TURKISH REPUBLIC OF NORTHERN CYPRUS
NEAR EAST UNIVERSITY
HEALTH SCIENCES INSTITUTE

Determination of Biofilm Formation and Antifungal Sensitivity in *Candida* Strains Isolated from Various Clinical Samples

FAWZI MANSOUR FAWZI ALQWASMI

MASTER OF SCIENCE THESIS

MEDICAL MICROBIOLOGY AND CLINICAL MICROBIOLOGY PROGRAM

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MENTOR

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STATEMENT (DECLARATION)

The data presented in this thesis was obtained in an experiment carried out in the microbiologylaboratory/NearEastUniversityHospital.Iplayedamajorrole inthepreparation and execution of the experiment, and the data analysis and interpretation are entirely my own work.

I am aware of and understand the NEAR EAST UNIVERSITY'S policy on plagiarism and I certifythatthisthesisismyownworkIhadnounethicalbehaviorinallstagesfromtheplanning of the thesis until writing, except where indicated by the references, and the work presented in it has not been submitted in support of another degree or qualification from this or any other university or institute oflearning.

FAWZI MANSOUR FAWZI ALQWASMI

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS AND SYMBOLS

%: Percentsign

°C: Celsius

ABC: The ATP-bindingcassette

ABCD: Amphotericin B colloidaldispersion

ABLC: Amphotericin B lipidcomplex

AFST:Antifungalsusceptibilitytesting

AIDS: Acquiredimmunodeficiencysyndrome

AMB: AMPHOTERICIN B

AMI: Antibody-mediatedimmunity

ATCC: Americantypeculturecollection

BCE:BeforetheCommonEraorBeforetheCurrentEra

BMD: Brothmicrodilution

BPs: Break Points

BSIs: Bloodstreaminfections

CAS: CASPOFUNGIN

CAUTI: Catheter-associatedurinarytractinfections

CDC: CentersforDisease Control andPrevention

CLABSI: Central-line-associatedbloodstreaminfections

CLSI: TheClinicalandLaboratoryStandardsInstitute

CMA:Corn Meal Agar

CMC:Chronicmucocutaneouscandidiasis

CMI: Cell-mediatedimmunity

CNS: Thecentralnervoussystem

CRBSI: Catheter-relatedbloodstreaminfections

CSF: Cerebrospinalfluid

CW: Cell wall

DC: Disseminatedcandidiasis

DNA:Deoxyribonucleicacid**E**

C:Esophagealcandidiasis

ECDC: TheEuropean Center forDisease Control

EPS: Extracellularpolymericsubstances

ER: Emergencyroom

ESCMID: TheEuropeanSocietyforClinicalMicrobiologyandInfectiousDiseases

et

al.:Andothers**EUCAST**:TheEuropeanCommitteeforAntimicrobialSusceptibilit

yTesting **FDA**: TheFoodandDrug Administration

FLU: FLUCONAZOLE

GMS: Grocott'sMethenamine Silver

HIV: Human ImmunodeficiencyVirus

hr: hour

ICU: Intensivecareunit

IDSA: InfectiousDiseasesSociety of America

IgA: Immunoglobulin A

IgG: Immunoglobulin G

IgM: Immunoglobulin M

ITR: ITRACONAZOLE

IUD: intrauterinedevice

IV: Intravenous

kg: Kiloigram

KOH: Potassiumhydroxide

L-AmB: LiposomalformulationAmphotericin B

L: Liter

lbs: A pound

LPCB:Lactophenolcottonblue

mcg /µg: Microgram

MCZ: MICONAZOLE

mg: Milligram

mg/L: Milligrams per liter

MH-GMB: Mueller–Hintonagarsupplementedwith 2% glucoseand 0.5 µg/mLmethylenebluedye

MH: Mueller-Hintonagar

MIC: Minimum inhibitoryconcentration

mL: Milliliter

mm: Millimeter

n:Number

NAC: Non- albicansCandida

NaCl: Sodiumchloride

NCCLS: TheNationalCommitteeforClinicalLaboratoryStandards

NICU: Neonatalintensivecareunit

nm: nanometer

No: Number

OPC: Oropharyngealcandidiasis

P-value: Probabilityvalue

PAS: PeriodicAcid–Schiff

PCR:Polymerasechainreaction

PD: Pharmacodynamics

PDA: PotatoDextroseAgar

pH:Potentialforhydrogen**P**

K: Pharmacokinetics

PLHA: People livingwith HIV/AIDS

PM: Plasmamembrane

PMNL: Polymorphonuclearleucocytes

QC: Qualitycontrol

R: Resistant

RPG: RPMI agarwith 2% glucose

RPMI: Roswell Park MemorialInstitute

RVVC: Recurrentvulvovaginalcandidiasis

S: Susceptible

S:Svedberg units

Saps: Secretedaspartylproteinases

SD: Standard deviation

SDA: SabouraudDextroseAgar

SDD: Susceptibledose-dependent

SPSS: Statistical PackagefortheSocialSciences

TOC:Tween-80, Oxgall,CaffeicAcid

TRM:TetrazoliumReductionMedium

TRNC: TheTurkishRepublic of NorthernCyprus

U:Unit**UT**:Urin

arytract

UTI:Urinarytractinfection

VOR: VORICONAZOLE

VVC:Vulvovaginalcandidiasis

yrs: Years

µg/mL:MicrogramperMilliliter

µm: Micrometer

Thesis Title: Determination of Biofilm Formation and Antifungal Sensitivity in Candida Strains Isolated from Various Clinical Samples

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ABSTRACT

Aim: This research was carried out to determine the distribution pattern isolated from different clinical specimens of *Candida* species and to studytheir antifungal susceptibility profileanalysisandtoshowtheabilityof*Candida*speciestoformbiofilm.**Materialsand Methods:** A total of 43 samples were collected from Near East hospital stocks. *Candida* samples were inoculated onSabouraud Dextrose Agar(SDA) to get pure colonies and the identificationwasdoneonHiCrome™*Candida*DifferentialAgar.Antifungalsusceptibility testing of isolated *Candida* species was done using VITEK 2 system. To show the ability of*Candida*speciestoformbiofilm,theisolatedcolonieswereculturedonCongoRedAgar (CRA)using*Staphylococcusaureus*ATCC35556strainsasapositivecontrol. **Results:**In thisstudyoutof43specimensweretested.*C.krusei*wasthemostcommonspecies27.9%. In antifungal susceptibility pattern testing some of *Candida* species shows a resistance patterns for Vorikonazole 9.1%, AmphotericinB 7.0% and Flusitozine 4.7%. Most of*Candida* species were able to produce biofilm 86%. *C. krusei* 29%and *C. albicans* 27% were the most species producing biofilm. **Conclusion:** This study indicates that high percentages of *Candida* species produce biofilm which is play a role in antifungal resistance.

Key Words: *Candida* species, Antifungal Susceptibility Test, biofilm

Tez Başlığı:Çeşitli Klinik Örneklerden İzole Edilen *Candida* İzolatlarında Biyofilm Oluşumu ve Antifungal Duyarlılıklarının Belirlenmesi

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Anabilim Dalı: Tıbbi Mikrobiyoloji ve Klinik Mikrobiyoloji

Amaç: Çeşitli klinik örneklerden izole edilen *Candida* izolatlarının tiplendirilmesi ile biyofilm oluşumu ve antifungal duyarlılık durumlarını belirlenmesi amaçlanmıştır.**Metot:** Çeşitli klinik örneklerden izole edilen 43 *Candida spp* olarak tanımlanan Yakın Doğu Üniversitesi Mikrobiyoloji stok koleksiyonundan kullanılacaktır. İzolatların çeşitli antibiyotiklere karşı duyarlılıkları Phoenix otomatize sistemi kullanılarak tayin edilecektir. İzolatların tiplendirilmesi için Chrom Agar Besiyeri kullanılacaktır. Biyofilm oluşturma Congo Red Agar ile karşılaştırılacaktır. Elde edilen veriler SPSS programı kullanılarak istatistiksel olarak değerlendirilecektir**Sonuçlar:**Kırk-üç tane *Candida spp*. izolatı kullanıldı. *C. krusei* (27.9%) en çok izole edilen *Candida spp*. izolatı antifungal duyarlılık testleri sonucunda *Candida spp*suşlarında direnç oranları 9.1% vorikonazol, 7.0% ampfoterisin B and 4.7% flusitozine. Biyofilm sonuçlarımıza göre 86% *Candida spp* biyofilm pozitif oldukları saptanmıştır. 29% *C. krusei* ve 27% *C. albicans* biyofilm pozitif olarak saptanan *Candida spp.* olarak saptanmıştır. **Tartışma:** Çalışmamız sonucunda *Candida spp* izolatları arasında biyofilm üretiminin fazla olduğu ancak antifungal direnci ile ilişkisi olmadığı sonucuna varılmıştır**.**

Anahtar kelimeler: *Candida* species, Antifungal Duyarlılık Test, Biyofilm

CHAPTER ONE

1. Introduction

*Candida spp.*is fungus-related eukaryotic yeast found in humans as the gastrointestinal tract,genitourinarytract,andnormaloralflora. *Candidaspp.*isconsideredtobeoneofthe most commonfungicausingopportunistichumandiseaseswhenthehost'simmunesystem slows,especiallyinhospitalizedpatients(Mergonietal.2018;TsegaandMekonnen2019). Referring to the different forms of *Candida spp*. such as *Candida glabrata (C. glabrata), Candida dubliniensis (C. dubliniensis), Candida krusei (C. krusei), Candida parapsilosis (C. parapsilosis), Candida tropicalis (C. tropicalis), Candida guillermondii (C. guillermondii*),*Candida albicans (C. albicans)* were the most proven fungi in the oral cavity, and the commentary fungi that led to nosocomial infection. *C. albicans* was the mostrecurrentfungiisolatedfromhumanbloodcultureinthecaseofcandidiasis(adisease caused by *Candida spp*). However, *Candida* species may also cause other diseases, such as vaginities, in which vaginal candidiasis attacks women's genital tract which iscurrently the most prevalent disease caused by the fungus(Pérez-García et al. 2017; Mergoni et al. 2018; Tsega and Mekonnen2019).

