanoDrop ND1000 Spectrophotometer. To avoid DNA contamination, the RNA samples were treated with RNAse-free DNase I .First-strand cDNA was synthesized from 0.5 µg total RNA using the Moloney-Murine Leukemia Virus (MMLV) reverse transcriptase and random hexamer oligonucleotides. *C. albicans* ERG11 gene was amplified from the synthesized cDNA with primers. Moreover, actin (ACT) was established as a housekeeping gene to normalize the dissimilar RNA concentrations during RNA extraction. The RT reactions were performed in triplicate in a Thermocycler . The relative expression ratio was calculated by the conventional method based on concentration of PCR products as follows: fold change in target gene expression = target/reference ratio in experimental sample relative to target/reference ratio in untreated control sample. Gene with statistically significant ($P \leq 0.05$) variation and fold changes of $\geq$ 2-fold and $\leq$ 0.5 were classified as significantly up-regulated and down-regulated, respectively (Alizadeh et al).

3. AIMS AND OBJECTIVES

The aim of this study is to

1. Isolate *Candida* from diabetic patients with symptomatic candidiasis.

2. Speciate the *Candida* isolates obtained from clinical specimens phenotypically.

3. Study the prevalence of non- *albicans Candida* among the isolates.

3. Assess the anti fungal susceptibility of the isolated species of Candida.

4. Evaluate ERG11 expression among Fluconazole resistant isolates using molecular methods.

4. MATERIALS AND METHODS

4.1 Study Period:

This is a prospective cross sectional study undertaken over period of one year from June 2017 – July 2018

4.2 Study Place:

This study was carried out in the Department of Microbiology, Department of Medicine and Department of Obstretics and Gynaecology – Tirunelveli Medical College

4.3.1 Study Group:

Diabetic patients with symptomatic candidiasis were included in this study

4.3.2 Inclusion Criteria:

Diabetic patients with symptomatic candidiasis

4.3.3 Exclusion Criteria:

- Patients who were not suffering from diabetes
- Pregnant women
- Patients who were on cancer chemotherapy
- Patients suffering from HIV/AIDS.
- Patients who were on antibiotics
- Patients who were catheterized.

4.4 Ethical clearance

The study was started after getting ethical committee clearance from the institution.

4.5 Informed consent

Informed consent was obtained from all patients included in the study.

4.6 Proforma:

The proforma was filled with the details like name, age, sex, Fasting blood glucose levels , treatment status for diabetes and other parameters significant to the present study.

4.7 Specimen Collection:

- Vulvovaginal swabs were collected using cotton tipped swab. All swabs were immediately transported to the Microbiology Laboratory of the hospital for processing.
- For urine specimen, the patients were instructed to collect 10 mL of a clean-catch midstream urine specimen into a sterile screw-capped wide-mouth container. The container was labeled with a sample number and date and time of collection, and was immediately delivered to the microbiology laboratory of Tirunelveli Medical College Hospital.

4.8 Specimen Processing:

Specimens collected were subjected to standard mycological procedures.

4.8.1 Direct Microscopic examination:

One swab was used for the direct microscopic examination by Gram stain. For each specimen , smears were made on a dry, clean glass slide, air dried and heat fixed . The fixed smears were stained by Gram staining method and observed under the oil immersion for the presence of gram positive budding yeast cells with or without pseudohyphae

4.8.2 Culture on Sabourauds Dextrose Agar:

The culture medium used was Sabourauds Dextrose Agar with pH 5.6.Second swab was inoculated immediately into the plates and incubated at 28^0C and 37^0C for 24-72 hours. Isolates were identified by colony morphology on SDA plates. Growth appeared in 2 to 3 days as

creamy, white pasty colonies .The microscopic morphology of the colony on Gram stain was noted.

4.8.3 Species identification:

Isolates were speciated based on the following tests:

Germ Tube Test:

- 0.5ml of human serum was taken in a sterile test tube.
- A small portion of an isolated colony of the yeast to be tested was inoculated into the human serum.
- The test tube was incubated at 37^0C for two hours.
- After two hours of incubation, a drop of the yeast serum suspension was placed on a microscopic slide , overlaid with a cover slip and examined for the presence of germ tube under lowpower microscope
- If the test was positive within two hours, the isolate was considered as *Candida albicans / Candida dubliniensis*

Growth at 42^0 C:

All germ tube positive isolates were subcultured on SDA and incubated aerobically at 42^0C, to distinguish between *Candida albicans* and *Candida dubliniensis*

Candida albicans : Growth of candida present

Candida dubliniensis : Scanty / Absent

Corn Meal Agar (Dalmau Plate Culture Technique):

- An isolated colony from the primary culture media was taken using a straight wire and inoculated into cornmeal agar plate by making three parallel lines about half an inch apart at an 45^0 angle to the culture media.

- A sterile cover slip was placed over the surface of the agar, covering a portion of the inoculated streaks.
- Plate was incubated at 28^0C for 48 hours.
- After 48 hours , the areas where the cuts into the agar made, were examined first under the low power objective and then under the high power objective for the presence of Hyphae - true or pseudohyphae, Blastoconidia, Arthroconidia and Chlamyoconidia

Candida CHROM agar

- Isolates were subcultured on Sabourauds Dextrose agar prior to inoculation on chromogenic media.
- A single yeast colony was streaked onto the plates.
- Plates were incubated at 37^0 C in the dark.
- The results were read after 48 hours and the colour of the colony was noted.
- This helps in the presumptive identification of most commonly isolated *Candida species* especially *Candida albicans, Candida tropicalis, Candida krusei.*

Candida albicans - Light green

Candida tropicalis - Steel blue

Candida krusei – Whitish Pink

Candida parapsilosis - Purple

Candida glabrata – Cream coloured

Candida dubliniensis - Dark green

Sugar Assimilation test:

- A yeast suspension was made from a 24-48 hrs culture grown in a sugar free media, in to 2 ml of Yeast Nitrogen base by adding heavy inoculums.

- The suspension was added to 18 ml of molten agar cooled to 45^0 c and mixed well and the entire volume was poured in to a 90 mm sterile petri plate.
- The plate was allowed to set at room temperature until the agar surface hardens.
- With the help of sterile forceps, carbohydrate discs like glucose, sucrose, lactose, maltose, xylose and trehalose were placed on the surface of the inoculated agar.
- The plates were incubated at 30^0C for 48-96 hrs hr and were observed for the growth of yeast around the sugar discs, indicating assimilation of that particular carbohydrate.
- Each *Candida species* utilizes specific carbohydrate substrate and the characteristic carbohydrate profiles were used to identify the species(Annexure)

4.8.4 Antifungal Susceptibility testing:

Antifungal susceptibility test for the *Candida* isolates was done by Disk Diffusion method according to The National Committee for Clinical Laboratory Standards (NCCLS) *Method for Antifungal Disk Diffusion Susceptibility Testing of Yeasts; Approved Guideline.*NCCLS document M44-A

Inoculum Preparation:

- Inoculum was prepared by Direct Colony Suspension Method
- All organisms are subcultured onto Sabourauds Dextrose agar to ensure purity and viability and incubated at 35^0C.
- Inoculum was prepared by picking five distinct colonies of approximately 1 mm in diameter from the 24-hour-old culture of *Candida* species.
- Colonies were then suspended in 5 mL of sterile 8.5 g/L NaCl; (0.85% saline) and the resulting suspension was vortexed for 15 seconds and its turbidity was adjusted visually by adding sufficient sterile saline or more colonies to adjust the transmittance to that

produced by a 0.5 McFarland standard to yield a yeast stock suspension of 1 x 10^6 to 5 x 10^6 cells per ml which produced a semi-confluent growth with most *Candida* species isolates.

Inoculation of Test Plates:

- Within 15 minutes after adjusting the turbidity of the inoculum suspension, a sterile cotton swab was dipped into the suspension.
- The swab was rotated several times and pressed firmly against the inside wall of the tube above the fluid level to remove excess fluid from the swab.
- The dried surface of a sterile Mueller-Hinton + GMB agar plate was inoculated by evenly streaking the swab over the entire agar surface. This procedure was repeated by streaking two more times, rotating the plate approximately 60° each time to ensure an even distribution of inoculums and then the rim of the agar was swabbed.
- The lid was left ajar for three to five minutes to allow for any excess surface moisture to be absorbed before applying the drug-impregnated disks.

Application of Disks to Inoculated Agar Plates:

- Antimicrobial disks were dispensed onto the surface of the inoculated agar plate and each disk was pressed down to ensure its complete contact with the agar surface.
- The disks were placed individually so that they are no closer than 24 mm from center to center.
- The plates were inverted and placed in an incubator set to 35^0C (± 2^0C) within 15 minutes after the application of discs
- *Candida albicans* ATCC 90028 was used as the Quality control strain

Reading Plates and Interpreting Results:

- Each plate was examined after 20 to 24 hours of incubation and the resulting zones of inhibition were uniformly circular with a semiconfluent growth.
- The plate was held a few inches above a black, nonreflecting background illuminated with reflected light to measure the zone diameter to the nearest whole millimeter at the point at which there was a prominent reduction in growth.
- Pinpoint microcolonies at the zone edge or large colonies within the zone were ignored and the zones were interpreted as per NCCLS document M44-A(Annexure)

4.8 Detection of ERG11 Expression Using PCR:

PCR Requirements:

- PureFast® Bacterial/fungal DNA minispin purification kit -Lysozyme ,Lysozyme digestion buffer, Proteinase-K, Binding buffer, Wash Buffer-1, Wash Buffer-2, Spin columns with collection tube and elution buffer.
- HELINI 2X ReD dye
- ERG11 gene Primers were from HELINI Biomolecules, Chennai, India -5µl/reaction
- **2X Master Mix:** 2U of Taq DNA polymerase, 10X Taq reaction buffer, 2mM MgCl2, 1µl of 10mM dNTPs mix and Red Dye PCR additives.
- **Agarose gel electrophoresis consumables:** Agarose, 50X TAE buffer (Tris base, acetic acid and EDTA), 6X gel loading buffer and Ethidium bromide were from HELINI Biomolecules, Chennai.

PCR Product: 273bp

Candida DNA Purification:

1. 1ml of culture centrifuged at 6000rpm for 5min and supernatant discarded

2. Pellet was suspended in 0.2ml PBS.

3. 180µl of Lysozyme digestion buffer and 20µl of Lysozyme [10mg/ml] added and incubated at 37^0C for 15min.

4. 400µl of Binding buffer, 5µl of internal control template and 20µl of Proteinase K added and mixed well by inverting several times and incubated at 56^0C for 15min.

5. Then 300µl of Ethanol was added and mixed well.

6. Then the entire sample was transferred into the PureFast® spin column and centrifuged for 1 min, discarded the flow-through and placed the column back into the same collection tube.

7. Added 500µl Wash buffer-1 to the PureFast® spin column. Centrifuged for 30-60 seconds and discarded the flow-through and placed the column back into the same collection tube.

8. Added 500µl Wash buffer-2 to the PureFast® spin column. Centrifuged for 30-60 seconds and discarded the flow-through and then place the column back into the same collection tube.

9. Discarded the flow-through and centrifuged for an additional 1 min. This step was done to avoid residual ethanol.

10. Transferred the PureFast® spin column into a fresh 1.5 ml micro-centrifuge tube.

11. Added 100µl of Elution Buffer to the center of PureFast® spin column membrane.

12. Incubated for 1 min at room temperature and centrifuged for 2 min.

13. Discarded the column and stored the purified DNA at -20°C. Quality and quantity of extracted DNA was checked by loading in 1% agarose gel and 5µl of extracted DNA was used for PCR amplification.

PCR Procedure:

1. Reactions were set up as follows;

Components Quantity

- HELINI RedDye PCR Master mix 10µl
- HELINI Ready to use - Primer Mix 5µl
- Purified Purified DNA 5µl
- Total volume 20µl

2. Mixed gently and spun down briefly.

3. Placed into PCR machine and programmed it as given in PCR setup

PCR setup:

Initial Denaturation: 95ºC for 5 min

Denaturation: 94ºC for 30sec

Annealing: 58ºC for 30sec 35 cycles

Extension: 72ºC for 30sec

Final extension: 72º C for 5 min

Loading:

1. Prepared 2% agarose gel. [2gm of agarose in 100ml of 1X TAE buffer]

2. Ran electrophoresis at 50V till the dye reaches three fourth distances and observed the bands in UV Transilluminator.

Agarose gel electrophoresis:

1. Prepared 2% agarose gel (2gm agarose in 100ml of 1X TAE buffer and melted using microoven)

2. When the agarose gel temperature was around 60ºC, 5µl of Ethidium bromide was added.

3. Poured warm agarose solution slowly into the gel platform.

4. Kept the gel set undisturbed till the agarose solidified.

5. Poured 1X TAE buffer into submarine gel tank.

6. Carefully placed the gel platform into tank and maintained the tank buffer level 0.5cm above the gel.

7. PCR Samples were loaded after mixed with gel loading dye along with 10µl HELINI 100bp DNA Ladder. [100bp, 200bp, 300bp, 400bp, 500bp, 600bp, 700bp, 800bp, 900bp, 1000bp and 1500bp]

8. Ran electrophoresis at 50V till the dye reaches three fourth distance of the gel.

9. Gel viewed in UV Transilluminator and observed the band pattern.

5. RESULTS

A total of 102 specimens were collected from diabetic patients with symptomatic candidiasis. Out of these 102 specimens, 56 were vaginal specimens from diabetic females and 46 were urine specimens from diabetic males.

From these samples, a total of 29 *Candida* isolates were obtained and then speciated based on germ tube test, sugar assimilation, Dalmau plate technique and CHROM agar. These *Candida species* were subjected to antifungal susceptibility testing by disc diffusion method using the following antifungal agents: Fluconazole, Voriconazole and Itraconazole. The isolates which were found to be resistant to Fluconazole were further subjected to ERG11 PCR.

Data collected were entered in Microsoft Excel and analysed using statistical analysis software Statistical Package for Social Services (SPSS) v.16. Appropriate statistics were calculated. p values <0.05 were statistically significant.

