A prospective study on prevalence of *Candida* species in clinical samples

By

Umber Tasneem

Department of Microbiology

Faculty of Biological Sciences

Quaid-i-Azam University

Islamabad

2014

A prospective study on prevalence of *Candida* species in clinical samples

A dissertation submitted in the partial fulfillment of the requirements for the degree of

Master of Philosophy

In

Microbiology

By

UMBER TASNEEM

Department of Microbiology

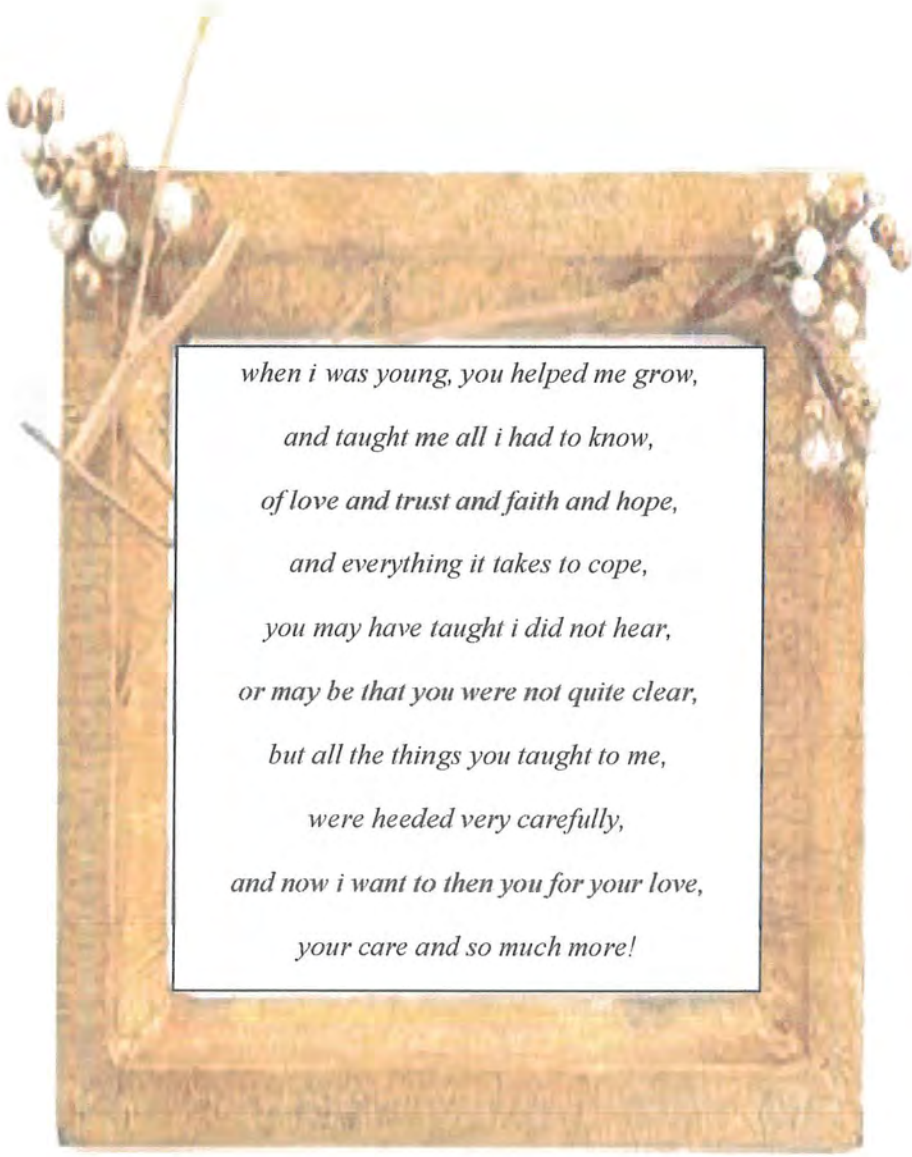
Faculty of Biological Sciences

Quaid-i-Azam University Islamabad

2014

IN THE NAME OF ALLAH THE MOST

BENEFICIENT AND EVER MERCIFUL



when i was young, you helped me grow,

and taught me all i had to know,

of love and trust and faith and hope,

and everything it takes to cope,

you may have taught i did not hear,

or may be that you were not quite clear,

but all the things you taught to me,

were heeded very carefully,

and now i want to then you for your love,

your care and so much more!

DECLARATION

It is stated that material contained in this thesis is my original work and has not been previously submitted in Quaid-I-Azam University or any other institution.

Umber Tasneem

CERTIFICATE

This thesis submitted by **Umber Tasneem** is accepted in its present form by the Department of Microbiology, Faculty of Biological Sciences, Quaid-I-Azam University, Islamabad, as fulfilling the thesis requirement for the degree of **Master of Philosophy** in Microbiology.

Internal Supervisor :

Dr. Rani Faryal

External Examiner:

Prof. Dr. Azra Yasmin

Chairman:

Prof. Dr. Fariha Hassan

Date: ***02-04-2014***

CONTENTS

List of Abbreviations

1	**UTI**	Urinary Tract Infection.
2	**ICU**	Intensive Care Unit
3	**GT**	Germ Tube.
4	**SDA**	Sabouraud Dextrose Agar.
5	**CLSI**	Clinical and Laboratory Standards Institute.
6	**EUCAST**	European Committee on Antimicrobial Susceptibility Testing.
7	**PCR**	Polymerase Chain Reaction.
8	**DNA**	De-oxyribo Nucleic Acid .
9	**ITS**	Internal Transcribed Spacer or Internal Transcribe Sequences.
10	**OPC**	Oro Pharyngeal Candidiasis.
11	**HIV**	Human Immunodeficiency Virus.
12	**VLBW**	Very Low Birth Weight.
13	**NICU**	Neonatal Intensive Care Unit.
14	**CSF**	Cerebral Spinal Fluid.
15	**RTI**	Respiratory Tract Infection.
16	**API**	Analytical Profile Index.
17	**YEPD**	Yeast Extract Peptone Dextrose.
18	**NSSI**	Non-Slip Slide Glass Incubation.
19	**CAID2**	Candida ID2
20	**CAID**	Candida ID
21	**CAC**	Chrome Ager *Candida*
22	**ATCC**	American Type Culture Collection.
23	**NCCLS**	National Committee for Clinical Laboratory.
24	**SHAM**	Salicylhydroxamic acid.
25	**RVVC**	Recurrent Vulvo-vaginal Candidiasis,
26	**PCR –RFLP**	PCR (Random Fragment Length Polymerization)
27	**MIC**	Minimal Inhibitory Concentration.
28	**GMMH**	Glucose Methylene Blue Muller Hinton

29	**ECVs**	Epidemiological Cutoff Values.
30	**RTPCR**	Real Time Polymerase Chain Reaction.
31	**RAPD**	Random Amplified Polymerase DNA.
32	**PCR RFLP**	PCR Restriction Fragment Length Polymerase
33	**QIH**	Quaid-e- Azam International Hospital.
34	**BD**	Becton Dickinson
35	**BA**	Blood Ager
36	**CA**	Chocolate Agar
37	**MCA**	Mackonkey Agar.
38	**CLED**	Cystine Lactose Electrolyte Deficient.
39	**PDA**	Potato dextrose ager.
40	**UK**	United Kingdom.
41	**USA**	United State of America .
42	**Pos**	Positive
43	**Neg**	Negative
44	**NB**	Nutrient broth.
45	**R**	Resistance
46	**S**	Sensitive
47	**YEPD**	Yeast Extract Peptone Dextrose.
48	**UV**	Ultraviolet .
49	**ATP**	Adenine Triphosphate
50	**CTP**	Cytosine Triphosphate.
51	**GTP**	Guanine Triphosphate.
52	**TTP**	Thiamine Triphosphate.
53	**GNR**	Gram negative Rods.
54	**Spp.**	Species
55	**CMA**	Corn Meal Agar.
56	***C.***	*Candida.*
57	**OPD**	Outdoor patients.
58	**IPD**	Indoor patients.
59	**ER**	Emergency.
60	**CCU**	Cardic Care Unit
61	**HVS**	High Vaginal Swab

62	**OT**	Oparation Theater.
63	**PDA**	Potato Dextrose Agar.
64	**QAU**	Quaid-i-Azam University

LIST OF TABLES

LIST OF FIGURES

ACKNOWLEDGEMENTS

Up and above everything else, I bow my head before Almighty ***"ALLAH"*** *alone, the most gracious merciful and compassionate, the omnipotent, the omniscient, the omnipresent, the only creator of the whole universe; whose bounteous blessing and exaltation flourished my thoughts and thrived my ambitions to eventually shape up the cherished fruit of my modest endeavors in the form of this manuscript. Next to Allah, all praise to his Holy Prophet* ***"HAZRAT MUHAMMAD (SAW)"****, the most perfect and exalted among and of ever borne on the surface of earth, whose eternal teachings would remain a source of guidance and inspiration for mankind forever.*

First I would like to thank Chairman of the department ***Prof. Dr. Safia Ahmed*** *for her cooperation with the research students.*

I deem it an utmost pleasure to express the heartiest gratitude to my very nice supervisor ***Dr. Rani Faryal, Department of Microbiology****, for her keen interest and guidance during my research work. I am also thankful to* ***Dr. Fariha Hassan*** *and* ***Dr Aamer Ali Shah, Department of Microbiology*** *for his continuous help during my research.*

I have set a light and ever burning flame of gratitude, warm thanks and appreciation to ***Dr. Col. Muhammad Farooq Khattak , HOD Pathology Department QIH*** *and* ***Dr. Farah Faqir, Consultant Microbiology Department QIH, Islamabad*** *for their kind cooperation, supervision, inspiring and sympathetic attitude and encouragement throughout my research work. Their vast knowledge and valued suggestions at all time polished and make this presentation worth. It appears to me as I was brought into the lap of this Hospital where I learned, I was matured and this hospital created a sense in my mind that how I have to move further. I am greatly thankful to all the staff of this hospital from the core of my heart.*

I express my sense of obligation to members of the supervisor committee and to all others for extending their keen interest, sincere guidance, continuous encouragement and sympathetic attitude during the whole span of the research work. I also extend my gratitude to specially my colleagues and seniors ***Mr. Zakir Hussain*** *and* ***Mrs. Aneela Mohsin, Supervisors Microbiology Department, Mr. Akram Ali, Miss. Rabia Durrani*** *and* ***Mr. Zafar Yasin.***

Thanks also to ***Mr. Shahid*** *and* ***Mr. Tanveer*** *Lab Assistants of* MRL *who cooperated us in our lab tasks. I would be failing in my duty if I don't express my cardiac and profound thanks*

to my very nice senior ***Muhammad Irfan, Miss. Nazia*** *for helping me in my research work, his continued encouragement, moral support, affection, and cooperation during the critical moments. I am also thankful to my very nice friends* ***Mis.s Nazia Jafri, Dr. Naima, Miss. Aster, Miss. Faiza Aslam, Mrs. Adeela Faisal*** *and* ***Miss. Hafsa Khalil*** *for her continuous encouragement throughout my research work.*

And at last but not the least, I deem it a great honour and privilege to record profound sense of gratitude and extend my compliments to my ever loving ***Ame Jan*** *and other family members* ***Sughra Tasneem, Syed Abul Hassan, Syed Fakhr -ul- Hassan*** *and* ***Syed Samir-ul-Hassan*** *for their encouragement and inspiration for higher ideas, moral support, their love and affection, amicable attitude and their countless prays for my glorious success about my pursuits throughout my life. I can do no more than to reaffirm my eternal devotion to all the members of my family.*

''I do appreciate all those who remembered me in their prayers and encouraged me throughout my life and education carrier''.

UMBER TASNEEM

ABSTRACT

ABSTRACT

Infections caused by opportunistic pathogens, *e.g.* yeasts are important reasons of morbidity and mortality throughout the world. *Candida* spp. are true opportunistic pathogens that enters in the circulation and deep tissues. This causes many deaths annually in Pakistan and is one of the serious public health concerns of study area. This work was conducted to study the prevalence of *Candida* species in clinical samples from Quaid-i-Azam International Hospital (QIH) Islamabad, Pakistan during March to December 2013. A total of 3007 clinical samples were collected from both in-door and out-door patients, in which only 255 were *Candida* positive. *Candida* species were isolated and identified using clinical, cultural, biochemical and molecular methods. Morphological identification was done by using different growth media. Further identification was done through gram staining and germ tube. Among 255 isolates, 128 were germ tube positive and 127 were germ tube negative. Comparison of two differential media *i.e.* Chrome agar *Candida* and Corn Meal agar was also done, showing that Corn Meal agar was better media for rapid identification of all *Candida* species. On the basis of growth on Chrome-agar and Corn Meal agar 128 (51%) isolates were *C. albicans*, followed by 30 (12%) *C. glabrata*, 26 (10%), *C. tropicalis*, 17 (7%) *C. krusei* and 19 (7.5%) were other *Candida* spp. while other *Candida* spp. were further identified on Corn Meal agar. Twelve (5%) isolates were of *C. parapsilosis*, 3 (2%) isolates were of *C. dubliniensis* and *C. lusitania* while 1 (0.4%) isolate was of *Trichosporoum* species. These results were further confirmed by using commercially available analytical profile index (API 10C) kit, which show 100% similarity with both differential media. Antifungal susceptibility tests of three predominantly used antifungals *i.e.* fluconazole, voriconazole and amphotericin B showed that *Candida* species showed had high resistance to fluconazole (14.5%) and increase sensitivity to amphotericin B (10%). The presence of *Candida* spp. was further confirmed by amplification of DNA of ITS region by PCR and sequencing. It is concluded that on the basis of present work *C. albicans* is the major specie among all *Candida* species causing candidemia. It is need of further work to give concentrated evidence in the prevalence of *Candida* infections in the local area.

INTRODUCTION

INTRODUCTION

Infections caused by opportunistic pathogens, *e.g.* yeasts, are important reasons of morbidity and mortality because of modifications in the immune system and hostile hospital environment. Yeast like fungi is the fourth major cause of blood stream infections (Ece *et al.*, 2012). *Candida* is a fungal pathogen isolated from clinical samples. These are constituent of normal flora of human beings (Pethani *et al.*, 2012). *Candida* spp. are true opportunistic pathogens that exploit recent technical advances to enter in the circulation and deep tissues (Gordana *et al.*, 2008).

Candida species causes many diseases, such as superficial mucocutaneous diseases to invasive infections, which also includes *Candida* peritonitis, systemic candidiasis and hepatosplenic candidiasis (Gordana et *al.*, 2008). *Candida* species can also cause simple mucocutaneous lesion to even deadly infections (Pethani *et al.*, 2012). Mucocutaneous candidiasis can be dividede into genitourinary and non-genital diseases. *Candida* also causes urinary tract infections (UTIs) and inflammation of the glans penis (Achkar and Fries, 2010).

Evaluation of the international literature shows that high incidence of hospital acquired diseases are caused by *Candida* species alongside the emergence of new pathogenic *Candida* species in all group of ages and hospitals, is of high concern for clinicians, hygienists and biologists. Nosocomial infections due to *Candida* spp. are increased during 1980's. In large hospitals of the United States of America, *Candida* species ranks second in ($P < 0.0001$) septicemia. In ICUs of Europe, *Candida* species lies third in hospital acquired infections. The last era has also been noticeable by the nosocomial origin, seriousness and frequency of fungal infections. Depending on the related risk factor, the deaths due to *Candida* spp. infections ranges between 13 to 90% (Grimoud, 2003).

In Iran infections that are hospital acquired have marvelous financial and health costs, with an expected occurrence of 2,000,000 infections, 20,000 deaths and having cost of 2 billion in year (Diba *et al.*, 2012). The incidence of fungal infections is rising in patients

with weak immune system, widespread use of antifungal agents, catheterization and chemotherapy (Pethani *et al.*, 2012). Most important places of *Candida* infection are both surgical and medical ICUs of adult, pediatric, and neonatal (Gordana *et al.*, 2008). Supervision of lethal candidiasis remains hindered due to postponements in diagnosis and lack of consistent diagnostic procedures (Pethani *et al.*, 2012). Therefore choice of treatment, administration and diagnosis are different and need to be doubtful in the overall surroundings of infected patient (Achkar and Fries, 2010).

Amongst all *Candida* species, *C. albicans* is common pathogenic specie, which inhabits on the skin, the reproductive and gastrointestinal tract. *C. albicans* can be isolated from the reproductive tract of 20% to 30% of healthy un-pregnant women's (Achkar and Fries, 2010). *C. albicans* is vital fungal specie, present in 60% of all yeasts isolated in clinical specimens (Prates *et al.*, 2011).

Candida species are around about 200 now a days but human infections are caused by only few species, in which only five species *i.e. C. glabrata, C. tropicalis, C. albicans, C. parapsilosis* and *C. krusei* were the world wide major cause of 90% infection in blood stream (Zarrin and Mahmoudabadi, 2009)

C. parapsilosis has been a leading *Candida* species that causes candidiasis, specially neonatal candiduria (Achkar and Fries, 2010). *C. parapsilosis, C. tropicalis* and *C. albicans* are the commonly recovered yeast like fungi causing blood stream infections (Ece *et al.*, 2012). *C. parapsilosis* positive blood cultures have been rated second in Canada, Latin America and Europe however third in the USA (Yucesoy and Serhat, 2003).

Yeast cells are morphological changes from unicellular into Germ Tube (GT) in human sera, blood, cerebrospinal fluids and plasma. Many things were reported as GT inducers, in which serum is the most effective GT inducer *in-vivo* studies. GT is a typical morphology witnessed only in *C. albicans*, GT conformation is only available method for identification of *C. albicans* (Nakamoto, 1998). However, recently it has been that

C. dubliniensis is similar to *C. albicans* in many features, especially in GT and chlamydospore production (Kangogo *et al.*, 2011).

Clinical specimens in routine are primary diagnosed by direct wet preparations and morphological characteristics of colony on different media like Sabouraud Dextrose Agar (SDA) and Chrom agar. *Candida* infections caused by *C. pseudohypha* can be also confirmed on microscopic observation by direct wet preparations (Diba *et al.*, 2012).

Chromogenic media helps in direct and rapid recognition of *C. albicans* and other species of *Candida* in mixed bacterial and fungal cultures than enrichment and selective media (Willinger *et al.*, 2002). Many chromogenic media for isolation and identification of *Candida* spp. are available. Various colored colonies with different morphology are formed on Chrom agar due to a result of breakdown of chromogenic material in the media by specific enzymes of species (Yucesoy and Serhat, 2003). Chrom-agar *Candida* is a chromogenic media for primary identification of *C. tropicalis, C. albicans and C. krusei* (Willinger *et al.*, 2002).Culture methods are not as much as accurate to identify *C. albicans* and other *Candida* species as compared to bacterial media due to this concern, studies were performed on urine samples and on SDA reported increased number of non-*C. albicans* species (Achkar and Fries, 2010).

Unadventurous identification procedures are tough and time consuming (Verweij *et al.*, 2013). Automated systems and packaged kit systems are commonly used, but they are luxurious and are insufficient by their size (Yucesoy and Serhat, 2003). The new commercial schemes are generally simpler to understand, less laborious to established and allow swift identification of isolates than the predictable methods (Davey *et al.*, 1995). Therefore, swift commercially accessible identification techniques have been established which helps in identification of germ tube negative fungi within 48 hours (Verweij *et al.*, 2013).

Severe struggles to develop techniques for testing susceptibility of fungi, have given rise to the emergence of the Clinical and Laboratory Standards Institute (CLSI) M27-A3 (Pappas *et al.*, 2011). The CLSI and European Committee on Antimicrobial

Susceptibility Testing (EUCAST) are two internationally documented standard approaches for antimycotic susceptibility of *Candida* species using broth microdilution (BMD).

CLSI exposed that only 21.1 % of fungal species isolated from vagina are sensitive to antifungal fluconazole. Non-*C. albicans* spp. which are source of repeated incidents, for which azoles agents are less effective (Achkar and Fries, 2010). *C. albicans* is the most common yeast pathogen and more sensitive to antimycotic agents then other species like *C. krusei*, *C. tropicalis* and *C. glabrata* (Yucesoy and Serhat, 2003). *Candida krusei* and *C. glabrata* show less activity against azole group. Itraconazole is normally repeated in mucosal candidiasis, especially in those patients who faced failure in treatment with Fluconazole. Voriconazole is effective for both invasive and mucosal candidiasis (Pappas *et al.*, 2011).

Uncommon specie, like *C. dubliniensis* have been reported to be less susceptible to antimycotic agents (Yu *et al.*, 2012). Itraconazole, voriconazole, fluconazole and posaconazole show same activity in contradiction of most *Candida* species (Pappas *et al.*, 2011). Flucytosine and amphotericin B are the only universally accessible antifungal agents for long period of time. But now the pharmaceutical trades announce itraconazole, fluconazole, ketoconazole and the lipid formulations of amphotericin B (Rex *et al.*, 2013).

