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APPLICATION OF MOLECULAR TECHNIQUES TO STUDY THE ETIOLOGY AND EPIDEMIOLOGY OF *CANDIDA ssp.* INFECTIONS IN CENTRAL VIETNAM

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ATTESTATION OF AUTHORSHIP

I hereby declare that this submission is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person except that which appears in the citations and acknowledgements. Nor does it contain material, which to a substantial extent I have submitted for the qualification for any other degree of another university or other institution of higher learning.

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"No one who achieves success does so without the help of others"

Alfred North Whitehead

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LIST OF ABBREVIATION

AAT1a	Aspartate aminotransferase
ACC1	Acetyl-coenzyme A carboxylase
ADP1	ATP-dependent permease
AIDS	Acquired immunodeficiency syndrome
ALS	Agglutinin like sequence
BFIs	Bacteria fungi interactions
BMD	Broth microdilution
BSI	Blood stream infection
C	Clade
C. albicans	*Candida albicans*
C. non albicans	*Candida non albicans*
Candida spp.	*Candida* species
CC	Cluster Clade
CDC	Centers for Disease Control and Prevention
CDR	*Candida* drug resistance
CLSI	Clinical and Laboratory Standards Institute
DSTs	Diploide sequence typing
EDTA	Ethylenediaminetetraacetic acid
EPA	Epithelial adhesin
ERGs	Ergosterol biosynthetic
EUCAST	European Committee on Antimicrobial Susceptibility Testing
HC Hospital	Hue Central Hospital
H. pylori	*Helicobacter pylori*
HIV	Human Immunodeficiency Virus
HUMP	Hue University of Medicine and Pharmacy
HWP	Hyphae wall protein
ICU	Intensive Care Unit

ITS	Internal transcribed spacer
LOH	Loss of heterozygosity
MALDI - TOF MS	Matrix assisted laser desortion ionization time-of- flight mass spectrometry
MDR	Multidrug resistance
MIC	Minimum inhibitory concentration
MLST	Multi Locus Sequence Typing
MP11b	Mannose phosphate isomerase
PCR	Polymera chain reaction
PFGE	Pulsed field gel electrophoresis
RAPD	Random amplified fragment length polymorphism
RFLP	Restriction fragment length polymorphism
rRNA	Ribosom RNA
SDA	Sabouraud dextrose agar
SYA1	Alanyl-RNA synthetase
SVP13	Vacuolar protein sorting protein
TAE	Tris-acetate -EDTA
UK	United Kingdom
USA	United States of America
UV	Ultraviolet
ZWF	Glucose-6-phosphate dehydrogenase

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ABSTRACT

The overall objective of this study was to apply a variety of molecular techniques to determine the etiology and epidemiology of *Candida spp.* infections in Central Vietnam.

MALDI-TOF Mass Spectrometry correctly identified 94.9% of 196 *Candida spp.* isolated strains. The remaining unidentified species (5.1%) were detected by ITS gene amplification and sequencing. A variety of *Candida* species were isolated, with the most common species being *C. albicans* (47.96%), followed by *C. tropicalis* (16.33%), *C. parapsilosis* (10.71%), *C. glabrata* (8.67%), *C. orthopsilopsis* (5.61%), *C . krusei* (3.57%) and others species.

Antibiotic susceptibility showed higher rates of resistance to fluconazole in *C. tropicalis* (56.67%). Two specific missense mutations in the ERG11 protein (Y132F and S154F) were detected from 26.67% of fluconazole resistant *C. tropicalis* isolates.

96.81% of *C. albicans* isolates carried all three virulence genes (*als1, hwp1, sap4*) regardless of the sample source, that was concordant with a higher frequency of *C. albicans* in mucosal candidiasis compared to a higher frequency of C. *non albicans* in candida colonizations.

A total of 12 *C. albicans* diploid sequence types (DSTs) were identified from ten different wards by Multi Locus Sequence Typing (MLST). Of the 12 identified DSTs, six were new DSTs assigned by this study. Based on *C. albicans* cluster analysis, 66.67% of the isolates clustered with previously known clades in global or Asian data, and 33.33% isolates were singleton. MLST results suggested a potential nosocomial transmision of *C. albicans* since the same DTS clones were found in different wards.