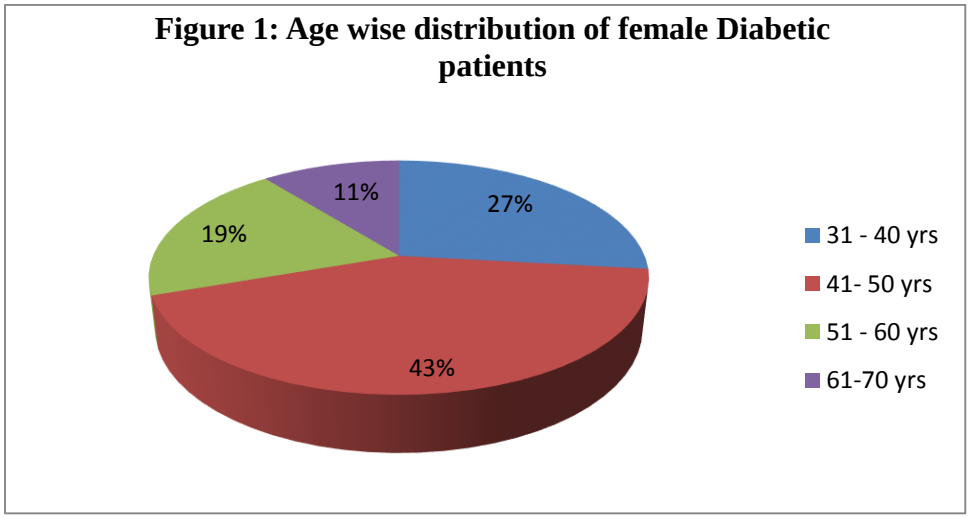
5.1. Description of study Group:

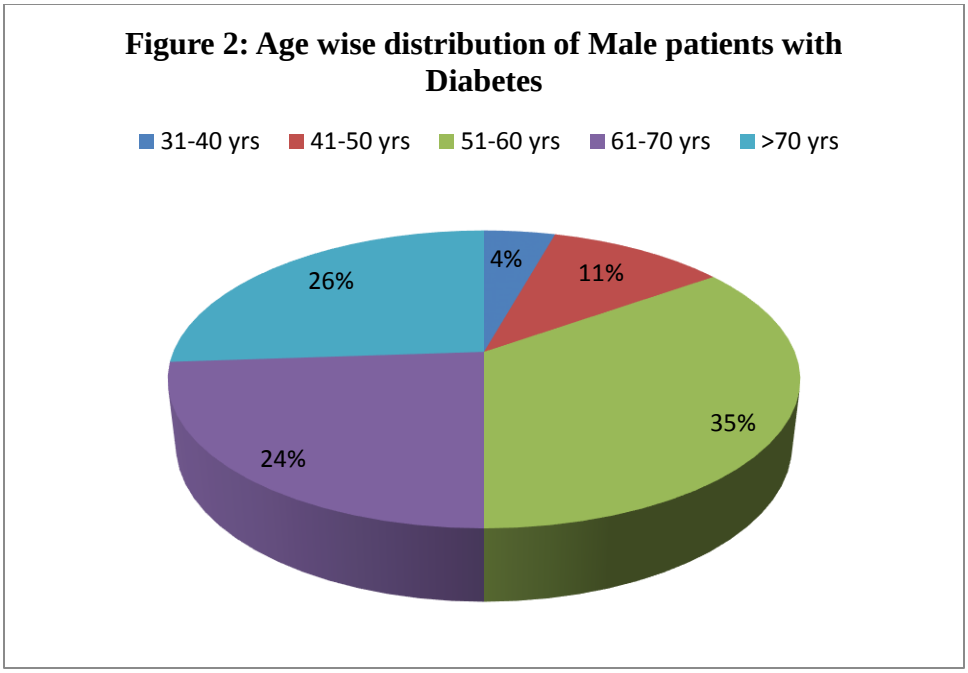
5.1.1. Age and gender distribution of the study population:

The mean age of the diabetic patients from whom *Candida* were isolated was 53.7 years with a SD of 12 years. The mean age of the male diabetic patients (61.8 years with SD 10 years) was significantly higher than that of female diabetic patients (47 years with SD 8.9 years) (p value < 0.001, Independent sample t test).

Table 1: Age and gender wise distribution of study group

AGE (in years)	Male patients with Diabetes		Feale patients with Diabetes		Total	
	No.	%	No.	%	No	%
31 - 40	2	4.3	15	26.8	17	16.7
41 - 50	5	10.9	24	42.9	29	28.4
51 - 60	16	34.8	11	19.6	27	26.5
61-70	11	23.9	6	10.7	17	16.7
>70	12	26.1	-	-	12	11.8
TOTAL	**46**	**100**	**56**	**100**	**102**	**100**





5.1.2. Symptoms of the study population:

The most common symptom of candidiasis among male diabetic patients was found to be dysuria (63%) and that among female diabetic patients was found to be vaginal discharge (66%) followed by pruritus and burning sensation.

Table 2: Symptoms of the study population

Female Diabetic patients		**Male Diabetic patients**	
Symptoms	**Frequency**	**Symptoms**	**Frequency**
Vaginal discharge	20	Dysuria	29
Pruritis and Burning sensation	26	Frequency and urgency	11
Vaginal discharge, Pruritis and Burning sensation	10	Dysuria ,Frequency and urgency	6
Recurrence of symptoms	3		

Figure 3: Distribution of female Diabetic patients based on their symptoms

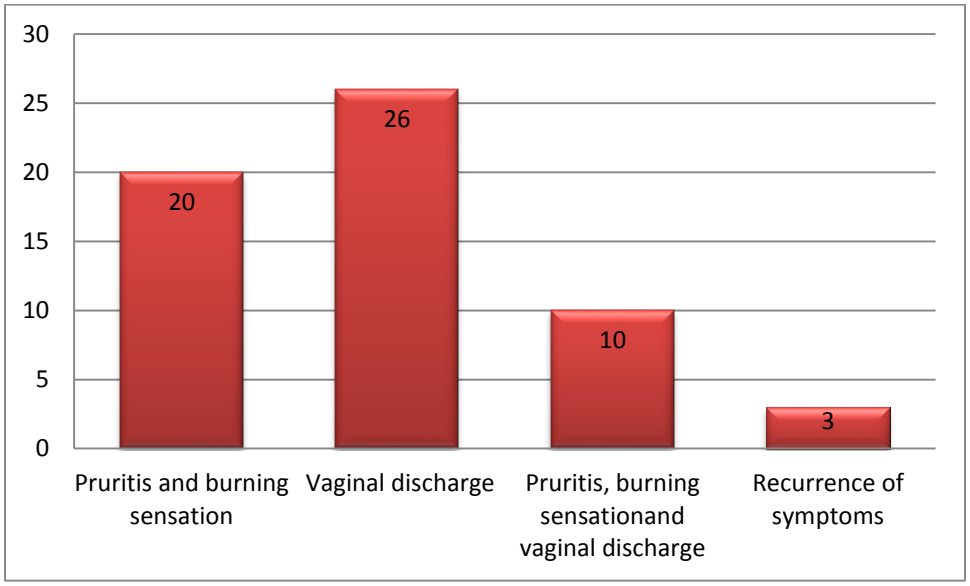
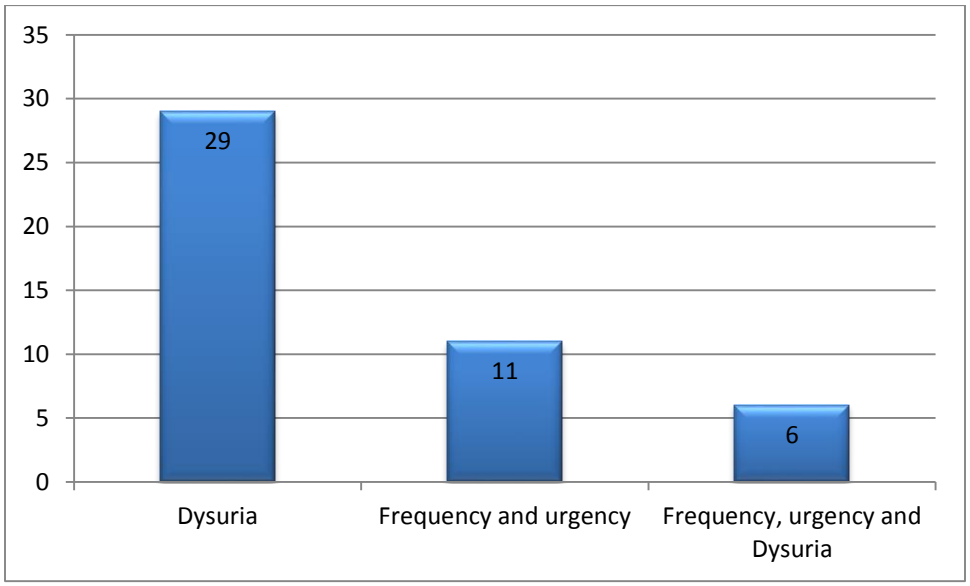


Figure 4: Distribution of male Diabetic patients based on their symptoms



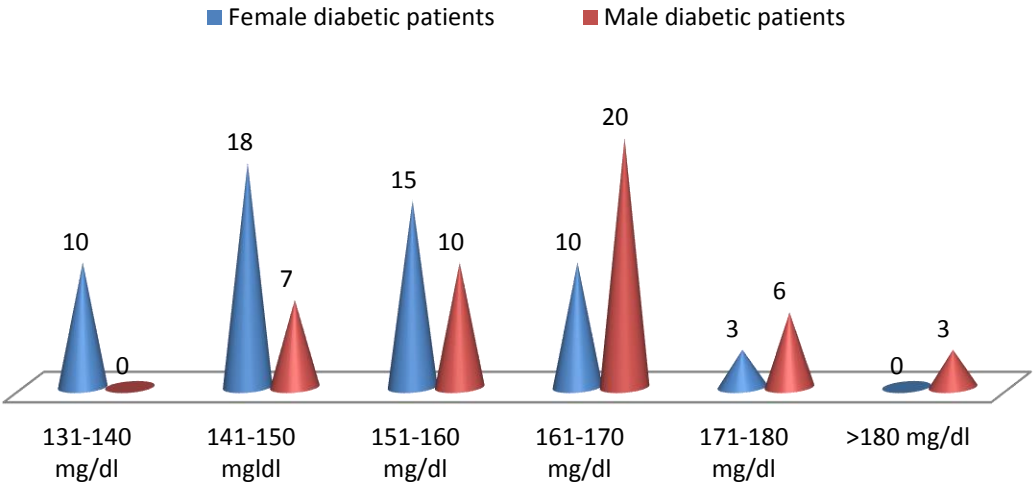
5.1.3. Fasting blood glucose level of the study population:

None of the diabetic population among the study population had fasting blood glucose level under control (i.e. <126mg/dl). The mean fasting blood glucose level of the diabetic patients was found to be 157 mg/dl (SD 12 mg/dl).

Table 3: Distribution of study group based on FBS level

Fasting blood glucose level in mg/dl	**Female Diabetic patients (N = 56)**		**Male Diabetic patients (N=46)**		**Total (N=102)**	
	Frequency	**%**	**Frequency**	**%**	**Frequency**	**%**
131-140	10	17.86	-	-	10	9.8
141-150	18	32.14	7	15.22	25	24.5
151-160	15	26.78	10	21.74	25	24.5
161-170	10	17.86	20	43.48	30	29.4
171-180	3	5.36	6	13.04	9	8.8
>180	-	-	3	6.52	3	2.9
Mean FBS	**152 mg/dl (SD – 11 mg/dl)**		**162 mg/dl (SD – 11 mg/dl)**			

Figure 5: Distribution of study group based on FBS levels



Fasting blood glucose level showed a statistically significant relationship with the age of the diabetic patients. It could be seen from the means plot (below) that the FBS levels steadily increases with increase in age of the diabetic patients (p value <0.001, One way ANOVA). There exist a significant positive correlation between the age of the patient and FBS levels (see scatter plot).