In a short period of time many molecular techniques such as multiplex PCR, nested PCR, fluorescent PCR, light-Cycler PCR and Taq-man PCR are developed for detection of *Candida* spp. with a high specificity and sensitivity (Allam and Ihab, 2012).

Based on differences in the rRNA, molecular identification techniques for fungi have been described recently, which help in rapid and accurate identification of *Candida* spp. (Xu *et al.*, 2013). For further identification of *Candida* isolates, Restriction Fragment Length Polymorphism is also used. In this technique amplified DNAs of *Candida* spp. are cut by using restriction enzyme *Msp*I (Mirhendi, 2006).

Between the 18 and 28s rRNA gene of *Candida* spp. and other micro-organisms, the ITS (Internal Transcribed Spacer 1 and 2) fragments are located. The variability in ITS regions plays important role in the phylogenic studies of many organisms (Orita *et al.*, 1989). Polymerase Chain Reaction (PCR) amplification of ITS2 region has been termed as a reliable and rapid method for characterization of yeast infections (Xu *et al.*, 2013).

Aims and Objectives

1. To isolate different *Candida* spp. in various type of clinical samples.
2. To find the prevalence of different *Candida* spp. in various type of clinical samples.
3. To find the presence of *Candida* species in various age groups.
4. To determine the isolation efficiency of commercially available media.
5. To compare isolation rate of *Candida* between media and germ tube.
6. To assess the antifungal sensitivity of isolates against commonly used antifungal.
7. To determine association of clino-pathological and demographic features with the *Candida* species.
8. To identify various *Candida* isolates on the basis of ITS (Internal Transcribe Sequences).

Review of Literature

REVIEW OF LITERATURE

Candida is one of the yeast cell, which produces buds and oval in shape, gram positive and form pseudo-hyphae in tissues and culture. *Candida* is one of the member of normal flora of human upper respiratory, gastrointestinal, and female genital tracts. Germ tube formation in serum is one of the rapid technique for primary identification of *Candida albicans.* Over the past two centuries the rate of infection of *Candida* spp. is increasing and other *Candida* species are replacing *C. albicans* at the majority of clinical sites like bloodstream infections. Due to the commensal quality of *Candida* species, it is found to be a cause of endogenous infection (Jha *et al.*, 2006)

2.1 Prevalence

In countries like Taiwan, hospital acquired yeast infections have drastically increased during the previous twenty years. This was due to the antifungal resistance against *Candida* species, which was recorded in 36.8% *C. albicans*, 30.8% *C. glabrata* and 46.5% *C. tropicalis* (Yun-Liang *et al.*, 2008).

Enwuru *et al.* (2008) carried out study to inspect the: survival of Oro-Pharyngeal Candidiasis (OPC), species allocation, profile of yeast that were susceptible to fluconazole isolated from oral samples of Human Immunodeficiency Virus (HIV) patients from Lagos Nigeria, between October, 2004 and June, 2005. C. *albicans* (40.5%) was the commonly isolated specie followed by *Candida tropicalis, Candida krusei, Candida glabrata* and *Candida neoformans.* Only 1 *Candida albicans* was germ-tube negative while 4 out of 13 isolates of C. *tropicalis* also showed germ tube positive. Total 74 isolates were checked for fluconazole sensitivity in which 78.4% were sensitive among them 86.7% were *C. albicans.* According to rank of susceptibility, *C. albicans* was the most prevalent then *Candida tropicalis* and *Candida krusei.*

Zarrin and Mahmoudabadi (2009) reported that the risk of *Candida* infections is increasing, due to the increase number of people at risk. The fourth major cause of blood stream infections in the USA were *Candida* species. However important geographic changes have been stated. Due to increased risk factors, the incidences of *Candida* species infections are increasing. Important geographic changes may be associated with medical interventions *i.e.* use of antifungals in prophylaxis.

Ariff *et al.* (2010) studied the clinical findings and spectrum of neonatal candidiasis in a tertiary care hospital, Karachi. Candidemia was found to b at a frequency of 0.9%. In neonates having very low birth weight (VLBW), the incidence was 46%. *C. albicans* was the most significant *i.e.* 55%. Identified risk factors were ventilation (> 7 days), period of hospitalization (> 7 days) and positive bacterial cultures.

Oberoi *et al.* (2010) analyzed the alteration in the epidemiology of antifungal and candidemia at tertiary care hospital in New Delhi, India. Fluconazole prescription was increased in candidemia. Cross-resistance to both voriconazole and fluconazole was checked in 11.3% isolates. The study described a change in *Candida* spp. and the appearance of novel species *C. haemulonii,* which was resistant to azole and amphotericin B.

The infections due to *C. tropicalis* are rising all the way through the world. Most of *Candida* spp. were resistant to azoles causing very high mortality and morbidity. Growth rate of *C. tropicalis* was higher in blood then urine and sputum. All 345 specimens were taken from urine, blood and sputum of patients admitted in intensive care units (ICUs) of many health care centers in India. Fifteen strains were isolated and among these high incidence of *C. tropicalis* infections that were resistant to azoles and to some extent to amphotericin B (Basu *et al.*, 2011).

Nejad *et al.* (2011) determined incidence of *Candida* species from oral cavity of infected humans going through from periodontitis in Iran. Total 172 specimens were taken from patients of 11-72 yrs. *C. albicans* (75%) was the dominant specie among all other species. Other species isolated were C. *glabrata (*12.5%), *C. tropicalis* (6.5%),

C. dubliniensis (4.0%) and *C. krusei* (2.0%). In this study, *C. albicans and C. dubliniensis* were further identified by chlamydospore formation, germ tube-test and Chrom-agar *Candida* (differential media). Germ tube test were positive in dark-green colonies (*C. dubliniensis)* and light-green colonies (*C. albicans*) on Chrom-agar *Candida.* Therefore, Chrom-agar *Candida* is an appropriate method for accurate and rapid recognition of yeast species and helpful in easy detection of *Candida* spp. in mixed cultures.

Lar *et al.* (2012) studied the distribution and occurrence of *Candida* species in HIV infected patients of Jos, Nigeria. A total of 248 subjects were studied for oropharyngeal candidiasis, in which 9.68% showed *Candida* growth. The age group of 36-45years patients showed maximum positive isolates. Percentage of positive isolates of *C. albicans* was 79.2%; *C. pseudotropicalis* 8.3%, while *C. parapsilosis, C. tropicalis* and *C .guilliermondi* 4.2%. Oropharyngeal candidiasis was less common in men than women. *C. albicans* still remained the foremost cause of oropharyngeal candidiasis. It was recommended that constant identification of yeasts in HIV and immune compromised patients should be done in order to improve the suitable treatment and minimize antifungal resistance.

Aspergillus and *Candida* spp. were found to be the common agents that cause invasive fungal infections in kids; however the main cause was still *C. albicans*. The risk factors and epidemiology were different in children admitted in the Neonatal Intensive Care Units (NICUs). In children, mortality rate for invasive candidiasis was less then invasive aspergillosis (Brissaud *et al.*, 2012).

Hammoud *et al.* (2012) determined newborns candidemia in NICU of the Maternity Hospital, Kuwait. *Candida* infection was present in 89 neonates where as 54 had insistent Candidemia. The percentage of fatality between persistent and non-persistent candidemia was 54 and 33% with higher percentage of females with persistent candidemia. All *Candida* spp. were susceptible to antifungals. It was determined that

persistent candidemia was common in neonates and was related with an improved risk of death.

Rajeswari *et al.* (2012) observed 56% incidence of *Candida* spp. through 6 types of clinical sources. The highest incidence was 68% from urinary catheter, 66.66% from intravenous tubes, 65% from venflon needles and 53.33% from blood bags. The *C. albicans* was principal isolates in all sources particularly in urinary catheter with 60.2% of other *Candida* species. Antifungal stability test of all isolates were done. Among them itraconazole resistance was most prevalent in *C. tropicalis* 83.3%, followed by *C. glabrata* 74.5% and fluconazole was more sensitive. All isolates had the capability to make biofilm, in which 37.4% isolates were strong biofilm manufacturer.

Parmeland *et al.* (2013) explored candidemia by *Candida* spp. in an institutional hospital during ten years 2000–2010. One hundred and eighty two patients contributed, in which *C. albicans* 102% was the major commonly identified specie. In other *Candida* species, *Candida glabrata* (32%), *Candida parapsilosis* (21%), *Candida tropicalis* (13%), *Candida krusei* (8%), *Candida kefyr* (3%), *Candida lusitania* and *Candida lipolytica* 2%, while *Candida famata*, *Candida guilliermondii*, *Candida inconspicua*, *Candida dubliniensis*, *Candida sake* and *Candida nivariensis* were 1%. Unexpectedly, this study showed that in spite of the ICUs *Candida* spp. distribution was similar from that of their earlier study in the 1998 to 2001, except *C. krusei*.

In Iceland a nation-wide study on candidemia was conducted in a period of 2000 to 2011, with the aim to resolve new techniques in occurrence of *Candida* spp., fungal species distribution, antimycotic use and their sensitivity patterns. Total 208 infections were recognized in 199 infected patients. A standard occurrence in this period was 5.7 cases per 100,000 populations per year, which was considerably high than 1990-1999 (4.3 cases per100,000 populations per year) survey. According to age, occurrence rate were considerably high in patients, 20.7 for <1 year and 18.1 for >60 yrs, and wide-ranging in gender. In males and females occurrence due to age *i.e.* >80 yrs was 28.6 and 8.3 respectively. Among all *Candida* species *Candida albicans, C. glabrata* and

C. tropicalis were the main isolates. Use of fluconazole was growing 2.4 times from 2000 -2011, with no increase in its resistance (Asmundsdottir *et al.*, 2013).

Alhussaini (2013) described that candiduria has increased considerably over the precedent few years. Kidney failure patients (20%) were anguish due to candiduria, in which males were less affected than females. *C. albicans* was commonly isolated specie among *C. krusei*, *C. tropicalis* and *C. glabrata*. Only *Candida albicans* showed germ tube positive test and chlamydospores assembly. All four *Candida* spp. isolated were further confirmed by sequencing of rRNA gene. Antifungal sensitivity showed that nystatin and amphotericin-B inhibited all *Candida* isolates while 50% - 67% of *Candida* strains were efficient to other antimycotic drugs. Sertaconazole and clotrimazole showed resistance to all isolates of *C. glabrata*.

2.2 Pathogenicity

Hurley and Stanley (1967) studied that systems of cell culture were appropriate for study of the pathogenicity of *Candida* species. The cytopathic properties of all species of *Candida* on epithelial cells of mouse and relationship between growth and death rate of fungi and its physical stage can be studied efficiently.

Kuleta *et al.* (2009) studied the virulence factors of the most prevalent pathogenic fungi in human *i.e. C. albicans*, *A. fumigates* and *C. neoformans*. Factors that contribute to virulence of *C. albicans* are discharge of hydrolytic enzymes, adherence, physical dimorphism and phenotypic switching while the virulence factors of *C. neoformans* are: melanin creation, capsule production and growth at 37°C. Making of pigments, oozing of toxins, hydrolytic enzymes and adhesion molecules present on the surface of cell are the reputed virulence factors of *A. fumigates*.

MacCallum (2012) discussed the models available with both systemic and mucosal infections to examine *Candida* virulence and evaluate the ability of various *Candida* strains to cause infections.

2.2.1 Biofilm Formation

Ramage *et al.* (2011) described the significance of fungal biofilms, mainly *C. albicans* and their resistance pathways. It is due to efflux push activity, extracellular matrix (ECM), cell compactness, pressure responses, general makeup and the over phase of drug targets in the cell.

2.2.2 Adherence Ability to Human Skin

Pasteur *et al.* (2011) analyzed the ability of adherence of *C. parapsilosis, C. albicans* and *C. tropicalis* to human skin, using Scanning Electron Microscope (SEM). The SEM analysis exposed that all above three species stick on the skin. C. *albicans* roofed at the whole skin in high amount than C. *parapsilosis* and *C. tropicalis.* Mucin like fabric covered the blastoconidia mostly in *C. albicans.* All *Candida* spp. have revealed characteristics similar to biofilm configuration which partially explains the dominance of *C. albicans* in cutaneous pathogenicity.

2.3 Isolation from Different Clinical Specimens

Jha *et al.* (2006) determined the isolation rate of *Candida* spp. from sputum specimens in Nepal. Total 462 specimens were examined, in which *Candida* species were isolated from 30 samples. *C. albicans* (70%) was the major isolate. In other species 13.33% was *C. tropicalis.* 10% *C.* krusei and 3.33% were *C. parapsilosis* and *C. stellatoidea.* It was concluded that in lower Respiratory Tract Infections (RTI), *Candida* was the third most common pathogen.

Kangogo *et al.* (2011) studied the phenotypic classification of *Candida* spp. from blood, stool, urine, Cerebral Spinal Fluid (CSF) and swabs of patients from different hospitals in Nairobi. In which *C. albicans* were (86.7%) but 3.8% were germ tube negative, whereas the rest of other *Candida* spp. were 13.3%. It was concluded that *Candida* spp. were of clinical important and some were fundamentally resistant to certain antifungals particularly in HIV infected patients.

Pethani *et al.* (2012) isolated and identified *Candida spp.* from different samples, as well as studied the occurrence of fluconazole resistance in Ahmedabad, India. A total of 62 specimens were examined. Out of 62 isolates *C. tropicalis* were 43.54%, *C. albicans* 30.64%, *C. guilliermondii* 11.29%, *C. parapsilosis* 8.06%, *C. kefyr* 4.84 %, and *C. glabrata* 1.62%. Thus, the main isolates of their study were the non albicans species. New-born were at high risk of candidemia because *C. guilliermondii* were isolated from their blood culture, while *C. albicans* were resistant to fluconazole *i.e.* 58%.

Yu *et al.* (Dec 2011) reported the first case of *C. dubliniensis* in Korea, appeared in a 64 years old woman. Symptoms were: incomplete seizure, regular fever and drowsiness. Germ tube positive and white colonies on Blood agar and Sabouraud Dextrose agar indicate growth of C. *dubliniensis*. C. *dubliniensis* was further identified using chemical and molecular techniques. The blood positive isolate *i.e.*, C. *dubliniensis* was less resistant to fluconazole, flucytosine, voriconazole and amphotericin B.

2.4 Rate of *Candida* spp. in Nosocomial Infections

Diba *et al.* (2012) isolated *Candida* spp. from nosochomial infections and IPD cases. Main isolates obtained were 66.6% *Candida*, 31.4% *Aspergillus* and only one case of *Alternaria* species. Among all IPD isolated yeasts; 60% *Candida albicans*, *Candida krusei* (17%), *Candida glabrata* (14.3%), *Candida tropicalis* (0.7%) and 3% *Candida parapsillosis*. Several infections due to *Candida* in mainly immune-compromised patients were due to contaminated ecological surface and hand touches.

2.5 Infections in Blood Stream

Candida spp. was considered as a main source of infections in blood stream and related with 80% of all fungal nosocomial infections. *Candida* species were the fourth most common reason of infections of blood stream in the USA. Non-*albicans* species were associated with at least 50% of all persistent infections and were different in clinical effects and susceptibility to antifungal drugs. The rough death rate of candidemia was

between 40 and 60%, due to which it was dare for all clinicians (Colombo and Guimaraes, 2003).

Romeo *et al.* (2011) reported the molecular description of *C. parapsilosis* obtained from the blood and catheter tips of patients in the Polyclinic Hospital, Italy. Among 97 *C. parapsilosis* isolates, 94 were recognized as *C. parapsilosis* and 3 isolates belonged to cryptic species. *C. orthopsilosis* were obtained from neonatal blood cultures. Therefore, *C. parapsilosis* was considered a significant hospital acquired pathogen.

Ece *et al.* (Sep 2012) studied the occurrence of *Candida* species in a tertiary-care hospital in Western Turkey. *Candida* species were taken from 337 samples. Mainly isolated strains were *Candida albicans* (38.6%), *Candida tropicalis* (13.9%), *Candida parapsilosis* (28.4%), *Candida glabrata* (7.4%) *and* 3.8% *Candida krusei. C. parapsilosis* was the main isolated strain in blood cultures and this may be due to the utilization of indwelling catheters.

2.6 Infection in Oral Cavity

Grimoud *et al.* (2003) studied the colony formation in the oral cavity by *Candida* spp. A total of 110 samples were examined from oral cavity, in which *Candida* spp. were grown in 67% of cases. *Candida albicans* was commonly recognized specie before *Candida glabrata.*

2.7 Vaginal Candidiasis

Sanchez (2006) investigated that in Mexico vaginal candidiasis is common due to different *Candida* species. Total 631 *Candida* species were recognized by API 20C system. The most repeatedly identified species were *C. glabrata* (35.9%), *C. albicans* (39.0%), and *C. tropicalis* (16.2%).

Rodriguez *et al.* (2012) determined the microbiological biochemical and molecular characters of four species of *Candida* in order to find vaginal candidiasis in Tuxtla. A total of 167 samples were taken from women, aged between 15- 45 years.

The frequency of candidiasis studied among these samples was 21%, in which many cases were asymptomatic. There is a higher dominance of *C. glabrata* than other *Candida* species. The disease occurrence was related with women belonged to poor families with vigorous sexual life.

Khan and Baqai (2010) isolated *Candida* from High Vaginal Swabs and studied *in-vitro* antimycotic activity against fluconazole, clotrimazole, and nystatin. Two hundred and fifty HVS were taken from many hospitals of Karachi. In which 40% of cases were positive for *Candida* species. Out of 100 samples, 30% were *Candida albicans*, 21% *Candida tropicalis*, 10% *Candida parapsilosis*, *Candida parakrusi*, *Candida glabrata* 8% and 3% *Candida krusei*. *In-vitro* antimycotic activity indicated that clotrimazole was more effective (70%) against *Candida* species. Fluconazole sensitivity was 36.2% in isolates and to nystatin was 63.5%. It was concluded, that *C. albicans* was the main isolate. Antimycotic susceptibility should be determined before treatment to avoid its failures

Taheri *et al.* (2011) performed a study in which 164 vaginal swabs were taken from mothers before delivery and from oral mucosa of all infants directly after birth. *C. albicans* and *C. albicans* were most frequent isolated species. There was considerable dissimilarity in other *Candida* spp. on the oral mucosa between cesarean and vaginally-born infants. There was no major dissimilarity in *Candida* spp. on the vaginal mucosa between mothers in both groups. Ketoconazole had greater antifungal action in all sets.

Isogai *et al.* (2010) acknowledged *Candida* spp. from HIV positive cases by Tobacco agar, Chrom agar, and PCR of amplified ITS rRNA region in Ethiopia. Oral rinses from 13 patients were collected. Both genotypic and phenotypic procedures were foremost to find out the species. These results showed that HIV-positive patients have elevated danger to *C. albicans* and other species infections in Ethopia.

2.8 Identification of Candida species

2.8.1 Germ Tube Formation

Nakamoto (1998) observed that *C. albicans* IFO 1385, produced germ tubes in corn meal (broth) at 37°C. In all *Candida* spp. Germ tubes were only formed in *C. albicans*. Therefore, under favorable circumstances, the rhythm of isolating *Candida albicans* was 100%, showing that germ tube recognition with non-slip slide glass incubation (NSSI) method is a fast and reliable method.

Kim *et al.* (2002) found that in all *Candida* spp. only *C. albicans* produce GT in serum-free Yeast Extract Peptone Dextrose (YEPD) media at 39°C, which helped in classification of *Candida albicans* from other species. Standard strains of *Candida*: two *Candida dubliniensis* and three *Candida albicans* were also cultivated. Two *Candida dubliniensis* produced GTs at 37°C in serum and are in yeast form at 39°C. Therefore, the sole GT production of *Candida albicans* at 39°C could be used as a procedure for fast and suitable recognition of *Candida albicans*.

Hudson *et al.* (2004) studied inducer of serum for GT formation in *Candida albicans*. In which the dialyzable main inducer was D-glucose and a second non-dialyzable percepetible inducer was tri-chloro acetic acid. However, 1.4% (v/v) of D-glucose promotes 50% GT production; the in-dialyzable inducer required 10 fold increase concentration. Serum was the efficient media for germ tube induction due to its pH *i.e.* 8.5 and has 2 type of inducers; which were dynamic at this pH.

2.8.2 Identification through Chromogenic Media

Yucesoy and Marol (2003) correlated the Chrom-agar and Bismuth sulphite glucose glycine yeast (Biggy) agar for recognition of *C. krusei, C. tropicalis, C. albicans and C. glabrata.* Total of 270 yeast strains were studied. The specificity and sensitivity standards were 99.4% for *C. albicans*, 100% for Chrom agar and 75.2% for Bismuth sulphite glucose glycine yeast agar. The specificity of other strains for Chrom agar was

100% and sensitivity was between 90.9 -100%. The specificity values were 95.4- 100% for Biggy agar and sensitivity was 66.6 and 100%, respectively. According to these results Chrom-agar *Candida* was simple and reliable method then Biggy agar for the presumptive detection of *Candida* spp.