Amplification of specific *H. pylori* gene (*ureA*) was applied to find a presence of this bacteria in *Candida* cells. Positivity was detected in 15.27% of *Candida spp.* (*C. albicans, C. tropicalis, C. orthopsilosis*) isolated from oral mucosa, sputum, vagina, and gastric fluid.

1. INTRODUCTION

1.1. *Candida* species

1.1.1. History

The original name of *Candida* comes from the Latin term “candidus”, meaning “glowing white,” which relates to the creamy and glistening white characteristic of yeasts colonies on culture media. This fungi is responsible for oral thrush, an infection that has been recognized for over 2000 years [1]. *Candida* infection, or ‘candidiasis’, is also known as ‘candidosis’ or ‘moniliasis’.

Candida albicans (*C. albicans*) was identified in the nineteenth century from three independent sources. First, in 1841, Fredrick Berg, a Swedish medical pratitioner, discovered that thrush was caused by fungus with filaments that dispersed into epithelial cells. Then in 1842, David Gruby, a medical practitioner in Paris, fully described the cells of thrush fungus and compared to that causing tinea. Thrush fungus was later named in 1853 as *Oidium albicans* by Charles Phillipe Robin. In 1868, Charles Quinquaud renamed the fungus to *Syringospora robinii*. The name was changed again to *Monilia albicans* in 1890 by Wilhelm Zopf. Around this time, detailed drawings of the fungus revealed the following characteristics: budding cell, pseudohyphae, hyphae, and dimorphism. In 1923, Christine Berkhout, a Dutch mycologist, changed the name of the fungus to *Candida albicans* [2, 3].

In addition to infecting the oral cavatities, *Candida* infects other parts of the body, including oesophageal, vaginal, cerebral, and intestinal lesions, etc. [2].

1.1.2. Taxonomy and mycology

The heterogeneous genus *Candida* belongs to the Kingdom: *Fungi*, Phylum: *Ascomycota*, Subphylum: *Ascomycotina*, Class: *Ascomycetes*, Order: *Saccharomycetales*, Family: *Saccharomycetaceae*, Genus: *Candida*. The genus contains approximately 200 species [4]

The taxonomy of the genus *Candida* is increasing overtime because of the reclassification of certain species (e.g., *Torulopsis glabrata* has been correctly identified as *C. glabrata*), and the discovery of new species such as *C. dubliniensis*, *C. orthopsilosis*, and *C. metapsilosis*. *C. orthopsilosis*, and *C. metapsilosis* were previously classified as part of the *C. parapsilosis* complex [5]. Futhemore, some species have been classified under *C. albicans*, including *C. claussenii* and *C. langeronii*. Recently, some new *Candida* species were distinguished from *C. albicans*, such as *C. albicans var. africana*, a new sucrose-negative variant of *C. albicans* that is closely related to *C. stellatoidea* type II [6].

More than 200 species of *Candida* have been described, most of which exist as saprophytes orgnisms. Half of the described species can not grow at 37^0C, making them unsuccesful as human pathogens. Approximately 30 species can infect humans. *C. albicans* is the most prevalent species, followed by other pathogenic species which include *C. glabrata, C. tropicalis, C. parapsilosis, C. krusei, C. guilliermondii, C. lusitaniae, C. kefyr, C. inconspicua, C. famata, C. rugosa, C. dubliniensis, C. norvegensis, C. lipolytica, C. sake, C. pelliculosa, C. apicola, C. zeylanoides, C. valida, C. intermedia, C. pulcherrima, C. haemulonii, C. stellatoidea, C. utilis, C. humicola, C. lambica, C. ciferrii, C. colliculosa, C. holmii, C. marina, and C. sphaerica* ect [7].

C. albicans, C. glabrata, C. parapsilosis, C. tropicalis and C. krusei account for 90-92% of all cases of candidiasis [8, 9]. The *Candida* pathogenic species list is expanding rapidly [7].