Biofilms are critical in medical environments, as implant surfaces or indwelling systems are often colonized by pathogens. A base with low humidity and some nutrients provide the niche for the production of biofilms (Kolter and Greenberg 2006).Biofilms exhibit multiple properties such as increased antimicrobial resistance, defensive barrier against hostdefensesystem,andmultidrugresistance(Ramageetal.2006).*Candidaspp.*biofilms are particularly resistant to different antifungal medications, and the processes causing biofilm tolerance are complex(Bink 2011).

Antifungal susceptibility testing has recently been developed to be standardized and is now available either from the Clinical and Laboratory Standards Institute (CLSI) which is previously known as the National Committee for Clinical Laboratory Standards (NCCLS)

and distributed to a specific class (M27-A3, M27-S3, M44-A, and M44-S2) or from the European antimicrobial Susceptibility Study Committee (EUCAST). CLSI and EUCAST also independently established basic guidelines; inhibition zones frequency (MIC) breakpoints, interpretive zone-diameter breakpoints, and AFST disk diffusion requirements(Alastruey-Izquierdo et al. 2015).

This research aimed to investigate the ability of *Candida* species to form biofilm and to investigate the antifungal efficacy and the resistance pattern on the *Candida* species.

2. GENERAL INFORMATION

HistoricalBackground

Dr.BernhardLangenbeck;amedicallectureratGöttingenUniversity,Germany,published the first description of *Candida spp*. in 1839 when he found a fungus in the throat sample from a patient who died of typhoid fever when mistaking the yeast as a typhoid-and not thrush-causing agent were “Finding of fungi on the mucous membrane of the gullet of a typhoid fever corpse” was his paper’s description(Knoke and Bernhardt 2006; Mccool 2010; Segal and Elad 2010; Mayser 2011; Silverman 2013; Mohamed Refai 2015; Deorukhkar 2018; Vila et al. 2020). In 1868, Hansen identified the fungus as a yeasted mycelial fungus and he approved "Monilia Candida" as probate name for it(Rippon 1988; Deorukhkar2018).

In 1874, Grawitz described the dimorphic nature of the fungus as budding yeast and mycelia forms(St Germain and Beauchesne 1991; Deorukhkar 2018). In 1862,candidiasis was firstly diagnosed by Zenker(Segal and Elad 2010; Deorukhkar 2018). The first involvementof*C.albicans*expressionwas in1853byCharlesPhilippeRobinwhichrefers to the pathogenic agent of thrush, while It was classified under the genus "Oidium" as Oidium albicans (Khan, Z. K. 1998; Segal and Elad 2010; Mohamed Refai 2015; Deorukhkar 2018; Vila et al. 2020). *C. albicans* could be isolated from many samples but the first isolation was in 1844 from a sputum sample referred to the patient have tuberculosis signs and symptoms, in 1849 they found it in avaginal swab from a patient with vulvovaginal candidiasis (VVC), while in 1853 Robin isolated it from patient with systemic candidal infection and a patient with a brain infection(Rippon 1988; Khan, Z. K. 1998; Segal and Elad 2010; Deorukhkar2018).

In 1954, the Eighth Botanical Congress in Paris accepted the binomial name of *C. albicans*as nomenconservandum. The name candida is a Latin word which mean toga Candida that also relates to the unique white robe of the Roman senators. The name creationmay refers to the white colonies of the agar medium of the *Candida* species of the oral lesions of aphthae or thrush(Segal and Elad 2010; Mayser 2011; Mohamed Refai 2015; Deorukhkar 2018; Vila et al. 2020).The fungus was identified as *Saccharomyces albicans*withinthegenus"*Saccharomyces*"in1877(SegalandElad2010;MohamedRefai 2015). In 1889, the fungus was identified as *Dematium albicans* by the genus *'Dematium'*. Aftera fewyears,Johan-OlsennamedthefungiofthegenusEndomycesasEndomycesin 1897 (Mohamed Refai 2015). In addition to, the fungus was classified refers to the genus "*Parasaccharomyces*" as *Parasaccharomyces albicans*, while Brownlie named thefungus referstothegenus"*Blastomyces*"as*Blastomycesalbicans*in1920(MohamedRefai2015).

After the discovery of *C.albicans*, Castellani made a detailed analysis of mycosis in 1912 and he suggested that other yeast species may be producing candidiasis and he made the first classification of other fungi that currently known as *C. tropicals*, *Candida kefyr*,and *C. guilliermondii*(Vincent 1992; Segal and Elad 2010; Deorukhkar 2018). In 1835, Veron identified the first incidence of esophageal candidiasis in newborns and believed that the newborns had developed the disease when they going through the womb and the vagina(Khan,Z. K. 1998;SegalandElad2010;Mayser2011;Deorukhkar2018).In1844, J.H. Bennett Detected similar fungi in a pneumothorax patient's sputum and lungs. Within two years, in 1846, Berg endorsed Langenbeck 's discovery that the disease was also the cause of thrush and it was verified for the first time that Esophageal candidiasis was a unique disorder. The association between the thrush and *C. albicans* was established defined by Berg, were clarified that *C. albicans* able to spread through feeding bottles within abnormal conditions which lead to the baby died because the exposure to candida bronchitis (Lynch 1994; Khan, Z. K. 1998; Mccool 2010; Segal and Elad 2010; Mayser 2011; Mohamed Refai 2015; Deorukhkar 2018).

Haussman revealed the similarities between the causative agent in both oral and vaginal candidiasisin1875,andhealsorevealedthetransmissionoftheinfectionfromthemother's vaginal lesion to the baby's mouth (Rippon 1988; Lynch 1994; Segal and Elad 2010; Deorukhkar 2018). The first description of candidal meningitis was in 1933 by Smith and Sano(Segal and Elad 2010; Deorukhkar 2018). Where Whelan et al.illustrated the subsequent creation of the diploid existence of *C. albicans* and the hereditary parasexual mechanism in 1980 (Whelan et al. 1980; Deorukhkar2018).

Referring to antifungal medications, The polyenees were the first clinically effective antifungal drugs, amphotericin B and nystatin introduced in the 1950s (Hazen and Brown 1951). Amphoteric molecules with a face of hydrophobic polyene and a base of hydrophilicity comprising many hydroxyl groups, while the mechanism of this drug causing intracellular brake down leading to release its components causing fungal death (Abuhammour and Habte-Gaber 2004). Flucytosine is a fungicidal medication, with a restricted range of activities; flucytosine was later discovered after the discovering of amphotericin B and was documented during 1963. While 5-fluorocytosine has been licensed forsystemiccandidosisChemotherapy(TitsworthandGrunberg1973;Dismukes 2000).Flucytosinespecificallyworksonthefungalgeneticmaterialleadingtoinhibit DNA synthesis (Odds et al. 2003). Azoles group went public in the late 1960s antifungal which isconsideredthe lessdangerousantifungalmedicationwithlowtoxicity.Azolesconsistof three main type clotrimazole,miconazole and ketoconazole. clotrimazole and miconazole werediscoveredin1970swhileKetoconazolein1980s(Maertens2004).Echinocandinwas releasedattheendofthe1990swhichisthenewestantifungalmedication,forpatientswith oropharyngeal candidiasis refusing azole antifungal echinocandin has been chosen as the most effective treatment (Walsh et al. 2004; Nevado et al.2005)

Taxonomy

Candida spp. was categorized under the taxonomic division of ascomycetes according to the modern classification dependent on the important characteristics like negative urease activity, fermentationofcarbohydratesbesidesinositol,andlackingofcapsule.Theyhave

β-glucans in their cell wall and it does not contain carotenoid pigment or starch. Recent advancements in molecular technology and research have led to improvements in the taxonomic status of some species of *Candida spp*. and the identification of new species, such as *C. dubliniensis*(Brandt 2002; Chin et al. 2016).

Referring to the discovery for a new *Candida* species there was improvement in thegenus taxonomy by the time which includes including *Candida orthopsilosis, C. dubliniensis, and Candida metapsilosis*(Bendel2011).

Morphology of *Candidaspp.*

As a heterogeneous population of eukaryotic, polymorphic, or dimorphic species, Genus *Candidaspp.*exists.Theyaresmallroundtoovalyeast-likecellsaerobic,thin-walled,that weigh around 4-6 μm that replicate asexually by budding called bud (blastoconidia). The cell consists of a single nucleus and it has membrane-bound organelles like vacuoles, mitochondria, and Golgi apparatus, but with no flagella. Most of *Candida* species are pseudohyphaeconsistofcurvedorbranchednotseparatedblastoconidia.Weretruehyphae andchlamydosporescouldonlybeproduced inspecificconditionssuchasoxygentension reduction(Bendel2011;Sudbery2011;Thompsonet al.2011;Silvaet al.2012;Polviet al. 2015; Mukaremera et al.2017).