Eraso *et al.* (2006) concluded that *Candida* ID2 (CAID2) media is a good applicant to change former media like *Candida* ID (CAID) and *Albicans* ID2 (ALB2), and compared it to Chrome Agar *Candida* (CAC), for presumptive and culture detection of *Candida* spp. in Spain. One hundred and two freshly isolated strains (*Candida, Geotrichum, Cryptococcus, Trichosporon and Saccharomyces*) from different clinical specimens and 345 stored strains from Pathogenic Fungi (NCPF), the American type culture collection (ATCC) and a collection center in Spain were analyzed. The slowest growth of colony was showed by ALB2. Colonies grew faster with stronger colour on CAID2. In stick collection 94.8% strains showed growth on CAID2 and CAID have 94.2% of growth. On both media CAC and *Albicans* ID 2 growth was 92.8%. Growth of 100% strains was showed by CAID2. Moreover CAID2 was good then CAC in faster growth, color, recognition of mixed type of cultures and primary discrimination between *Candida dubliniensis* and *Candida albicans*.

Nadeem *et al.* (2010) used Chrom-agar for primary detection of all *Candida* spp. from detached samples in Karachi, Pakistan. A total of 487 strains were analyzed. Chrom-agar *Candida* can simply recognize three *Candida* species on due to morphology, colonial color and precisely distinguished between them i.e. *C. tropicalis*, *C. krusei* and *C. albicans*. The specificity and sensitivity of this media for *Candida albicans*, *Candida tropicalis* and *Candida krusei* was calculated as 99%, 98%, and 100%. It was concluded that Chrom-agar *Candida* is helpful in quick recognition of *Candida* species, which could be very useful in rising suitable therapeutic policies and administration of patients.

2.8.4 Identification through Commercial Kits

Mashad and Mahmoud (2011) *in-vitro* compared marketable tests E-test and candifast kit for antimycotic suensitivity testing of *Candida* species to national committee for clinical laboratory standards (NCCLS) BMD technique. A total of 30 isolates were included. The overall proportion of conformity of E-test with normal method was 90% for each of amphotericin B and fluconazole. For amphotericin B the consistency of E-test with NCCLS was 100% in tested species apart from *C. glabrata* (85.7%) and *C. parapsilosis* (66.6%), while the results of candifast were 100% except for *C. glabrata* (85.7%). For fluconazole the fraction of concurrence of E-test was 100% for each of *C. tropicalis and C. glabrata* and was 92.8 for *C. albicans* and 66.6% for *C. parapsilosis.* The fraction of candifast was 100% except *Candida albicans* and *Candida glabrata* which was 92.8 and 85.7% respectively. E-test method was replacement but cannot be measured as a replacement for the NCCLS reference method.

Mondelli *et al.* (2012) identified the *Candida* spp. by the automated system Vitek-Biomerieux and physical technique. Ninety-eight samples of *Candida* spp. were studied, in which 13.5% of *C. parapsilosis* samples were different from the manual method, which were faultily identified by Vitek system as *C. tropicalis*, *C. lusitaneae*, and *Candida albicans.* One discrepancy was present in *Candida glabrata* and *Candida guilliermondii* which was recognized as *C. parapsilosis* and *Candida* spp. by Vitek. All *Candida albicans*, *Candida lusitaneae* and *Candida tropicalis* specimens were known appropriately.

Verweij *et al.* (2013) compared seven marketable yeast classification methods with that of a manual method and compare the costs of the marketable kits. In 52 clinical *Candida* isolates 19 species were identified correctly ranges between 59.6 and 80.8%. The maximum yield was obtained with API (Analytical Profile Index) *Candida* and Auxacolor *i.e.* 78.8% and 80.8%. Both the methods identified 93.1% of *Candida* spp. isolates properly. All seven methods were unsuccessful to recognize *C. catenulate, C. norvegensis, C .dubliniensis* and *C .haemulonii.* In contrast to Auxacolor, the API

Candida requires less time. It was suggested that API *Candida* and Auxacolor was the most helpful systems for classification of *Candida* spp. in clinical samples.

Xu *et al.* (2013) isolated *Candida* species from blood stream and compared API20C with PCR amplification and sequencing. Fifty eight isolates were obtained. The API20C was useful in the classification of *Candida* spp. and molecular techniques do not improve identifications complete using the API20C system.

2.9 Treatments

2.9.1 By Chemical Method (Salicylhydroxamic acid (SHAM))

Pang *et al.* (2010) controlled the development of six disease causing *Candida* spp. by ~13 mM Salicylhydroxamic acid (SHAM). 25mM SHAM repressed the O2 uptake speed of *Candida dubliniensis*, *Candida tropicalis* and *Candida krusei* upto 18 to 36%, but remains un-effective on *Candida parapsilosis* and *Candida glabrata.* SHAM considerably retarted the growth of *Candida* species. SHAM is helpful in the treatment of urolithiasis and urinary tract infections (UTIs) and SHAM is feasible in handling of *Candida* species diseases.

2.9.2 By Zatariamulti flora Extract

Farasat *et al.* (2012) recognized *C. albicans* isolated from Recurrent Vulvovaginal Candidiasis (RVVC) patients by PCR-RFLP (Random Fragment Length Polymerization) and its drug sensitivity to *Zatariamulti flora* extract. A total of 23 yeasts were analyzed, in which 53% were *Candida albicans*, 35% *Candida glabrata* and 13% *Candida krusei*. The minimal inhibitory concentrations (MICs) of the used *Z. multiflora* extract for three different *C. albicans* strains, was 17437.5, 8718.75 and 34875 µg/ml. *Candida* spp. were sensitive to *Z. multiflora* extract and hoped that in the future this herbal drug may be the substitute of chemicals drugs and be used to treat RVVC patients.

2.9.3 By Antifungal Activity

Lee *et al.* (2009) investigated the disk diffusion method with Glucose Methylene Blue Muller Hinton (GMMH) agar for identification of the fluconazole and voriconazole susceptibility of all *Candida* species and correlate these results with Epsilometer Test (E-test). The results of both antifungals after 24 h had a high correlation. Positive analytical value of the disk diffusion test of voriconazole on agar after 24h was 100% for *Candida* spp. and *Candida albicans.* Both methods were rapid, pure, economical and realistic for the early susceptibility testing of *Candida* species to fluconazole. And voriconazole

Satana *et al.* (2010) studied the antifungal sensitivity of 67 oral *Candida* species from effected patients with HIV, in Turkey. All species were susceptible to voriconazole. Sixty five were effective against Amphotericin B, 50 to Itraconazole, 64 to Ketoconazole and 66 to Fluconazole. A total of 8.96% resistance was observed. No resistance was found to voriconazole and fluconazole.

Agha *et al.* (2011) recognized different strains of fungi and their antifungal sensitivity in immune-compromised patients. One hundred and sixty five samples were collected. *C. glabrata* was screened from 39 samples, in which resistance to fluconazole was 13%. Despite of *C. albicans*, C. *glabrata* was also difficult to treat in immune-compromised patients because of its resistance to antifungals.

Jeddy *et al.* (2011) described antifungal sensitivity of *Candida* isolated from HIV infected patients in Chennai, India. Thirty six oral samples were taken. *Candida* spp. was the major isolate in HIV positive patients and 21 samples were resistant to fluconazole due to more exposure to the drug.

Moris *et al.* (2012) studied occurrence and antifungal sensitivity pattern of *C. parapsilosis* isolated from oral cavity of HIV affected patients. In 318 *Candida*, 15 were recognized as *C. parapsilosis* by Polymerase Chain Reaction (PCR) and RFLP. In this study known isolates of *C. metapsilosis* were also obtained. Antifungal tests specified

that all were sensitive to Fluconazole, Amphotericin B, Itraconazole, Ketoconazole, Caspofungin and Voriconazole.

Pfaller and Diekema (2012) refined and standardized antifungal sensitivity of *Candida,* which help in organizing *Candida* infections. Rationale of 24-h reading time for *Candida* and Epidemiological Cutoff Values (ECVs) were also developed. The Clinical Breakpoints (CBPs) for echinocandins, fluconazole and voriconazole were revised. This review helped in the development of antimycotic susceptibility testing using CLSI and BMD technique.

A new way for sensing fluconazole resistance in *Candida* spp. is by X-Plate skill. Investigation of 1383 clinical samples with VVC exposed, that this technology was capable to notice and specify the *Candida*. The occurrence and fluconazole susceptibility outlines of the clinical isolates using this technique were extremely similar to available microbroth dilution way (Chadwick *et al.*, 2013).

2.10 Molecular Techniques

Nnadi *et al.* (2012) isolated and performed molecular categorization of C. *africana* from Jos, Nigeria. During investigation two unusual *C. albicans* isolates were found. Molecular investigation showed that both isolates belonged to family of *Candida africana,* which is new anticipated specie of *Candida* correlated to *Candida albicans.*

2.10.1 Polymerase Chain Reaction (PCR)

Looke *et al.* (2011) developed a rapid and inexpensive genomic DNA extraction procedure from yeast cells for application based on PCR. This process does not require any dangerous chemicals, enzymes, or extreme temperatures, and is useful for immediate analysis of many samples. DNA extracted by this technique is appropriate for RT qPCR, colony PCR, and DNA sequencing for amplification of ≤ 3500 bp DNA fragments.

2.10.2 Multiplex PCR

Rad (2012) identified *Candida* spp. from vaginal isolates by multiplex PCR in Mahdieh hospital, Iran. Total 191 isolated species were taken. *C. glabrata* and *C. albicans* were the two main causes of vulvovaginal candidiasis. Multiplex PCR is a reliable and rapid technique to classify *Candida* spp.

G. Liguori *et al.* (2011) compared nine methods for *C. albicans* classification with multiplex PCR with the ATCC strains. A total of 390 yeast strains were obtained from vaginal and oral swabs. Only 253 isolates were *C. albicans* by germ tube, and multiplex PCR. Comparison of phenotypical methods was between 81.9% and 87.7%. Maximum and minimum values of specificity were, 96.8% (*Candida* ID2) and 75.1% (Chrom agar). It was concluded that all the methods tested showed satisfactory levels of sensitivity and specificity with respect to the multiplex PCR; therefore if molecular techniques are not accessible all could be helpful for *C. albicans* identification.

2.10.3 Real Time Polymerase Chain Reaction (RTPCR)

Selvarangan *et al.* (2003) reported a RTPCR technique for identification of six *Candida* species straight from BACTEC (blood culture) system. Deoxyribonucleic acid (DNA) of 62 BACTEC positive samples were extracted and the results of this technique was 100% similar with morphological and biochemical features. *C. albicans* (22) was the major isolate. Other species were *Candida parapsilosis* (10), *Candida tropicalis* (1), *Candida glabrata* (22), *Candida krusei* (2) and Candida *lusitaniae* (1).

Metwally *et al.* (2007) developed a swift test to distinguish between fluconazole resistant and sensitive species of *Candida i.e. C. tropicalis, C. parapsilosis, C. albicans, C. dubliniensis, C. krusei* and *C. glabrata* by RT-PCR from positive blood-culture bottles. The results were 100% analogous with those of phenotypic detection carried out at the same time. RT-PCR technique is less time consuming as compared with a predictable phenotypic process, from ~72 to 3 h.

Using seven different DNA extraction procedures from *C. albicans*, *Candida*-specific RT-PCR was used for its identification in blood. Out of seven procedures, Master Pure reagent kit gave considerably privileged yield of nucleic acid while high purity was given by Yea-Star Genomic DNA kittechnique. More notably, *C. albicans* DNA was detected with maximum sensitivity through the YeaStar method (Metwally *et al.*, 2008).

2.10.4 Rolling-Circle Amplification Based Method

Zhou *et al.* (2008) detected and identified *Candida*, *Scedosporium* spp. and *Aspergillus* by using Rolling-Circle Amplification based method. This technique used species-specific padlock probes besieged to the ITS-2 region of the fungal rDNA gene complex. The attempt was swift (2 hours) and precise. Of 28 fungal isolates *Candida* (16), *Scedosporium* spp. (6) and *Aspergillus* (6) were identified properly.

2.10.5 Random Amplified Polymorphic DNA (RAPD)

Rocha *et al.* (2008) recognized and differentiate *Candida* spp. from peads by random amplified polymorphic DNA (RAPD). Thirty-four isolates were analyzed by RAPD. Regarding the only fluconazole resistant *C. tropicalis* was different from the other *Candida* species. The data were inadequate to insist that the only dissimilarity was the susceptible to fluconazole. It was concluded that the RAPD technique might be used to verify *Candida* spp. recognized by microbiological procedures.

2.10.6 PCR Restriction Fragment Length Polymerase (PCR RFLP)

Present phenotypic recognition methods of *Candida* spp. are lengthy so molecular techniques were used. A total of 170 specimens were examined, in which 52 were positive for *Candida* species. By API20 C AUX, maximum isolates were of *C. albicans* (44.2%), *Candida tropicalis* (25%), *Candida glabrata* (11.5%), *Candida krusei, Candida stellatoidae,* and *Candida kefyr* (1.9%). Categorization by RF PCR showed 100 % conformation with API in case of *Candida glabrata, Candida trobicalis, Candida*

guilliermondiand and *Candida krusei* while 96% in *Candida albicans*. Isolates of *Candida kefyr*, *Candida parapslosi* and *Candida lusitaniae* cannot be recognized by RF-PCR at species level. It was concluded that *C. albicans* was the most significant *Candida* spp. and other species were rising as a vital pathogens. It was also concluded that using the *MpsI* (restriction enzyme) PCR RFLP is a good and fast identification system (Allam and Salem, 2012)

METHODS AND MATERIALS

MATERIALS AND METHODS

This present study was performed in Microbiological Laboratory, Department of Pathology, Quaid-i-Azam International Hospital (QIH) and Department of Microbiology, Quaid-i-Azam University (QAU), Islamabad, Pakistan.

3.1. Sample Collection

The study was carried out for a period of 10 months (March to December 2013). During this period 3007 samples were collected from QIH for the purpose of isolation and identification of different *Candida* species causing candidiasis. All specimens were collected and handled according to standard protocols.

3.2 Sample Preservation

Fresh blood samples were collected in a blood culture bottles containing Cary-Blair's transport media from patients. Bottles containing samples were placed in BACTEC9120 (Becton Dickinson, BD) for further analysis and later on cultured on two types of selective media.

Urine, HVS, stool, tissues, bronchial leavage, tracheal secretion and other swabs like pus, wound and throat were streaked immediately on different selective media for further isolation and characterization of the causative agents.

Samples were collected from both male and female patients, visiting the hospital. Before the conduction of study, these patients were informed about the purpose of study and their background information regarding the health status and other related questions. All information's were gathered by in person interview or by parents/guardians of the children in a form of questionnaire.

3.3 Isolation of Clinical Isolates of *Candida* species

Before isolation of *Candida* spp. from different clinical samples different type of media were prepared. In Blood agar (BA) (CM OO55, Oxoid) (Appendix) 32 gm were

dissolved in 800 ml and autoclaved it for 20 mins at pressure of 1.5 and 121°C. After autoclaving when the media was cooled at 45°C, in sterile condition human or sheep blood was added and pours it in petri plates. Allowed it to solidify at room temperature. After solidification it was used for inoculation of different clinical samples. Chocolate Agar (CA) (CM OO55, Oxoid) was also prepared according to the above procedure but in CA, blood was poured at a temperature of 70°C. Due to which all red blood cells were lysed and give brown colour instead of red.

MacConkey (MC) Agar (Appendix) (CMO 115, Oxoid) 43 gm in 800 ml, Cystine Lactose Electrolyte Deficient (CLED with indicator CM0423, Oxoid) medium (Appendix) 28.96 in 800 ml and Sabouraud Dextrose Agar (SDA) (Appendix) (CM0041,Oxoid) 52 gm in 800 ml and autoclaved them for 20 mins at pressure of 1.5 and 121°C. After autoclaving when the media was cooled at 45°C, pour it in petri plates and allowed to solidify at room temperature. After solidification it was used for inoculation of different clinical samples.

All samples from patient were collected aseptically and cultured on five different types of media *.i.e.* urine specimens were inoculated on CLED by streaking and by BACTERURITEST strips (Mast Diagnostics, U.K). In BACTERURITEST strips less than or equal to100,00 organisms per ml, in which a colony count of less than o equal to 25 bacilli, and less than or equal to 30 for gram positive cocci. It is recommended that if colony count is less than or equal to 20 in spite of its type should be important. Growth of bacteria less than 10,000 organisms/ml produce only few colonies on media and which is considered as no growth.

Samples from throat, pus and wound were taken aseptically from patients by sterile swabs and were streaked directly on BA and MC Agar.

The BACTEC 9120 is an instrument which helps in quick identification of microorganisms in blood samples. Blood sample which is to be checked for any type of microorganism is injected into the bottle, which is placed inside the BACTEC for reading

after regular interval and proper incubation. Sensors are present in each bottle which show response to CO2, which is produced during the microbial metabolic pathway present in blood or the intake O2 required for microbial growth. Instrument monitored the sensors after every 10mins for checking of its fluorescence, which is directly proportional to CO2 production and O2 consumption inside the bottle. Positive result shows the presence of living microorganisms in that bottle. Those samples were then taken out from instrument and streaked on BA and MA, respectively.

Ear swab and vaginal samples were also taken aseptically in swabs containing Amies Medium (mwe, medical wire, UK) for specimen collection and transportation. Both type of swabs were streaked on BA, MA and SDA.

Sputum sample (not saliva), infected tissues (In a container containing 8.5% normal saline), and all body fluids (Ascetic, Pleural, Cerebral Spinal Fluid) were collected through special aseptical techniques with great care and were processed on BA, MA and CA while tracheal secretion was streaked all four type of media.

Fungal cultures were cultured on SDA and placed on both 25°C and37°C, while other samples were incubated for 24-48 hours at 37°C. After incubation colonies of *Candida* were observed. Observed colonies were further examined for morphology by Gram staining.

3.4 Gram Staining

Under aseptic conditions a smear of *Candida* specie from each positive sample was prepared on glass slide and heat fixation was done. Crystal Violet was flooded on slide and leave it for 1min. Then rinse it with water and Gram's Iodine was flooded and washed the slide with water after 1min. Then 95% ethyl alcohol was used for decolorization of slide. After washing again Safranin was flooded for 45sec and rinse the slide with water. Slide was then air dried and observed under the 100X lense of microscope. All chemicals used were from MERCK, Karachi, Pakistan and their composition were given in appendix.

3.5 Germ Tube test

Using a sterile loop, a small portion of a pure colony of *Candida* species were inoculated in to sterile test tubes containing 0.5ml of serum. The test sera were aseptically collected from fresh human blood by centrifugation. Centrifugation is a process use to remove cellular debris from blood to separate cell free plasma or serum. The resulting mixture was incubated at a temperature of 37°C for not more than 2 hrs. At 10 minutes intervals, a drop of mixture prepared was put on a clean microscopic glass slide, covered it with a small cover slip and carefully examined under microscope, using the 10X and 40X objective lenses.

3.6 Identification of *Candida* species

Chrome agar (17 gm in 1000 ml) and Corn meal agar (1.7 gm in 100ml) was also prepared according to the above protocol. After 24-48 hours for identification of different *Candida* species, colonies were shifted on two types of differential media *i.e.* Chrom Agar-*Candida* (Chromc agar Microbiology, France) (Appendix) and Corn Meal Agar (CM0103, Oxoid) (Appendix) and incubate the plates for 24- 48 hrs at 37 °C.

3.7 Preservation of Different *Candida* species

After primary identification on both media, all *Candida* species were streaked on Potato Dextrose Agar (PDA) (39 gm in 1000 ml) (Appendix) for the aim of obtaining their pure culture and again incubate them at 37°C for 24 to 48 hours. After 24-48 hrs pure colonies obtained were preserved in Nutrient Broth (NB) (13gm in 1000 ml) (Appendix) containing 70% glycerol for molecular characterization.

3.8 Analytical Profile Index 10 *Candida* (API 10C)

After identification of seven *Candida* strains on Chrom agar and Corn Meal Agar, Analytical Profile Index 10 *Candida* (API 10C) (Biomurex, France) was used for further identification. API0C is a standardized system for *Candida* spp. in which 10 different biochemical tests were included.