1.1.3. Morphogenesis

Genus *Candida* constitute a heterogeneous group of eukaryotic, dimorphic, or polymorphic organisms [10, 11]. All *Candida* species grow as yeast cells or blastoconidia under general culture conditions between 25^0C and 35^0C, with growth augmented by increased sugar or fat content in the media. Yeast cells are approximately 2-10µm in the largest dimension, round to oval, and reproduce by budding. *C. albicans* is among the larger yeast at 4-6 x 6-10µm, whereas *C.*

glabrata and *C. parapsilosis* are among the smallest at 1-4 x 2-9µm and 2-4 x 2-9µm, respectively [5]. They multiply principally by the production of blastoconidia (buds). As normally, blastoconidia of *Candida spp.* in microbiota gut of being human or animal exits in round, oval shape, but they could have different morphogenesis depending on the species [10, 12]. When blastoconidia are produced from one another in a linear fashion without separating, a structure termed a pseudohypha is formed. Under certain circumstances, such as growth under reduced oxygen tension, some yeasts may produce true hyphae. Most members of the genus produce filamentous forms (pseudohyphae or true hyphae). *C. parapsilosis* forms pseudohyphae but not true hyphae. *C. dubliniensis*, *C.tropicalis* and *C. albicans* form true hyphae [5, 12]. The presence of budding yeasts, pseudohyphae or hyphae in infected tissue are usually indicative of candidiasis [13, 14]. *C. glabrata* is the only pathogenic species that does not produce filamentous forms, existing exclusively as blastoconidia [12].

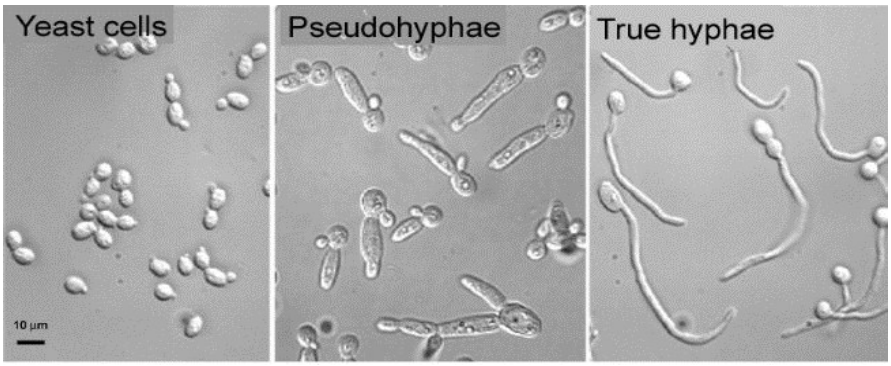

Figure 1.1. *Candida spp.* morphology

(www.tcd.ie/Biology_Teaching_Centre/assets/pdf/by2205/by2205-webgalleries2011/by2205-gallery1/candida.pdf)

1.1.4. Cell biology and enzymology

Candida spp. growth characteristics, metabolic features and enzymology characteristics are the same to those of eukaryotes and especially similar to *Saccharomyces cerevisiae* [10].

Polysaccharides are an essential compound in the cell walls of *Candida* species [10, 15]. *Candida* cell walls are composed of mannans, glucans and a small amount of chitin (Figure1.2) [10, 16, 17]. These components are closely bound to polypeptides and proteins found on the cell membrane [16]. Three types of adhesion molecules were observed [18]: (1) glycoproteins which are expressed specifically on the surface of hyphae form, (2) the protein moiety of glycoproteins which binds to host glycosides containing fucose or N-acetyl glucosamine, and (3) the polysaccharide portion of a mannoprotein. Furthermore, the structure of mannan polysaccharides found on the walls of *Candida* plays a potent role in its pathogenicity [10, 19]. *C. albicans* mannan masking of glucan and yeast hypha morphogenesis are required for disruption of host processes that function to inactivate pathogens, leading to survival and escape of this fungal pathogen from within host phagocytes [20].