In the following figure 1, a sample of a patient with Candidiasis shows pseudohyphae and hyphae (Thompson et al.2011)

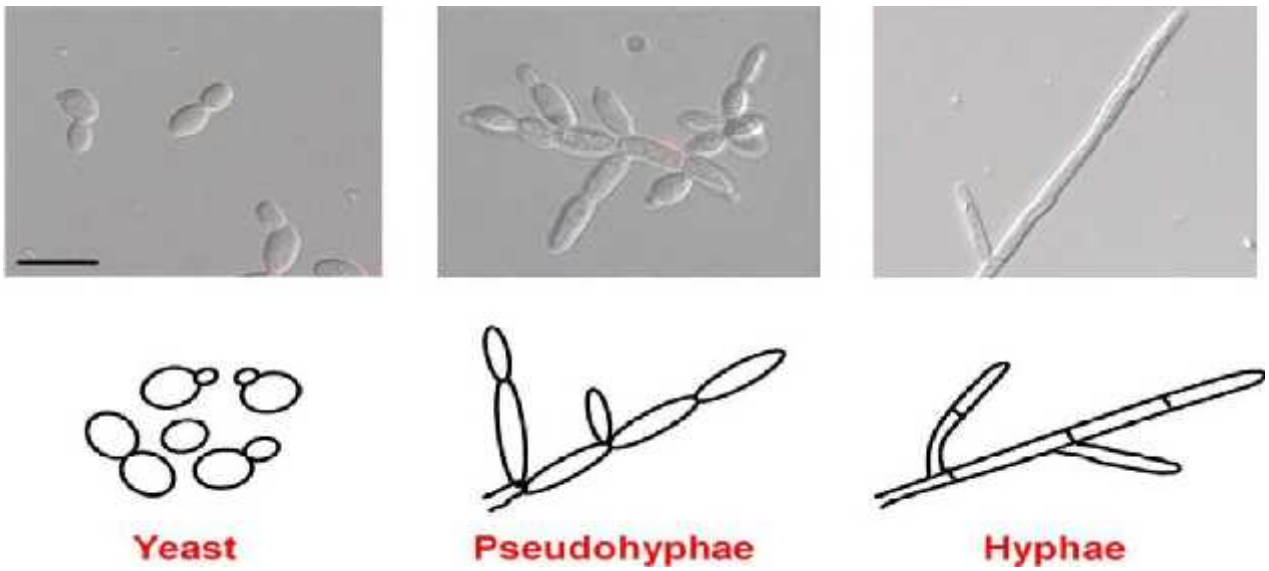

Figure 1. Candidiasis pseudohyphae and hyphae in a patient sample

The Laboratory Diagnostic of*Candida*Species

Diagnosticandrecognition*Candidaspp*.organismsareknowntobeasignificantchallenge for both microbiologists and clinicians. Providing a timely and accurate diagnosis of *Candida*speciesisthusacrucialandessentialprerequisitetoestablishtheeffectivetherapy for *Candida* infection(Deorukhkar 2014a; Posch et al.2017; Sturaro et al.2018; Deorukhkar2018).Themostimportantstandardphenotypesaregrownutilizingtraditional methods including testing for germ tubes, enzyme testing, chromogenic testing and fermentation and commercial methods, including molecular techniques like Polymerase chine reaction (PCR)(Neppelenbroek et al.2014).Reasonable specimens should be obtainedfrominfectionsitesforsuccessfulisolationandexactidentificationofthespecies *Candida spp*. and transported to the laboratory within two hours of collectability, suitable and timely sample processing and sample inoculation on the required culture medium and incubation at a suitable temperature(Shivaprakash 2015; Deorukhkar2018).

There are many type of sample used in the direct examination of *Candida* species with deferent protocols. Cerebrospinal fluid (CSF) sample selection must be conducted by

a practitioner in aseptic circumstances. The application of severe caution in fluid selection withoutanycontamination,asanyCSFisolate isknowntobeimportant.Sendingadequate volume for the every necessary test and do not fill up the containers, since it produces an aerosol upon opening. Two mL is a proposed amount for fungalculture.

Urine specimen should be collected early in the morning from the center of the voiding withanapproximateamountof5-10mL.Inthecaseofaurinarycathetercontainer,thetest must be obtained using an aseptic strategy and must be gathered in a sterile syringe and subsequentlytransferredtoasteriletestbottle.Coveredspecimens:Stockrefrigerated(4 °C) or at room temperature. Covered specimens must be kept refrigerated (4 °C). Transfer to the lab within 12 hours. Referring to the sputum, before breakfast and by using mouth rinse and wash, the sample will be collected early in the morning within sterile container. In addition, there are another types of specimens like stool, blood and pus (Shivaprakash 2015; Deorukhkar 2018).

DirectExamination

It offers a simple diagnosis of *Candida spp*. clinical samples specifically(Chander; J. 2017).A good screening procedure for liquid and mucoid clinical specimens is direct microscopicinspection.Itissimpletoproduce10percent ofthewetquantityofpotassium hydroxide(KOH)ortousedifferentstainingtechniques.Directmicroscopicanalysisrelies mainly on the form of clinical sample(Shivaprakash 2015; Deorukhkar 2018).For the visualizationofyeastcellsandotherfungalcomponents,a10percentKOHwetvolumeof preparation is useful. In the case of specimens such as skin, nail scratching, and mucosal secretions that were rough or slimy, specimens should be washed by KOH before slide preparation because by eliminating protein residues in the specimens, it enhances the demonstration of fungal species. KOH wet preparation quantity rendered on a clean slide by applying a drop of a sterile saline solution (0.85 percent NaCl), place the sample on it and blend it well then protected with slide coverslips. Explore them under the microscope at varying magnifications, at high and low brightness(Deorukhkar 2014a; Deorukhkar 2018).

Forstainedsmears,theyeaststrainsandpseudohyphaeforfirmspecimenssuchasbiopsies ormaceratedtissuearebetterdemonstrated.Gramstain,PeriodicAcid-Schiff(PAS)stain, andGrocott'sMethenamineSilver(GMS)stainaredifferentstainingstrategiesthatcanbe used (Deorukhkar 2014a; Deorukhkar 2018). Gram staining is the most effective method used in sample manifestation to be able to see the yeast cells such as: sputum, purulent discharges, gastric cleaning, lung aspirates, menstrual drainage and urine. While this type of stains used for pseudohyphae and Gram positive *Candida* species(Chander; J.2017).

Fluorescent microscopy could be used to distinguish fluorescent Candida cells in the white Calcofluor stained tissue (CFW). Furthermore, in the regular screening test of obstetrics and gynaecology, a Pap smear reveals yeast cells of *Candida spp*. The other diagnostic staining approach used for tissue parts is histopathological tests such as PAS, H&E and GMS stain(Chander; J. 2017).

Phenotypicmethods

Yeast cell could be identified by many types of pursuers such as sabouraud dextrose agar (SDA) media, chromogenic medium, corn meal agar (CMA), carbohydrate fermentation, germ tube test (Abdulla and Mustafa 2020).

Sabouraud Dextrose Agar(SDA)

Theselectiveanddifferentialmaterialisnotdescribedby SDA(Samaranayakeetal. 1987; Deorukhkar 2018). SDA is the commonest medium being used for significant Candida isolation. The *Candida* genus, as a fastidious species, grows on most laboratory media for the purpose of isolation of fungi. The consequence is Candida's rise, but not everything, because of the low pH of several bacteria (Odds 1991; Deorukhkar 2014a; Deorukhkar 2018).

SDA is used to inhibit bacterial growth and cycloheximide from penetration of saprotrophic fungus as an antibiotic aid, such as tetracycline and gentamicin (Deorukhkar 2018). Although certain *Candida* species including *C. krusei*, *C. tropicalis*, and *C. parapsilosis*consideredsaprotrophicfungusappearstobecycloheximidesusceptible.SDA

is antibiotic free for that it is now the most common media in *Candida* species (López-Martínez 2010; Deorukhkar 2014a; Deorukhkar 2018).

Candida spp. could be seen on this medium with specific properties such as: soft, creamy, convex, pasty colony and after the incubation it may appear as a ground. In addition to SDA, the *Candida spp*. can be used for isolation and separation, as can Potato Dextrose Agar, Malt Peptone Agar, Pagano-Levin Agar, and Nickerson Medium. *Candida spp*. progresses well in the basic blood agar and in the cortex, which may also differentiate *Candida spp*.(Deorukhkar 2014a).

Blood cultures have some big drawbacks; however, the existing "gold standard" form of diagnosinginvasivecandidiasisisregarded.Theprocedureusedregularlytodevelopblood is relatively cruel and can require many days to be positive. Blood cultures are negative in comparison to nearly 50 % of patients with reported invasive candidiasis(Reiss and Morrison 1993; Perfect 2013; Deorukhkar 2018).

Chromogenic medium (CHROMagar)

CHROM agar is a selective and differential medium used to promote yeast cell recognition. This media consist of chromogenic substrates the work directly with the secreted enzymes from the fungal or the microorganism which leads to change the colony color and every species of *Candida spp*. has a specific color and colony characteristics. Besides, the ability of the chromogenic medium to identify the mixed yeast sample that contains many *Candida* species in a short time (Sumitra Devi and Maheshwari 2014; Khadka et al. 2016; Tang et al. 2019; Borman et al. 2020 Jun 11).

*C.albicans*appearassmoothcoloniesoflight greencolor,*C.tropicalis*asbluetometallic blue colored elevated colonies, *C.glabrata*look like cream to white smooth colonies, as purple fluffy colonies, *C. krusei* emerges, *C. parapsilosis* occurs as colonies pale cream- colored colonies and *C. dubliniensis* looks to be a dark green colony. *Candida pelliculosa* forms pink colonies (Khadka et al. 2016; Tang et al. 2019; Abdulla and Mustafa 2020; Borman et al. 2020 Jun11).