Pure colonies obtained after 24-48 hours obtained were subjected to API10C index. An incubation box was prepared by adding about 5ml sterilized water to make a humid environment. The name of strain was recorded on the elongated flap of incubation box. Strip was taken out from the packing and placed inside the box. For standardization of the inoculum density for a susceptibility test, equivalent to a 3.0% McFarland standard (Annexure) was used. Then fungal suspension was prepared in 5ml of normal saline (0.85 percent NaCl solution). For this purpose fresh culture of 24 hours old was used. Two to three well isolated colonies were taken with the help of platinum wire loop. After shacking the bacterial suspension was compared to 3.0% McFarland standard. By using a sterile syringe all wells of the strip were filled according to the instructions of API 10C manual. All tests were filled half (only tubes were filled). An anaerobic environment was created in GLU, GAL, SAC, TRE, RAF and URE, tubules by putting a drop of mineral oil overlay. Lid of box was closed and placed it in incubator for 24hrs at 37 °C.

3.9 Antifungal Sensitivity Test

The Kirby-Bauer method was used for antimicrobial susceptibility testing, recommended by CLSI (Wikler *et al.*, 2006). Inoculum was set by preparing saline (0.85% sodium chloride) solution of well isolated colonies from PDA agar plates of 18-24 hrs old. The saline suspension was prepared according to the 0.5% McFarland standards. Glucose Methylene Blue Mullar Hinton (GMM) media (Appendix) was prepared. By the help of spreader lawn was prepared on the surface of GMM agar, from the above prepared inoculum.

Three antifungal discs *i.e.* 20µg Amphotericin B (Liofilchem, Italy), 01µg Voriconazole (Oxoid, England) and 25µg Fluconazole (Liofilchem, Italy), were placed on the GMM agar plate. Each disc was pressed down to ensure complete contact with the agar surface. All three antifungal discs were placed on prepared lawn on plates in 24cm. All plates were then inverted and sited in incubator at 37°C for 24 to 48 hrs.

3.10 Genotypic Characterization

3.10.1 DNA Extraction from *Candida* spp. for PCR

DNA was extracted from *Candida* spp. from protocol discussed by Deak *et al.* (2000) with slight modification. *Candida* species were inoculated for 16 -18 hrs at 37°C on Yeast Extract Peptone Dextrose (YEPD) broth (Appendix) except tryptone-glucose-yeast extract (TGY). By centrifugation all cells were settle down at bottom on 14,000 rpm (revolution per minutes) except rpm of 16,000 for 4 mins and then washed and re-suspended in 100µl of sterile distilled water. After repetition of the same step mention above, cells were again washed and again suspended it. After washing, boiled the suspension for not more than 10mins and centrifugation was done at 14,000 rpm for 6 mins. The part of the supernatant was used for analysis of PCR.

After DNA extraction 1.5% agarose gel was prepared, in which 0.4gm of agarose (Invitrogen) was dissolved in sterile distilled water of 36 ml and then add 1X Tris Borate EDTA (TBE) buffer, 4ml (Appendix) in a flask. Heat it for 50 sec in micro-wave oven. When the molten gel has cooled at about 50°C, 3µl of Ethidium Bromide (Appendix) was added. The molten gel was then poured in a caster (Power Pac Basic, BioRed) and combs were placed correctly. After solidification at 30 mins, gel was kept p in a tank containing IX TBE buffer. All extracted DNA samples (3µl) were mixed with 3µl of loading dye (Bromophenol Blue) and loaded them inside the wells. Voltage of The 110 volts was applied for 20 mins. After 20 minutes, the gel was then removed from the tank and was transferred to UV (Ultraviolet) transilluminator (Biometra TI2) for looking of DNA bands.

3.10.2 Polymerase Chain Reaction (PCR) Reaction

3.10.2.1 Primers

After DNA extraction of different *Candida* strains isolated from all positive samples of candidiasis, PCR amplification of Inter Transcribe Sequences (ITS1- ITS2) of rDNA

regions was achieved by a pair of primer *i.e.* ITS1 (forward GGT GAA CCT GCG G-3') and ITS4 (reverse, 5'-TCC TCC GCT TAT TGA TAT GC-3') (Fermentans, Germany).

3.10.2.2 Amplification Conditions

A basic PCR set up requires several components and reagents (Annexure). For DNA amplification take autoclaved PCR tubes and label these according to the code of strains. PCR amplification was performed in two types of methods *i.e.* by using Prepared Master Mix and by combining several components of a master mix. In both conditions a final volume of 25 µl was prepared.

In case of prepared master mix, each reaction consists of 5 µl from 5X FIREPOL Prepared Master Mix (Fermantas, Germany) (Appendix), 3ul DNA template, 0.4 µl Reverse and forward primer each and 16ul of PCR water where in other method each reaction contains 3µl of DNA as a template, 0.5µl of both primers, 0.4µl of dNTPs (100mM dATPs, dCTPs, dGTPs, dTTPs in 100mM of TE buffer) (Solid Biodyne), 0.2ul Taq DNA polymerase (Roche Diagnostics, Germany), 2.5ul 10× PCR buffer, 1.5ul Magnasium Chloride and 16.4ul PCR water.

Amplifications process was completed t in thermal cycler (Biometra). The amplification process contains 36 cycles. Each cycle consist of primary denaturation step for 5mins at 95°C, second step of denaturation was completed in 45sec at 95°C, annealing of primers was completed in 1min at a temperature of 52.5°C, primary extension was completed in 1min at 72°C and final extension was for 5mins at 72°C . Then the strip was placed in the PCR machines for DNA amplification and to test the amplification of DNA, the agarose gel electrophoresis was performed.

3.10.2.3 Gel Electrophoresis

This technique is used to check whether DNA is amplified or not. In order to prepare 2.0 % agarose gel, 0.8 gm of agarose was weighed and dissolved in solution of 36ml sterile distilled water and TBE buffer (4 ml) in a glass flask. After heating in microwave oven, the molten gel was cooled at about 50°C, ethidium bromide was added. The molten gel s

was mixed completely. Comb was placed carefully for proper formation of wells in the gel. The molten gel was poured in the caster and allowed it to solidify for 30mins. Gel was placed in a gel tank (Power Pac Basic, Biored) containing IX TBE buffer after solidification and carefully removed the comb. First load the DNA marker of 1kb and then all DNA samples (5µl) with 0 desired amount of loading dye. Closed the lid and voltage of 110 V was applied for 45 mins. After 45 minutes, the gel was placed in UV trans-illuminator for visualizing and photograph of amplified DNA. According to Allam and Salem, (2012) expected amplified PCR products were 510- 871bp (535bp of *Candida albicans*, 871bp of *Candida glabrata,* 524 bp of *Candida tropicalis,* 510 bp of *Candida krusei,* 608bp of *Candida guilliermondii* and 520 bp of *Candida parapsilosis.*

RESULTS

RESULTS

4.1 Sampling

This study was carried out at Microbiology Lab., Pathology Department, QIH and Microbiology Department of QAU, Islamabad Pakistan, during the period of March to December 2013.

4.2 Isolation of *Candida* species from clinical samples:

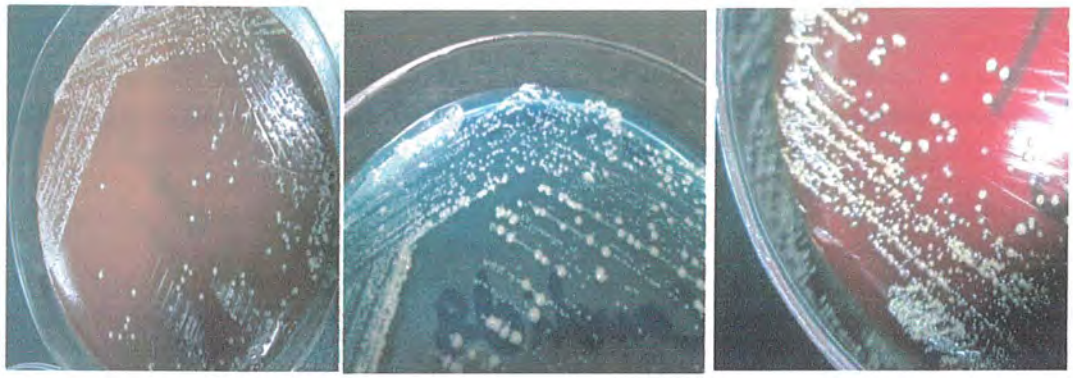
Total 3007 clinical samples were collected from patients of QIH. All samples were cultured on different media such as BA, MA, SDA, CA and CLED agar. Colonies were observed after 24-48 hours of incubation at 37°C. *Candida* species produced smooth, creamy, pasty and convex colonial growth with frustrating odor on SDA. On BA and CA *Candida* spp. produced convex, whitish smooth colonies with appendages while on CLED agar small convex and whitish colonies were observed (Figure 4.1). MA is a selective media for GNR (Gram Negative Rods). So growth of GNR both Lactose fermentors and Non Lactose fermentors observed on MA is not included in our study. Only *Candida* positive samples were included, which were not observed on MA.

4.3 Gram Staining:

In all samples on the basis of morphology on different media, only two hundred and fifty five samples were gram stained to check the morphological characteristics of *Candida* species under microscope. It was observed that *Candida* spp. were oval budding yeast and Gram positive shown in Figure 4.2. In some cases pseudohypha were also observed under 100 X of lenses.

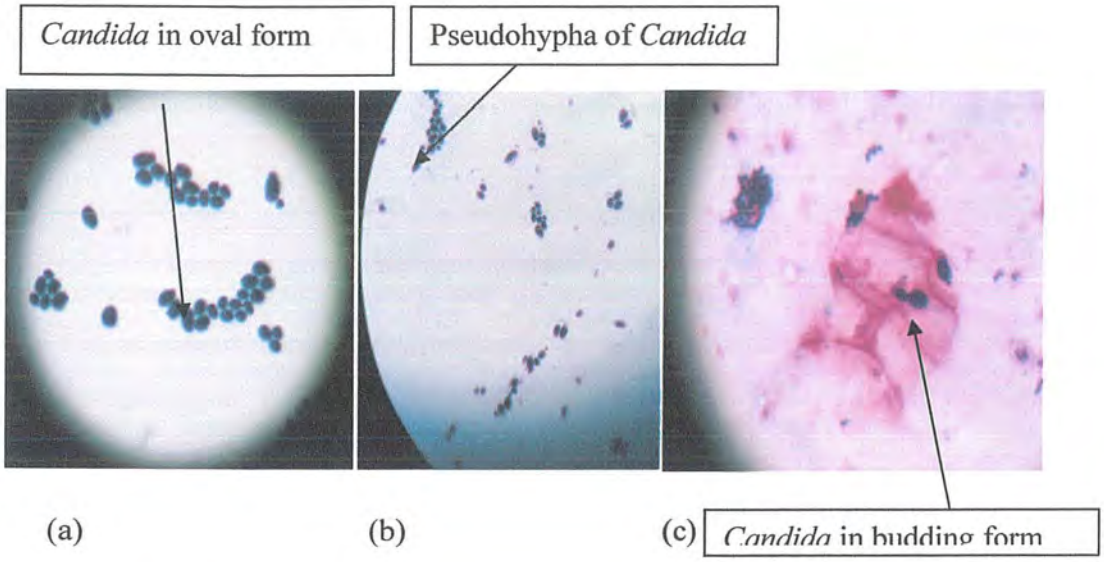
4.4 Germ Tube Test:

After identification on the basis of microscopy, germ tube test was performed to distinguish between germ tube positive and germ tube negative species of *Candida*. In all positive samples 128 were positive and 127 were negative. Latter on it was confirmed by two different differential media that all GT positive species were *Candida albicans* produced hyphae while non-*Candida albicans* did not show production of hyphae (Figure 4.3, Table no 4.1)



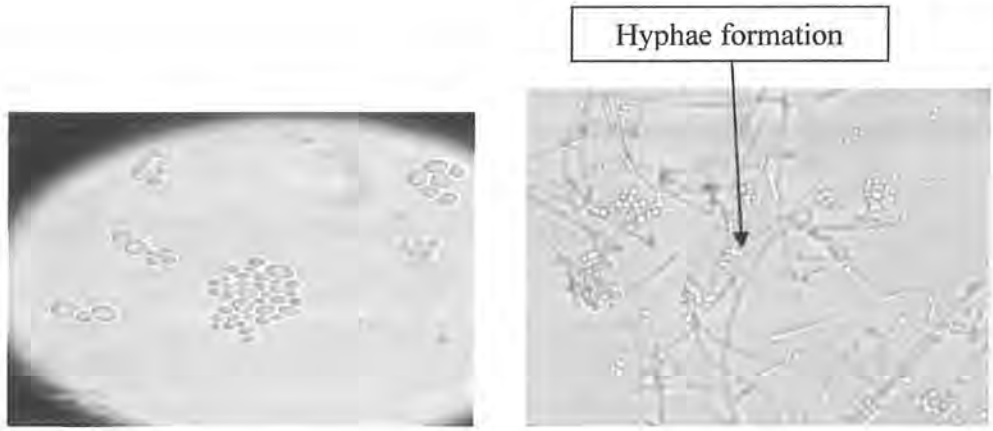
(a) (b) (c)

Figure 4.1: Colonial morphology of *Candida* species on different media (a) Growth of *Candida* spp. (C73) on Chocolate agar (b) Growth of *Candida* spp. (C184) on CLED agar (c) Growth of *Candida* spp. (C128) on Blood agar



(a) (b) (c)

Figure 4.2: Gram staining of *Candida* species (100X). (a) Growth of *Candida* spp. (C73) from urine in oval shape (b) Pseudohypha of *Candida* (C184) in urine (c) Budding yeast (*Candida*) in Sputum (C128)



(a) (b)

Figure 4.3: Identification of *Candida* species through Germ tube. (a) Germ tube negative *Candida* without hyphae (b) Germ tube positive *Candida* with hyphae.

4.5 Identification of *Candida* species:

Further identification after germ tube was done on two type of differential media used for *Candida i.e.*CMA and Chrome agar. After incubation of all *Candida* positive samples for 1 to 2 days at 37°C, different colours of colonial growth were observed on Chrome agar-*Candida*. Dark green colour of colonies indicated the growth of *C. dubliniensis*, light green color indicated growth of *C. albicans,* blue colour indicated *C. tropicalis*, pink color indicated *C. glabrata* and purple colour indicated *C. krusei,* while colorless colonies showed growth of other *Candida* species which were conformed on Corn Meal agar. On the basis of growth on both media *i.e.* CMA and Chrome agar, *C. albicans* were 128 (51%), *C. glabrata* were 30 (12%), *C. tropicalis* were 26 (10%), *C. krusei* were 17 (7%) and other species were 19 (7.5%) while in Corn Meal agar *Candida* spp. were further identified. Twelve (5%) species were *C. parapsilosis*, 3 (2%) were C. *lusitania* and C. *dubliniensis* while *Trichosporoum* specie was 1 (0.4%) (Figure 4.4, Table no 4.2).

4.6 Preservation of *Candida* species:

All *Candida* species were cultured on potato dextrose agar (PDA) and YEPD broth for preservation after identification on two differential media. After incubation *Candida* develops as cream, smooth, pasty convex colonies with irritating smell on PDA like SDA (Figure 4.5).

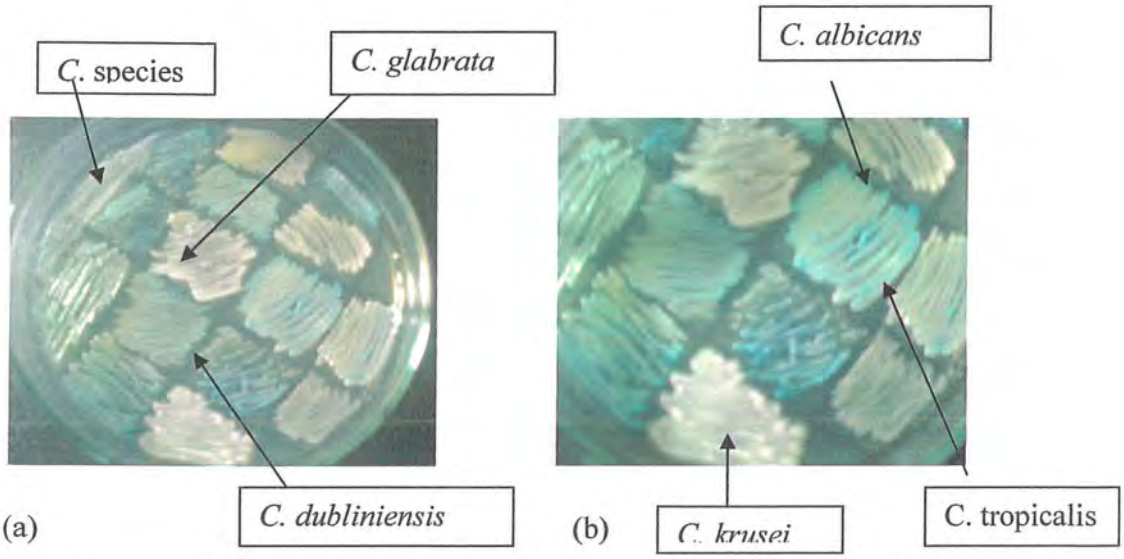


Figure 4.4: Growth of different *Candida* spp. on Chrome agar- *Candida* (a) Dark green colour: *C. dubliniensis*, Pink color: *C. glabrata,* Colorless colonies: *Candida* spp. (b) Light green color: *C. albicans,* Blue colour: *C. tropicalis*, Purple colour: *C. krusei.*

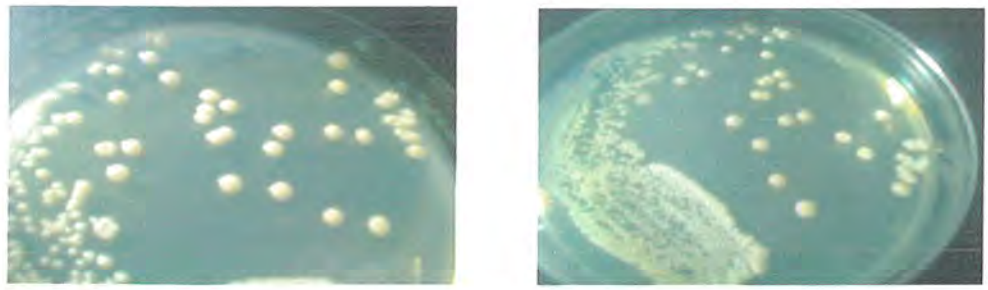


Figure 4.5: Pure creamy, smooth pasty and convex colonies of isolate number (a) C. *albicans* (C4) and (b) *C. glabrata* (C36) on Potato Dextrose Agar (PDA).

4.7 Analytical Profile Index 10 *Candida* (API 10C):

All *Candida* positive samples identified through germ tube and two differential media *i.e.*Chrom agar and Corn Meal agar were further confirmed through Analytical Profile Index 10 *Candida* (API 10C, Bio Meurex) system. The results observed from the API strip, were converted into numerical values. The generated numbers from API 10C system was (7012) for *Candida albicans,* (7100) for *Candida glabrata*, (7005) for *Candida tropicalis*, (7114) for *Candida dubliniensis*, (7100) for *C. parapsilosis* and (7717) for *Trichosporoum* specie. These numbers were then checked in the catalogue book (Table No 4.3) and was identified which is identification up to specie level. The results of 10 biochemical tests performed for *Candida* spp. were shown in (Figure 4.6-4.11, Table no 4.4).

4.8 Antifungal Sensitivity Test

Antifungal sensitivity was checked by disc diffusion method, in which three antifungals (fluconazole, voriconazole and amphotericin B) are used. The sensitivity of *Candida* spp. was checked with help of list of break points of zone of inhibition *i.e.*fluconazole is resistant, if zone of inhabitation is less then 13mm. Similarly for amphotericin B was resistant on less then 12mmof zone and voriconazole at less then and equal to 12mm (CLSI M27-A3, 2008). Results of zone of inhibition showed by ATCC strain of *C. albicans* 90029 was, voriconazole 32mm, fluconazole 28mm and amphotericin B 20mm. The results showed among three routinely used antifungals that all strains of *Candida* were more resistant to fluconazole (14.5%) followed by, voriconazole (13%) and amphotericin B (10%). *C. albicans* was more resistant strain among all isolated *Candida* strains (Figure 4.12, Table no 4.5).

Table No 4.4: Results of different *Candida* strains identified through API 10C in all3007 clinical samples.