Phospholipids and sterols are dominant in lipids structure of *Candida spp.* Ergosterol is the major membrane sterol. These lipids provide the site of action for the synthesis of enzymes involved in cell wall morphogenesis and antifungal action. Lipid alterations can occur during a yeast to mycelium transition [21]. Additionally, *Candida spp.* are constantly changing the structure of enzymically active proteins such as enolase and N-acetyl glucosaminidase, ubiquitin like epitopes and a protein related to the heat shock protein family (hsp70 and hsp 90) [10, 11].

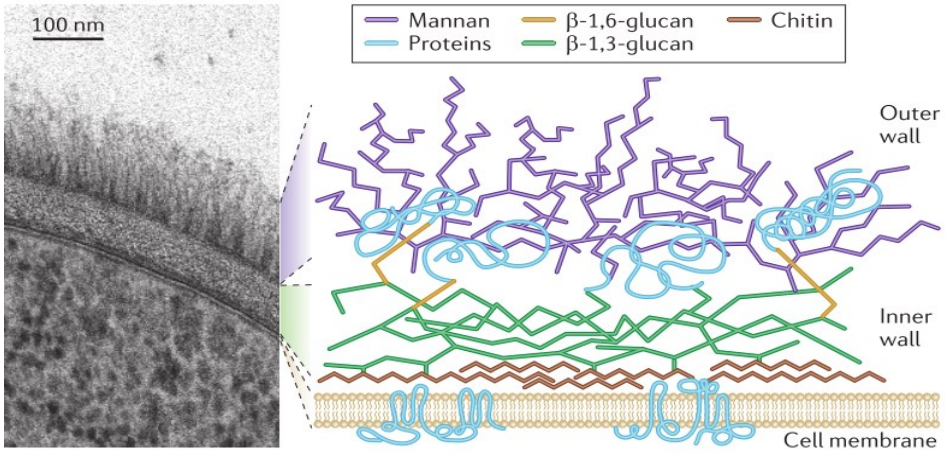

Figure 1.2. Structure of the *C. albicans* cell wall [19].

Candida spp. can grow in wide a pH range, from below 2.0 to nearly 10 [22], and under microaerophilic and even anaerobic conditions as well as the more normal aerobic atmospheres of incubation. Glucose, galactose and sucrose are all substrates for growth of the fungus, and nitrogen requirements can be met by relatively low concentrations of ammonium ions.

Many enzymes of *C. albicans* and *C. non albicans* have been characterized. Secreted aspartyl proteinases (SAP), one of the most studied enzymes, produce by *C. albicans*, *C. parapsilosis*, *C. tropicalis*, *C. dubliniensis*, *C. guilliermondii*, *C. kefyr*, *C. lusitaniae*, and *C. krusei* [23-25]. These enzymes produce non specific proteolysis of host proteins involved to defend against infection. Their different profiles of pH dependent irreversible denaturation may partially explain differences in virulence of *Candida* species [25]. The *sap* gene family in *C. albicans* includes at least 10 isoenzymes which are referred to as SAP1 through SAP10 [25]. Different SAPs are associated with a different location within the yeasts and different pathogenocity [25]. SAP1-3 expression is dominant in mucosal and cutaneous candidosis, whereas SAP 4-6 may be important for systemic disease [10, 25] and could induce apoptosis of epithelial cells by a novel Trojan horse mechanism [26].

C. albicans, *C. dubliniensis*, *C. glabrata*, *C. krusei*, *C. lusitaniae*, *C. parapsilosis*, and *C. tropicalis* also produce phospholipases [10, 27]. These enzymes play an important role in controlling of yeast growth, remodeling of fungal cell membranes and spreading in host tissues through hydrolysis of phospholipids [28]. Phospholipase B is important in *C. albicans* virulence, and it is secreted by the yeast during the infection process [28, 29].

1.1.5. *Candida spp.* from gut commensal to pathogen

Candida species are human commensals [30], commonly found on the mucosal surfaces of gastrointestinal and genitourinary tracts, skin, and under fingernails, and belong to human mycobiota (Figure 1.3) [5, 10, 31]. Moreover, *C. albicans* is also isolated from various sources, such as the atmosphere, fresh water, sea water and soil [10].