In2010,theabilityofCHROMagartodistinguishbetween*Candida*specieswasdone by Nadeem. The findings suggested that chromogenic medium would easily classify three *Candida* species specifically *C.albicans*, *C. tropicalis* and *C. krusei*. Chromogenic agar sensitivity and specificity for each one of them were as follows: 99% *C. albicans*, 98% *C. tropicalis*,and for*C.krusei*itwas100%.Anotherstudyin2013conductedthatallCandida specieshaveadefermorphologyandcolonycoloronchromogenicagar.Dependentonthe morphology and color changes Candida species arranged as the following: (70%) for *C. albicans, (16.6%) for C. glabrata, (6.7%) for C. krusei and C. tropicalis* (6.7%)(Nadeem et al. 2010; Manikandan and Amsath 2013; Sanjeev Kumar2013).

Germ tubeformation

Germ tube examination "Reynolds-Braude phenomena" it is one of the most common laboratoryteststoclarify*Candida*speciesspecifically*C.albicans*.Germtubeexamination identified by the presence of filamentous hyper formation which is called blastoconidy. *C. albicans*and*C.dubliniensis*demonstratedasthecommonest*Candida*speciestoforming germ tube after the incubation of patient serum for 2-4 hours under specific temperature (37 °C). The development of germ tubes may be determined by different influences, such as the existence of other types of *Candida*, bacterial infection, medium design, inoculum concentration and temperature of incubation(Neppelenbroek et al. 2014).

In 2005, study was done to differentiate the ability of *C. albicans* to form germ tube and the study done by using human, rabbit, sheep, cow and goat blood serum. Rabbit or sheep sera were the most recommended as a result more than human blood. While human blood may lead cause transmission of the infectious pathogens. In 2012 another study was done between*C.dubliniensis*and*C.albicans*to discusstheabilityofthebothtoformgermtube on various media in addition tohuman plasma, trypticase soy broth, pooled human serum, horse serum, egg white and peptone water. The study results conduct that trypticase soy broth is the perfect medium to produce germ tube of each *C. albicans* and *C. dubliniensis* (J O etal.2005).

Chlamydospore formation

This procedure is less arbitrary than the germ tube examination, but more time-consuming.*C. albicans*and *C. dubliniensis*, and quite occasionally *C. tropicalis*. Chlamydospores are believed to be formed by *C. tropicalis*. In C. albicans, big, refractile-walled, terminal chlamydospores could be identified with this Dalmau plate culture technique.Whendevelopedoncornmealagarorricestarchagar.Whilenutrient-deficient media are corn meal agar and rice starch agar, other growth media have been proposed, such as sunflower seed agar, casein agar, tobacco agar, Staib agar, and recently mustard agar. In *C. albicans*chlamydospores are shaped independently at the hyphal ends, while pairs, triplets or wider clusters are sometimes attached in *C..dubliniensis*. It is possible to use morphological features and various growth trends on corn meal agar for evolution of certain medically significant Candida spp. (Chander; J.2017).

Epidemiology of Candidiasis

There is a huge range of diseases caused by Candida species. While the infection returned to Candida species called Candidiasis and these infections could be reneged as the following: Superficial, Mucocutaneous, Cutaneous, Oropharyngeal, Vulvovaginal, InvasiveandSystemicinfectionsmayhappened(Balsdon1993;Pappas2006;Ortegaet al. 2011; Jabra-Rizk et al.2016).

SuperficialCandidiasis

Superficial Candidiasis is the most widespread Candidal infection. Candida can be found in mucosa and skin as a commensal. Eyes, oropharynx, and gastrointestinal tract was the most common superficial infection(Balsdon 1993; Segal and Elad 2010; Jayatilake 2011). (2017)

Cutaneous Candidiasis

Candida species can induce many different forms of cutaneous infections like nail, hair, and skin. Where it can also cause interdigital candidiasis, diaper rash (perianal) in infants, moisture and discoloration of the skin and chronic mucocutaneous Candidiasis even skin

foldswhichisseeninfemales.Cutaneouscandidiasisdoesnotactivatetheimmunesystem since the infection is contained in the stratum corneum and it doesn't have the potential to enter the tissues of the body. *C. albicans* considered to be the commonest species that lead to cutaneous candidiasis where*C. parapsilosis* is the second one (Elias Anaissie 2009; Goetz et al. 2010; Segal and Elad 2010; Zarei MAHMOUDABADI and Izadi 2011; Jagdish Chander 2017). Onychomycosis was commonly seen in patients with cutaneous candidiasis, it was isolated from water and diabetes mellitus.In a high number of cases, onchomycosis was highly correlated with nail thickening, ridging, and discoloration(Anaissie 1992; Jayatilake et al. 2009; Segal and Elad2010).

MucocutaneousCandidiasis

Candida species are capable to cause infections in the non sterilized area of the mucosal layer such as oral candidiasis or oral thrush. This is mainly referred to as hereditary abnormalities in the immune system leading to host T-cells deficiency(Jagdish Chander 2017; Romero et al. 2017). *C. albicans* considered to be the commonest species causing mucocutaneous candidiasis. There was a study in 2013 showed the prevalence of mucocutaneouscandidiasisin*Candida*species,andresultswereasthefollowing:(76.6%) of the cases for *C. albicans*, ,*C. krusei as (6.7%)* , (5%) for*C. parapsilosis and* (11.7%) for *C. tropicalis*(Sougata 2013).

OropharyngealCandidiasis

Oropharyngeal candidiasis (OPC) contributes to mouth-and- throat infections. Where its opportunisticinfectioncausedbytheoral*Candida*speciesShiftfromharmlessmicrobiota to pathogenic *Candida spp*.leading to (OPC)(Akpan and Morgan 2002; Gerald Mandell 2009).*C. albicans* have been extracted from 80% of oral ulcers and lesions for that *C. albicans* considered to be the commonestspecies in the oral cavity (Millsop and Fazel2016).

This type of infection mostly could be seen in the elderly, denture wearing, immunocompromised and diabetic patients(Akpan and Morgan 2002). Erythematous Oropharyngeal candidiasis and Pseudomembranous candidosis are the two main types of OPC. Were erythematous OPC responsible for the Smooth red patches on the hard or soft

palate and Pseudomembranous candidosis responsible to the smooth white papular ulcers(Odds 1988; Lekshmi L 2015).

VulvovaginalCandidiasis

*C.albicans*wasthecommonest speciesthatleads tovulvovaginalcandidiasis(VVC).Where *Candida* species could be found in female vagina as harmless commensal from 10% to 25%(Achkar and Fries 2010; Segal and Elad 2010; Jagdish Chander2017).

Vulvovaginal candidiasis is a terminology referred to the symptomatic inflammation causedby*Candida*species.Around40-50percentofwomensufferfromchronicinfections and less than 5% of the adult female community has recurrent VVC attacks(Sobel 1997) . There are popular signs for VVC include curd-like pus, swelling, burning pain, sexual dysfunction,anddyspareunia.Inadditionto,insomecases,theythoughtthattheincreases intheglycogeninthefemalevaginarelatedtohighreproductivehormonesecretionwhich leads to form an appropriate environment containing carbon to enhance *Candida spp.* growth (Achkar and Fries 2010; Segal and Elad2010)

Invasive Candidiasis

Invasive candidiasis is one of the most serious candida infections with high mortality and morbidity. This kind of infection defer than all other *Candida* infections. Invasive candidiasiscauseslocalizedinfectionsinspecificpartsofthehumanorganssuchasbones, blood, heart, and eyes. Candidemia is the most widespread type of invasive candidiasis with a high mortality rate. Where *C. albicans* the most common species causing Candidemia(Nucci and Anaissie 2001; Wisplinghoff et al. 2004; Pfaller and Diekema 2007; Kullberg and Arendrup2015).

In Northern Europe and the United States, *C. glabrata* and *C. parapsilosis* was demonstrated to be the second common cause ofCandidemia(Falagas et al. 2006). Candiduria is one of the most common hospitalized infections related to *Candida* species. Candiduria is an opportunistic infection with a high mortality rate of 30% - 40%. *C. albicans* were the most commonly cause of candiduria followed by *C. glabrata* and *C.*

parapsilosis(Morgan et al. 2005; Ortega et al. 2011; Behzadi et al. 2015; Gajdács et al. 2019).

SystemicCandidiasis

Systemiccandidiasisisanopportunisticinfectionthathappenedwithhospitalizedpatients. Which is consist of many *Candida* infection and disseminated diseases (Urinary tract infections, pulmonary candidiasis, candidal meningitis, oropharyngeal and upper gastrointestinal tract infections, Endocarditis, Myocarditis, Pericarditis, candidal pneumonia, laryngeal candidiasis, symptomatic cystitis, pyelonephritis, urinary fungus balls, candida osteomyelitis, central Nervous System candidiasis and ocular candidiasis) caused by deferent *Candida* species(Lasday and Jay 1994; Kim et al. 2006; Shah et al. 2008; Falcone et al. 2009; Pappas et al. 2009; Gamaletsou et al. 2012; Pfaller and Castanheira 2016). Disseminated candidiasis happened when the patient undergoes strict antibiotic therapy, immunosuppressive treatment even catheterization, intravenous catheters, acute burns, and diabetes mellitus may cause disseminated candidiasis. Also, around (6%) Central Nervous System involvement will occur in the patients facing systemic candidiasis (Shankar et al.2016).

In2013,thestudyshowedthedistributionof*Candida*speciescausinginvasivecandidiasis inPakistan.Theresultshowsthatthemost commononeinadultswas*C.tropicalis*as38% andthesecondspecieswere*C.parapsilosis*as(17.8%),*C.glabrata*as(15.9%) where (12.3%) for*C. albicans.* In neonates, *C. tropicalis* was the commonest species with (36%) followed by *C. albicans* with (21%). In children, the most common species was *C. albicans, C. tropicalis* and *C. parapsilosis* with (31.9%), (26.4%), (19.4%) respectively(Farooqi et al.2013).