Figure 6: Means plot showing mean FBS levels among various age groups

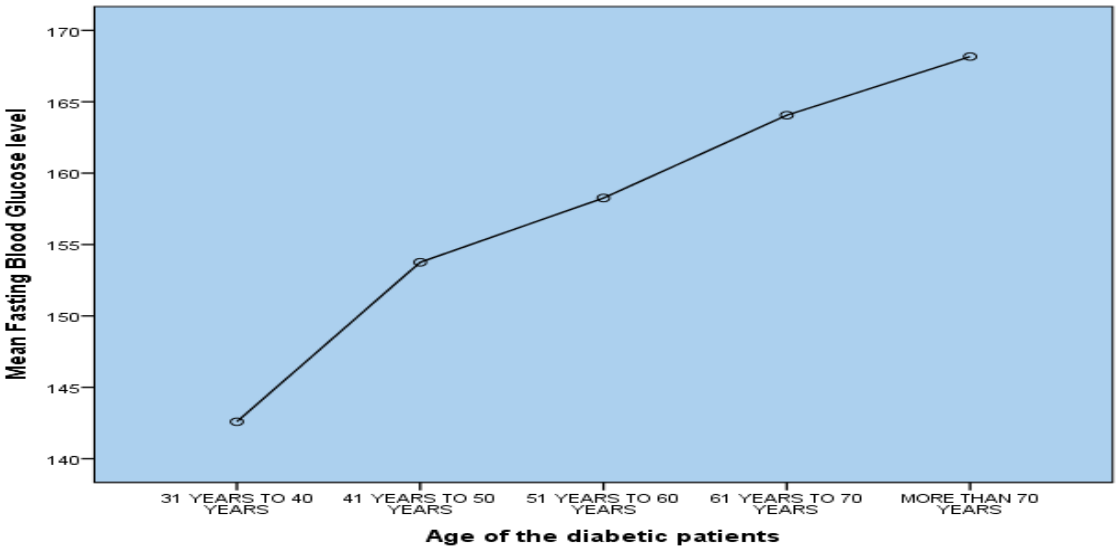
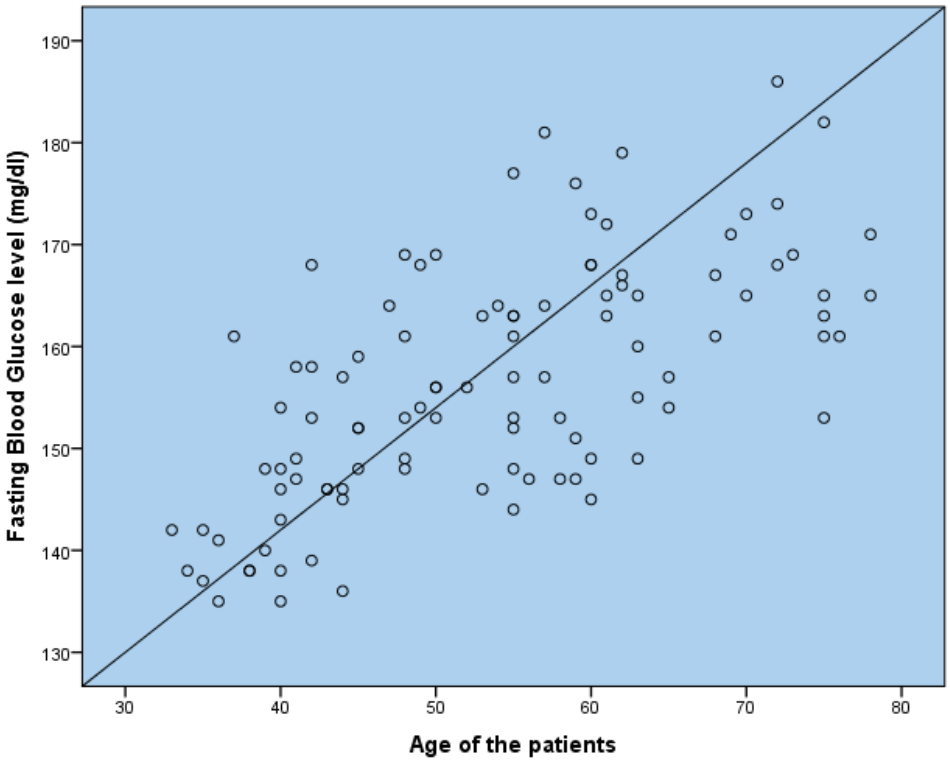


Figure 7: Scatter plot showing FBS levels among various age groups



5.1.4. Treatment status of the study population:

Only 47% of the diabetic patients were on treatment. The mean age of the diabetic patients on treatment (62 years with SD 9.5 years) was found to be significantly higher than that of the diabetic patients who were not on treatment (46 years with SD 8.6 years) (**p value < 0.001**).

Table 4: Distribution of Diabetic study group based on their treatment status for Diabetes

Treatment status	**On treatment**		**Not on treatment**		**Test**	**p value**
	No.	%	No.	%		
Male	29	63	17	37	Chi square test, χ^2 = 8.593 df = 1	**0.005** (significant)
Female	19	34	37	66		
Total	48	47	54	53		
Mean age (in years) (SD)	62 (9.5)		46 (8.6)		Independent sample t test	**<0.001** (significant)

5.2. Isolation of Candida:

5.2.1. Gram Staining:

On gram staining, 19 smears were found to be positive for *Candida*. Out of the 19 positive smears, 15 were vaginal swab smears and only 4 were urine smears. 5 smears were positive for budding yeasts with pseudohyphae, 14 for budding yeasts only and 83 were found to be negative. Nearly 27% of the vaginal swabs of the vaginal smears were positive for *Candida* on gram staining, whereas only 8.6% of the urine smears were positive for *Candida* on gram staining.

Table 5: Gram stain results of vaginal and urine smears

Smear	Gram staining		
	Budding yeasts with pseudohyphae	Budding yeasts only	Negative
Vaginal swabs (n = 56)	3 (5.4%)	12 (21.4%)	41 (73.2%)
Urine (n = 46)	2 (4.3%)	2 (4.3%)	42 (91.3%)
Total (N = 102)	**5 (4.9%)**	**14 (13.7%)**	**83 (81.4%)**

Figure 8 :Gram stain results of vaginal swab smears of female Diabetics

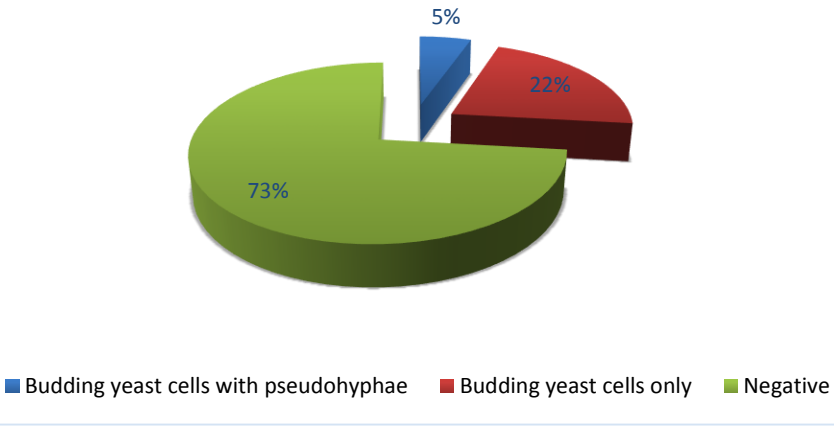
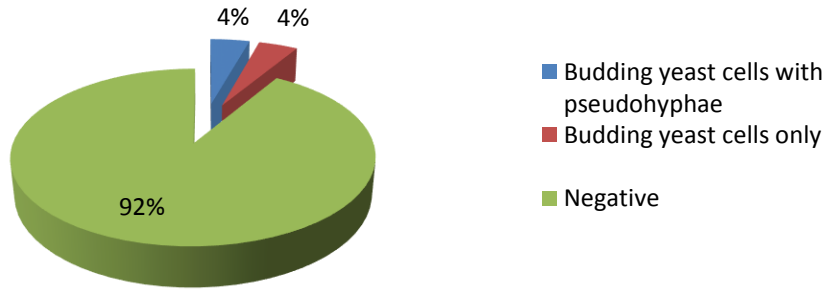


Figure 9: Gram stain results of urine smeras of male Diabetic patients



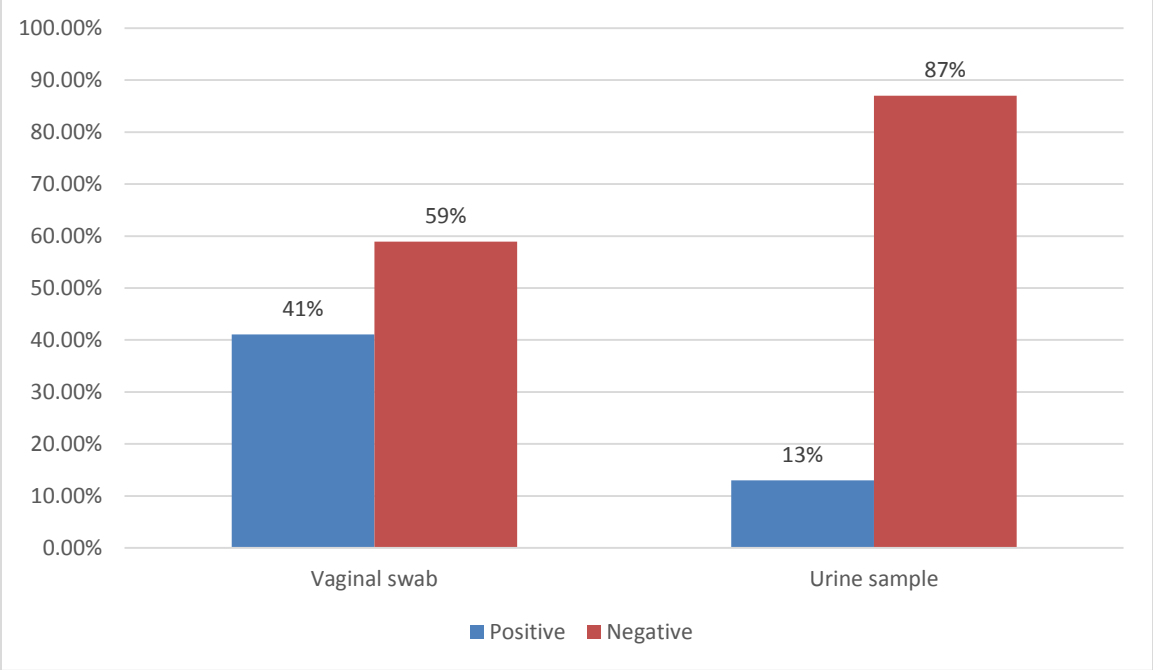
5.2.2. Culture:

A total of 29 samples were found to be positive for *Candida* on culture. Around 41% of vaginal swabs were positive whereas only 13% of the urine samples were found to be positive for *Candida* by culture.

Table 6: Culture results for *Candida* among the study group

Smear	Culture results	
	Positive	**Negative**
Vaginal (n = 56)	23 (41.1%)	33 (58.9%)
Urine (n = 46)	6(13%)	40(87%)
Total (N = 102)	**29 (28.4%)**	**73 (71.6%)**

Figure 10: Prevalence of VVC and Candiduria among Diabetic females and males



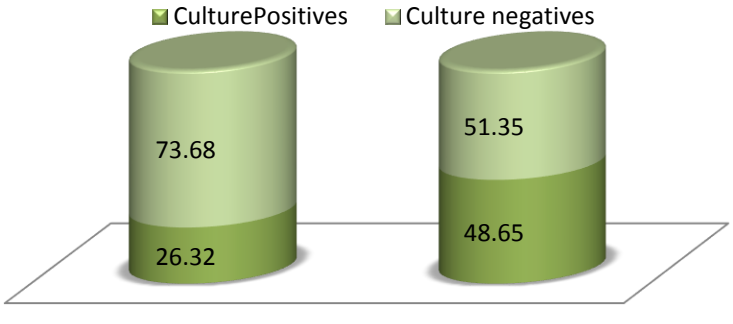
5.2.3. Culture results Vs treatment status:

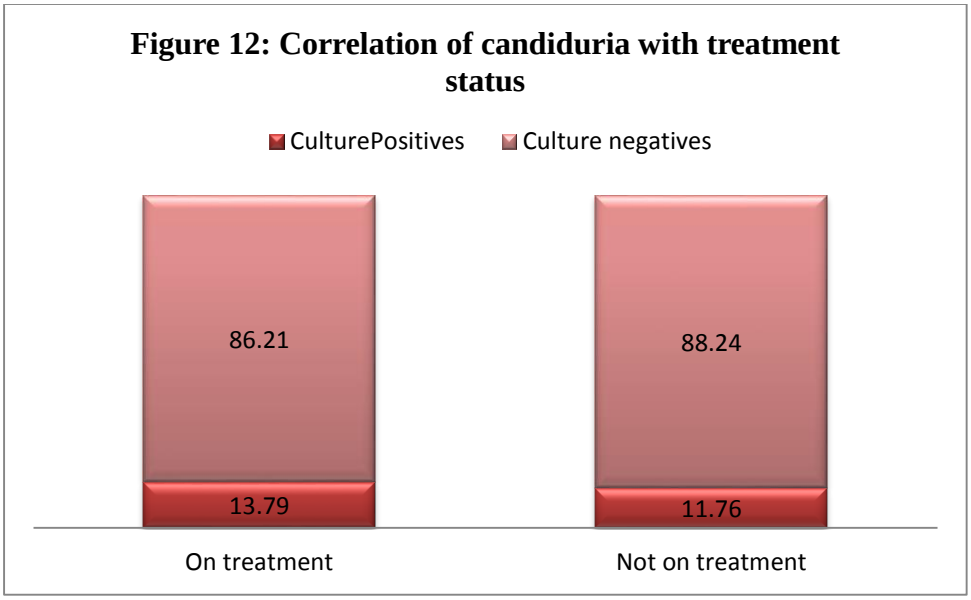
The culture results for *Candida* did not show any significant association with age and fasting blood glucose levels of the diabetic patients. However, there is significant relationship between the culture results and the treatment status of the patients. Nearly 37% of the diabetic patients who were not under treatment had candidiasis, whereas only 19% of the diabetic patients on treatment had candidiasis.

Table 7: Treatment for Diabetes Vs Candidiasis

Treatment status	Culture		Total	Test	p value
	Positive	Negative			
On treatment	9 (18.8%)	39 (81.3%)	48	Chi square test, $\chi^{2\ value}$ = 4.176 df = 1	**0.04 (significant)**
Not on treatment	20 (37%)	34 (63%)	54		
Total	29	73	102		

Figure 11: Correlation of Vulvovaginal candidiasis with treatment status





5.2.4. Gram stain Vs Culture

Culture results showed that *Candida* was isolated from nearly 28.4% of the specimens collected from diabetic patients, whereas gram staining found only 18.6% of the specimens to be positive for *Candida.*

Table 8: Isolation of *Candida*: Gram stain Vs culture

Gram stain	Culture		Total (N=102)
	Positive	Negative	
Positive	19 (65.5%)	0	19
Negative	10 (34.5%)	73 (100%)	83
Total	29	73	102

The above table shows that only 65.5% of specimens found to be culture positive were identified as positive for *Candida* on gram staining. Whereas, all specimens found to be culture negative were found to be negative for *Candida* on gram staining.

This shows that the gram staining is highly specific (100%) and only 65.5% sensitive in isolating *Candida*.