The prevalence of *Candida* colonization varies depending on site and population sampled, and sampling method. It is estimated that between 25-40 % of people are colonized by *C. albicans* at any given point [11], this rate is approximately 6% (2-37%) among healthy person for oral *Candida* colonization and approximately 47% (13-76%) in hospitalized patients [10]. These figures could be higher in patient having high risks factors. For example, oral carriage rates may be higher in certain situations such as in HIV infected patients with low CD4 counts, denture users with denture stomatitis, diabetic patients, patients receiving antineoplastic chemotherapy and children [10]. It is believed that 100% of humans may carry one or more *Candida* species in the gut from the duodenum to the colon. The numbers of yeasts carried at any point in the gut can increase to levels that may become detectable in the mouth and feces in illness or other situations where the host's microbial suppression mechanisms become reduced.

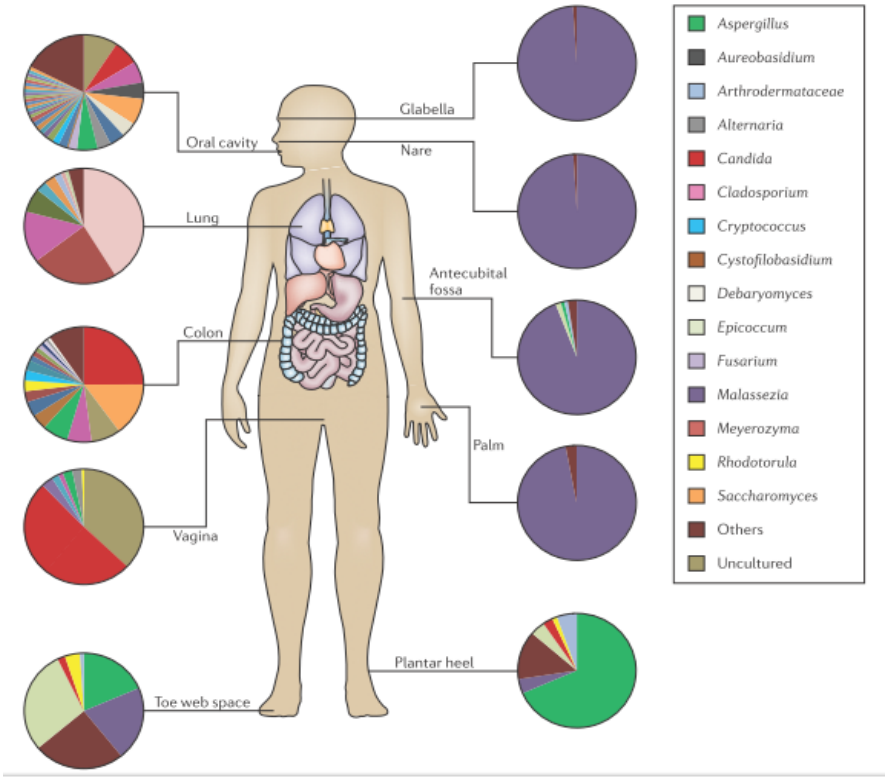

Figure 1.3. The human mycobiota [31]

Candida spp. become pathogens in situations where the host's resistance to infection is lowered locally or systemically [11, 32]. As opportunistic pathogens,

they can damage local mucosal epithelium and sometimes, systemic infections in which they can spread to all major organs and colonize in these organs. There are several steps in tissue invasion by *C. albicans*: (1) adhesion to the epithelium; (2) epithelial penetration and invasion by hyphae; (3) vascular dissemination, which involves hyphal penetration of blood vessels and seeding of yeast cells into the bloodstream; and (4) endothelial colonization and penetration during disseminated diseases [19]. This process is shown in Figure 1.4.