In 2013, there was another study about the incidence of Candida species with in immunodeficient patients and the species was isolated from the lower respiratory tract. The commonest isolated species was demonstrated: *C. albicans* as (80%) were *C. tropicalis, C. dubliniensis* and *C. guilliermondii* as the second common isolated species with (12.5%), (5.0%), (2.5%) respectively. Were the highest age group was between 25 and 34(Ogba et al. 2013). Referring to pulmonary tuberculosis patients caused by *Candida* species in 2013,the

prevalence of co-infection related to different *Candida* species was reported as follows: (50%) for *C. albicans* as the most common one, was (20%) for each *C. tropicalis* and *C. glabrata*(Kalietal.2013).In2012,astudywasdoneondifferenttypesof*Candida*species were taken fromdeferent clinical samples within Intensive Care Unit patients between (2009 and 2011). The study administrated that candidemia was the most clinical investigation betweenpatients.*C.tropicalis*werethemostcommonspecieswith(49.0%)andthesecond most common species was *C. albicans, C. guilliermondii ,C. glabrata*(26.6%), (13.5%),(7.8%) respectively *and C. krusei* with (3.8%) (Paswan et al.2012).

Virulence factors

Adhesins andInvasins

The first step in the infection development is adherence and it is necessary for the organism's survival in the host. Every *Candida* spp has a specific ability to adhesions. Where in *C. albicans* it have specific proteins (adhesins) that allow *C.albicans* to adhere to the living host cells(Mayer et al. 2013) In the case of adhesins, they consist of several surface proteins such as non-covalent wall- associated proteins, cell surface-associated proteases (Sap9 and Sap10), hypha-associated GPI- linked protein (Hwp1),GPI-linked proteins (Eap1, Iff4 and Ecm33), others like fibronectin and fibrinogen and agglutinin-like sequence proteins (ALS) andthe integrin- like surface protein Int1. Were ALSproteins consist of eight classes: ALS1, ALS2, ALS3 to ALS7 and ALS9(Mayer et al. 2013) (2019,2017.2). Hypha- associated adhesin (ALS3) and Ssa1 are important for adhesion processes were they allow the penetration to the host cell and tissue invasion.

Tissue colonization of *Candida spp*. happens where there are malfunctions of the host immune system, changes in natural microbial flora, or deficiencies in the structure of the host tissue. This leads to the disruption of the host outer cell envelope(Calderone and Fonzi 2001; Naglik et al. 2011; Mayer et al. 2013; Wibawa 2016).

Secretion of Extracellular HydrolyticEnzymes

After adhesion presses, *Candida spp.* can secrete hydrolytic enzymes where virulence factor is essential, which facilitates the penetration of *Candida spp*. in the host cells and

helps *Candida spp*. to adapt to the host cell environment. The hydrolytic enzymes consist ofthreemajortypesproteinases,phospholipases,hemolysins,andlipases(Silvaetal.2012; Mayer et al. 2013; Sardi et al. 2013; Pawar P.R.2014).

Proteinases

In 1965, Staib. eere first one noted the secretion of aspartyl proteinase, and since then its activity has been correlated with virulence. The importance of proteinases is the ability to hydrolyzehostsepithelialproteinandmucosalbarrier,enhancethe*Candidaspp.*adherence to the host cells and depredate host cellsimmunoglobulins(Staib 1966; Naglik et al. 2003; Tan et al.2010).

Phospholipases

Phospholipasesconsistofnumerousandbasicesterbondscleavedaccordingtotheseester bonds, phospholipase was distributed within four groups (A, B, C, and D) and there are seven phospholipase genes five of the them was explained in detail (*PLA*, *PLB1*, *PLB2*, *PLC1*, *PLC2*, *PLC3*and *PLD1*). While phospholipases returnees to group B, which cause interruption in the host membrane. Besides, the ability of phospholipases to prepare specific receptors allowing the attachment of yeast cell to host cells(Niewerth andKorting 2001;Mavoretal.2005;Khanetal.2010;Mayeret al.2013;Kalaiselvi2014;Deorukhkar and Saini 2015).

Lipase

In 1965, Werner was the first scientist who defined lipase. Lipases are made up of 10 members arranged from LIP1 to LIP10. Lipases enhance lipids breakdown from the nutrient materials, even it allows the yeast cells to adhere to the host cells and tissue, Activate non-specific immune responses by raising cell-mediated immunity and self defencemechanismagainstothernormalflora(Stehretal.2004;Gácseretal.2007;Mayer et al.2013).

Hemolysin

Hemolysin activity developed the ability of hemolysin enzyme, hemolysin enable the *Candida spp*. to pass through the host cells and to continue living in these cells. The yeast cell has the capability to transfer iron to energy. However, in human cells iron can only be seen in hemoglobin where free iron couldn't be found in the human cells. For that, they bind to blood cells and then break them down to obtain iron using hemolysin enzymes. The level of hemolysin produced depends on the species and even the *Candida* strain(Moors et al. 1992; Luo et al. 2001; Rossoni et al. 2013; Wibawa 2016).

Biofilm

One of the most significant virulence factors returned to Candida species especially *C. albicans* is the ablity to form biofilm. Biofilm is a member of microbial cells accumulate on each other onasurfacesformingabuffyextracellular matrixtosurviveonitandthesesurfacescould be living or nonliving surfaces (biotic or nonbiotic). Such as endotracheal tubes, stents, shunts, implants, pacemakers and mostly catheters(Fanning and Mitchell 2012; DeorukhkarandSaini2016).Thedevelopment ofbiofilmisacontinuouscyclewithmany steps: 1) adhesins proteins responsible about the adhesion of yeast cells to the host cells and tissue, 2) increase the cells number by cell multiplication, 3) colonization by the developmentofhyphalcells,4)aggregationanddevelopmentofextracellular matrixmaterial and the last step, 5) lyses of biofilm complex and yeast cells released(Finkel and Mitchell 2011; Silva et al. 2012; Mayer et al.2013).

TherearemanytranscriptionfactorsresponsibleforbiofilmformationsuchasBrg1,Bcr1, Tec1, Ndt80, Rob1, and Efg1(Fanning and Mitchell 2012; Nobile et al. 2012).Many processes are consider to be active in biofilm antimicrobial resistance like 1) sluggish penetration of the antifungal or an antimicrobial agent in the biofilm, 2) inappropriate microenvironment in the biofilm may lead to sluggish the growth, 3) the evidence of the existence a small amount of highly resistant yeast cells and in the last 4) reactions in the adaptive stress(Mah and O'Toole 2001; Stewart and Costerton 2001; Pugliese and Favero 2002).

C. albicans are deemedto becomethe commonest*Candida* species because of the high opportunistic ability and the good biofilm activity in a variety of substrates(Mukherjee et al. 2005). A study in 2011was showing a comparison in the biofilm formation within *C. albicans* and *C. parapasilosis*, which estimated that *C. albicans* has a very high abilitytoform biofilmmorethan*C.parapasilosis*.In2009,thestudyreflectsthatother*Candida* species (*C. parapasilosis, C. glabrata and C. tropicalis*) can form a biofilm. However, referring to the *Candida* species biofilm extracellular matrix proteins and carbohydrates components, *C. parapasilosis* contains significant quantities of carbohydrate and the protein level is low. In contract with *C. glabrata* and *C. tropicalis* biofilm content, where they have a lower level of carbohydrates and higher proteins level(Baillie and Douglas 1999; Silva et al. 2009; Villar-Vidal et al. 2011; Silva et al.2012).

Biofilm formation within the medical equipment can play an important partwithin morbidity and death rate of the patients. Were more than 45 million medical devices par year in the United States were reported with biofilm contamination and the percentage of biofilm formation in Candidemia infected patients was around 83.3%(Bouza et al. 2014).

Thebiofilmisveryresistantto commonlyusedantifungaldrugs.Inthiscase,these diseases require direct intervention to remove the yeast cells from the infected medicinal equipment, which may include surgery and large doses of antifungal (Mukherjee et al. 2005).

AntifungalResistance

Antifungal agents: agents have a therapeutic activity lead to eliminate or inhibit *Candida* infections (Kanafani and Perfect 2008; Pfaller 2012).

There is two class with five major groups related to antifungal drugs:

1) Systemic antifungal drugs consist of A) Polyenes (Amphotericin B), B) Azole derivatives (Imidazole like Miconazole, Ketoconazole) and Triazole like Itraconazole,Posaconazole, Fluconazole,Ravuconazole and Voriconazole),C) Echinocandin like Anidulafungin, Capsofungin and Micafungin.

2) Topical antifungal drugs consist of A) Azoles–Imidazole: Oxiconazole, Sulconazole, Miconazole, Econazole, Butaconazole, Bifonazole, Tiaconazol, Terconazole, Clotrimazole, Ketoconazole , Fenticonazole, Isoconazole. B) Polyene antibiotics: Amphotericin B, Nystatin,Rimocidin, Hitachimycin, Filipin, Hamycin, Pimaricin and Natamycin.In addition, Cicloporox olamine, Benzoic acid, Undecyclinic acid, Povidone iodine, Sodium thiosulphate, Triacetin, Gentian violet, Quinidochlor,Tolnaftate.

3) Systemic antifungal drugs for superficial infections consist of A) Heterocyclic benzofurans like Griseofulvin and Corticofunvi, B)Allylamine like Butenafine, Terbinafine andNaftifinetifun.