Serial #	Tests	*C. albicans*	*C. glabrata*	*Trichosporoum* spp.	*C. tropicalis*	*C. dubliniensis*	*C. parapsiloss*
1	GLU	Pos	Pos	Pos	Pos	Pos	Pos
2	GAL	Pos	Neg	Pos	Pos	Pos	Pos
3	SAC	Pos	Neg	Pos	Pos	Pos	Pos
4	TRE	Neg	Pos	Pos	Neg	Pos	Pos
5	RAF	Neg	Neg	Pos	Neg	Neg	Neg
6	βMAL	Neg	Neg	Pos	Neg	Neg	Neg
7	αAMY	Pos	Neg	Pos	Neg	Pos	Neg
8	βXYL	Neg	Neg	Neg	Neg	Neg	Neg
9	βGUR	Neg	Neg	Neg	Neg	Neg	Neg
10	URE	Neg	Neg	Pos	Pos	Neg	Neg
11	βNAG	Pos	Neg	Pos	Neg	Neg	Neg
12	βGAL	Neg	Neg	Pos	Pos	Pos	Neg
Number Generated		7012	7100	7717	7005	7114	7100

Table No 4.5: Results of sensitivity pattern of three routinely used antifungals (Fluconazole, Voriconazole and Amphotericin B) for all 255 *Candida* positive isolates by disk diffusion method. Pos: Positive, Neg: Negative

Serial No	Candida strains	Voriconazole (Resistant) (18-23mm)	Fluconazole (Resistant) (15-20mm)	Amphotericin B (Resistant) (8-12mm)
1	*C. albicans*	24	27	14
2	*C. glabrata*	2	4	2
3	*C. tropicalis*	2	2	0
4	*C. krusei*	2	2	6
5	*C. parapsilosis*	3	2	0
6	*C. lusitania*	0	0	0
7	*C. dubliniensis*	0	0	0
8	*Trichosporoum* spp.	0	0	0

4.9 Demographic Data

Analysis of demographic data through questionnaire collected from patients revealed that educated people were effected more than the uneducated ones. People above primary level were considered as educated ones. Males, females and children's are involved in this study. Low educational level of parents could also contribute to candidemia in children's.

4.9.1 Gender Distribution

In all positive samples, more females than males were infected by *Candida* species, isolated from about 167 (65%) females and 88 (35%) males (Figure 4.13, Table No 4.6).

4.9.2 Age Distribution of Patients

Among all clinical samples, positive samples of candidiasis were found in all age group of 1- 101years. In female *Candida* infections were high up to 40 years and gradually declined with increase in age while in male patients *Candida* infections is generally decreased up to 60 years and increased from 60 years up to 101 years shown in Figure 4.14, Table No 4.6.

4.10 Distribution on the Basis of Source:

Three thousand and seven clinical samples were collected in sterile conditions from different patients visiting various wards in hospital and streaked on different media. After incubation at 37°C for 24-48 hrs colonies were observed. Only 255 samples were positive for *Candida* species infections, in which incidence of *Candida* was high in urine 36% (n=92) and lowest incidence was found in throat swab, stool, body fluid, fungal culture and tissue 0.4% (n=1). More females (68.4%) than males (31.6%) were infected by *Candida* species (Figure 4.15, Table No 4.7).

4.11 Distribution on the Basis of Location:

All positive samples were collected from patients of different locations of hospital, showed highest number of Outdoor Patients (OPD) *i.e.*87, followed by Intensive Care Unit (ICU) 72, Indoor Patients (IPD) 60, Emergency (ER) 11, Cardiac Care Unit (CCU) 9, Day Care 8, Neonatal Intensive Care Unit (NICU) 7 and Operation Theater (OT) 1. Percentage of infected females (69%) is higher than males (31%) (Figure 4.16, Table no 4.8).

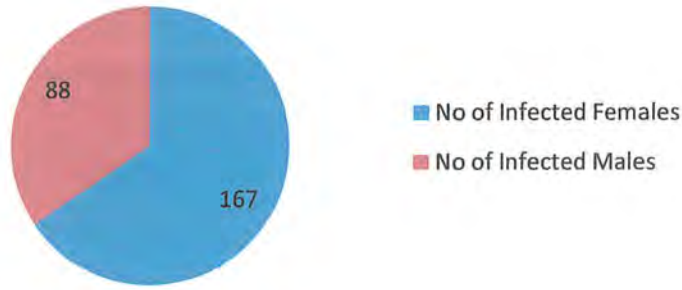


Figure 4.13: Gender distribution of *Candida* positive isolates showing highest percentage of female patients then male patients.

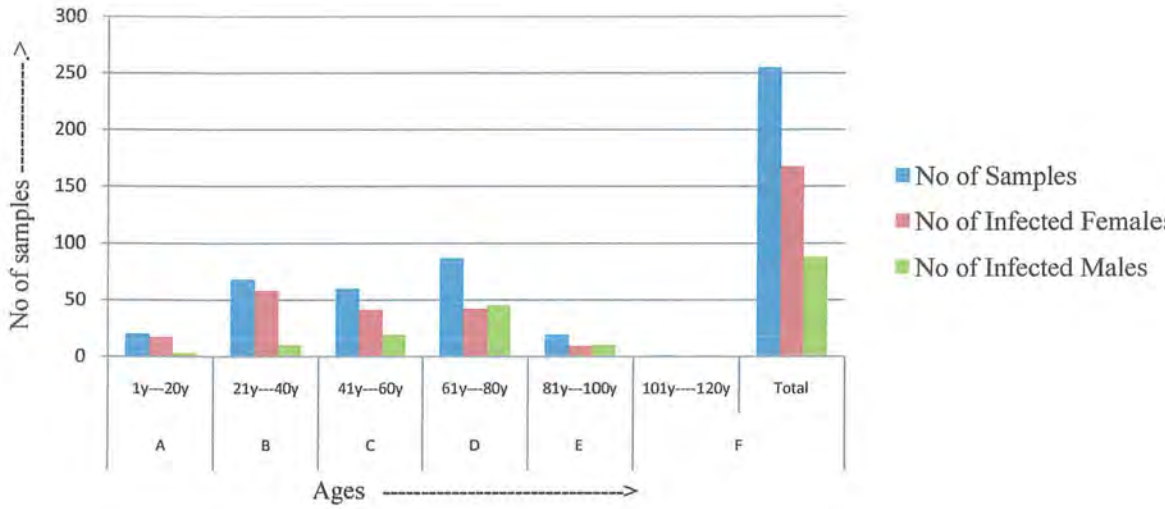


Figure 4.14: Age distribution of all *Candida* positive patients showing highest percentage of female (21-40 yrs) and males (61-80yrs).

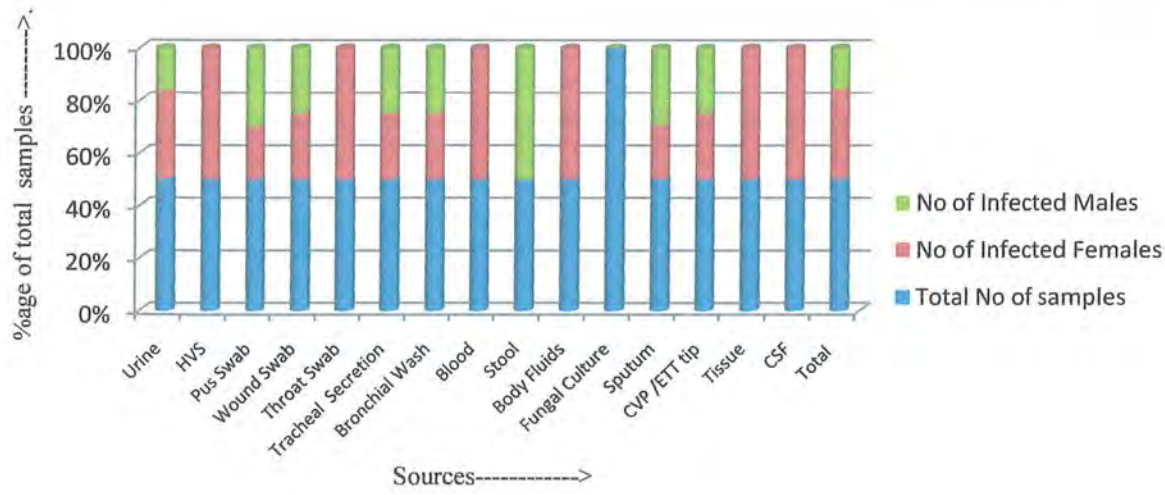


Figure 4.15: Distribution of *Candida* positive samples on the basis of source showed high incidence of *Candida* in urine samples and low incidence in throat swab etc. Percentage of infected female is higher than male.

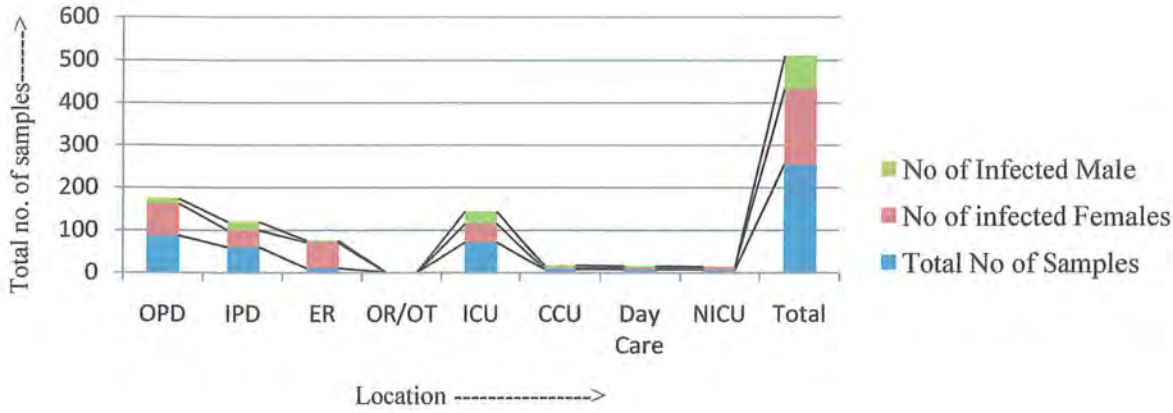


Figure 4.16: Distribution of *Candida* positive samples on the basis of location showed high incidence of *Candida* in outdoor patients and low incidence was observed in patients of Operation Theater.

4.12 Genotypic Characterization

3.12.1 DNA Extraction from *Candida* spp. for PCR:

After extraction of DNA, it was run on 1.5% agarose gel along with loading dye (Bromophenol Blue).When loading dye moved upto certain distance on gel. It was then taken and placed in UV Transilluminator for visualization different bands of DNA (Fig 4.17).

4.12.2 Amplification of DNA of *Candida* spp. by PCR through ITS:

After completion of PCR reaction, the PCR product was run in 2% agarose gel along with 1kb DNA ladder (Generular). After visualization of amplicon bands, these were compared with marker bands as each band showing specific size. From those sizes it was confirmed that the amplicon lies in region of 500bp that were the length of *Candida* spp. DNA (Fig 4.18).

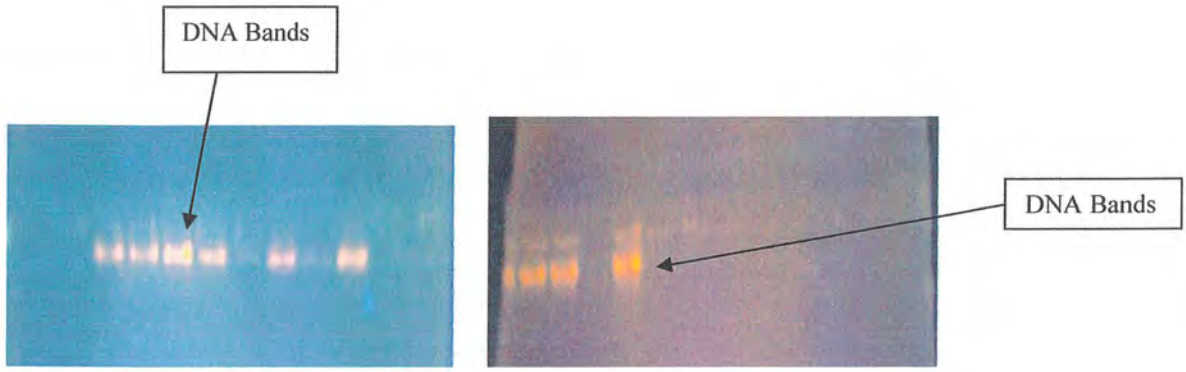


Figure 4.17: DNA bands of different species of *Candida* on 1.5 % agarose gel through UV Transilluminator.

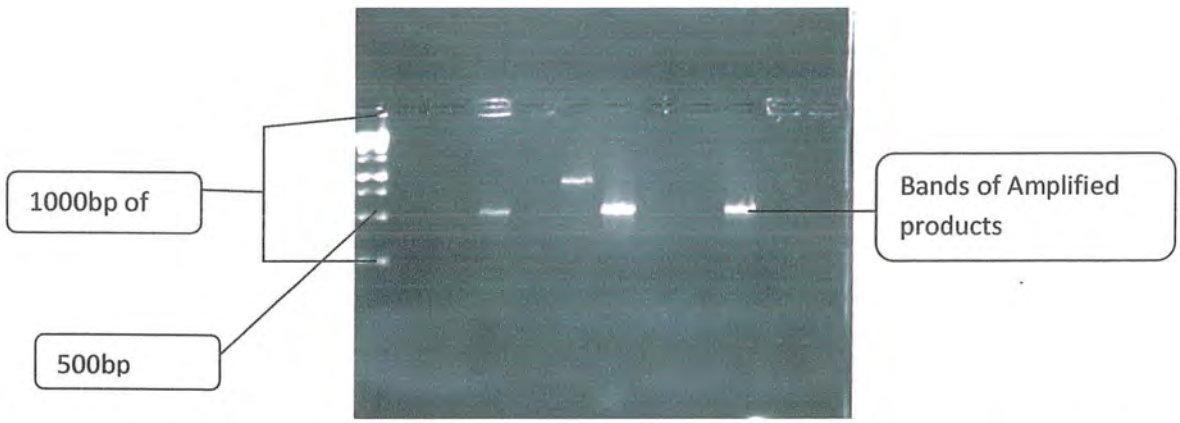


Figure 4.18: Bands of amplified products (500bp) of different species of *Candida* on 2.0 % agarose gel through UV Transilluminator.

Figure 4.19: Bands of amplified products of different species of *Candida* on 2.0 % agarose gel through UV Transilluminator.

DISCUSSION

DISCUSSION

Candida is an oval budding yeast cells, Gram positive and produces pseudohypha in culture and tissues (Jha *et al.*, 2006). In the previous two decades, the occurrence of *Candida* spp. infection has gradually increased and *Candida albicans* is the commonly isolated specie in all *Candida* from all clinical specimens.

All samples were taken from both indoor patients and outdoor patients visiting QIH. In present study, results showed that *Candida* species produced creamy, convex and flat colonies haing annoying smell on differential media like SDA. Similar study was carried out by Kangogo *et al.* (2011) and Al-Hussaini *et al.* (2013) that colonies of *Candida* were cream to white in color, glabrous, smooth, and yeast-like in appearance after growth on SDA. All *Candida* isolates showed good growth on SDA.Growth of *Candida* spp. on SDA was also observed by Pethani *et al.* (2012). The media mostly used for the isolation of *Candida* species is SDA. It is a general media that supports the growth of fungi. However, SDA is not a differential media and colonies of *Candida* spp. grown on this agar cannot be easily differentiated from each other.

It was observed in gram staining that *Candida* species are budding and oval yeast, having gram positive pseudo-hypha under 100X of lense. This results conforms the work of Al-Hussaini *et al.* (2013) that microscopic morphology showed spherical to sub spherical budding yeast cells of isolates producing pseudohyphae. Diba *et al.* 2012 also confirmed *Candida* infections by microscopic observation of *Candida* pseudohypha in wet preparations.Gram staining is a widely used techniquefor stain clinical important *Candida* species (Pethani *et al.*, 2012).

Germ tube of all positive samples was performed, in which 128 were positive and 127 were negative. All GT positive species were *Candida albicans* having chlamydospores while chlamydospores were absent in GT negative *Candida* species showed similarity with other workers like Kangogo *et al.* (2011), Nakamoto (1998) and Lar *et al.* (2012), who found that microscopic morphology showed spherical to sub spherical budding yeast cells. According to Kangogo *et al.* (2011) germ tube test indicated that 86.1% of *C. albicans* were germ tube positive and 13.9% were germ tube negative. Germ tubes were formed by all 111 strains of *Candida albicans* and absent in other *Candida* species (Nakamoto, 1998). Pethani *et al.* (2012) also used

germ tube technique for primary identification of *Candida* spp. In this study more germ tube were positive due to high incidence of *C. albicans* which is germ tube positive. Therefore it is conclude that rate of infections due to *C. albicans* was high then non- *Candida albicans* infections in this area.

On the basis of growth and colony colour on Chrome-agar *C. albicans* was the leading pathogen as showed in various studies (Lar *et al.*, 2012, Kangogo *et al.*, 2011 and Grimoud *et al.*, 2003). Pethani *et al.* (2012) found dissimilar results from our study because in their results *C. tropicalis* was the major isolate followed by *C. albicans*. Diba *et al.* (2012) and Pethani *et al.* (2012) used CMA and Chrom agar *Candida* for primary diagnosis of *Candida* spp.

Diba *et al.*, 2012 also isolated *C. albicans, C. tropicalis, C. glabrata, C. krusei, C. guilliermondii* and *C. parapsilosis* from different clinical samples. Different clinical samples were investigated by Otag *et al.* (2005), in which *Candida albicans* was the most common specie among all fungi. Ergon *et al.* (2005) isolated *Candida* species from clinical specimens in which 53.3% was *Candida albicans,* 14.5% was *Candida tropicalis*, 12.2% was *Candida glabrata* and 6.5% was *Candida parapsilosis.*

Our results are also similar with Gultekin *et al.* (2010) and Allam and Ihab (2012). They discussed that the most commonly identified specie was *Candida albicans* and *Candida tropicalis.* Willinger *et al.* (2002) also used Chrome agar for primary identification of all *Candida* species while Yucesoy and Serhat (2003) and Al-Hussaini *et al.* (2013) used CMA for primary identification of all *Candida* species.

In both media 35 isolates showed controversies in our study. In 7 isolates growth of *C. albicans* was observed on Chrome agar-*Candida* while growth of *C. dubliniensis* was observed on Corn Meal agar. Other 6 isolates were *C. tropicalis* on Chrome agar and *Candida albicans* on CMA. Three species were of *Candida glabrata, Candida albicans, Candida tropicalis and Candida glabrata* on Chrome agar while growth of *C. krusei, C. tropicalis, and C. parapsilosis* was observed on Corn Meal agar. In Chrome agar *C. krusei* was replaced by *C. tropicalis* and *C. glabrata* while *Candida tropicalis* was replaced by *Candida krusei, Candida dubliniensis* and *Candida glabrata* on CMA. Dissimilar results obtained were due to composition of

both differential media and difference in indicators used in media. Due to which identification of different strains is different. So that prevalence of infections due to *C. albicans* was high then non- *Candida albicans* in this area, which is a major cause of increase rate of *C. albicans* in our study.

Further identification of all samples was done by API 10C. Results obtained showed 90% similarity with both differential media. Yucesoy and Serhat (2003) and Kangogo *et al.* (2011) used API 20 C AUX for identification of *Candida* species. Davey *et al.* (2005) compared API 20C system with Auxacolor identification system. Auxacolor correctly identified isolates upto 85.7% while 88.6% was identified by API20C. Seventy four percent and 3.7% was false identification by API 20C and Auxacolor. Ece *et al.* (2012) used the ID32C yeast identification system (Biomerieux, France). Willinger *et al.* (2002) stated that the API20C is very useful technique for recognition of all *Candida* species from positive blood culture and molecular techniques did not increased the identifications confirmed by API20C. Allam and Ihab (2012) used API 20 C AUX, in which *Candida albicans* were 44.2%, *Candida tropicalis* 25%, 11.5% *Candida glabrata* 11.5% *and Candida krusei* were 1.9%. *Candida stellatoidae* and *Candida kefyr* were also isolated. Our results were dissimilar from others, due to use of different identification system *i.e.* API 10C, which is not used so frequently for identification. Due to unavailability of other identification systems our results did not match with others.

Disc diffusion method was used for antimycotic susceptibility testing. It was concluded that in all three antifungals *Candida* species were less sensitive to fluconazole (14.5%). Resistance of voriconazole was (13%) and amphotericin B was (10%). Highest resistance was shown by *Candida albicans.* Our results showed similarity with Pethani *et al.* (2012).This study showed resistance to fluconazole in *C. albicans.* Al-Hussaini *et al.* (2013) revealed that by *In-vitro* antifungal sensitivity test all *Candida* strains were resistant to nystatin and amphotericin B. Itraconazole, clotrimazole, ketoconazole, fluconazole, tioconazole and sertaconazole were sensitive to 50% - 67% of *Candida* species. All *C. glabrata* isolated were resistant to sertaconazole and clotrimazole. Khan and Baqai (2010) identified *Candida* from HVS and checked *in-vitro* antifungal susceptibility of clotrimazole, nystatin and fluconazole against *Candida. In-vitro* antifungal susceptibility results showed that

clotrimazole is effective against 70% of *Candida* spp.. fluconazole was effective against 36.2% and Nystatin disc was 63.5% effective. Fluconazole is more resistant then voriconazole and amphotericin B due to self medication and increase use of fluconazole of patients visiting QIH. Change conditions, area, various infections of patients and use of antifungals are the major factors that contribute in the antifungal resistance of our area. Moreover *C. albicans* is the common cause of candidiasis which is more resistant to fluconazole, that's why resistant to fluconazole is more as compared to other two commercial available antifungals *i.e.* voriconazole and amphotericin B.