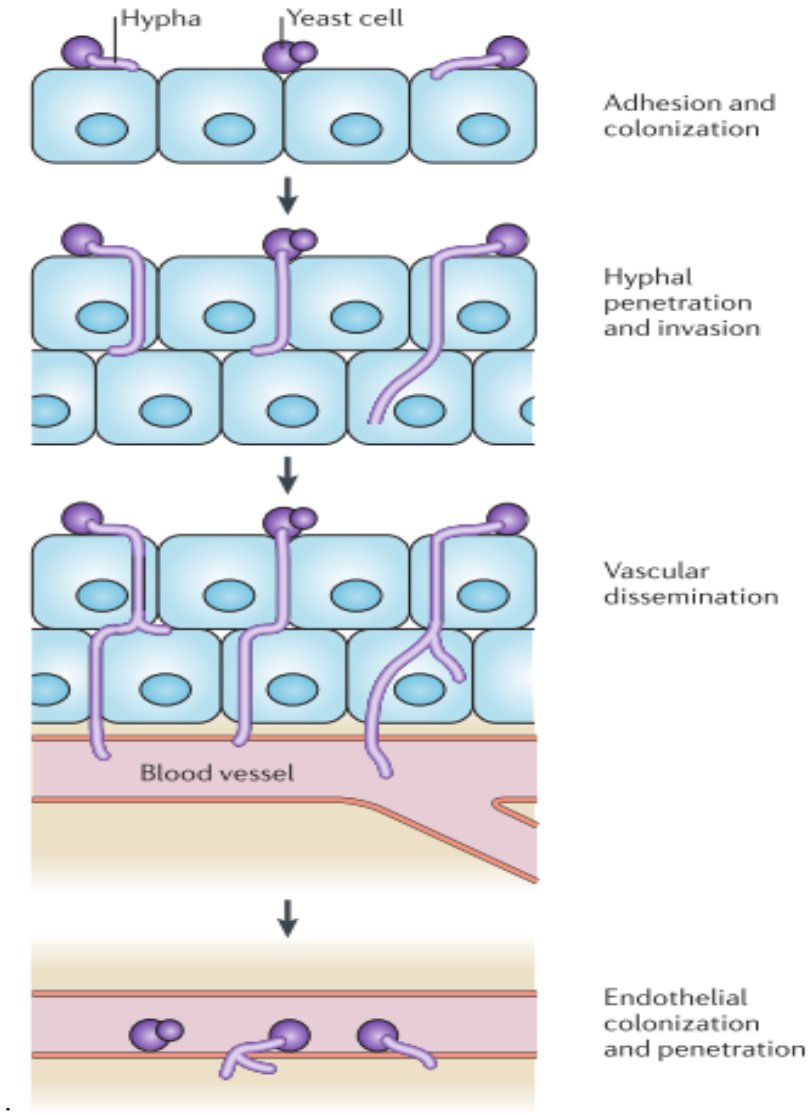

Figure 1.4. The steps of *C. albicans* tissue invasion [19].

The switch from commensalism to pathogenesis in *Candida spp.* depends on both fungal and host factors. Some essential fungal factors include adhesion, dimorphism, biofilm formation and hydrolytic enzyme [32].

1.1.6. Candidiasis epidemiology

Candidiasis refers to infection caused by any of more than 200 species of the genus *Candida*. This fungi is capable of causing several different types of diseases, ranging from commonly encountered superficial infections (oral candidiasis, intertriginous candidiasis, ungual and periungual candidiasis, angular chelitis, vaginitis, chronic mucocutaneous candidiasis) to rare, candidaemia, systemic diseases [32-34]. Systemic infections are normally caused by endogenous *Candida spp.* escaping the gastrointestinal tract and circulating via the bloodstream to infect deep tissues such as the lungs, kidney, and liver [5]. Candidiasis is usually caused by *C. albicans*, and some time by other species (*C. parapsilosis*, *C. tropicalis*, *C. krusei*, *C. glabrata*, *C. stellatoides* etc.).

Candida spp. are the fourth most common cause of nosocomial bloodstream infections (BSI) in the United States with a 35% to mortality rate. According to Centers for Disease Control and Prevention (CDC), approximately 46.000 healthcare associated *Candida* infections occur among hospitalized patients in the United States each year with 30% of patients with candidemia with drug resistant *Candida* die during hospitalization. Additionally, drug resistant *Candida* infections results in millions of dollars in excess costs to U.S. healthcare expenditures each year [35].