Referringtoallantifungaldrugs,thereisjustthreemajortypesusedfor*Candida*infections such as: azoles, polyenes, echinocandins and antimetabolites (Kumar et al.2016).The majority of antifungal function dependent on the ability to affect fungal cells in the host bodysuchas:Azoleagentshaveafungistatic impactthatpreventsworkingofErg11p,14-demethylase lanosterol. Which lead to poor biosynthesis of ergosterol and fungal lipid membrane and echinocandins affects on the flexibility of the fungal cell wall. Were polyenes attached to ergosterol of the cell membrane causing lethal membrane pores and thisporesleadstocelldeath.5-flucytosinedefinedashighlytoxicinhibitorofnucleicacid biosynthesis were allylamines considered as squalene epoxidase inhibiter (Yuzo and Yuri 1987; McClellan et al. 1999; Vermes et al. 2000; Douglas 2001; Laniado-Laborín and Cabrales-Vargas). However, there is some *Candida* species resistance to a group of these drugs such as: *C. glabrata* resistance to azole drugs were other studyshows the ability of *C. glabrata* to resist echinocandin, *C. krusei* and *Candida auris* both can resistance to fluconazole were *Candida auris* defined as multi drug resistance (Pfaller et al. 2012; Cho et al. 2014; Chowdhary et al. 2014; Arendrup and Patterson 2017; Pfaller et al. 2017).

C. albicans reflects as one of the most common species resistance to antifungal drugs that refers to a specific resistance mechanisms which is efflux of antifungal agents reflects in many genes *MDR*1, *CDR*1, *CDR*2 and *ERG*11P (Lanosterol 14α- demethylase) enzymethat leadtoinhibitaminoacidsaturation(Sardietal.2011).ReferringtoWHOand

Global epidemiological data, *C. albicans* nowadays was highly resistance to azoles antifungal drugs. It is very widely between 2% to 50% in Korea, Denmark and South Africa. Where in Iran, *C. albicans* antifungal drugs sensitivity was as the follow: 75% for fluconazole, 80% for ketoconazole, 67% for voriconazole and 61% for itraconazole. In 2013,studyshowedthat*C.albicans*sensitivetofluconazoleandamphotericinBas84.2% and 92.1% respectively (Mondal et al. 2013; WHO 2014; Tan et al.2015).

VITEK (bioMérieux, Inc.) and disk diffusion methods (CLSI M44A2) were demonstrated as the most common laboratory test to identify the sensitivity of *Candida* species to antifungal drugs. In addition, there is other less common laboratory test used to identify the sensitivity of *Candida* species to antifungal drugs such as Sensititre YeastOne, agar dilution methods and SensiQuattro *Candida* EU(r) (Lee et al. 2001; Lee et al. 2009; 2009; Alastruey-Izquierdo et al. 2015).

In 2014, study discussed antifungal susceptibility and the prevalence of *Candida* species isolated from different clinical samples. *C. albicans* was the most frequent species as(65.0%) and the second common species was *C. tropicalis as* (24.3%) and *C. krusei as* (10.7%).There was a high resistance percentage for antifungal drugs like fluconazole, clotrimazole,ketoconazoleandamphotericinBwith(12.62%),(7.76%),(0.97%),(0.97%) respectively and nystatin was the only resistance pattern (C. Sajjan et al. 2014).There are manystudiesconductedin2013.Thestudy,theywereworkingonthesusceptibilitypattern of antifungal taken from patients with mucocutaneous candidiasis. *C. albicans* was sensitive forfluconazole with (91.30%), for *C. tropicalis* (57.14%), for *C. parapsilosis* (50%)and for other species like *C. krusei* they are resistant forfluconazole. which voriconazole and itraconazole sensitive for all *Candida* species(Sougata Kumar Burman 2013).Thestudyin2013,describetheantifungalsusceptibilitypattern inpatients infected with *C. dubliniensis* recovered from HIV. Referring to *Candida* species, *C. dubliniensis* was the most common isolated with25.33% were 15.79% of *C. dubliniensis* resistant to fluconazole, 13.16% of *C. dubliniensis species* was resistant to clotrimazole and for ketoconazole and itraconazole10.53% species was resistant (More et al.2013).The

resistance pattern for fluconazole and nystatin was only seen in non *albicans Candida*(Amar et al. 2013).

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CHAPTER TWO

MATERIALS AND METHODS

Theresearchwasperformed intheMicrobiologyLabortheNearEast UniversityHospital in the Turkish Republic of Northern Cyprus. A total of 43 samples for the analysis were collectedbetweenSeptember2019andSeptember2020.Thesampleswerecollectedfrom different clinical specimens of admitted patients from different hospital units such as Sputum,CSF, Aspirate, Urine, Catheter, Blood, Vagen and Wound. The study procedure was accepted by the Science Evaluation Committee of the Near EastUniversity.

Phenotyping identification of Candidaspecies

SDA used to get pure colonies and it takes 24-48 hours to appear. After colonies appear, CandidacolonieswereculturedonHiCrome™*Candida*DifferentialAgarbasedonspecific colors for eachspecies.

SDA was prepared as per the manufacturer's directions as follows:

1) Suspend 65.0 grams in 1000 ml purified/distilledwater.
2) Heat to boiling to dissolve the mediumcompletely.
3) Sterilize by autoclaving at 15 lbs. pressure (121°C) for 15minutes.
4) Cool to 45-50°C. Mix well and pour into sterile Petriplates.

The media were prepared referring to the manufacturer's instructions as follows:

1) Suspend 42.72 grams in 1000 ml purified/distilledwater.

2) Heat to boiling to dissolve the medium completely with noAUTOCLAVE.
3) Cool to 45-50°C. Mix well and pour into sterile Petriplates.
4) Prepared media was kept at 4ºC and used within three days.
5) Isolates to be identified in chromogenic media were subcultured twice on SDA before inoculation.
6) A single *Candida* isolate colony was inoculated, and the plate was incubated for 48 to 72 hours at37ºC.
7) The results were conduct based on colonies color and colonies'morphology.

Biofilmformation

The formation of biofilm was done by cultivation of all *Candida* samples on Congo Red Agar (CRA). *Staphylococcus aureus* ATCC35556 strains was used as a positive control were the results appears as brownish black color on the media after incubation period for 48 hours at 37 °C(Mariana et al. 2009).

Congo Red Agar (CRA) was prepared according to the manufacturer's directions as follows:

1) Suspend 40.0 grams in 1000 ml purified/distilledwater.
2) Sterilize by autoclaving at 15 lbs. pressure (121°C) for 15minutes.
3) Adding Congo Red stain after or beforecooling
4) Cool to 45-50°C. Mix well and pour into sterile Petriplates

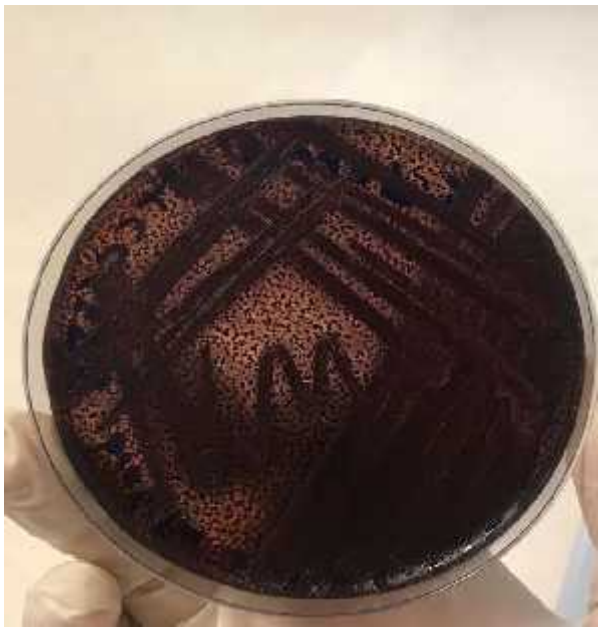

Figure 2.*Staphylococcus aureus* ATCC35556 strains positive control

Figure 3.*C. albicans* biofilm positive

Antifungal susceptibilitytest

The antifungal susceptibility of *Candida spp*. was performed using VITECK 2 system.

1) From the primary isolation medium, some colonies showing similar morphologyweretakenforpreparedirectcolonysuspensionandsuspendedby using a flamed loop or by using a sterile cotton swab, the yeast cells was taken and suspended in 4-5 ml sterile saline solution (0.85%NaCl).
2) Mix the cell suspension using a vortex mixer for 15seconds.
3) when the turbidity is visible, the suspension density is adjusted by the use of a spectrophotometer. The turbidity needs to be balanced to 0.08 to 0.10 at an absorbance of 625 nm for the 0.5 McFarlandstandard.
4) The prepared suspension was posted into a VITEK 2cassette
5) The cassette placed into the VITEK 2 instrument and the results was read automatically after 12-48h.
6) Antifungal susceptibility card test was used for every organism. While every patient have a singlecard
7) The drug concentration ranged from 0.25–16 µg/ml for AmphotericinB,1–64 µg/ml for Flusitozine and 0.125–16 µg/ml for voriconazole.

StatisticalAnalysis

Qualitativeandquantitativedatavaluesalongwiththepercentage foreverysinglesample. The Chi-square test is tested as appropriate on the association between two or more variables. Pictorial explanations of the major results of the study were rendered using an appropriate statistical graph. A $P<0.05$ was deemed significant. All statistical analyses were done using statistical packages SPSS version 25.0 (SPSS Inc. Chicago, IL,USA).

Ethics Committee

Ethical approval of the study was taken from Near East Ethical Committee with the permission number NEU/2020/7-989 Since our study was retrospective, informed consent form was not used.

CHAPTER THREE

4. Results

In this study, 43 Samples was obtained from various body locations. The following figure 2showsthaturinesampleswerethemajorsamplefollowedbysputumandaspirate.Thefollowing Table 1 reflects the percentage of the collected samples. Urine samples (n=13, 30.2%), sputum samples (n=10, 23.3%), aspirate samples (n=10, 23.3%), blood (n=5, 11.6%), CSF samples (n=2, 4.7%) and vagen sample (n=1, 2.3%), wound sample (n=1, 2.3%) and catheter samples (n=1, 2.3%) respectively(Table1).