Among all 255 positive specimens, percentage of females patients was high than male patients. Due to increased cases of vaginal candidiasis, infection in female patients is hidh then male patients visiting hospital. Similar results were carried out by Grimoud *et al.* (2003). Their results showed that 77.3% were women and 22.7% were men. In our study *Candida* infections were present in the age of 1to101yrs. In female patients *Candida* infections were raised upto 40 yrs and become less with increase in age group due to hormonal changes while in males *Candida* species infections decreased up to the age of 60 yrs and raise with increase in age due to weak immune system. Our results were similar up-to certain level with Al-Hussaini *et al.* (2013), who showed that the age of *Candida* infected persons ranging from 27 to 80 years. Pethani *et al.* (2012) observed that neonates were at higher risk of developing candidemia. The high prevalence of candidiuria in female confirms the work of Passos *et al.* (2005) who found that the percent of candiduria in females was 61.6% compared to 38.4% in males. More recently, Rashwan *et al.* (2010) observed candiduria in 34.4% females and 14.9% in males. In our results percentage of female patients is high due to increase cases of vaginal candidiasis in patients visiting QIH, may be due to problem in personal hygienic conditions.

In all positive samples percentage of *Candida* infections was high in urine samples (92) and its incidence was low in other samples like stool, tissues, throat swab, body fluids and fungal cultures (1). Our results were dissimilar with Allam and Ihab (2012) who showed that the highest percentage of *Candida* was (60%) from oral swabs followed by (34%) peritoneal dialysate, (24%) urine, (23%) sputum and (10%) pus. Similar results were presented by Ece *et al.* (2012), who isolated 42.7% *Candida*

species from urine, 45.9% from blood culture, 3.8% from tracheal secretion and 7.4% from wound swabs. Kangogo *et al.* (2011) also presented results having 33.3% urine, 16.7% sputum, 8% tracheal aspirate and 4.7% blood (4.7 %).More isolates were from vaginal swabs because number of female patients is high, an indication that vaginal-candidiasis is very common. Few isolates were isolated from blood and CSF because these are normally body fluids in sterile conditions, in which *Candida* meningitis and candidaemia is not common.

Using ITS primers, PCR product lies in the region of 500 to 800bp fragments on 2% agarose gel electrophoresis. Our results were similar with Allam and Ihab (2012), who demonstrated that by using the ITS1 and ITS4 primer pair amplification product 510-871 bp. were observed. Orita *et al.* (1989) also observed fragments of 500 to 600 bp on 1.5% agarose gel. Diba *et al.* (2012) also used ITS for identification of *Candida* spp. through PCR. Romeo *et al.* (2011) used ITS region for PCR amplification and sequencing. Variety in sizes of band is due to change in incidence of isolates obtained through candidiasis.

CONCLUSION

CONCLUSIONS

- Major conclusions of this research work are:

1) *C. albicans* (51%) is the major isolate among all other *Candida* species isolated.
2) All *C. albicans* are germ tube positive and other species are germ tube negative.
3) *C. glabrata* (12%) is the second high isolate followed by *C. tropicalis* (10%), *C. krusei* (7%), *C. parapsilosis* (5%), *C. dubliniensis* (2%), C. *lusitania* and *Trichosporoum* species (0.4%).
4) In antifungal susceptibility fluconazole is more resistant and sensitivity of amphotericin B is high.
5) In our study female patients (65%) are more infected then male patients (35%).
6) Age distribution of all *Candida* positive patients showing highest percentage of female (21-40 yrs) and males (61-80yrs).
7) Among all clinical samples urine (36%) showed high incidence of *Candida* infections.
8) Outdoor Patients *i.e.* 87 (both male and females) showed high incidence of candidiasis.
9) Among two differential media *i.e.* Corn Meal agar and Chrome agar-*Candida*, Corn Meal agar is quick and reliable method for primary identification of all *Candida* species.

FUTURE PROSPECTIVES

FUTURE PROSPECTIVES

- Future perspectives of our study are:

1. Resistant genes of *Candida* species could be studied.
2. Morphological and physiological factors playing role in its prevalence should be investigated.
3. Detail epidemiological studies could be studied which can help in control of candidiasis in both males and females.

REFERENCES

- Achkar, J. M., and Bettina, C. F. (2010) *Candida* infections of the genitourinary tract. Clinical Microbiology reviews; 23: 253–273

- Agha, M. A., Shaheen, A.A., Shaheen, S., Muhammad, N. Z., Muhammad, R. K., Muhammad, A, M. (2011). *Candida glabrata*: An emerging threat for the immunocompromised. Gomal Journal of Medical Sciences: 9(1): 115-118.

- Alhussaini, M.S., Noha F. ET., Ahmad, M.M. (2013). Phenotypic and molecular characterization of *Candida* species in urine samples from renal failure patients. Science Journal of Clinical Medicine; 2(1) : 14-25

- Allam, A. A., and Ihab M. S. (2012). Evaluation of rapid molecular identification of clinically important *Candida* spp. isolated from immune-compromised patients using RF-PCR. Journal of American Science;8(2)

- Ariff, S., Ali, F. S., Sajid, B.S., Reema, S. (2010). Clinical spectrum and outcomes of neonatal candidiasis in a tertiary care hospital in Karachi, Pakistan. Journal of Infection in Developing Countries; 5(3):216-223.

- Asmundsdottir, LR., Erlendsdottir, H., Gottfredsson, M. (2013). Nationwide study of candidemia, antifungal use, and antifungal drug resistance in Iceland, 2000 to 2011. Journal of Clinical Microbiology; 51(3):841-8.

- Basu, S., Debasrita, C., Subrata K. D. and Satadal, D. (2011). Biological characteristics of nosocomial *Candida tropicalis* Isolated from different clinical materials of critically Ill Patients at ICU. International journal of microbiological research; 2 (2): 112-119

- Bii, C.C., M. C. Kangogo., G. Revathi and M. W. Wanyoike. (2009).Genotypes of *Candida albicans* from clinical sources in Nairobi Kenya. African Journal of Microbiology Research; 3(9):475-477.

- Brissaud, O., Julie, G., Jerome, H., Olivier, T., and Theoklis, Z. (2012). Invasive fungal disease in PICU: epidemiology and risk factors. Annals of Intensive Care; 2 (6):4-8.

- Chadwick, SG., Schuyler, JA., Vermitsky, JP., Adelson, ME., Mordechai, E., Gygax, SE. (2013). X-Plate technology: a new method for detecting fluconazole resistance in *Candida* species. Journal of Medical Microbiology.(1).

- Colombo, AL., Guimarães, T. (2003). Epidemiology of hematogenous infections due to *Candida* species. Journal of Venomous Animals and Toxins including tropical diseases; 36(5):599-607.

- Davey, K. G., P. M. Chant., C. S. Downer., C. K. Campbell., D. W. Warnock. (1995). Evaluation of the Auxacolor system, a new method of clinical yeast identification. Journal of Clinical Pathology; 48:807-809

- Deak, T., J. Chen., and L. R. Beuchatl (2000). Molecular Characterization of *Yarrowia lipolytica* and *Candida zeylanoides* isolated from poultry. Applied and Environmental Microbiology; 66(10): 4340–4344.

- Diba, K., M. Rhaimirad, K. Makhdoomi and Z. Khorshidvand (2012). Identification of *Candida* species isolated from hospital acquired infections cases and hospital indoor environments. African Journal of Microbiology Research; 6(19): 4164-4168.

- Ece, G., Pinar, S., Gulgun, A., Sabri, A., Sukran, K. (2012). The Evaluation of the distribution of yeast like fungi '*Candida* species' at a tertiary care center in Western Turkey. International Journal of Medical Sciences; 9(7):617-620

- Eraso, E., Maria, D., Moragues, M. VV., Ismail, H. S., Nagore, GG., Jose, P. and Guillermo Q. (2006). Evaluation of the new chromogenic medium *Candida* ID 2 for isolation and identification of *Candida albicans* and other medically important *Candida* species. Journal of Clinical Microbiology; 44(9): 3340–3345.

- Ergon, M., Yucesoy, M. (2005). Evaluation of species distribution of yeasts isolated from intensive care units during the four years period. Bulletin of Microbiology; 39(3):309-18.

- Enwuru, C.A., A. Ogunledun., N. Idika., N.V .Enwuru., F. Ogbonna., M. Aniedobe., and A. Adeiga. (2008). Fluconaole resistant opportunistic oro-Pharangeal *Candida* and Non *Candida* yeast like isolates from HIV infected patients attending ARV clinics in logos, Nigeria. Journal of African Health and Science; 8(3): 142-148.

- Farasat, A., Mohammad. S., Milad, M., Shima, A.R., Younes, G., Mohammad, A. E., Mosayeb, R., (2012). Identification of *Candida albicans* isolated from recurrent vulvovaginal candidiasis (RVVC) patients by PCR-RFLP method and its drug sensitivity to *Zataria. multiflora* extract. European Journal of Experimental Biology; 2 (6): 2004-2009

- Grimoud, A.M., Nicole M., Helene B., Sandrine A., Jean P. L and Gérard C. (2003). Colonization of the oral cavity by *Candida* species: risk factors in long-term geriatric care. Journal of Oral Science; 45(1):51-55.

- Gordana, M. B. M., M. M. Svetlana., M., Goloaorbino K. (2008). The Imporatnce of genus *Candida* in human samples. Mycology, Mycotoxicology and Mycoses; 114: 79-95.

- Gultekin B, Eyigor M, Telli M. (2010). ANKEM Dergisi.; 24(4):202-208.

- Hammoud, MS., Al-Taiar, A., Fouad, M., Raina, A., Khan, Z.(2012). Persistent candidemia in neonatal care units: risk factors and clinical significance. International Journal of Infectious Diseases; S1201-9712(12) 01313-6.

- Hudson, D.A., Quentin, L. S., Rebecca, J. S., Gillian, E. N., Pat, J. B. E., Patrick, A. S. and Peter, C. F.(2004). Identification of the dialysable serum inducer of germ-tube formation in *C. albicans*. Journal of Microbiology; 150:3041–3049

- Hurlaey, R., and Valeriec, S. (1968). Cytopathic effects of pathogenic and Non-pathogenic Species of *Candida* on cultured mouse epithelial cells: Relation to the growth rate and morphology of the fungi. Journal of Medical Microbiology; 2: 63-74.

- Isogai, H., Andargachew. M., Erimias, D., Henok, T., Afework, K., Koichi, K., Takeshi, N., Emiko, I. (2010). Identification of *Candida* species from human immunodeficiency virus-infected patients in Ethopia by combination of CHROM agar, Tobacco agar and PCR of amplified internally transcribed rRna spacer region. The Journal of Applied Research.;10 (1) :2-8

- Jeddy, N., K. Ranganathan., Uma, D., and Elizabeth, J. (2011). A study of antifungal drug sensitivity of *Candida* isolated from human immunodeficiency virus infected patients in Chennai, South India. Journal of Oral Maxillofacial Pathology; 15(2): 182–186.

- Jha, BK., Dey, S., Tamang, MD., Joshy, ME., Shivananda, PG., Brahmadatan, KN.(2006).Characterization of *Candida* species isolated from cases of lower respiratory tract infection .Kathmandu University Medical Journal;4(3):290-294

- Kim, D., Woon, S.S., Kyoung, H.L., Kyunghoon, K., Joo, Young, P., and Choon, M. K. (2002).Rapid differentiation of *Candida albicans* from other *Candida* species using its unique germ tube formation at 39°C. Journal of Wiley Inter Science; 19: 957–962.

- Kuleta, J.K., Maria, R.K., and Andrzej, K. (2009).Fungi pathogenic to humans: molecular bases of virulence of *Candida albicans*, *Cryptococcus neoformans* and *Aspergillus fumigates*. The Journal of the Polish Biochemical Society and of the Committee of Biochemistry and Biophysics Polish Academy of Sciences; 56 (2): 211–224.

- Khan, F., and Rakhshanda B. (2010). In-Vitro antifungal sensitivity of

fluconazole, clotrimazole and nystatin against vaginal candidiasis in female of child bearing age. Journal of Ayub Medical College Abbottabad; 22 (4):197-200.

- Kangogo, MC., Wanyoike MW., Revathi G and Bii CC. (2011). Phenotypic characterization of *Candida albicans* from clinical sources in Nairobi, Kenya. African Journal of Health Sciences: 19:19-23.

- Lar, P.M., Pam, K.V., Tiri, Y., Shola, O., Agabi, Y., Dashen, M.M., Mawak, J.D. (2012). Prevalence and distribution of *Candida* species in HIV infected persons on antiretroviral therapy in Jos. Journal of Medicine and Medical Science; 3(4): 254-2509

- Lee, K.K., Donna, M. M., Mette, D. J., Louise, A. W., Frank, C. O., Neil, A. R. G., and Carol, A. M. (2011). Elevated cell wall chitin in *C. albicans* confers echinocandin resistance *In-Vivo*. Journal of Antimicrobial Agents and Chemotherapy; 56(1): 208–21

- Liguori, G., V. D. Onofrio, F. Galle, A. Lucariello , L. Albano, M.R. Catania, M. Guida. (2010).*Candida albicans* identification: comparison among nine phenotypic systems and a multiplex PCR. Journal of preventive medicine and hygiene; 51: 121-124

- Looke ,M, K., K, Kristjuhan.(2011). Extraction of genomic DNA from yeasts for PCR-based applications. Biotechniques; 50(5):325-8.

- MacCallum, M.D. (2012). Hosting Infection: Experimental Models to Assay Candida Virulence. International Journal of Microbiology; 1-12.

- Mashad A.E., and Mohamed, T. M. (2011).Commercial tests for *in vitro* antifungal susceptibility testing of *Candida* species compared to standard (NCCLS) broth microdilution. International Journal of Medicine and Medical Sciences; 3(4): 100-104

- Metwally, L., G. Hogg., P. V. Coyle., R. J. Hay., S. Hedderwick., B. McCloskey., H. J. O'Neill., G. M. Ong., G. Thompson., C. H. Webb. and R. McMullan.(2007). Rapid differentiation between fluconazole-sensitive and -resistant species of *Candida* directly from positive blood culture bottles by real-time PCR. Journal of Medical Microbiology; 56:964–970

- Metwally, L., Fairley D.J., P. V. Coyle., R. J. Hay., S. Hedderwick., B. McCloskey., H. J. O'Neill., C. H. Webb., W. Elbaz and R. McMullan.(2008).Improving molecular detection of *Candida* DNA in whole blood: comparison of seven fungal DNA extraction protocols using real-time PCR. Journal of Medical Microbiology, 57, 296–303.

- Mirhendi PCR- Detection of *Candida albicans* in blood using a new primer pair to diagnosis of systemic candidiasis. Iranian Journal of Public Health; 32:1-5., SH., and K Makimura. (2006).

- Mondelli, AL., Niéro, M. L., Bagagli, E., Camargo, CH., Bruder, N. A., Sugizaki, MF., Carneiro, MV., Villas, B. PJF. (2012) *Candida* spp.: manual identification (reference method) and automated identification (Vitek system platform). The Journal of Venomous Animals and Toxins including Tropical Diseases; 18(3): 335-339.

- Moris, DV., Melhem, MS., Martins, MA., Souza, LR., Kacew, S., Szeszs, MW., Carvalho, LR., Pimenta, R. MV., Berghs, HA., Mendes, RP.(2012). Prevalence and antifungal susceptibility of Candida parapsilosis complex isolates collected from oral cavities of HIV-infected individuals. Journal of Medical Microbiology; 61(12):1758-65.

- Nadeem, S.G., Shazia, T. H.,and Shahana, U. K.,(2010). Use of CHROM agar *Candida* for the presumptive identification of *Candida* species directly from clinical specimens in resource-limited settings. Libyan Journal of Medicine; 5: 2144 1-6.

- Nakamoto, S. (1998). Germ tube formation of *Candida albicans* in corn meal broth using the non-slip slide glass incubation method. Yonago Acta medica ;41:65–72
- Nejad, B. S., Ablollah, R. and Fereshteh, M. (2011).Prevalence of *Candida* species in the oral cavity of patients with periodontitis. African Journal of Biotechnology; 10(15): 2987-2990.
- Nnadi, N, E., Grace, M. A., Fabio, S., Mark, O, O., Ifeoma, B. E., Giuseppe, C. and Orazio, R. (2012). Isolation and molecular characterization of *Candida africana* from Jos, Nigeria. Medical Mycology Month 2012; Early Online: 1–3.
- Oberoi, J. K., Chand,W., Neeraj, G., Reena, R., S. Datta and Kamaljeet, P. (2010). Non-*albicans Candida* species in blood stream infectionsin a tertiary care hospital at New Delhi, India. Indian Journal of Medical Research; 136: 997-1003
- Orita, M., Iwahana, H., Kanazawm, H. (1989). Detection of polymorphism of human DNA by gel electrophoresis as single strand conformation polymorphism. Yonago Acta medica; 86: 2766-2770.
- Otag, F. *et al.* (2005): An outbreak of Pichia ohmeri infection in the paediatric intensive care unit: case reports and review of the Literature. Mycoses 48, 265-269.
- Pang, S. Y. M., Stephen, T., and Simon, B. (2010). Inhibition of the growth of pathogenic *Candida* spp. by salicylhydroxamic acid. International Journal of Biological and Life Sciences; 6(1): 1-7.
- Pappas, G. P., Carol, A. K. David, A. Daniel, K. Benjamin, Jr., Thierry F. C, John, E. E, Jr., Scott, G. F, John F. F, Bart-Jan K, Luis OZ, Annette, C. R, John H. R, Thomas J. W, and Jack D. S. (2011). Clinical infectious diseases; 48:503–35
- Parmeland, L., Gazon, M., Guerin, C., Argaud, L., Lehott, J.J., Bastein, O., Allaouchiche, B., Michallet, B., Picot, S., Bienvenue, AL. ((2013). *Candida*

albicans and non-*Candida albicans* fungemia in an institutional hospital during a decade. Journal of Medical Mycology. 51 (1): 33-7

- Passos, XS; Sales, WS; Maciel, PJ., Costa, CR.; Miranda, KC. And Lemos, Jdc. A (2005): Candida colonization in intensive care unit patients' urine. memorias do Instituto Oswaldo Cruz; 100:925-928.
- Pasteur, A.R., Y. Ullmann., and I. Berdicevsky (2011). The Pathogenesis of *candida* infections in a human skin model: Scanning electron microscope observations. International Scholarly Research Network; Volume 1-6

- Pethani, J. D., Dr. Jigar Gusani ,Dr.Sanjay Rathod, Dr. Parul D Shah. (2012).*Candida* species identification from clinical specimens and its changing pattern in a tertiary care hospital. Indian Journal of Applied Basic Medical Sciences;14.19: 83-91

- Pfaller, M. A., and D. J. Diekema. (2007). Epidemiology of invasive candidiasis: a Persistent Public Health Problem. Clinical Microbiology Reviews; 20 (1):133–163.

- Prates, R. A., Ilka, T. K., Martha S. R., George P. T. and Michael R. H.(2011). Influence of multidrug efflux systems on methylene blue-mediated photodynamic inactivation of *Candida albicans*. Journal of Antimicrobial Chemotherapy; 66: 1525–1532.
- Rad, M.M., Ameneh, S. Z., Maryam, A. Z., Mahkam, T., and Yasaman, M. (2012). Identification of *Candida* Species Associated with vulvovaginal Candidiasis by Multiplex PCR. infectious diseases in obstetrics and gynecology; :1-5.
- Rajeswari, P., P. Vijayalakshmi., D. Jegadeeshkumar. (2012). Prevalence of Biofilm Producing MDR *Candida albicans* and non-*Candida albicans* Isolate from medical devices. International Journal of Applied Biology and Pharmaceutical Technology; 3(4):254-263.