In the world, it estimated that there are 72.8 million *Candida* opportunistic infection cases per year [36]. The increasing rate of invasive fungal pathogens is related to the increasing number of critically ill patients and the use of a broad spectrum of antibiotics, surgical procedures, cytotoxic therapy with prolonged neutropenia, other immune suppressive therapies, indwelling invasive devices, and intensive care support. *Candida spp.* are the most prominent invasive fungal infection in critically ill adult patients as well as in neonatal patients treated in the

Intensive care units (ICU), and the second cause of invasive fungal infection in severely immunocompromised patients such as those with cancer and recipients of hematopoietic stem cell and bone marrow or solid organ transplantation. It is estimated that 0.5-1% patients having high risk factors will contract *Candida* bloodstream infection, which contributed to 8-10% of all nosocomial bloodstream infections. Approximately 20-50% of these patients will die as a result of the infection, and an additional 10-40% will die from underlying disease (Figure 1.5) [37].

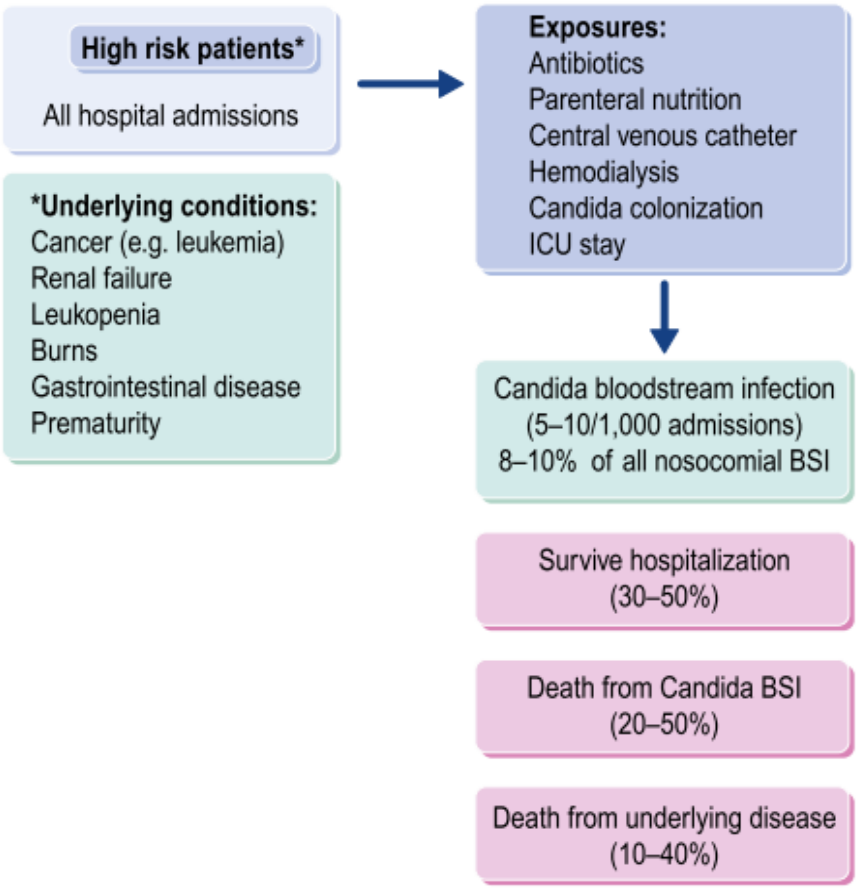

Figure 1.5. Hospital - acquired Candida infections [37]