Figure 13: Gram stain Vs Culture for detection of *Candida* among vaginal samples

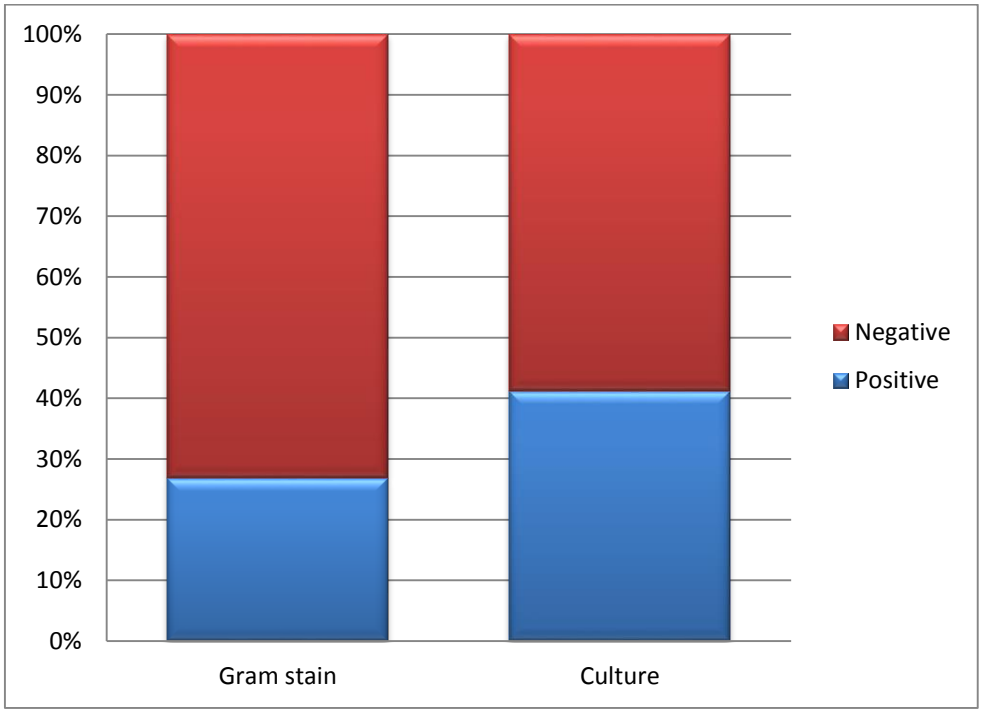


Figure 14: Gram stain Vs Culture for detection of *Candida* among urine samples

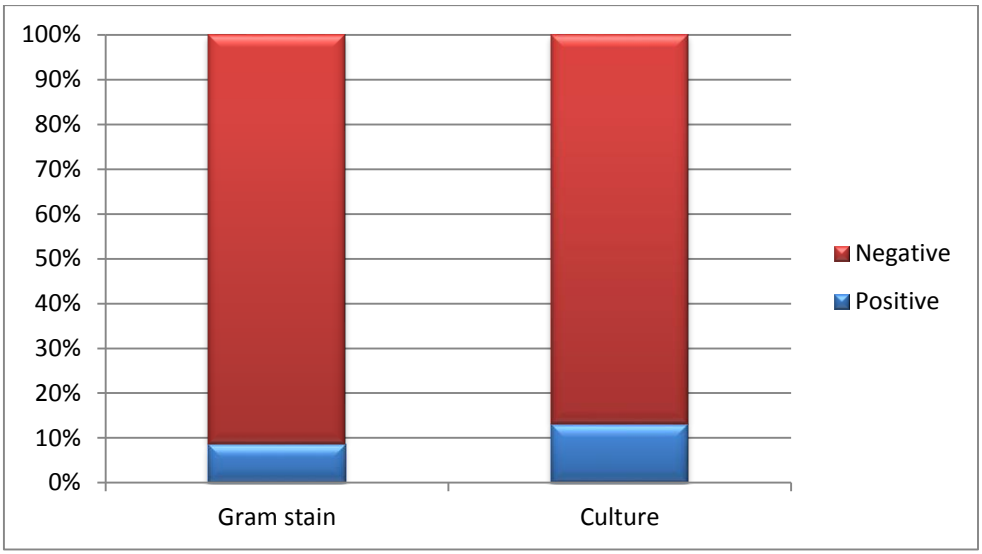


Table 9: Comparison of culture positivity with FBS levels among Diabetics

Fasting blood glucose level in mg/dl	Vulvovaginal candidiasis		Candiduria	
	Culture Positives	Culture negatives	Culture Positives	Culture negatives
130-140	4 (40%)	6 (60%)	-	-
141-150	6 (33.3%)	12 (66.7%)	-	7 (100%)
151-160	8 (53.3%)	7 (46.7%)	-	10 (100%)
161-170	4 (40%)	6 (60%)	4 (20%)	16 (80%)
171-180	1 (33.3%)	2 (66.7%)	2 (33.3%)	4 (66.7%)
>180	-	-	-	3 (100%)

The above table shows that that 66.7% of female diabetics with the FBS range of 141-150mg/dl and 171-180mg/dl were culture negatives in this study. Majority (53.3%) of culture positives were in the FBS range of 151-160mg/dl. In case of diabetic males, only 33.3% and 20% of those with the FBS range of 171-180mg/dl and 161-170mg/dl respectively had candiduria and the rest were culture negatives.

5.3. Speciation of Candida:

Speciation of *Candid*a was done by using CHROM agar, Dalmau plate technique, Germ tube test and sugar assimilation tests.38% of the culture positive isolates were found to be *Candida albicans*. Remaining 62% were non-*albicans Candida* which included *C.tropicalis* (24%), *C.parapsilosis* (10.3%), *C.glabarata* (20.7%) and *C. krusei* (7%).

Table 10: Distribution of *Candida* species among male and female diabetics

Species	**Vulvovaginal candidiasis**		**Candiduria**		**Total**	
	No.	%	No.	%	No	%
Candida albicans	9	39.1	2	33.3	11	37.9
Candida tropicalis	6	26.1	1	16.7	7	24.1
Candida glabrata	4	17.4	2	33.3	6	20.7
Candida parapsilosis	2	8.7	1	16.7	3	10.3
Candida krusei	2	8.7	-	-	2	6.9
Total	23	100	6	100	29	100

Figure 15 : *Candida albicans Vs Non-albicans* among Genitourinary candidiasis

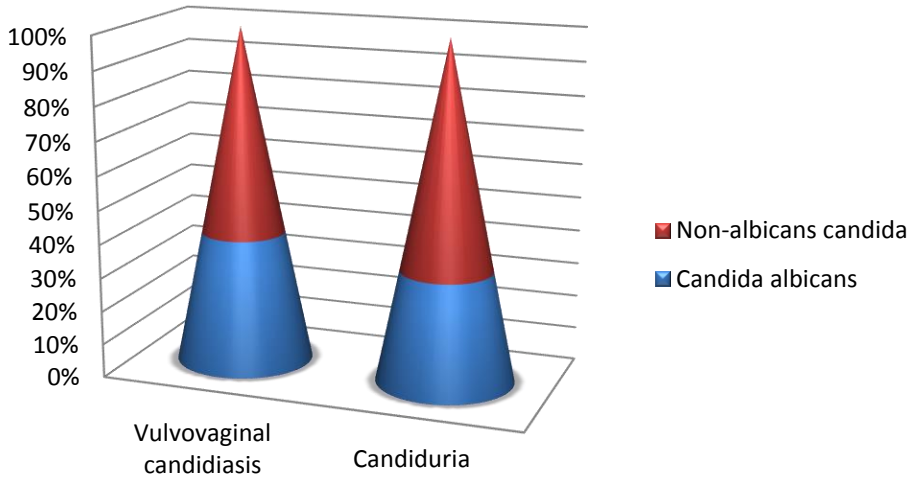


Figure 16 : Distribution of *non albicans candida* among Vulvovaginal candidiasis

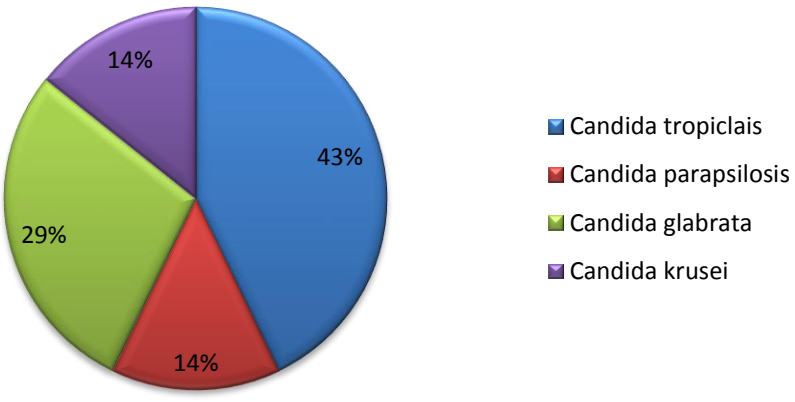
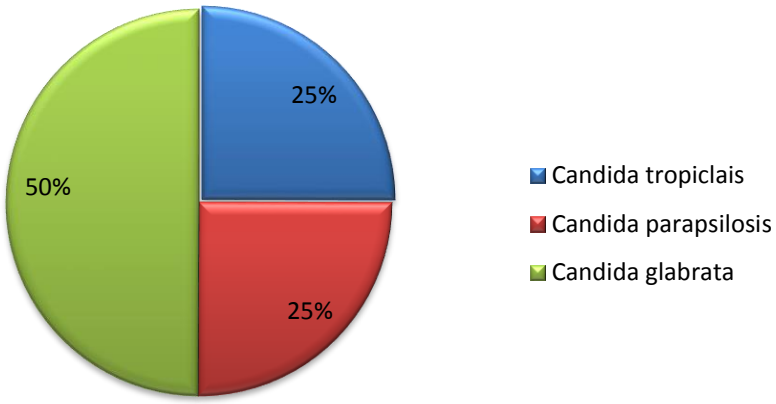


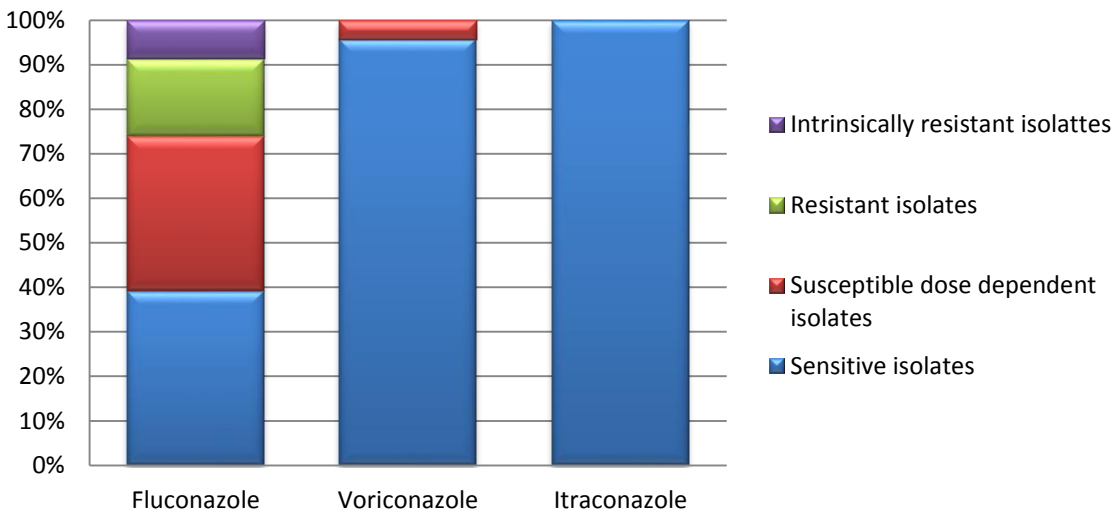
Figure 17 : Distribution of *non albicans candida* among Candiduria



5.4. Anti Fungal Susceptibilty:

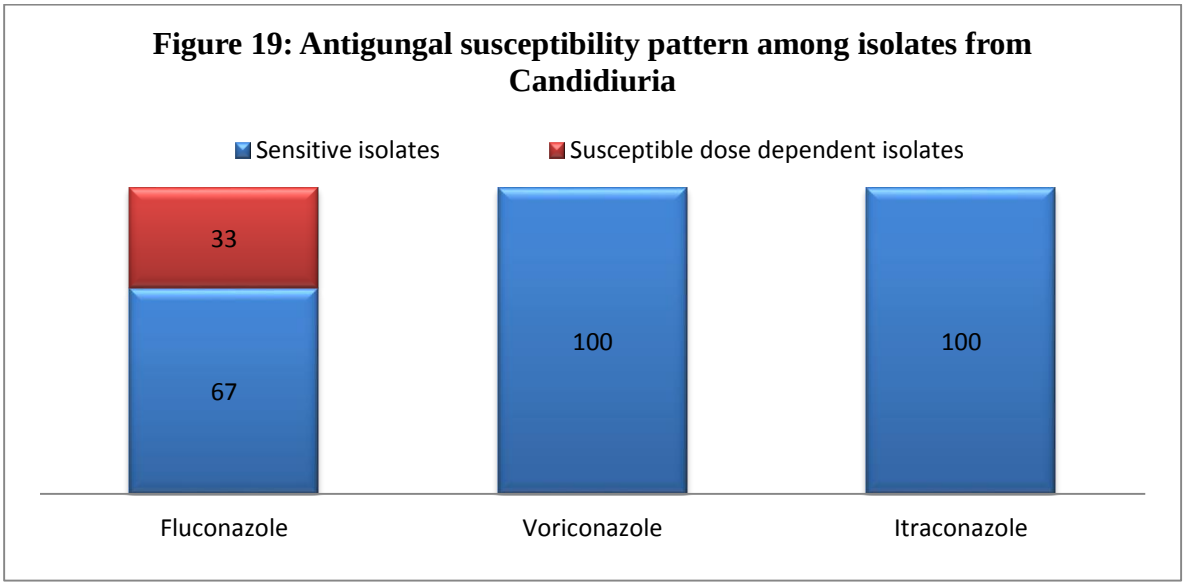
All the 29 *Candida* isolates were subjected to antifungal susceptibility testing by disc diffusion method using Fluconazole,Voriconazole and Itraconazole. Highest level of sensitivity (100%) was seen for Itraconazole followed by Voriconazole. Highest level of resistance (17.4%) was seen against Fluconazole. All the isolates were found to be sensitive to Itraconazole. 28 isolates were sensitive to Voriconazole and one isolate was susceptible dose dependent to Voriconazole.