- Ramage, G., Ranjith, R., Leighann, S., and Craig, W. (2012). Fungal Biofilm Resistance. International Journal of Microbiology; Volume 2012: 1-14

- Rashwan, N.M. Mohamed. AK.A. Saif., El-Deen, S. Ahmed, E.H. and Imail, S.A. (2010): Pattern of Candida urinary tract infections among cancer patients in south Egypt. Cancer Institute of Bulletin of Pharmaceutical Sciences, Assiut University, 33:121-130

- Rex, J. H., Michael A. P, John N. G, Marilyn S. B, Ana El. Mahmoud A. G, Michael L, Frank C. O, Michael G. R, Thomas J. W and Arthur L. B. (2013). Development of interpretive breakpoints for antifungal susceptibility testing: conceptual framework and analysis of *In Vitro—In Vivo* correlation data for Fluconazole, Itraconazole and *Candida* Infections. Journal of Clinical Infectious Diseases; 24:235-47

- Rocha, B. A., Gilda, M. B., Del, N., Lidia, Y., Mariana, V. BS., Alexander, Roberto, P., and Thelma, S. O. (2008). Identification and differentiation of *Candida* species from pediatric patients by random amplified polymorphic DNA. Journal of Venomous Animals and Toxins including Tropical Diseases; 141(1):1-5.

- Rodríguez, S.M., Peggy, E.A.G., Víctor, M. V. V., Beatriz, X.C.,Yolanda del, C. PL. (2012). Prevalence of *Candida* spp. in wosmtuedny ionf the City of Tuxtla Gutierrez, Chiapas. International Biotechnology Color Journal; 2(2):6-14.

- Romeo, O., Demetrio, D., Barbara, C., Antonio, C., Giuseppe, C. (2012). Molecular Characterization of italian *Candida parapsilosis* isolates reveals the cryptic presence of the newly described species *Candida orthopsilosis* in the blood culture from New Borns. Diagnostic of Microbiology and Infectious Disease; 72 (2012): 234.

- Sánchez, R., R., Flores, P. R., Arriaga, A. (2006). Identification of *Candida* species causing vaginitis in mexican patients. Journal of Infectious Disease and

Clinical Microbiology ; 24(10):634-6.

- Satana, D., Gonca E.G., and Zayre, E. (2010).The antifungal susceptibilities of oral *Candida* spp. isolates from HIV-infected patients. African Journal of Microbiology Research; 4(17):1831-1835.

- Selvarangan, R., Uyen, B., Ajit, P. L and B, T. Cookson. (2003). Rapid Identification of commonly encountered *Candida* species directly from blood culture bottles. Journal of Clinical Microbiology; 41(12):5660–5664.

- Taheri, J. B., Hamed, M., Saeedeh, M., Sedigheh, B., Fatemeh, N., Naser, V., Mahin, B., Maryam, B., Nika, B. (2011). Evaluation of *candida* isolation from vaginal mucosa of mothers and oral mucosa of neonates on the basis of delivery type. African Journal of Microbiology Research; 5(17):5067-5070.

- Verweij, P E., I. M. Breuker., A.J.M.M. Rijs., J.F.G.M. Meis. (2013). Comparative study of seven commercial yeast identification systems. Journal of Clinical Pathology ;52: 271-273.

- Wikler, M.A., F.R. Cockerill, W.A. Craig, M.N. Dudley, G.M. Eliopoulos, D.W. Hecht, J.F. Hindler, D.E. Low, D.J. Sheehan, F.C. Tenover, J.D. Turnidge, M.P. Weinstein, B.L. Zimmer, M. J. Ferraro and J.M. Swenson. (2006). Performance standards for antimicrobial susceptibility testing; sixteenth informational supplement. Clinical and Laboratory standards Institute; 26(3): 78-82

- Willinger, B., Cornelia, H. Brigitte, S. and Mammad, M. (2002). Performance of *Candida* ID, a new chromogenic medium for presumptive identification of *Candida* Species, in comparison to Chrome agar *Candida*. Journal of Clinical Microbiology; 39: 3793–3795.

- Xu, J., B. C. Millar., J. E. Moore., R. Mc Clurg., M. J. Walker., J. Evans., S .Hedderwick., R. McMullan. (2013).Comparison of API20C with molecular identification of *Candida* spp. isolated from bloodstream infections. Journal of Clinical Pathology;55:774–777

- Yang, YL., Ming, F. C., Ya, W. C., Tzuu, G. Y., Hsin, C., Sai, C. L., Bruno, M.H. C., Fan, C.T., Tun ,C. C., Yu, H. H., Zhi, Y. S., Chung, H. H. C., Ju, Y. L., and Hsiu, J. L.(2008). Host factors do not influence the colonization or infection by fluconazole resistant *Candida* species in hospitalized patients. Journal of Negative Results in Bio-Medicine; 7 (12):1477-5751.

- Yu. Nae, M.D., Ryoun, K. M.D., and Mi Kyung, L. M.D. (2012). The First Korean Case of Candidemia due to *Candida dubliniensis*. Annals of Laboratory Medicine; 32:225-228

- Yucesoy, M., and Serhat M. (2003). Performance of Chrome agar *Candida* and biggy agar for identification of yeast species. Annals of Clinical Microbiology and Antimicrobials, 2:8

- Zarrin, M., Ali, Z. M. (2009). Invasive candidiasis; a review article. Jundishapur Journal of Microbiology; 2(1): 1-6 1

- Zhou, X., Fanrong, K., Tania, C. S., Hui, W., Yiqun, D., and Sharon, C. A. C. (2008). Practical method for detection and identification of *Candida*, *Aspergillus*, and *Scedosporium* spp. by use of Rolling-Circle Amplification. Journal of Clinical Microbiology; 46(7): 2423–2427

APPENDIX

APPENDIX

Composition of Various Media

i. MacCONKEY Agar (CM0115, OXOID) (g/l)

Peptone	20.0 g
Lactose	10.0 g
Bile Salt	31.5 g
Sodium Chloride	5.0 g
Neutral Red	0.03 g
Crystal Violet	0.001 g
Agar	15.0 g
Distilled Water	1.0 Liter

pH: 7.1± 0.2

ii. Blood Agar Base (CM0055, OXOID) (g/l)

Lab-Lemco Powder	10.0 g
Peptone	10.0 g
Sodium Chloride	5.0 g
Agar	15.0 g
Distilled Water	1000 ml

pH: 7.3 ± 0.2

iii. **Nutrient Broth (CM0001, OXOID) (g/l)**

Lab-Lemco Powder	1.0 g
Yeast Extract	2.0 g
Peptone	5.0 g
Sodium Chloride	5.0 g

pH: 7.4± 0.2

iv. **CLED Medium with Andrade Indicator (XM0423, OXOID) (g/l)**

Peptone	4.0 g
Lab-Lemco Powder	3.0 g
Tryptone	4.0 g
Lactose	10.0 g
L-cystine	0.128 g
Bromothymol Blue	0.02 g
Android Indicator	0.1 g
Agar	15.0 g
Distilled Water	1000 ml

pH: 7.5± 0.2

v. Sabouraud Dextrose Agar (CM0041, OXOID) (g/l)

Mycological Peptone	10.0 g
Glucose	40.0 g
Agar	15.0 g
Distilled Water	1000 ml

pH: 5.6 ± 0.2

vi. Corn Meal Agar (CM0103, OXOID) (g/l)

Corn Meal Extract (From 50g whole in maize)	2.0 g
Agar	15.0 g
Distilled Water	1000 ml

pH: 6.0 ± 0.2

vii. Glucose Mueller Hinton Methylene Blue Agar (g/l)

Mueller Hinton Agar	38.0 g
Glucose	10.0 g
Methylene Blue	70 µl
Distilled Water	1000 ml

pH: 7.3 ± 0.

viii. **Mueller Hinton Agar (CM0337, OXOID) (g/l)**

Beef, Dehydrated Infusion form	300.0 g
Casine Hydrolysate	17.5 g
Starch	1.5 g
Agar	15.0 g
Distilled Water	1000 ml

pH: 7.3 ± 0.2

ix. **Potato Dextrose Agar (CM0139, OXOID) (g/l)**

Potato Extract	4.0 g
Dextrose	20.0 g
Agar	15.0 g
Distilled Water	1000 ml

pH: 5.6 ± 0.2

x. **Yeast Extract Peptone Dextrose Broth (g/l)**

Yeast Extract	200 g
Peptone	40.0 g
Dextrose	20.0 g
Distilled Water	1000 ml

xi. Chrome Agar-Candida (CA220, Chrome Agar Microbiology) (g/l)

Peptone	10.2 g
Chromogenic Mix	22.0 g
Chloramphenicol	0.5 g
Agar	15.0 g
Distilled Water	1000 ml
pH: 6.1 +/- 0.2	

Composition of Stains and Solutions

i. Crystal Violet

Solution A:

Crystal Violet	2.0 g
Ethanol 95%	20 ml

ii. Gram Iodine Solution

Iodine Crystals	1.0 g
Potassium Iodide	2.0 g
Distilled Water	300 ml

iii. Safranin Solution

Stock Solution:

Safranin	2.5 g
Ethanol 95%	100 ml

iv. 70 % Glycerol

80-90 % Glycerol	30 ml
Distilled Water	70 ml

v. API 10C

Reagents:

API NaCl 0.85% medium	5ml
API suspension medium	5 ml

API10C reagent kit

Mineral oil

v. $BaSO_4$ 3.0 percent McFarland Standards:

A $BaSO_4$ 3.0 percent McFarland standards were prepared as follows:

A 3.0 ml aliquot of 0.048 mol/L $BaCl_2$ (1.175 percent w/v $BaCl_2$. $2H_2O$) was added to 97.0 ml of 0.18 mol/L H_2SO_4 (1 percent v/v) with constant stirring to maintain a suspension. The correct density of the turbidity standard was verified by using a spectrophotometer with a 1 cm light path and matched cuvette to determine the absorbance. The absorbance at 625 nm was adjusted at 0.008 to 0.10 for the 3.0 percent McFarland standards. The Barium Sulfate suspension was transferred in 4 to 6 ml aliquots into screw-cap tubes of the same size as those used in growing or diluting the bacterial inoculum. These tubes were tightly sealed and stored in the dark at room temperature. The Barium Sulfate turbidity standard was vigorously agitated on a mechanical vortex mixer before each use and inspected for a uniformly turbid appearance.

vi. PCR (Polymerase Chain Reaction)

Components and Reagents:

- DNA template that contains the DNA region (target) to be amplified.
- Two primers that are complementary to the 3' (three prime) ends of each of the sense and anti-sense strand of the DNA target.
- Taq polymerase or another DNA polymerase with a temperature optimum at around 70 °C.
- Deoxynucleoside triphosphates (dNTPs; also very commonly and erroneously called Deoxynucleotide triphosphates), the building blocks from which the DNA polymerases synthesizes a new DNA strand.
- Buffer solution, providing a suitable chemical environment for optimum activity and stability of the DNA polymerase.
- Divalent cations, Magnesium or Manganese ions; generally Mg^{2+} is used, but Mn^{2+} can be utilized for PCR-mediated DNA mutagenesis, as higher Mn^{2+} concentration increases the error rate during DNA synthesis.
- Monovalent cations Potassium ions.

vii. 5X FIREPol Master Mix (Fermentas)

- FIRE POL DNA polymerase
- 5X Reaction Buffer B (O.4 M Tris HCL, 0.1 M Ammonium Sulphate, 0.1% w/v Tween 20)
- 12.5 mM Magnasium chloride
- 1mM dNTPS of each (200Mm dATPs, 200mM dCTPs, 200mM dTTPs, 200mM dGTPs)
- Blue Dye migration equivalent to 3.5-4.5 kb DNA fragment.
- Yellow Dye migration in excess of primers in 1% agarose gel 35-45 bp

- Components that increase sample density for direct loading
- Storage at -20°C

viii. 1X Tris-Boric Acid-EDTA (TBE)

Tris (pH 7.6),	89 mM
Boric acid	89 mM
EDTA	2 mM

10X Stock (1 liter) -- dissolve in 600 ml distilled water:

Tris base	108 g
Boric acid	55 g
0.5 M EDTA (pH 8.0)	40 ml

Fill to a final volume of 1 liter with distilled water.

Table No 4.1: Results of germ tube test for all 255 clinically isolated *Candida* positive samples.

Neg: Negative , **Pos:** Positive

Serial #	Names on plate	Germ tube	Serial #	Names on plate	Germ tube	Serial #	Names on plate	Germ tube
1	C1	Neg	40	C43	Pos	79	C86	Pos
2	C2,C11	Neg	41	C44	Neg	80	C87	Pos
3	C3,29,96	Pos	42	C45,165	Neg	81	C89	Pos
4	C4	Neg	43	C46,182	Neg	82	C90	Pos
5	C5,77,189	Pos	44	C47	Pos	83	C91,114	Pos
6	C6	Neg	45	C48	Neg	84	C92	Neg
7	C7	Pos	46	C49,176	Pos	85	C93,98	Neg
8	C8	Pos	47	C50	Pos	86	C94	Pos
9	C9	Pos	48	C51	Pos	87	C97	Pos
10	C10	Pos	49	C52	Pos	88	C99	Pos
11	C12,52	Neg	50	C53	Pos	89	C101	Pos
12	C13	Pos	51	C54,153	Neg	90	C103	Pos
13	C14,60,63	Pos	52	C55	Pos	91	C104	Neg
14	C15	Pos	53	C56	Pos	92	C106	Neg
15	C16	Pos	54	C57	Pos	93	C107	Pos
16	C17	Neg	55	C58,95	Neg	94	C108	Pos
17	C18	Neg	56	C59	Pos	95	C109,213	Pos
18	C19	Pos	57	C61	Pos	96	C110	Neg
19	C20	Neg	58	C62,88,105	Pos	97	C112	Pos
20	C21	Neg	59	C64,83,102	Pos	98	C113	Pos
21	C22	Pos	60	C65	Pos	99	C115	Neg
22	C23	Pos	61	C66	Pos	100	C116	Neg
23	C24	Pos	62	C67	Pos	101	C117	Neg
24	C25	Pos	63	C68	Neg	102	C119	Pos
25	C26	Pos	64	C69	Pos	103	C120	Pos
26	C27	Pos	65	C70	Neg	104	C121	Pos
27	C28,145	Pos	66	C71	Neg	105	C122	Pos
28	C30,228	Pos	67	C72	Pos	106	C123	Neg
29	C31	Pos	68	C73	Pos	107	C124	Neg
30	C32,40	Pos	69	C74	Neg	108	C125	Pos
31	C33	Pos	70	C75	Pos	109	C126	Neg
32	C34	Pos	71	C76	Neg	110	C127	Neg
33	C35,100	Pos	72	C78	Neg	111	C128	Pos
34	C36	Neg	73	C79,242	Pos	112	C129	Pos
35	C37	Neg	74	C80	Neg	113	C130	Pos
36	C38	Neg	75	C81	Neg	114	C131	Pos
37	C39	Neg	76	C82	Neg	115	C132	Pos
38	C41	Pos	77	C84,227	Pos	116	C134	Pos

39	C42,184	Neg	78	C85	Pos	117	C135	Pos
118	C136	Pos	159	C184	Neg	200	C237	Neg
119	C137,190	Pos	160	C185	Pos	201	C239	Pos
120	C138.414	Pos	161	C186	Neg	202	C241	Neg
121	C139	Pos	162	C187	Pos	203	C243	Neg
122	C140	Pos	163	C188	Pos	204	C244	Neg
123	C142	Pos	164	C191	Pos	205	C245	Neg
124	C143	Neg	165	C192	Neg	206	C246	Neg
125	C144,225	Neg	166	C193	Pos	207	C247	Neg
126	C146	Neg	167	C194	Pos	208	C248	Neg
127	C147	Neg	168	C195	Pos	209	C249	Pos
128	C148	Neg	169	C196	Pos	210	C250	Neg
129	C149	Pos	170	C197,226	Pos	211	C251	Neg
130	C150	Pos	171	C198,221	Neg	212	C252,259	Neg
131	C151	Neg	172	C199,209	Neg	213	C253	Neg
132	C152	Neg	173	C200	Pos	214	C254	Neg
133	C154	Neg	174	C201	Pos	215	C255	Pos
134	C155	Pos	175	C202,238	Neg	216	C256	Neg
135	C156	Pos	176	C203	Neg	217	C257	Pos
136	C157	Pos	177	C204	Neg	218	C258	Neg
137	C158	Pos	178	C205	Neg	219	C260	Neg
138	C159	Neg	179	C206	Neg	220	C261	Neg
139	C160	Pos	180	C207	Neg	221	C262	Neg
140	C161	Neg	1818	C208	Pos	222	C263	Neg
141	C162	Pos	182	C210	Neg	223	C264	Neg
142	C163	Pos	183	C211	Neg	224	C265	Neg
143	C164	Neg	184	C212	Neg	225	C266	Pos
144	C166	Neg	185	C216	Pos	226	C267	Neg
145	C167	Neg	186	C217	Pos	227	C268	Pos
146	C168,171,229	Neg	187	C218	Neg	228	C269	Neg
147	C169	Pos	188	C219	Pos	229	C270	Pos
148	C170,214	Neg	189	C220	Neg	230	C271	Pos
149	C172,240	Pos	190	C222	Neg	231	C272	Pos
150	C173,215	Neg	191	C223	Pos	232	C273	Pos
151	C174	Neg	192	C224	Neg	233	C274	Neg
152	C175	Neg	193	C230	Pos	234	C275	Pos
153	C177	Neg	194	C231	Neg	235	C276	Neg
154	C178	Neg	195	C232	Pos	236	C277	Pos
155	C179	Neg	196	C233	Pos	237	C278	Pos
156	C180	Pos	197	C234	Pos	238	C279	Pos
157	C181	Neg	198	C235	Neg	239	C280	Neg

158	C183	Pos	**199**	C236	Pos	**240**	C281	Neg
241	C282	Neg	**247**	C288	Neg	**253**	C294	Neg
242	C283	Pos	**248**	C289	Neg	**254**	C295	Neg
243	C284	Pos	**249**	C290	Neg	**255**	C296	Pos
244	C285	Pos	**250**	C291	Pos			
245	C286	Pos	**251**	C292	Neg			
246	C287	Neg	**252**	C293	Neg			

Table No 4.2: Identification of all 255 positive samples of candidiasis through two differential media *i.e.* Chrom Agar- *Candida* and Corn meal agar.