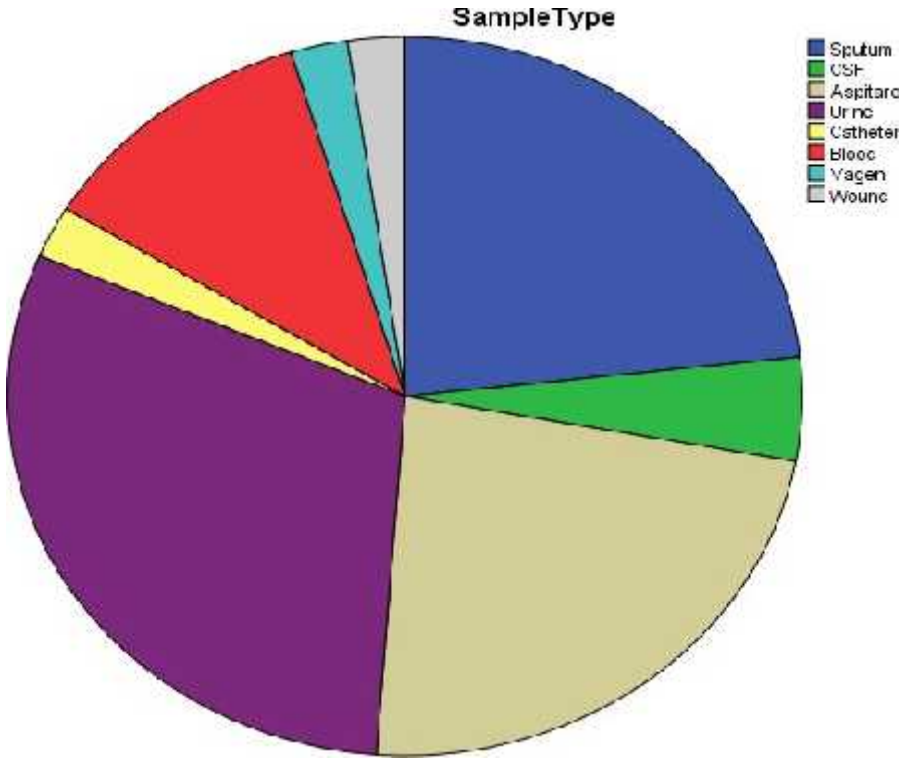

Figure 4. The distribution of the specimens obtained from various body locations.

Table 1. The distribution of the specimens obtained from various body locations.

Sample type	Number	Percentage
Urine	13	30.2
Sputum	10	23.3
Aspirate	10	23.3
Blood	5	11.6
CSF	2	4.7
Vagen	1	2.3
Wound	1	2.3
Catheter	1	2.3

Referring to biofilm formation, most of *Candida spp*. were able to produce biofilm(n=37, 86%)while(n=6,14%)couldnot(Table2).*C.krusei*and*C.albicans*werethemostspecies producing biofilm (n=11, 29%) and (n=10, 27%) respectively; followed by *C. tropicalis* with (n=9, 24.3%) and *C. glabrata* (n=7, 18.9%) (Table3).

Table 2. The total biofilm formation in *Candida* species.

Biofilm formation	Number	Percentage
Positive	37	86
Negative	6	14

Table 3. Biofilm formation distributed in *Candida* species.

Candida species	Number	Percentage
Candida krusei	11	29
Candida glabrata	7	18.9
Candida tropicalis	9	24.3
Candida albicans	10	27

Flusitozine was the most sensitive antifungal within *Candida* species with (n=41, 95.3%) and the second most sensitive antifungal was AmphotericinB (n=40, 93%) followed by Vorikonazole. Vorikonazole showes low sensitivity patterns within *Candida* species (n=20, 90.9%). (Table 4).

Antifungal resistance were showed in Table 5.

Table 4. Antifungal sensitivity patterns distributed in *Candida* species.

Antifungal sensitivity patterns	Number	Percent
AmphotericinB	40	93
Flusitozine	41	95.3
Vorikonazole	20	90.9

Table 5. Antifungal resistance patterns distributed in *Candida* species

Antifungal resistance patterns	Number	Percentage
AmphotericinB	3	7
Flusitozine	2	4.7
Vorikonazole	2	9.1

- **According to statistical analysis;**

✓ No signifigance difference between biofilm and *Candida* species(p=0.083)

✓ No signifigance difference between biofilm and Amphotericin B resistance(p=0.630)

✓ No signifigance difference between biofilm and flusitozine(p=0.738)

✓ No signifigance diference between biofilm and vorikonazole (p= 0.340)

CHAPTER FOUR

4. Discussion

Candida species infections have risen and become increasingly complex to control due to increasedimmunogenicdisorders,abuseofbroad-spectrumantibiotics,increasedusageof immunosuppressive medications, starvation, endocrine disorders, widespread use of medical equipment, ageing and an rise in the number of patients (Silva et al. 2017). In addition, the whole development of infectious diseases has experienced major improvements during the lasttwo decades (Deorukhkar 2014b). Fungi previously considered non-pathogenic or less virulent are now recognized as a major cause of morbidity and mortality in immunocompromised and critically ill patients (Deorukhkar 2014a).

C. albicans and non-albicans Candida (NAC) populations have been strongly associated with several opportunistic fungal infections (Martins et al. 2015). In the past decades, 92–95% of all cases of *Candida* infection have been induced by the five most widespread agents, *C. albicans* and non-albican Candida (NAC) species *C. glabrata, C. parapsilosis, C. krusei, and C. tropicalis*(Pappas et al. 2015; Berkow and Lockhart 2017).

Many researches around the world have shown a rise in the incidence of *Candida* infections. The sixth to tenth causative agent of nosocomial Blood Stream infections is recognised in Europe for Candida species. In the USA, Candida species is reported as the 2ndcausativeagentforcatheter-associatedurinarytractinfections,the3rdcausativeagent for bloodstream infections in patients with intensive care unit and catheter-associated bloodstream infections, and the 4th causative agent for health-associated bloodstream infections

*Candida*speciesisolationinurinesamplesgenerallyputsaclinicianandmicrobiologistin a conundrum about whether the candiduria symbolizes just *Candida* colonization or infectionofthelowerorupperurinarytract,aswellasascendingpyelonephritisandsepsis infectionsoftherenal*Candida*(Bukhary2008).Inourstudy(n=13,30.2%)ofthecollected specimens were urinesamples.

Inarecentstudy,thepercentageof*C.albicans*isolatedfromtheurinesamplewas(23.7%), whereas 76.3% of isolated *Candida* species were belonged to NAC species (Deorukhkar 2018). As per Fisher's study in 2011, >50% of *Candida* isolates from the urine sample belong to NAC species (Fisher 2011). In our study, *C.krusi*was the commonest species with12samples,27.9%followed*byC.glabrata*,*C.tropicalis*and*C.albicans*with(n=11, 25.6%), (n=10, 23.3%) and (n=10, 23.3 %(respectively. Candidiasis infections causedby *C. tropicalis* have significantly increased on a global scale, proclaiming this organism as emerging pathogenic yeast. Among NAC, *C. tropicalis* was the predominant species in various recent studies. Pahwa*et al.* (2014) reported this observation with (38.7%) of NAC isolateswere*C.tropicalis*(Nirkhiwaleetal. 2014).Inthisstudy,therateofisolationof*C. glabrata* among urine samples was 18.4%. Sobel *et al.* (2000) and Deorukhkar (2018) reportedasimilarpercentage(20%)of*C.glabrata*amongUTIintheirresearches.Whereas deFreitas*etal.*(2014)reported*C.glabrata*in61.3%ofnosocomialcandiduria(deFreitas *etal.*,2014).Thedistributionof*Candida*speciesisolatedfromurinespecimenswasseenin many studies. First study indicate that *C. tropicalis* was the most common isolated species(24.2%)followedwith(23.7%)for*C.albicans*,(20%)for*C.glabrata*,*(*19.6%)for *C. krusei* (Deorukhkar, 2018). Second study *C. albicans* was the most dominant specie with (44%) followed by *C. glabrata, C. krusei and C. tropicalis with (*26%), (8%) and (11%) respectively. Other study in 2013 shows that *C.tropicalis*themost common specie inthisstudywith*(*45.4%)followedby*C.albicans*,*C.glabrata*and*C.kruseiwith(*30.3%), (9.1%) and (15%) respectively. Study in 2012 *C. albicans* was the dominant specie with (53.3%)followedby*C.glabrata*,*C.kruseiandC.tropicaliswith(*24.4%),(2.2%),(3.7%) respectively.Anotherstudyin2012*C.albicans*wasalsothecommonestspeciewith(44%) followed by *C. glabrata, C. krusei and C. tropicalis with (*18%), (6%) and (20%) respectively.Laststudyindicateshows*C.albicans*,*C.glabrata*,*C.kruseiC.tropicalis*

*with (*35.6%), (8.9%), (6.7%), (22.2%) respectively (Deorukhkar, 2018; Kobayashi *et al.*, 2004; Ozhak-Baysan *et al.*, 2012; Paul *et al.*, 2004; R. *et al.*, 2013; Zarei-Mahmoudabadi *et al.*, 2012).