Figure 18: Antigungal susceptibility pattern among isolates from Vulvovaginal candidiasis



Among the 6 *Candida* isolates obtained from candiduria , 4(66.7%) were sensitive and 2(33.3%) were Dose dependent susceptible to Fluconazole. All the 6 isolates were sensitive to both Itraconazole and Voriconazole.

Figure 19: Antigungal susceptibility pattern among isolates from Candidiuria



5.4.1. Fluconazole resistance Vs *Candida species*

Figure 20: Fluconazole susceptibility pattern of *Candida albicans and non- albicans Candida* isolates of Vulvovaginal candidiasis

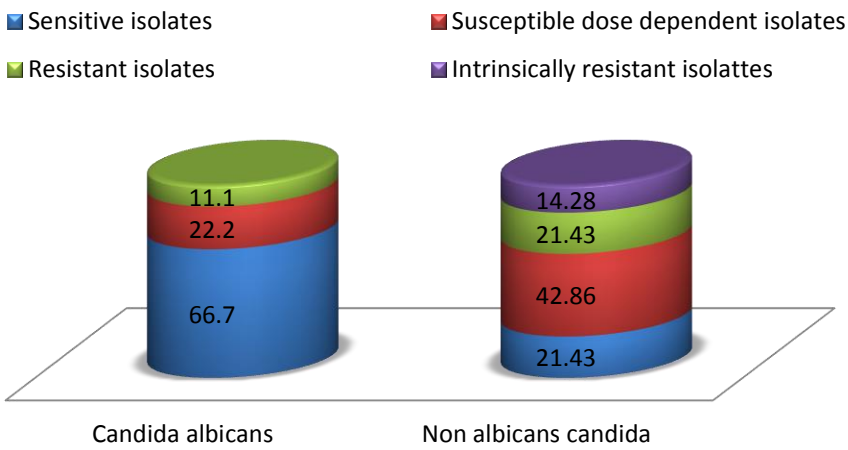


Table 11: Fluconazole susceptibility pattern of isolates of Vulvovaginal candidiasis

Candida species	**Sensitive**	**Susceptible Dose Dependent**	**Resistant**
Candida albicans	6(66.7%)	2(22.2%)	1(11.1%)
Candida tropicalis	2(33.3%)	1(16.7%)	3(50%)
Candida glabrata	-	4(100%)	-
Candida parapsilosis	1(50%)	1(50%)	-

5.4.2. Fluconazole resistance Vs Treatment status and FBS

Resistance to Fluconazole was high among those who were not on treatment. None of the diabetic patients on treatment was found to be resistant to Fluconazole, whereas nearly 17.4 % of the isolates from patients not on treatment were found to be resistant to Fluconazole (p value – 0.40, Fishers exact test). Resistance to Fluconazole did not show any significant relationship with fasting blood glucose (p value – 0.36, Independent sample t test).

Figure21 : Comparison of Fluconazole susceptibility pattern with Diabetic treatment status among Vulvovaginal candidiasis patients

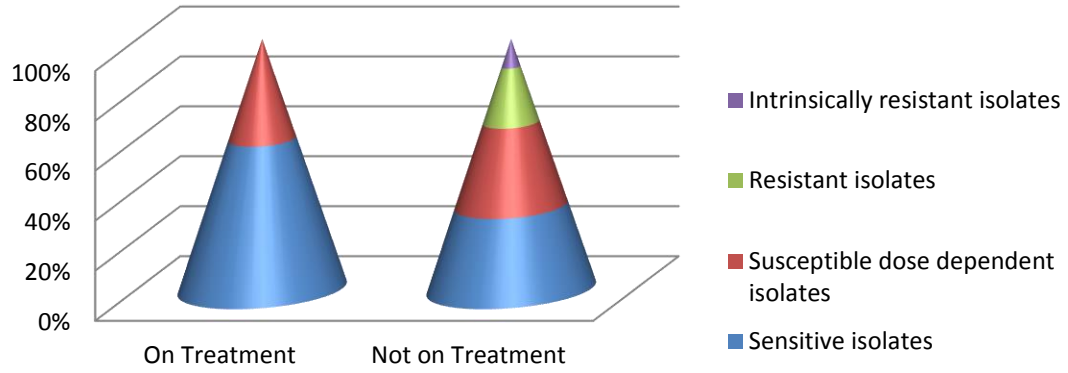
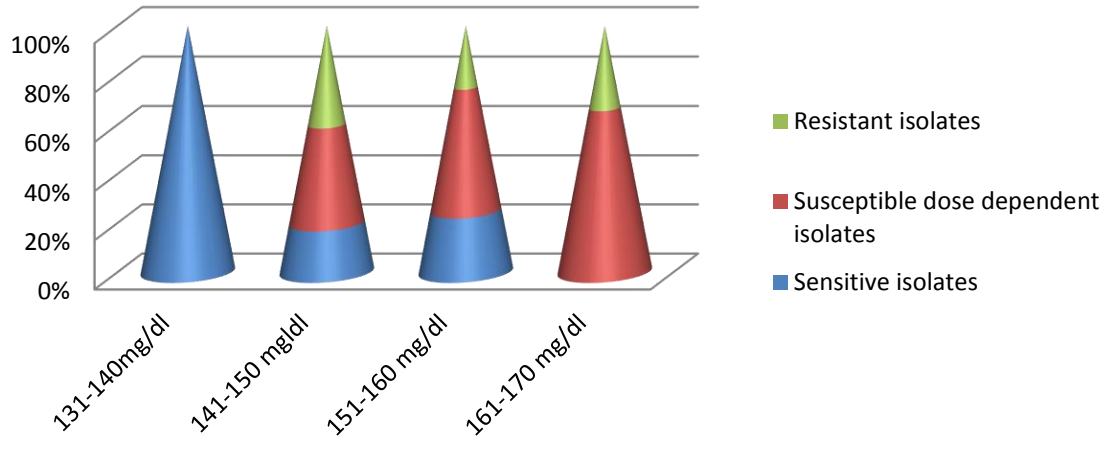


Figure 22 : Correlation of Fluconazole susceptibility to FBS levels of diabetic women who were not on Treatment



The 4 Fluconazole resistant isolates were isolated from patients who were not on any form of treatment for diabetes. Among the 4 isolates , one of them was *Candida albicans* (25%) and the rest were *Candida tropicalis* (75%).

5.5 Detection of Mechanism of Resistance Using PCR:

Table 12: ERG11 PCR in Fluconazole resistant isolates

Fluconazole resistant isolates(n=4)	**PCR**	
	Positives	Negatives
Candida albicans(n=1)	-	1 (100%)
Candida tropicalis(n=3)	1(33%)	2 (67%)

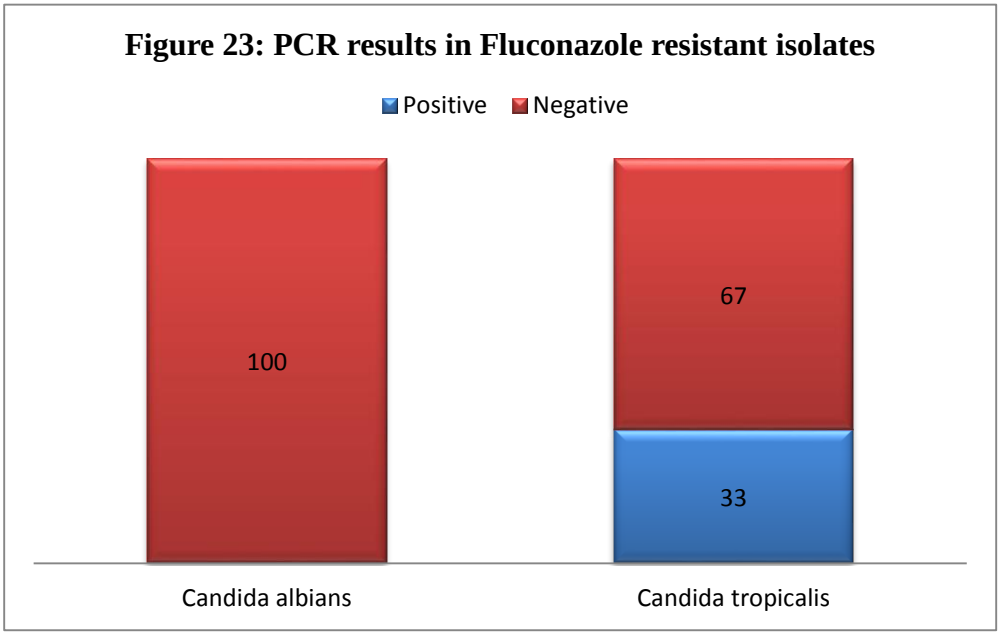


Figure 23: PCR results in Fluconazole resistant isolates

The four Fluconazole resistant isolates were subjected to molecular methods for detection of the mechanism of azole resistance using PCR done to detect the point mutation C1214T and one was found to be positive. Increased ERG11 gene expression due to point mutation results in the production of a large amount of 14 α-lanosterol demethylase, favoring the continuous synthesis of ergosterol and the maintenance of cell integrity which in turn allows the fungus to resist the action of the drug.

6. DISCUSSION

Candidiasis has emerged as an alarming opportunistic infection with an increase in a number of patients among the diabetics who have immune dysfunction. The intensity of infection is more severe and the response to therapy is slow in diabetic patients. Over the last decade, *non-albicans Candida* associated with human infections have increased and in recent times have developed resistance to anti‑fungal agents, in particular to the azole compounds. The present study was carried out to determine the relative contribution of *C. abicans* and non-*albicans species* in diabetic patients with symptomatic candidiasis , their antifungal susceptibility profile and role of ERG11 expression in azole resistance.

In our study, 102 specimens were collected out of which 56 were vaginal specimens from diabetic females and 46 were from urine specimens from diabetic males. The mean age of the diabetic patients from whom specimens were collected in our study was 53.7 years with a SD of 12 years. The mean age of the male diabetic patients was 61.8 years with a SD of 10 years among which the majority (34.8%) of them were between 51-60 years of age (**Table 1** and **Figure 1**). The female diabetic patients in our study had a mean age of 47 years (with SD 8.9 years) and 42.9 % fell between 41-50 years of age (**Table 1** and **Figure 2**). The lower mean age in females may be due to the fact that most of the patients attending the outpatient department belong to the reproductive age group.

In our study, the chief complaint among the symptomatic female diabetic patients was vaginal discharge which accounts for about 46.4% followed by pruritis and burning sensation (35.7%). These results were in accordance with the study by Rathod et al which showed a higher rate of complaints of vaginal discharge (31%) followed by pruritis (29%). Both Vaginal discharge and pruritis with burning sensation were present in 17.9% of the diabetic females. Out of the 56 diabetic females, only three (5.4%) complained of recurrence of symptoms within 1year (**Table2**

and **Figure 3**). The positive predictive values of these symptoms for predicting vulvovaginal candidiasis were low. A minority of women with these symptoms were subsequently diagnosed with vulvovaginal candidiasis (53.85%, 25%and 50% respectively). The low positive predictive value of symptoms indicate that the patients should not be treated based on symptoms alone which may inadvertently leads to treatment of patients without arriving at a correct diagnosis.

Among the 46 symptomatic diabetic males, 63 % complained of dysuria followed by frequency with urgency of micturition (23.95%). All these symptoms were present in 13% of the diabetic males (**Figure 4**). All these symptoms were consistent with the symptoms of *Candida* cystitis as shown in studies by Achkar et al and Bukhary ZA et al.

The mean fasting blood glucose level of the diabetic patients was found to be 157 mg/dl (SD 12 mg/dl). Table 3 shows that the mean fasting blood glucose in diabetic males was 162mg/dl with a SD of 11 mg/dl and that of diabetic females was 152mg/dl with a SD of 11mg/dl. This disparity may be due to difference in the age groups taken into study, their dietary habits, physical activity index, patient education and adherence to treatment regimen.

Table 3 and **Figure 5** shows that majority (32.14%) of diabetic females had their fasting blood glucose levels between 141-150mg/dl and 43.48% of the diabetic males had a fasting glucose level in the 161-170mg/dl range. **Figure 6 and 7** shows that the FBS levels steadily increases with increase in age of the diabetic patients and there is a significant positive correlation between the age of the patient and FBS levels.

But studies by De et al and Eid M et al which showed that younger subjects showed a poor glycemic control with high HbA1C levels. Both these studies also show that patients with recently diagnosed diabetes (duration of disease < 5 years) had the best glycemic control. The

discordance between the studies may be attributed to the fact that the duration of diabetes, dietary habits and physical status are not included in our study.

The mean age of the diabetic patients on treatment (62 years with SD 9.5 years) was found to be significantly higher than that of the diabetic patients who were not on treatment (46 years with SD 8.6 years). Among the 47% who were on treatment, 63% were males and 34% were females (**Table 4**). This difference in treatment status between males and females may be attributed to the fact that Diabetes is still considered a social stigma among some females in rural India and they have low awareness about their disease, its complications, adherence to medication and other self care practices.

In our study only 19 (18.6%) of smears were found to be positive for *Candida* by gram staining. Of these, 15 were vaginal smears and only 4 smears were from urine. **Table 5** and **Figure 8** shows that 73.2% of the vaginal smears were negative on gram staining and only 26.8% were found to be positive which is higher than that of the study by Vijaya et al which showed a positivity of only 7.3%.Among the positives, 21.4% showed budding yeast cells and 5.4% showed budding yeast cells with pseudohyphae. Urine smears for *Candida* only showed a positivity rate of 8.6% .**Figure 9** shows that both the budding yeast cells and budding yeast cells with pseudohyphae were present in 4.3% of the total urine smears. Gram stain is a valuable method in rapid accurate diagnosis of symptomatic VVC and even superior to culture as it demonstrates the invasive forms of the yeast.