Serial #	Names on plate	Chrome agar results	Corn meal results	Serial #	Names on plate	Chrome agar results	Corn meal results
1	C1	*C. glabrata*	*C. glabrata*	37	C39	Candida spp.	C. lusitania
2	C2,C11	*C. krusei*	*C. krusei*	38	C41	*C. albicans*	*C. albicans*
3	C3,29,96	*C. albicans*	*C. albicans*	39	C42,184	*C. glabrata*	*C. glabrata*
4	C4	*C. albicans*	*C. glabrata*	40	C43	*C. albicans*	*C. albicans*
5	C5,77,189	*C. albicans*	*C. albicans*	41	C44	*C. krusei*	*C. tropicalis*
6	C6	*C. tropicalis*	*C. albicans*	42	C45,165	*C. tropicalis*	*C. tropicalis*
7	C7	*C. albicans*	*C. albicans*	43	C46,182	*C. glabrata*	*C. glabrata*
8	C8	*C. albicans*	*C. albicans*	44	C47	*C. albicans*	*C. albicans*
9	C9	*C. albicans*	*C. albicans*	45	C48	*C. glabrata*	*C. glabrata*
10	C10	*C. albicans*	*C. albicans*	46	C49,176	*C. albicans*	*C. albicans*
11	C12,52	*C. krusei*	*C. krusei*	47	C50	*C. albicans*	*C. albicans*
12	C13	*C. albicans*	*C. albicans*	48	C51	*C. albicans*	*C. albicans*
13	C14,60,63	*C. albicans*	*C. albicans*	49	C52	*C. albicans*	*C. albicans*
14	C15	*C. albicans*	*C. albicans*	50	C53	*C. albicans*	*C. dubilensis*
15	C16	*C. albicans*	*C. albicans*	51	C54,153	*C. tropicalis*	*C. glabrata*
16	C17	*C. glabrata*	*C. glabrata*	52	C55	*C. albicans*	*C. albicans*
17	C18	*C. krusei*	*C. glabrata*	53	C56	*C. albicans*	*C. albicans*
18	C19	*C. albicans*	*C. albicans*	54	C57	*C. albicans*	*C. albicans*
19	C20	*C. krusei*	*C. krusei*	55	C58,95	*C. tropicalis*	*C. tropicalis*
20	C21	*C. krusei*	*C. krusei*	56	C59	*C. albicans*	*C. albicans*
21	C22	*C. albicans*	*C. albicans*	57	C61	*C. albicans*	*C. albicans*
22	C23	*C. albicans*	*C. albicans*	58	C62,88,105	*C. albicans*	*C. albicans*
23	C24	*C. albicans*	*C. albicans*	59	C64,83,102	*C. albicans*	*C. albicans*
24	C25	*C. albicans*	*C. albicans*	60	C65	*C. albicans*	*C. albicans*
25	C26	*C. albicans*	*C. albicans*	61	C66	*C. albicans*	*C. albicans*
26	C27	C. albicans	C. albicans	62	C67	*C. albicans*	*C. albicans*
27	C28,145	C. albicans	C. albicans	63	C68	*C. glabrata*	*C. glabrata*
28	C30,228	C. albicans	C. albicans	64	C69	*C. albicans*	*C. albicans*
29	C31	C. albicans	C. albicans	65	C70	*C. krusei*	*C. krusei*
30	C32,40	C. albicans	C. albicans	66	C71	*C. tropicalis*	*C. albicans*
31	C33	C. albicans	C. albicans	67	C72	*C. albicans*	*C. albicans*
32	C34	C. albicans	C. albicans	68	C73	*C. albicans*	*C. albicans*
33	C35,100	C. albicans	C. albicans	69	C74	*C. glabrata*	*C. glabrata*
34	C36	C. glabrata	C. glabrata	70	C75	*C. albicans*	*C. albicans*
36	C38	C. albicans	C. albicans	72	C78	*C. glabrata*	*C. glabrata*
73	C79,242	*C. albicans*	*C. albicans*	114	C131	*C. albicans*	*C. albicans*

74	C80	*C. tropicalis*	*C. tropicalis*	115	C132	*C. albicans*	*C. albicans*
75	C81	*C. albicans*	*C. albicans*	116	C134	*C. albicans*	*C. albicans*
76	C82	*C. glabrata*	*C. glabrata*	117	C135	*C. albicans*	*C. albicans*
77	C84,227	*C. albicans*	*C. albicans*	118	C136	*C. albicans*	*C. albicans*
78	C85	*C. albicans*	*C. albicans*	119	C137,190	*C. albicans*	*C. albicans*
79	C86	*C. albicans*	*C. albicans*	120	C138.414	*C. albicans*	*C. albicans*
80	C87	*C. tropicalis*	*C. albicans*	121	C139	*C. albicans*	*C. albicans*
81	C89	*C. albicans*	*C. albicans*	122	C140	*C. albicans*	*C. albicans*
82	C90	*C. tropicalis*	*C. albicans*	123	C142	*C. albicans*	*C. albicans*
83	C91,114	*C. albicans*	*C. albicans*	124	C143	*C. krusei*	*C. krusei*
84	C92	*Candida* spp.	*C. lusitania*	125	C144,225	*C. albicans*	*C. tropicalis*
85	C93,98	*C. tropicalis*	*C. albicans*	126	C146	*C. tropicalis*	*C. albicans*
86	C94	*C. albicans*	*C. albicans*	127	C147	*C. glabrata*	*C. glabrata*
87	C97	*C. albicans*	*C. albicans*	128	C148	*C. krusei*	*C. krusei*
88	C99	*C. albicans*	*C. albicans*	129	C149	*C. albicans*	*C. albicans*
89	C101	*C. albicans*	*C. albicans*	130	C150	*C. albicans*	*C. albicans*
90	C103	*C. albicans*	*C. albicans*	131	C151	*C. glabrata*	*C. glabrata*
91	C104	*Candida* spp.	*C. parapsilosis*	132	C152	*C. krusei*	*C. krusei*
92	C106	*C. krusei*	*C. krusei*	133	C154	*Candida* spp.	*C. dubliniensis*
93	C107	*C. albicans*	*C. albicans*	134	C155	*C. albicans*	*C. albicans*
94	C108	*C. albicans*	*C. albicans*	135	C156	*C. albicans*	*C. albicans*
95	C109,213	*C. albicans*	*C. albicans*	136	C157	*C. albicans*	*C. albicans*
96	C110	*C. tropicalis*	*C. tropicalis*	137	C158	*C. albicans*	*C. albicans*
97	C112	*C. albicans*	*C. albicans*	138	C159	*Candida* spp.	*C. parapsilosis*
98	C113	*C. albicans*	*C. albicans*	139	C160	*C. albicans*	*C. albicans*
99	C115	*Candida* spp.	*C. parapsilosis*	140	C161	*C. tropicalis*	*C. tropicalis*
100	C116	*C. glabrata*	*C. krusei*	141	C162	*C. albicans*	*C. albicans*
101	C117	*C. albicans*	*C. albicans*	142	C163	*C. albicans*	*C. albicans*
102	C119	*C. albicans*	*C. albicans*	143	C164	*C. krusei*	*C. glabrata*
103	C120	*C. albicans*	*C. albicans*	144	C166	*C. glabrata*	*C. tropicalis*
104	C121	*C. albicans*	*C. albicans*	145	C167	*C. glabrata*	*C. glabrata*
105	C122	*C. albicans*	*C. albicans*	146	C168,171,229	*C. glabrata*	*C. glabrata*
106	C123	*C. tropicalis*	*C. tropicalis*	147	C169	*C. albicans*	*C. albicans*
107	C124	*C. tropicalis*	*C. tropicalis*	148	C170,214	*C. glabrata*	*C. krusei*
108	C125	*C. albicans*	*C. albicans*	149	C172,240	*C. albicans*	*C. albicans*
109	C126	*Candida* spp.	*C. parapsilosis*	150	C173,215	*C. tropicalis*	*C. dubliniensis*
110	C127	*C. krusei*	*C. krusei*	151	C174	*C. glabrata*	*C. glabrata*
111	C128	*C. albicans*	*C. albicans*	152	C175	*C. krusei*	*C. krusei*
112	C129	*C. albicans*	*C. albicans*	153	C177	*C. krusei*	*C. tropicalis*
113	C130	*C. albicans*	*C. albicans*	154	C178	*C. krusei*	*C. krusei*
155	C179	*C. tropicalis*	*C. tropicalis*	195	C232	*C. albicans*	*C. albicans*

156	C180	*C. albicans*	*C. dubliniensis*	196	C233	*C. albicans*	*C. albicans*
157	C181	*C. glabrata*	*C. glabrata*	197	C234	*C. albicans*	*C. albicans*
158	C183	*C. albicans*	*C. albicans*	198	C235	*C. glabrata*	*C. glabrata*
159	C184	*C. glabrata*	*C. glabrata*	199	C236	*C. albicans*	*C. albicans*
160	C185	*C. albicans*	*C. tropicalis*	200	C237	*C. tropicalis*	*C. tropicalis*
161	C186	*C. krusei*	*C. krusei*	201	C239	*C. albicans*	*C. albicans*
162	C187	*C. albicans*	*C. albicans*	202	C241	*C. tropicalis*	*C. tropicalis*
163	C188	*C. albicans*	*C. dubliniensis*	203	C243	*C. krusei*	*C. krusei*
164	C191	*C. albicans*	*C. albicans*	204	C244	*C. glabrata*	*C. glabrata*
165	C192	*C. glabrata*	*C. glabrata*	205	C245	*Candida* spp.	*C. parapsilosis*
166	C193	*C. albicans*	*C. albicans*	206	C246	*Candida* spp.	*C. parapsilosis*
167	C194	*C. albicans*	*C. dubliniensis*	207	C247	*C. krusei*	*C. krusei*
168	C195	*C. albicans*	*C. albicans*	208	C248	*C. tropicalis*	*C. tropicalis*
169	C196	*C. albicans*	*C. dubliniensis*	209	C249	*C. albicans*	*C. albicans*
170	C197,226	*C. albicans*	*C. albicans*	210	C250	*C. glabrata*	*C. glabrata*
171	C198,221	*C. glabrata*	*C. glabrata*	211	C251	*C. tropicalis*	*C. tropicalis*
172	C199,209	*Candida* spp.	*C. parapsilosis*	212	C252,259	*Candida* spp.	*C. dubliniensis*
173	C200	*C. albicans*	*C. tropicalis*	213	C253	*C. glabrata*	*C. glabrata*
174	C201	*C. albicans*	*C. albicans*	214	C254	*Candida* spp.	*C. dubliniensis*
175	C202,238	*C. tropicalis*	*C. tropicalis*	215	C255	*C. albicans*	*C. albicans*
176	C203	*C. tropicalis*	*C. krusei*	216	C256	*C. glabrata*	*C. glabrata*
177	C204	*C. tropicalis*	*C. tropicalis*	217	C257	*C. albicans*	*C. albicans*
178	C205	*C. glabrata*	*C. parapsilosis*	218	C258	*C. tropicalis*	*C. tropicalis*
179	C206	*C. glabrata*	*C. glabrata*	219	C260	*C. tropicalis*	*C. tropicalis*
180	C207	*C. glabrata*	*C. krusei*	220	C261	*Candida* spp.	*C. parapsilosis*
181	C208	*C. albicans*	*C. albicans*	221	C262	*C. glabrata*	*C. parapsilosis*
182	C210	*C. tropicalis*	*C. parapsilosis*	222	C263	*C. glabrata*	*C. parapsilosis*
183	C211	*C. glabrata*	*C. glabrata*	223	C264	*Candida* spp.	*C. parapsilosis*
184	C212	*C. tropicalis*	*C. parapsilosis*	224	C265	*C. tropicalis*	*C. tropicalis*
185	C216	*C. albicans*	*C. dubliniensis*	225	C266	*C. albicans*	*C. albicans*
186	C217	*C. albicans*	*C. tropicalis*	226	C267	*C. tropicalis*	*C. tropicalis*
187	C218	*Candida* spp.	*C. parapsilosis*	227	C268	*C. albicans*	*C. albicans*
188	C219	*C. albicans*	*C. albicans*	228	C269	*C. tropicalis*	*C. tropicalis*
189	C220	*C. tropicalis*	*C. glabrata*	229	C270	*C. albicans*	*C. albicans*
190	C222	*C. krusei*	*C. krusei*	230	C271	*C. albicans*	*C. albicans*
191	C223	*C. albicans*	*C. albicans*	231	C272	*C. albicans*	*C. albicans*
192	C224	*C. krusei*	*C. krusei*	232	C273	*C. albicans*	*C. albicans*
193	C230	*C. albicans*	*C. albicans*	233	C274	*Candida* spp.	*C. lusitania*
194	C231	*C. tropicalis*	*C. tropicalis*	234	C275	*C. albicans*	*C. albicans*
235	C276	*C. glabrata*	*C. glabrata*	246	C287	*C. tropicalis*	*C. tropicalis*
236	C277	*C. albicans*	*C. albicans*	247	C288	*C. glabrata*	*C. glabrata*
237	C278	*C. albicans*	*C. albicans*	248	C289	*Candida* spp.	*Trichosporoum*

238	C279	*C. albicans*	*C. albicans*	**249**	C290	*C. tropicalis*	*C. tropicalis*
239	C280	*C. tropicalis*	*C. parapsilosis*	**250**	C291	*C. albicans*	*C. albicans*
240	C281	*C. glabrata*	*C. glabrata*	**251**	C292	*C. tropicalis*	*C. tropicalis*
241	C282	*Candida spp.*	*C. parapsilosis*	**252**	C293	*C. tropicalis*	*C. tropicalis*
242	C283	*C. albicans*	*C. albicans*	**253**	C294	*C. tropicalis*	*C. tropicalis*
243	C284	*C. albicans*	*C. albicans*	**254**	C295	C. glabrata	C. glabrata
244	C285	*C. albicans*	*C. albicans*	**255**	C296	*C. albicans*	*C. albicans*
245	C286	*C. albicans*	*C. albicans*				

Table No 4.3 Reference table of API 10C showing reference ranges and test details

READING TABLE

TESTS	ACTIVE INGREDIENTS	QTY (mg/cup.)	REACTIONS/ENZYMES	RESULTS	
				NEGATIVE	POSITIVE
1) GLU	D-glucose	1.4	Acidification (GLUcose)	violet grey-violet →	yellow green / grey
2) GAL	D-galactose	1.4	Acidification (GALactose)		
3) SAC	D-saccharose	1.4	Acidification (SACcharose)		
4) TRE	D-trehalose	1.4	Acidification (TREhalose)		
5) RAF	D-raffinose	1.4	Acidification (RAFfinose)		
6) βMAL	4-nitrophenyl-ßD-maltopyranoside	0.08	ß-MALtosidase	colorless	pale yellow-bright yellow
7) αAMY	2-chloro-4-nitrophenyl-αD maltotrioside	0.168	α-AMYlase	colorless	pale yellow-bright yellow
8) βXYL	4-nitrophenyl-ßD-xylopyranoside	0.095	ß-XYLosidase	colorless-very pale yellow / blue / green **	pale yellow-bright yellow
9) βGUR	4-nitrophenyl-ßD-glucuronide	0.063	ß-GlUcuRonidase	colorless / blue / green	pale yellow-bright yellow
10) URE	urea	1.68	UREase	yellow-pale orange	red
11) βNAG (in tube no. 8) *	5-bromo-4-chloro-3-indoxyl-N-acetyl-ßD-glucosaminide	0.09	N-Acetyl-ß-Glucosaminidase	colorless / yellow	blue / green **
12) βGAL (in tube no. 9) *	5-bromo-4-chloro-3-indolyl-ßD-galactopyranoside	0.0815	ß-GALactosidase	colorless / yellow	blue / green

* Tubes 8 and 9 are bi-functional : tube 8 : βXYL (test no. 8) / βNAG (test no. 11)
tube 9 : βGUR (test no. 9) / βGAL (test no. 12)

** Any trace of green in cupule 8 = βXYL (–) βNAG (+)

• The quantities indicated may be adjusted depending on the titer of the raw materials used.

Table No 4.6: Age and Gender Distribution of all *Candida* positive patients, showing highest percentage of female patients then male patients.

Group	Age	No. of Samples	No. of Affected Females	% of Affected Females	No. of Affected Males	% of Affected Males
A	1y-20y	20	17	85%	03	15.0%
B	21y-40y	68	58	85.30%	10	14.70%
C	41y-60y	60	41	68.30%	19	31.60%
D	61y-80y	87	42	48.30%	45	51.70%
E	81y-100y	19	09	47.30%	10	52.70%
F	101y-120y	01	00	0.0%	01	100.0%
	Total	**255**	**167**	**65.50%**	**88**	**34.50%**

Table No 4.7: Distribution of *Candida* positive samples on the basis of source showed high incidence of *Candida* in urine samples and low incidence in throat swab etc. Percentage of infected female is higher than male.

Source	No. of Samples	Positive samples	No. of Infected Females	% of Infected Females	No. of Infected Males	% of Infected Male
Urine	991	92	58	63%	34	37%
HVS	456	66	66	100%	00	0%
Sputum	176	34	13	38.2%	21	61.8%
Trachea Secretion	65	26	12	40%	14	60%
Bronchial Wash	35	12	05	42%	07	48%
Pus Swab	93	10	03	30%	07	70%
CVP /ETT tip	25	04	02	50%	02	50%
Wound Swab	70	02	01	50%	01	50%
Blood	785	02	02	100%	00	0%
CSF	80	02	02	100%	00	0%
Throat Swab	45	01	01	100%	00	0%
Stool	40	01	00	00%	01	100%
Body Fluids	86	01	01	100%	00	0%
Fungal Culture	15	01	00	00%	01	100%
Tissue	45	01	01	100%	00	0%
Total	**3007**	**255**	**167**	**65.4%**	**88**	**34.6%**

Table No 4.8: Distribution of *Candida* positive samples on the basis of location showed high incidence of *Candida* in outdoor patients and low incidence was observed in patients of Operation Theater.

Location	No. of Samples	Positive samples	No. of Infected Females	% of Infected Females	No. of Infected Males	% of Infected Male
OPD	753	87	69	79.30%	18	20.70%
ICU	596	72	41	57%	31	43.0%
IPD	426	60	39	65%	21	35%
ER	378	11	6	54.50%	05	45.50%
CCU	106	09	02	22.30%	07	77.70%
Day Care	208	08	04	50.0%	04	50.0%
NICU	435	07	06	85.70%	01	14.30%
OR/OT	105	01	00	0.0%	01	100.0%
Total	**3007**	**255**	**167**	**65.5%**	**88**	**34.5%**

OPD: Outdoor Patients **ICU:** Intensive Care Unit **IPD:** Indoor Patients **ER:** Emergency

CCU: Cardiac Care Unit **NICU:** Neonatal Intensive Care Unit **OR/OT:** Operation Theater

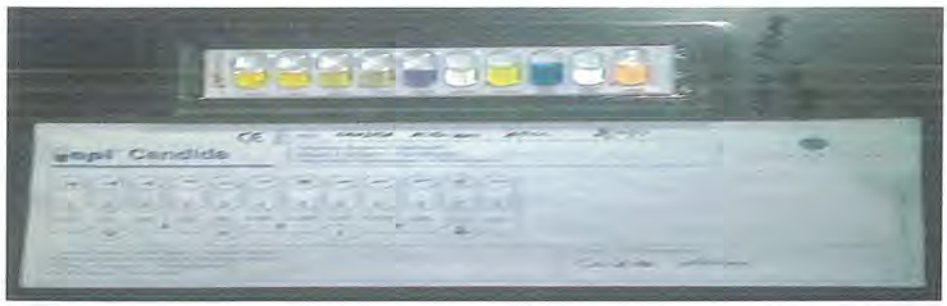


Figure 4.6: Results of API10 C of strain 286 (*Candida albicans*) showing 100% similarity with both differential media

Figure 4.7: Results of API10 C of strain 292 (*Candida tropicalis*) showing 98% similarity with both differential media

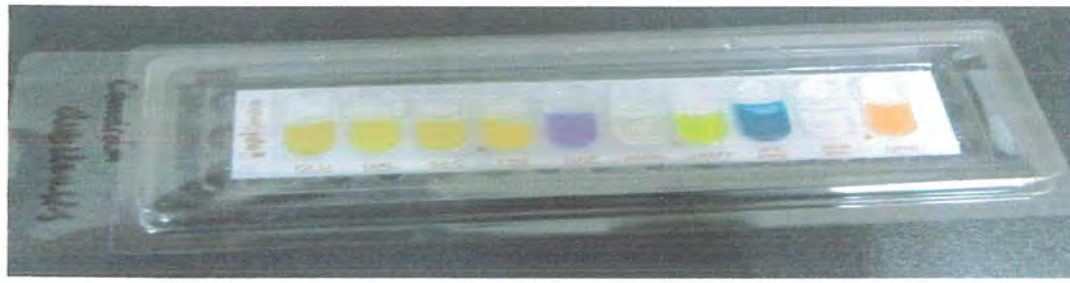


Figure 4.8: Results of API10 C of strain 154 (*Candida dubliniensis)* showing 99% similarity with both differential media

Figure 4.9: Results of API10 C of strain 104 (*Candida parapsilosis)* showing 98% similarity with both differential media

Figure 4.10: Results of API10 C of strain 104 (*Candida glabrata)* showing 100% similarity with both differential media

Figure 4.11: Results of API10 C of strain 289 (*Trichosporoum* spp.*)* showing 100% similarity with both differential methods

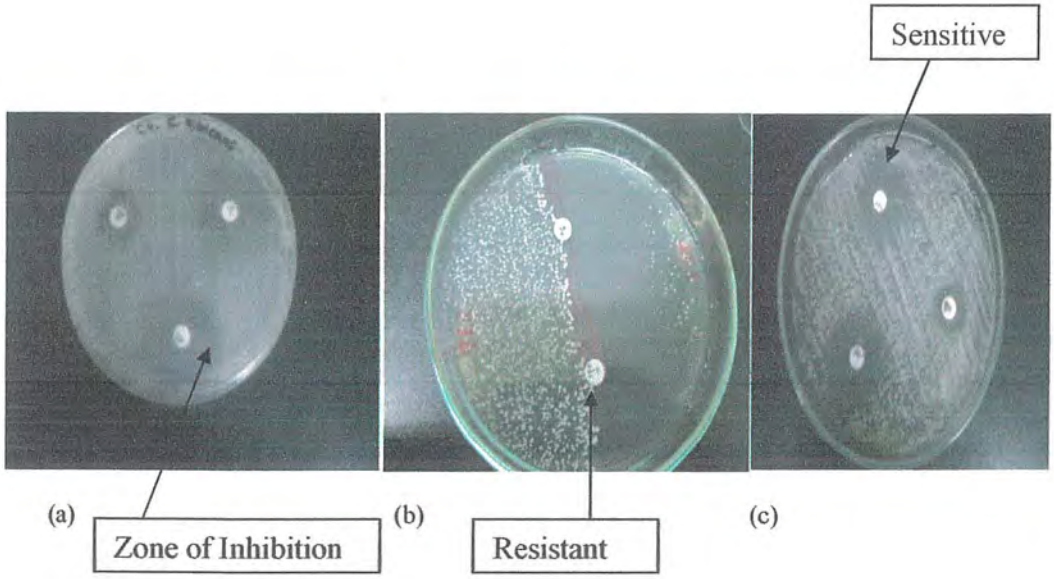


Figure 4.12: Results of antifungal sensitivity of (a) C4 (*Candida albicans*), (b) C253 (Candida *glabrata*) and C254 (*Candida dubliniensis*) (c) C6 (*Candida tropicalis*).