The distribution of *Candida species isolated from* sputum specimens was seen in many studies. First study indicate that *C. albican was the most common isolated species* (26.7%) followed with (26%) for *C. glabrata, (*5.7%) for *C. krusei and (*11.5%) for *C. tropicalis* (Deorukhkar, 2018). Second study *C. albicans* was the most dominant specie with (89.3%) followed by *C. glabrata, C. krusei and C. tropicalis with (*0%), (0%) and (10.7%) respectively. Other study in 2013 shows that *C. albicans*the most common specie in this study with *(*46%) followed by *C.glabrata, C. krusei and C. tropicalis* with (25%), (10%) and (7%) respectively. Study in 2012 *C. albicans* was the dominant specie with (87%) followed by *C. glabrata and C. tropicalis* with (3.6%) respectively. other study in 2010,*C. albicans* was also the commonest specie with (74.4%) followed by *C. glabrata, C. krusei and C. tropicalis with (*3.9%), (3.2%) and (7.1%) respectively. In last study *C. albicans, C. glabrata and C. tropicalis with (*78%), (4%), (6%) and (12%) respectively (Agwu *et al.*, 2012; Baradkar & Karyakarte, 1999; Deorukhkar, 2018; Kliemann *et al.*, 2008; Luque *et al.*, 2009; Mane *et al.*, 2010; Mulu *et al.*, 2013).

Study in 2018 was done to indicate the ability of *Candida* species to produce biofilm and show antifungal susceptibility patterns. *C. albicans* (45.5%) was found to be the most prevalent species, accompanied by *C. tropicalis* (28.88%), *C. krusei* (20 %), *C. glabrata*(3.33%),and*C.parapsilosis*(2.22%).ThespeciesofCandidawasextractedfrom urine (43%), BAL / sputum (18.88%), high vaginal swab (8.88%), suction tips (7.77%), blood and wound swabs (6.66%), fluid (3.33%), bile aspirate (2.22%), and deep tissue (1.11%). A greater percentage of females were infected than males, and candidiasis was more vulnerable in the age range of 51 to 60 years. A larger percentage of biofilm isolates of *C. albicans* were produced, followed by *C. parapsilosis, C. tropicalis*, and *C. krusei*. However, *C. glabrata* showed no biofilm development. Amphotericin B was 100% susceptible to all Candida isolates. The next successful medication of 81.11 %resistance wasvoriconazole.24.44%ofthestrainswerefluconazoleimmune(MarakandDhanashree 2018)

Candida susceptibility to antifungal agents can have significant consequences on infection control (Sanglard & Chances, 2002). A mixture of factors linked to the host, an antifungalagent,orthecausativeagentitselfisresponsibleforthistolerance(JohnH.Rex et al., 2001). There is little or limited patient-to-patient spread of resistant isolates since candidiasis isnotaviralinfection.ResistancecannotbespreadbyplasmidsintheCandida genus, unlike bacterial infections. Therefore, tolerance frequently develops in Candida isolate after exposure to an antifungal drugs (Arendrup,2013).

According to the recommendations issued by the Infectious Diseases Society of America (IDSA), antifungal resistance research is effective for both *C. albicans* and NAC organisms. Susceptibility testing for *C. albicans* is appropriate in patients with active candidemia or other forms of disseminated candidiasis, while susceptibility testing with NAC species is extremely relevant in patients who have obtained a prophylactic or therapeutic dose of any azole-class antifungal medication (Pappas *et al.*, 2015).M27-A3 for macrobroth and microbroth dilution and M44-A for diskdiffusion resistance monitoring of yeast and yeast-like fungi (J. H. Rex et al., 2008; Sheehan et al., 2004) have been certified by the Clinical and Laboratory Quality Institute (CLSI) Although the CLSI comparison broth microdilution approach is theoretically difficult and time-consuming, alternateapproachessuchasdiscdiffusionandE-testingarenowwidelyfollowedbymany resource-limited hospital labs (John H. Rex *et al.*,2001).

In this research, to assess the antifungal resistance profile of *Candida* isolates, the disc diffusion technique using Mueller-Hinton agar supplemented with 2 percent glucose and 0.5 µg / mL methylene blue dye (MH-GMB) was implemented. The Agar Disc Diffusion System was developed by Meiset al. (2002), and it was further enhanced by Barry et al. (2002), (M. A. Pfaller et al., 2004). The disc diffusion technique is convenient and economicalcomparedtotheCLSIgenericmicrobrothdilutionmethod.Themethodofdisc diffusion and the routine Kirby-Bauer method for measuring bacterial susceptibility are identical, so it is simple to use in regular laboratories with a strong daily (John H. Rex *et al.*,2001).

Oxman*etal.*(2010)andDeorukhkar(2018)reportedtheresistancetofluconazolewasseen in 34.6% of *Candida* isolates. Resistance to fluconazole was also observed in species considered innately susceptible to fluconazole such as *C. albicans* and *C. tropicalis*. Fluconazole resistance was reported in various studies as well. It was seen in 37.5% of *Candida* isolates in Gupta *et al.* (2001) study, 11.7% of *Candida* isolates in Xess*et al.* (2007)study,36%of*Candida*isolatesinKothari*etal.*(2009)study,in30.8%of*Candida* isolatesinGiri*etal.*(2013)study,in5.4%ofCandidaisolatesinKirazandOz(2011)study, in 2.5% of *Candida* isolates in Fleck *et al.* (2007) study, and in 1.9% of *Candida* isolates in yang *et al.* (2006). Warning that clear species recognition might not be adequate to predictCandidaspeciessusceptibilitytrendsforfluconazole(Oxmanetal.,2010).In2002, Krcmery and Barnes reported that during fluconazole therapy and prophylaxis, more than 20 percent of Candida organisms establish tolerance (Krcmery & Barnes, 2002). Vanden Bosscheetal.(1992)andSanglardet al.(1999) havewarnedthat bothinnateandacquired toleranceis likelytobestrong,particularlyinplaceswheretheuseoffluconazoleisstrong (Bossche *et al.*, 1992; Sanglard *et al.*, 1999).In this analysis, 92.5% of Candida isolates demonstrated tolerance to voriconazole. In the NAC genus, voriconazole tolerance was strongest in *C. tropicalis* (100%). Whereas Deorukhkar (2018) recorded that 5.3% of Candida isolates and 7.4% of *C. tropicalis* among NAC species were immune to voriconazole (Deorukhkar, 2018). Negri *et al.* (2012) reported the rates of voriconazole resistance in *C. tropicalis* isolates from Latin America, Europe, and North America were 1.7%, 3.6%, and 2%, respectively (Negri *et al.*, 2012).

In 2019, study was done to determine Antifungal resistance pattern using broth dilution which is includes amphotericin B, 5-flucytosine, fluconazole, itraconazole,voriconazole, posaconazole, anidulafungin, micafungin, and caspofungin. *C. albicans* was the commonest specie in this study with (65%) accompanied by *C. glabrata* and *C. parapsilosis* with (19% and 10%). In elderly patients *C. glabrata* was the most frequent, wereinyoungchildren*C.parapsilosis*thecommonest.Resistencepatternwassemistobe low within Candida species. In contrast, *C. glabrata* was the less sensitive to fluconazole (Lindberg et al.2019)

In 2017, study was done to determine antifungal susceptibility pattern of *non- albicans* species in cancer patients. *C. tropicalis* was the most frequent specie with (41.9%) with higherresistancepatternoverallwhichisresistancetofluconazolewith(13.9%)(Wuetal. 2017).In2016,studyconductsantifungalsusceptibilitypatternofCandidaspeciesisolated from vagina.*C. albicans* was the commonest specie with (88.2%) followed by *C. glabrata* and *C. kafyr* with (8.8%) and (2.9%) respectively. Caspofungin was only resisted by one isolated *C. albicans*. Whereas, susceptible to fluconazole at MIC range 1-0.25 µg/ml. Clotrimazole considered as the best vaginal drugs that inhibit (88.2%) of *C. albicans*(Rezaei-Matehkolaei et al.2016).

CONCLUSION

Phenotypic identification by specific *Candida* media allowed accurate identification of speciesandthisprocedurewaschosentobeareliableandlowcosteffectivetechniquebut this procedure takes time. Biofilm formation in *Candida spp*. gave them some resistance patterns. Whereas, most of *Candida spp*. in this study were able to Form biofilm.Phenotypic identified *Candida spp*. was confirmed by Chromogenic medium whichisgivingaspecificcolorforeachspecies.WeproposedVITEC2systemstoidentify antifungal susceptibility which is more sensitive and specificmethod.

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Appendix-1

EK-1048-2020

YAKIN DOĞU ÜNİVERSİTESİ
BİLİMSEL ARAŞTIRMALAR ETİK KURULU

ARAŞTIRMA PROJESİ DEĞERLENDİRME RAPORU

Toplantı Tarihi : 23.01.2020
Toplantı No : 2020/76
Proje No :989

Yakın Doğu Üniversitesi Tıp Fakültesi öğretim üyelerinden Doç. Dr. Meryem Güvenir'in sorumlu araştırmacısı olduğu, YDU/2020/76-989 proje numaralı ve **"Çeşitli Klinik Örneklerden İzole Edilen Candida İzolatlarında Biyofilm Oluşumu ve Antifungal Duyarlılıklarının Belirlenmesi"** başlıklı proje önerisi kurulumuzca değerlendirilmiş olup, etik olarak uygun bulunmuştur.

1. Prof. Dr. Rüştü Onur (BAŞKAN)
2. Prof. Dr. Nerin Bahçeciler Önder (ÜYE) KATILMADI
3. Prof. Dr. Tamer Yılmaz (ÜYE) KATILMADI
4. Prof. Dr. Şahan Saygı (ÜYE)
5. Prof. Dr. Şanda Çalı (ÜYE)
6. Prof. Dr. Nedim Çakır (ÜYE)
7. Prof. Dr. Nurhan Bayraktar (ÜYE)
8. Doç. Dr. Nilüfer Galip Çelik (ÜYE) KATILMADI
9. Doç. Dr. Emil Mammadov (ÜYE)
10. Doç. Dr. Mehtap Tınazlı (ÜYE) KATILMADI