Culture positivity for *Candida* was found in 41.1% of diabetic females in our study (**Table 6** and **Figure 10**). Studies by Goswami et al and Shrivastav et al showed a culture positivity of 46%

and 47.5% respectively. Peer *et al* reported a prevalence rate of 35.5% and Faraji *et al*. reported a prevalence of vulvovaginal candidiasis as 20% among diabetic women in their studies.

Table 6 and **Figure 10** also shows that *Candida* was isolated by culture from 13% of urine samples from diabetic males which is lower than that of the study by Yismaw et al which showed a prevalence of 17.1% in symptomatic diabetics. Pandey et al reported a higher prevalence of candiduria (43.75%) in his study. Similarly, Goyal et al and Zarei et al reported candiduria in 2.36% and 16.5%, of their study populations, respectively.

The culure positivity for *Candida* did not show any significant association with age and fasting blood glucose levels of the diabetic patients but nearly 37% of diabetics who were not any treatment had candidiasis whereas candidiasis was present only in 18.8% diabetics who were on treatment (**Table 7**) which shows there is a significant relationship between the occurrence of candidiasis and the treatment status of patients for diabetes. **Figure 11** shows that 26.32% of diabetic females who were on treatment and 48.65% who were not on any form of treatment for diabetes suffered from vulvovaginal candidiasis respectively. **Figure 12** shows that candiduria was present in 13.79% who were on treatment for diabetes but only 11.76% of males with untreated diabetes had candiduria.

28.4% of the specimens were found to be positive for *Candida* by culture whereas only 18.6% of the samples were found to be positive by gram staining. **Table 8** shows that only 65.5% of culture positives were also found to be positive by gram staining. **Figure 13** and **14** shows the lower sensitivity of gram stain compared to culture in detecting *Candida*. Even though gram staining is highly specific in our study, it is only found to be 65.5% sensitive by our study.

Lower sensitivity of gram stain compared to culture was showed in studies by Vijaya et al (41.41%), Bello et al (70%) and Goswami et al(77%).Though gram stain can detect the invasive

form of the yeast , given the fact that *C. glabrata* and some *Candida albicans* mutants cannot produce hyphae but clearly can cause candiduria confirming that the presence of hyphae is not a valid marker for infection. In addition its low sensitivity could also restrict its use in routine practice. So a combination of culture and gram stain is the ideal approach for the diagnosis of candidiasis.

Table 9 shows that 66.7% of female diabetics with the FBS range of 141-150mg/dl and 171-180mg/dl were culture negatives in our study. Majority (53.3%) of culture positives were in the FBS range of 151-160mg/dl. In case of diabetic males, only 33.3% and 20% of those with the FBS range of 171-180mg/dl and 161-170mg/dl respectively had candiduria and the rest were culture negatives.

The speciation of the isolates was done by using CHROM agar, Dalmau plate technique, Germ tube test and Sugar assimilation tests. CHROM agar is a simple and rapid test for speciation and has a additional advantage of identification of *Candida* from mixed cultures while sugar assimilation test takes upto 48-96 hours. The species obtained from vulvovaginal candidiasis were *Candida albicans* (39.1%), *Candida tropicalis* (26.1%), *Candida glabrata* (17.4%), *Candida parapsilosis* (8.7%) and *Candida krusei (8.7%).* The distribution of *Candida* species isolated from diabetic patients with vulvovaginal candidiasis showed a majority of *Candida non albicans* which was 61.9% (n=14) whereas *Candida albicans* isolates constituted only 39.1% (n=9) as shown in **Table 10** and **Figure 15**. Among the non *albicans Candida* (n=14*), Candida tropicalis* was the commonest isolate 42.85% (n=6) in our study as shown in Figure 16 followed by *Candida glabrata* 28.57% (n=4), *Candida parapsilosis* 14.29% (n=2) and *Candida krusei* 14.29% (n=2)(**Figure 16**).

Shrivastav et al isolated a majority of non-*albicans Candida* isolates in his study and overall 12 (21.1%) *Candida albicans*, 15 (26.3%) *Candida glabrata*, 24 (42.1%) *Candida krusei* and 6 (10.5%) *Candida tropicalis* were isolated. In symptomatic group of diabetic patients *Candida albicans* was predominant as 10 (17.5%) followed by *Candida glabrata* 8 (14%), while in asymptomatic group of patients *Candida krusei* 19 (33.3%) was predominant.

Candida glabrata was isolated in 68 (61.3%) , C*andida albicans* in 32 (28.8%) and *Candida tropicalis* in 4(3.6%) of diabetic patients with vulvovaginal candidiasis by Ray D et al. Study by Faraji R et al showed that the predominant *Candida species* isolated in diabetics with vulvo-vaginal candidiasis were *Candida glabrata* (39%), *Candida albicans* (26%) and *Candida tropicalis (17%).*

Among isolates obtained from candiduria, only 33.3% isolates were *Candida albicans* and the rest were non-*albicans Candida*. As shown in **Figure 17**, 50% of isolates were *Candida glabrata* and the rest was contributed equally by *Candida tropicalis* and *Candida parapsilosis.*

Study by Pandey et al showed that the major *Candida species* isolated were *Candida tropicalis 34% (17)* followed by *C. albicans 30% (15), C. parapsilosis 16% (8), C. glabrata 14% (7) and C. krusei 6% (3)* which indicated that non- *albicans Candida* were emerging in urine of diabetic patients and Falhati M et al isolated *Candida glabrata* (n=19, 50%), *C. albicans* (n=12, 31.6%), *C. krusei* (n=4, 10.5%), *C. tropicalis* (n=2, 5.3%), and *C. kefyr* (n=1, 2.6%) from urine of 305 diabetic outpatients. In both these studies majority of the isolates were non-*albicans Candida* species.

Fluconazole is still considered the first-line treatment for *Candida* infections because it offers the advantages of oral formulations, favorable bioavailability, and low level of toxicity. The Fluconazole resistance of *Candida* species has been increasingly reported in the past decade. The

antifungal susceptibility testing done by Kirby-Bauer disk diffusion method on isolates from Vulvovaginal candidiasis showed that 39.1% (n=9) were sensitive, 34.8% (n=8) were susceptible dose dependent (SSD) and 17.4% (n=4) were resistant to Fluconazole in our study. *Candida krusei* which constituted 8.7% (n=2) of the isolates were considered resistant as they are intrinsically resistant to Fluconazole. 28 (95.7%) isolates were sensitive to Voriconazole and only one isolate (4.3%) which was *Candida krusei* showed intermediate resistance. All the isolates were sensitive to Itraconazole (**Figure 18**).

Study by Kalaiarasan et al showed that 38 isolates (74.5%) were susceptible, 8 (15.7%) isolates were resistant and 5 (9.8%) isolates were S-DD to Fluconazole and all *Candida* species were found susceptible to Voriconazole. Fluconazole was found to be sensitive in 36 (33.3%) patients, SDD in 5 (4.6%) patients and resistant in 67 (62%) of patients with VVC in a study by Khan M et al and 64% isolates were found to be sensitive, 16% were SSD and 20% were found to be resistant by Khadka S et al.

The increased resistance and dose dependent susceptibile isolates in our study compared to the above studies may be attributed to the presence of large number of *Candida glabrata* and *Candida tropicalis* in a small number of isolates.

Among the 6 isolates from candiduria, 66.7% (n=4) were sensitive and 33.3% (n=2) were susceptible dose dependent (SSD) to Fluconazole (**Figure 19**) and the 2 susceptible dose dependent isolates were found to be *Candida glabrata*. All the isolates were sensitive to both Voriconazole and Itraconazole in our study. Yashavanth et al noted that among 33 candiduria isolates in his study 66.66% were susceptible, 9.1% were SSD and 24.24% were resistant (which also include *Candida krusei*) to Fuconazole and 72.72% were susceptible, 9.1% were SSD and

18.18% were resistant to Voriconazole .The above study also showed that only 66.6% of NAC were susceptible while 80% of *Candida albicans* were sensitive to Fluconazole. A study by Datta P et al showed that 62.7% candiduria isolates were resistant to Fluconazole.

Among the *Candida albicans* isolates, 66.7% were sensitive, 22.2% were susceptible dose dependent and only 11.1% were resistant to Fluconazole whereas among NAC species only 21.43% were sensitive, 42.86% were susceptible dose dependent, 21.43% were resistant and the rest were intrinsically resistant to Fluconazole. 33.3%, 16.7% and 50% of *Candida tropicalis* isolates were sensitive, SDD and resistant to Fluconazole respectively. All the isolates of *Candida glabrata* were reported as SDD as they cannot be reported sensitive to Fluconazole as per CLSI guidelines. Among the *Candida parapsilosis* isolates half were sensitive and the rest were SDD to Fluconazole. **Table 11** and **Figure 20** shows that majority of the SDD and resistant isolates were *non-albicans Candida* compared to *Candida albicans*. Among 17.4% of Fluconazole resistant isolates, 75% were *Candida tropicalis* and 25% were *Candida albicans.*

However in a study done by Mohanty et al in 2006, none of the isolates were resistant to Fluconazole while 30% were found to be susceptible dose dependent. The majority of the SSD isolates were *Candida glabrata* which is similar to the findings in our study. The finding in our study is contrary to the study by Khan M et al which showed that Fluconazole was sensitive in 37.7% , SDD in 8.8% and resistant in 53.3% of *Candida albicans* isolates where as only 38.8% of *C. tropicalis* were resistant and the rest were sensitive to Fluconazole.37.5% of *Candida glabrata* isolates were found to be sensitive, while the rest were resistant to Fluconazole.

But a study by Deorukhkar et al showed that Fluconazole resistance was more common in *C. tropicalis* (29.5%), *C. glabrata* (27.3%) and *C. kefyr* (25%) isolates. 12.7% of *C. glabrata*

isolates were dose dependent susceptible to Fluconazole. The higher Fluconazole resistance among *Candida tropicalis* isolates noted in the study by Deorukhkar et al is similar to the results obtained in our study.

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Figure 21 shows that none of isolates from the diabetic patients on treatment were found resistant whereas 17.4% of isolates from untreated diabetes were found to be resistant to Fluconazole. No significant relationship was found between Fluconazole resistance and fasting blood glucose levels as shown in **Figure 22**.

In one study, one *Candida tropicalis* isolate which was resistant to Fluconazole was found to be positive by PCR among the four tested resistant isolates (**Table 12** and **Figure 23**). Although ERG gene is present in all species of *Candida*, the primer used in this study was designed to detect C1214T point mutation within the gene that leads to S405F aminoacid substitution as shown by Kelly et al, Pam et al and Perea et al, which was associated with resistance hence the detection of gene in some isolates and not in others. Point mutation in the ERG gene results in ERG11 over expression which in turn results in increased ergosterol synthesis ultimately counteracting the mechanism of action of azoles.

7. SUMMARY

102 samples were collected in this study conducted in Tirunelveli Medical college,Tirunelveli from the period of June 2017 to July 2018 of which 56 were vaginal specimens from diabetic females and 46 urine samples were collected from diabetic males.

- ♣ The mean age of the diabetic patients from whom specimens were collected in this study was 53.7 years with a SD of 12 years.
- ♣ The chief complaint among the symptomatic female diabetic patients was vaginal discharge (46.4%) and that of symptomatic diabetic males was dysuria (63%).Recurrent episodes were present in 5.4% of diabetic females.
- ♣ There was a significant positive correlation between the age of the patient and FBS levels in this study.
- ♣ The mean age of the diabetic patients on therapy for diabetes was found to be significantly higher than that of the diabetic patients who were not on treatment. Among the 47% who were on treatment, 63% were males and only 34% were females.
- ♣ 18.6% and 28.4% of the specimens were found to be positive for *Candida* by gram staining and fungal culture respectively. Gram stain is positive in only 65.5% of culture positives.
- ♣ 41.1% of diabetic females had Vulvovaginal candidiasis whereas only 13% of diabetic males had candiduria in this study.
- ♣ The culture positivity for *Candida* did not show any significant association with age and fasting blood glucose levels of the diabetic patients but there is a significant relationship in this study between the occurrence of candidiasis and the treatment status of patients for diabetes.

- ♣ The isolates obtained from vulvovaginal candidiasis were *Candida albicans* (39.1%), *Candida tropicalis* (26.1%), *Candida glabrata* (17.4%), *Candida parapsilosis* (8.7%) and *Candida krusei* (8.7%) and among candiduria isolates , only 33.3% isolates were *Candida albicans* and the rest were non-*albicans Candida*.
- ♣ The antifungal susceptibility testing of isolates from vulvovaginal candidiasis shows that 39.1% were sensitive, 34.8% were susceptible dose dependent (SSD) and 17.4% were resistant to Fluconazole while most of the isolates were sensitive to Voriconazole and all the isolates were sensitive to Itraconazole.
- ♣ Candiduria isolates showed 66.7% susceptibility and 33.3% dose dependent susceptibility to Fluconazole and 100% susceptibility to Voriconazole and Itraconazole.
- ♣ Among 17.4% of Fluconazole resistant isolates, 75% were *Candida tropicalis* and 25% were *Candida albicans*. No significant relationship was found between Fluconazole resistance and fasting blood glucose levels in this study.
- ♣ Only one isolate was positive for PCR done to indirectly detect ERG11 overexpression among the four Fluconazole resistant isolates.

8. CONCLUSION

The present study demonstrated the importance of species identification and susceptibility testing for antifungals in *Candida* isolated from diabetic patients as uncontrolled diabetes mellitus causes metabolic alterations such as increased levels of glycogen, which can significantly increase colonization and infection by *Candida.* 60.9 % of non-*albicans Candida* were isolated in this study, among which only 21.4 % of the isolates were sensitive to Fluconazole that are generally used for the management of Vulvovaginal candidiasis. Isolation of non-*albicans Candida* have clinical implication due to their reduced susceptibility to various antifungals, thus highlighting the importance of isolation, species identification and antifungal susceptibility of *Candida* prior to initiation of therapy for proper selection of antifungal agent. The judicious use of antifungal agents is very important for the prevention of emergence and spread of drug resistant *Candida* species.

This study indicates that non- *albicans Candida* are emerging as a cause of candiduria in diabetic patients and its isolation from clinical specimens can no longer be ignored as nonpathogenic isolate nor can it be dismissed as a contaminant. The emergence of non-*albicans Candida* isolates increases the need for evaluation of urine samples of diabetic patients and prescription of appropriate antifungal therapy.

Antifungal susceptibility testing should be used to calculate clinical response and accordingly local antibiograms can aid in empirical assortment of antifungals, guiding options for long-term therapy and are meant for alternative regimens in testing of isolates from recurrent infections.

Alteration in sterol biosynthesis, analysis of expression level of *ERG11* gene and analysis of gene sequences can be further investigated in clinical isolates of Fluconazole-resistant *Candida* species. Further studies are required to explore the molecular mechanisms that could be targeted to control Fluconazole resistance.

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10.ANNEXURE

I - PROFORMA

PROFORMA

S.No: OP No.

Name of the patient :

Age : Sex :

Address :

Compliants:

Pruritis / Vaginal discharge/ Burning micturition

Dysuria/Frequency/Urgency

Past History :

H/o similar complaints in the past : Yes/No

No. of episodes:

Laboratory Investigations :

FBS level-

Mycological Investigations

Specimen – Vaginal Swab/Urine

Microscopy

Gram stain :

Culture on SDA

Colony morphology-

Gram stain-

Speciation:

Culture on CHROM agar-

Germ tube test-

Growth on Cornmeal agar-

Sugar assimilation test:

Sugars	Glucose	Lactose	Maltose	Sucrose	Trehalose	Xylose
Interpretation						

Antifungal Susceptibility testing:

Antifungal discs	Fluconazole(25µg)	Voriconazole(1µg)	Itraconazole
Interpretation			

ERG PCR:

II – PREPARATION OF MEDIA

1. Sabouraud's Dextrose Agar :

Composition

Ingredients	Gms / Litre
Dextrose (Glucose)	40.000
Mycological peptone	10.000
Agar	15.000

65.0 grams of the dehydrated media was suspended in 1000 ml of distilled water and boiled to dissolve the medium completely. Media was sterilized by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Media was cooled to 45-50°C and then poured into sterile Petri plates. Final pH was adjusted to 5.6±0.2

2. HiCrome Candida Differential Agar :

Composition

Ingredients	**Gms / Litre**
Peptone, special	15.000
Yeast extract	4.000
Dipotassium hydrogen phosphate	1.000
Chromogenic mixture	7.220
Chloramphenicol	0.500
Agar	15.000

42.72 grams of the dehydrated media was suspended in 1000 ml of distilled water and boiled to dissolve the medium completely as the media should not be autoclaved. Media was cooled to 45-50°C and then poured into sterile petri plates. Final pH was adjusted to 6.3±0.2.

3. Corn meal agar:

Composition

Ingredients	**Gms / Litre**
Corn meal	50.000
Agar	15.000

17 grams of the dehydrated media was suspended in 1000 ml of distilled water and boiled to dissolve the medium completely. Sterilized the medium by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Media cooled to 45-50°C and then poured into sterile Petri plates. Final pH was adjusted to 6.0±0.2.

4. Yeast Nitrogen Base Agar:

Composition

Ingredients	**Gms / Litre**
Part A	
Agar	40.000
Part B	
Ammonium sulphate	5.000
L-Histidine hydrochloride	0.010
DL-Methionine	0.020

DL-Tryptophan	0.020
Biotin	0.000002
Calcium pantothenate	0.0004
Folic acid	0.000002
Inositol	0.002
Niacin	0.0004
p-Amino benzioc acid	0.0002
Pyridoxine hydrochloride	0.0004
Riboflavin (Vitamin B2)	0.0002
Thiamine hydrochloride	0.0004
Boric acid	0.0005
Copper sulphate	0.00004
Potassium iodide	0.0001
Ferric chloride	0.0002
Manganese sulphate	0.0004
Sodium molybdate	0.0002
Zinc sulphate	0.0004
Monopotassium phosphate	1.000
Magnesium sulphate	0.500
Sodium chloride	0.100
Calcium chloride	0.100

40 grams of Part A was suspended in 900 ml of distilled water and boiled to dissolve the medium completely. Media was sterilized by autoclaving at 15 lbs pressure (121°C) for 12 minutes. Media was cooled to 50°C and aseptically admixed with sterile part B solution and then the plates are poured. Final pH was adjusted to 5.4±0.2

Part B was prepared in 10x strength by suspending 6.75 grams in 100 ml of distilled water and warmed to dissolve the medium completely. Sterilized the medium by filtration and was kept refrigerated until use.

5. Mueller Hinton Agar, 2% Glucose with Methylene blue agar:

Composition

Ingredients	**Gms / Litre**
Beef infusion	300.000
Casein Acid Hydrolysate	17.500
Starch	1.500
Glucose	20.000
Methylene blue	0.0005
Agar	17.000

58 grams of dehydrated media was suspended in 1000 ml of distilled water and boiled to dissolve the medium completely. Media was sterilized by autoclaving at 15lbs pressure (121°C) for 15 minutes. Media was cooled to 45-50°C before pouring into petri plates. Final pH adjusted to 7.3±0.1.

6. McFarland 0.5 Turbidity standard

- Prepare this turbidity standard by adding 0.5ml of 1.175% $BaCl_2$ to 99.5ml of 1% H_2 SO4 with constant stirring to maintain a suspension.
- Verify the correct density of the turbidity standard by using a spectrophotometer. The absorbance at 625nm should be 0.08 to 0.10 for the 0.5 McFarland standard
- Distribute 4 to 6ml into screw capped tubes and tightly seal these tubes and store them in the dark at room temperature.
- Vigorously agitate this turbidity standard on a mechanical vortex just before use.

III – INTERPRETATION TABLES

1.Sugar Assimilation Pattern:

Candida Species	Glucose	Lactose	Maltose	Sucrose	Xylose	Trehalose
C. albicans	+	+	+	+	+	+
C. tropicalis	+	-	+	+	+	+
C. parapsilosis	+	-	+	+	+	+
C. glabrata	+	-	-	-	+	+
C. krusei	+	-	-	-	+	-

2. Interpretation of zone diameters in disk diffusion method as per NCCLS M44 A2-2009 for Fluconazole :

Candida species	**Interpretation of Zone diameters for Fluconazole (25µg)**		
	Susceptible	Susceptible Dose Dependent(SDD)	Resistant
C. albicans	≥ 17mm	15-16mm	≤ 13mm
C. tropicalis	≥ 17mm	15-16mm	≤ 13mm
C. parapsilosis	≥ 17mm	15-16mm	≤ 13mm
C. glabrata	-	≥ 15mm	≤ 14mm
C. krusei	-	-	-

3. Interpretation of zone diameters in disk diffusion method as per NCCLS M44 A2-2009 for Voriconazole:

Candida species	Interpretation of Zone diameters for Voriconazole (1µg)		
	Susceptible	Intermediate	Resistant
C. albicans	≥ 17mm	15-16mm	≤ 14mm
C. tropicalis	≥ 17mm	15-16mm	≤ 14mm
C. parapsilosis	≥ 15mm	13-14mm	≤ 12mm
C. glabrata	-	-	-
C. krusei	≥ 15mm	13-14mm	≤ 12mm

4. Interpretation of zone diameters in disk diffusion method for Itraconazole:

Interpretation of Zone diameters for Itraconazole (10µg)		
Susceptible	Susceptible Dose Dependent(SDD)	Resistant
≥ 20mm	12-19 mm	≤ 11mm

IV MASTER CHART

S.No	AGE	SEX	SPECIMEN	SYMPTOMS*	FBS (mg/dl)	ON TREATMENT	GRAM STAIN**	CULTURE	SPECIES***	FLUCONAZOLE	VORICONAZOLE	ITRACONAZOLE	ERG PCR
1	34	Female	Vaginal swab	3	138	No	3	Pos	1	S	S	S	-
2	48	Female	Vaginal swab	3	149	No	3	Pos	2	R	S	S	Neg
3	45	Female	Vaginal swab	2,3	159	Yes	2	Pos	2	S	S	S	-
4	45	Female	Vaginal swab	3	148	No	3	Neg	-	-	-	-	-
5	42	Female	Vaginal swab	3	153	No	3	Neg	-	-	-	-	-
6	35	Female	Vaginal swab	3	142	No	3	Neg	-	-	-	-	-
7	42	Female	Vaginal swab	3,4	168	No	2	Pos	4	SDD	S	S	-
8	40	Female	Vaginal swab	2	148	No	3	Neg	-	-	-	-	-
9	57	Female	Vaginal swab	3	157	Yes	1	Pos	3	S	S	S	-
10	55	Female	Vaginal swab	3	177	Yes	2	Pos	1	S	S	S	-
11	44	Female	Vaginal swab	2,3	145	No	2	Pos	1	SDD	S	S	-
12	55	Female	Vaginal swab	3	148	Yes	3	Neg	-	-	-	-	-
13	41	Female	Vaginal swab	3	158	No	2	Pos	1	R	S	S	Neg
14	37	Female	Vaginal swab	3	161	No	2	Pos	5	IR	SDD	S	-
15	40	Female	Vaginal swab	2	138	No	3	Neg	-	-	-	-	-
16	33	Female	Vaginal swab	2,3	142	No	3	Neg	-	-	-	-	-
17	62	Female	Vaginal swab	3,4	167	No	1	Pos	2	R	S	S	Pos
18	53	Female	Vaginal swab	2	146	Yes	3	Neg	-	-	-	-	-

S.No	AGE	SEX	SPECIMEN	SYMPTOMS*	FBS (mg/dl)	ON TREATMENT	GRAM STAIN**	CULTURE	SPECIES***	FLUCONAZOLE	VORICONAZOLE	ITRACONAZOLE	ERG PCR
19	39	Female	Vaginal swab	2	148	No	3	Pos	2	SDD	S	S	-
20	47	Female	Vaginal swab	2	164	No	3	Neg	-	-	-	-	-
21	63	Female	Vaginal swab	3	155	Yes	2	Pos	4	SDD	S	S	-
22	41	Female	Vaginal swab	3	147	No	3	Neg	-	-	-	-	-
23	48	Female	Vaginal swab	2	148	No	3	Neg	-	-	-	-	-
24	49	Female	Vaginal swab	2	154	No	3	Neg	-	-	-	-	-
25	44	Female	Vaginal swab	3	146	No	3	Neg	-	-	-	-	-
26	38	Female	Vaginal swab	3	138	No	3	Neg	-	-	-	-	-
27	36	Female	Vaginal swab	2,3	141	No	3	Pos	1	S	S	S	-
28	45	Female	Vaginal swab	2	152	No	2	Pos	4	SDD	S	S	-
29	59	Female	Vaginal swab	2,4	147	Yes	2	Pos	3	SDD	S	S	-
30	55	Female	Vaginal swab	2,3	161	Yes	3	Neg	-	-	-	-	-
31	42	Female	Vaginal swab	3	139	No	3	Neg	-	-	-	-	-
32	50	Female	Vaginal swab	3	153	Yes	3	Neg	-	-	-	-	-
33	49	Female	Vaginal swab	2	168	Yes	3	Neg	-	-	-	-	-
34	40	Female	Vaginal swab	2	135	No	3	Neg	-	-	-	-	-
35	35	Female	Vaginal swab	3	137	No	3	Pos	1	S	S	S	-
36	40	Female	Vaginal swab	2	143	No	3	Neg	-	-	-	-	-
37	43	Female	Vaginal swab	2,3	146	No	3	Pos	2	R	S	S	Neg

S.No	AGE	SEX	SPECIMEN	SYMPTOMS*	FBS (mg/dl)	ON TREATMENT	GRAM STAIN**	CULTURE	SPECIES***	FLUCONAZOLE	VORICONAZOLE	ITRACONAZOLE	ERG PCR
38	48	Female	Vaginal swab	3	153	No	2	Pos	4	SDD	S	S	-
39	36	Female	Vaginal swab	3	135	No	3	Neg	-	-	-	-	-
40	44	Female	Vaginal swab	3	157	Yes	3	Neg	-	-	-	-	-
41	39	Female	Vaginal swab	3	140	No	2	Pos	2	S	S	S	-
42	44	Female	Vaginal swab	3	136	No	2	Pos	1	S	S	S	-
43	61	Female	Vaginal swab	3	165	Yes	3	Neg	-	-	-	-	-
44	69	Female	Vaginal swab	2,3	171	Yes	3	Neg	-	-	-	-	-
45	42	Female	Vaginal swab	2,3	158	Yes	3	Neg	-	-	-	-	-
46	55	Female	Vaginal swab	2,3	163	No	1	Pos	1	SDD	S	S	-
47	65	Female	Vaginal swab	2	157	Yes	3	Neg	-	-	-	-	-
48	55	Female	Vaginal swab	2	153	No	3	Pos	5	IR	S	S	-
49	53	Female	Vaginal swab	3	163	Yes	3	Neg	-	-	-	-	-
50	63	Female	Vaginal swab	2,3	160	Yes	3	Neg	-	-	-	-	-
51	41	Female	Vaginal swab	2	149	No	3	Neg	-	-	-	-	-
52	60	Female	Vaginal swab	2	173	Yes	3	Neg	-	-	-	-	-
53	57	Female	Vaginal swab	2	164	Yes	3	Neg	-	-	-	-	-
54	38	Female	Vaginal swab	3	138	No	3	Neg	-	-	-	-	-
55	50	Female	Vaginal swab	2	156	No	3	Pos	1	S	S	S	-
56	43	Female	Vaginal swab	2	146	No	3	Neg	-	-	-	-	-

S.No	AGE	SEX	SPECIMEN	SYMPTOMS*	FBS (mg/dl)	ON TREATMENT	GRAM STAIN**	CULTURE	SPECIES***	FLUCONAZOLE	VORICONAZOLE	ITRACONAZOLE	ERG11 PCR
57	40	Male	Urine	1	154	No	3	Neg	-	-	-	-	-
58	40	Male	Urine	1	146	No	3	Neg	-	-	-	-	-
59	57	Male	Urine	1,5	181	Yes	3	Neg	-	-	-	-	-
60	48	Male	Urine	1	169	Yes	3	Neg	-	-	-	-	-
61	45	Male	Urine	1	152	No	3	Neg	-	-	-	-	-
62	48	Male	Urine	1	161	No	3	Neg	-	-	-	-	-
63	75	Male	Urine	5	182	Yes	3	Neg	-	-	-	-	-
64	62	Male	Urine	5	179	Yes	1	Pos	1	S	S	S	-
65	70	Male	Urine	5	165	Yes	3	Neg	-	-	-	-	-
66	58	Male	Urine	1	153	No	3	Neg	-	-	-	-	-
67	78	Male	Urine	1,5	165	Yes	3	Neg	-	-	-	-	-
68	61	Male	Urine	1	172	Yes	3	Neg	-	-	-	-	-
69	59	Male	Urine	1	151	No	3	Neg	-	-	-	-	-
70	56	Male	Urine	1	147	No	3	Neg	-	-	-	-	-
71	62	Male	Urine	1	166	Yes	3	Neg	-	-	-	-	-
72	72	Male	Urine	5	186	Yes	3	Neg	-	-	-	-	-
73	70	Male	Urine	1,5	173	Yes	3	Neg	-	-	-	-	-
74	68	Male	Urine	5	167	Yes	3	Neg	-	-	-	-	-
75	63	Male	Urine	1	149	Yes	3	Neg	-	-	-	-	-

S.No	AGE	SEX	SPECIMEN	SYMPTOMS*	FBS (mg/dl)	ON TREATMENT	GRAM STAIN**	CULTURE	SPECIES***	FLUCONAZOLE	VORICONAZOLE	ITRACONAZOLE	ERG11 PCR
76	61	Male	Urine	1	163	Yes	3	Neg	-	-	-	-	-
77	55	Male	Urine	5	152	No	3	Neg	-	-	-	-	-
78	60	Male	Urine	1	145	No	3	Neg	-	-	-	-	-
79	58	Male	Urine	1	147	No	3	Neg	-	-	-	-	-
80	72	Male	Urine	5	168	Yes	3	Neg	-	-	-	-	-
81	75	Male	Urine	5	153	Yes	3	Neg	-	-	-	-	-
82	76	Male	Urine	1,5	161	Yes	2	Pos	4	SDD	S	S	-
83	55	Male	Urine	1	157	No	3	Neg	-	-	-	-	-
84	75	Male	Urine	5	163	Yes	3	Neg	-	-	-	-	-
85	60	Male	Urine	1	149	No	3	Neg	-	-	-	-	-
86	63	Male	Urine	1	165	Yes	3	Neg	-	-	-	-	-
87	50	Male	Urine	1	156	No	3	Neg	-	-	-	-	-
88	55	Male	Urine	1	163	Yes	3	Pos	1	S	S	S	-
89	73	Male	Urine	1,5	169	No	1	Pos	3	S	S	S	-
90	75	Male	Urine	1	161	Yes	3	Neg	-	-	-	-	-
91	65	Male	Urine	1	154	Yes	3	Neg	-	-	-	-	-
92	72	Male	Urine	5	174	Yes	3	Neg	-	-	-	-	-
93	60	Male	Urine	1	168	Yes	3	Neg	-	-	-	-	-
94	59	Male	Urine	1	176	Yes	3	Neg	-	-	-	-	-

S.No	AGE	SEX	SPECIMEN	SYMPTOMS*	FBS (mg/dl)	ON TREATMENT	GRAM STAIN**	CULTURE	SPECIES***	FLUCONAZOLE	VORICONAZOLE	ITRACONAZOLE	ERG11 PCR
95	60	Male	Urine	1	168	Yes	3	Neg	-	-	-	-	-
96	68	Male	Urine	5	161	Yes	3	Neg	-	-	-	-	-
97	54	Male	Urine	1	164	No	3	Pos	2	S	S	S	-
98	52	Male	Urine	1	156	No	3	Neg	-	-	-	-	-
99	75	Male	Urine	1	165	Yes	3	Neg	-	-	-	-	-
100	78	Male	Urine	1,5	171	Yes	2	Pos	4	SDD	S	S	-
101	50	Male	Urine	1	169	Yes	3	Neg	-	-	-	-	-
102	55	Male	Urine	1	0	No	3	Neg	-	-	-	-	-

***Symptoms:** Dysuria -1, Pruritis & Burning Sensation -2, Vaginal Discharge -3, Recurrence of Symptoms-4, Frequency And Urgency -5

****Gram Stain:** Budding Yeast Cells And Pseudohyphae -1, Budding Yeast Cells Only -2, Negative-3

*****Species:** *Candida albicans* -1, *Candida tropiclais* -2, *Candida parapsilosis*-3, *Candida glabrata*-4, *Candida krusei* -5

V. COLOUR PLATES

Figure 1: Growth of different species of *Candida* on CHROM agar

Figure 2: Positive Germ tube test

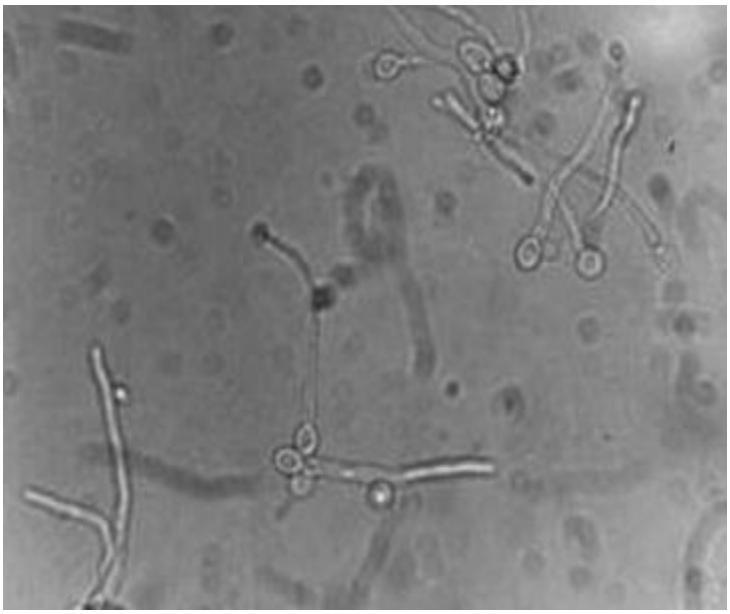


Figure 3: Morphology of *Candida tropicalis* on Corn meal agar

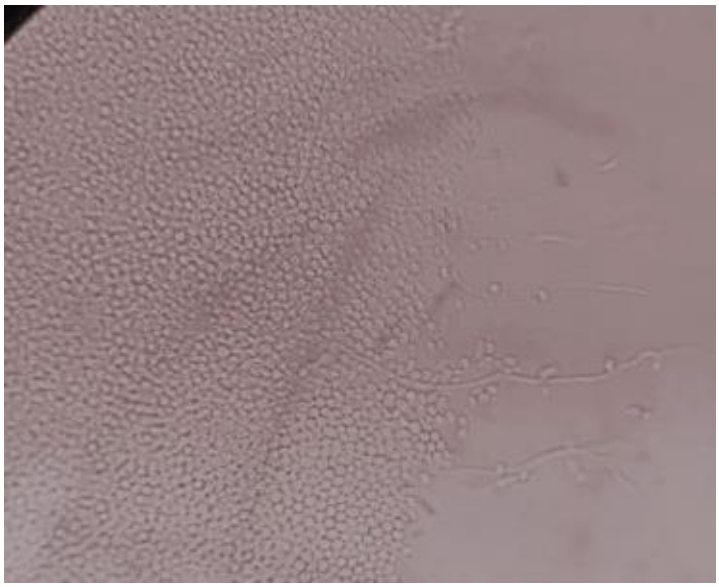


Figure 4: Morphology of *Candida glabrata* on Corn meal agar

Figure 5: Sugar assimilation pattern of *Candida albicans*

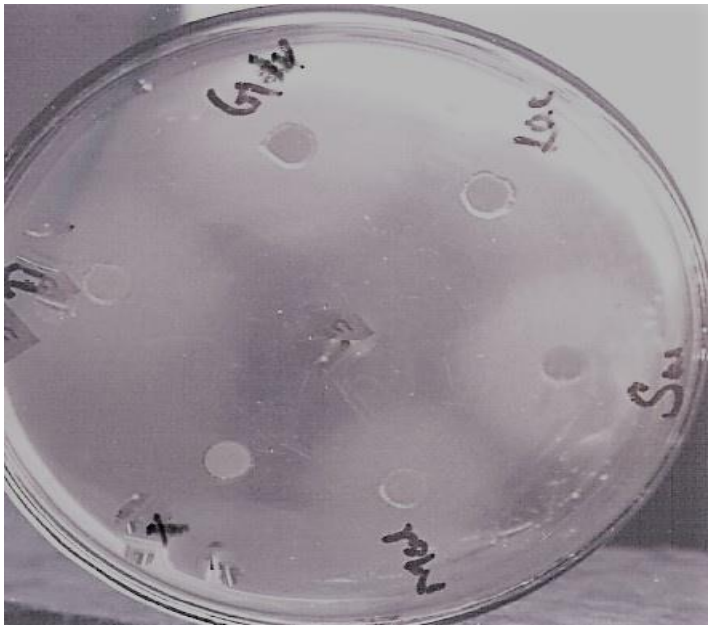


Figure 6: Fluconazole sensitive isolate on Mueller-Hinton Agar with 2% Glucose and 0.5 µg/mL Methylene Blue Dye (GMB) Medium

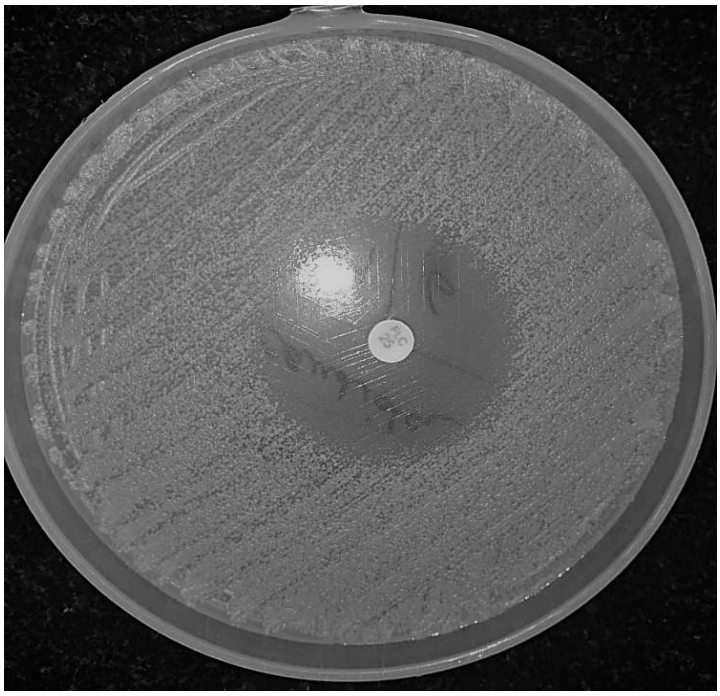
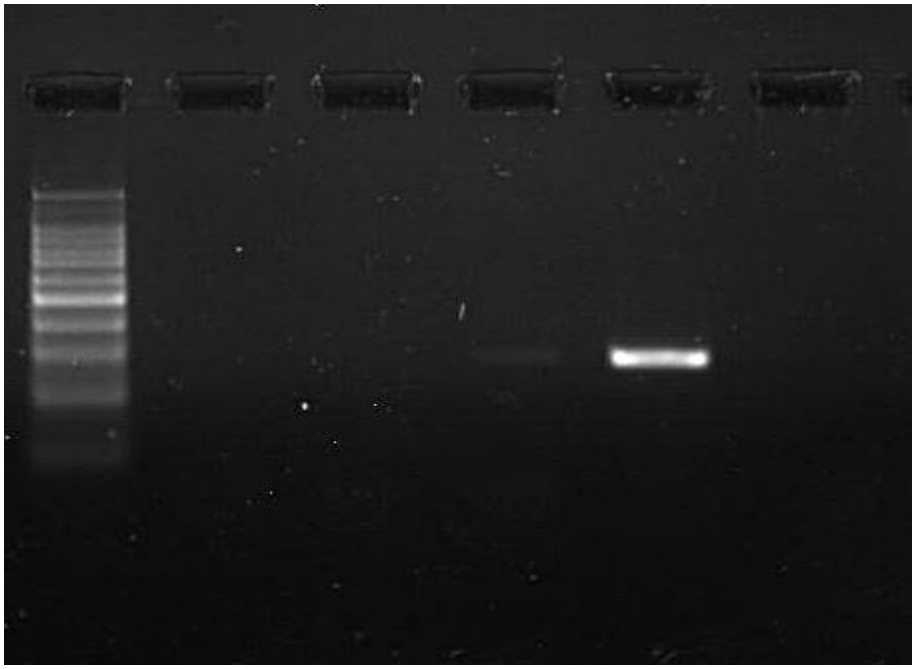


Figure 7: Fluconazole resistant isolate on Mueller-Hinton Agar with 2% Glucose and 0.5 µg/mL Methylene Blue Dye (GMB) Medium

Figure 8: ERG PCR: DNA Ladder- 1 -2 -3 (Negative isolates) – 4 (Positive isolate)-Negative control



VI- ABBREVIATIONS

ATCC - American Type Culture Collection

CLSI – Clinical Laboratory Standards Institute

DNA - Deoxyribonucleic Acid

DM – Diabetes mellitus

ERG - Ergosterol

GMB – Glucose with Methylene Blue

IR – Intrinsic Resistance

MHA - Mueller Hinton Agar

MIC - Minimum Inhibitory Concentration

NAC- Non-*albicans Candida*

NCCLS – The National Committee for Clinical Laboratory Standards

PCR – Polymerase Chain Reaction

SDA – Sabouraud's Dextrose Agar

SDD - Susceptible Dose Dependent

VVC- Vulvovaginal candidiasis

YNB- Yeast Nitrogen Base