Although *C. albicans* is the most common cause of invasive fungal infections, the increasing number of infections from *C. non albicans* species is reported as a major source of infection [7, 38]. The ARTEMIS Global Antifungal Surveillance Program determined that *C. albicans* was the most common cause of

invasive fungal infections (63-70%), followed by *C. glabrata* (44%), *C. tropicalis* (6%), and *C. parapsilosis* (5%) [39]. However, the species distribution of *C. non albicans* varies by location and istitution of source reports. In most surveys conducted in US and Europe, *C. glabrata* is the second most common *Candida* species leading to invasive fungal infections [9] [40]. By comprison, in Asian - Pacific countries and Latin America, *C. tropicalis* and *C. parapsilosis* are the second and third most common *Candida* species, respectively [40]. Worldwide, there is a decrease in frequency of *C. albicans* and an increase in *C. parapsilosis* and *C. tropicalis,* while the frequency of *C. glabrata* and *C. krusei* has remained unchanged. In addition, patient characteristics and time period using antifungal therapy also have an influence on the species distribution of this genus. *C. albicans* is more frequent in patients aged 18 years and younger, the frequency of *C. parapsilosis* decreases with age, and *C. glabrata* is more common in the elderly [9].

1.1.7. Diagnosis of candidiasis

Candidiasis diagnosis

For *Candida spp.* isolated from sterile sites, including blood and peritoneal fluids, intravenous line tips and tissue, should be identified to species level by sending to a specialized laboratory if necessary, exception of bronchoscopy fluid [41]. It is very important to classify between candida colonization and candidiasis for *Candida spp.* isolated from unsterile sites, such as urine, vaginal secretion, gastrointestinal fluid, stool, ect. In these cases, the observation of hyphae or pseudohyphae by direct microscopy can help to detect the infection. In addition, not all *Candida spp.* form filaments during infection (e.g. *C. glabrata*), and microscopy in such cases will show only yeast cells [42].

According to The European Society of Clinical Microbiology and Infectious Diseases (ESCMID) [42], candidiasis diagnostic was recommended as follow:

Table 1.1. Summary of recommendations by *Candida* disease, specimen and test evaluated [42]

Disease	Specimen	Test	Recommendation
Candidaemia	Blood	Blood culture	Essential investigation
	Serum	Mannan/anti mannan	Recommended
		β-D glucan	Recommended
Invasive candidiasis	Blood	Blood culture	Essential investigation
	Serum	B-D glucan	Recommended
	Tissue and sterile body fluid	Direct microscopy and histopathology	Essential investigation
		Culture	Essential investigation
Chronic disseminated Candidiasis	Blood	Blood culture	Essential investigation
	Serum	Mannan/anti mannan	Recommended
		B-D glucan	Recommended
	Tissue and sterile body fluid	Direct microscopy and histopathology	Essential investigation
		Culture	Essential investigation
Oropharyngeal and oesophagic candidiasis	Swab	Culture	Essential investigation
	Biopsy	Direct microscopy and histopathology	Essential investigation
		Culture	Essential investigation
Vaginal candidiasis	Swab	Direct microscopy	Essential investigation
		Culture	Essential investigation
		Commercial test	Use validated test only

Candida spp. phenotypic identification

Different methods have been developed and used since 1950s for *Candida spp.* identification [34]. The gold standard technique of phenotype identification is based on culturing strains followed by identification of different phenotypic characteristics. The detection techniques includes germ tube test, chromogenic test, enzymatic test, and fermentation tests [43, 44].

The most convenient and common methods for *Candida* species identification is carbohydrate assimilation and/or enzyme detection. This method proceed in strips or plates, which are commercially available. These commercial availbe test include the API 20C AUX (bioMerieux-Vitek, France), the API Candida (bioMerieux, France), the Auxacolor (Sanofi Diagnostics Pasteur, France), and the Uni Yeast Tek kit (Remel Laboratories, Lenexa, Kansas, USA). These tests use an increase in turbidity (API 20C AUX) or the production of color (API Candida, Auxacolor, Uni YeastTek) in each of a series of wells containing different substrates to produce a particular biochemical profile. The profile produced is read and translated into a numerical code that is deciphered using the manufacturer's reference manual. However, a disadvantage of these tests is that they are unable to differentiate between *C. albicans* and several species such as *C. dubliniensi*, *C. tropicalis*, *C. lusitaniae* [45].

Additional methods for *Candida spp* identification was chromogenic media such as CHROMagar Candida (France), Oxoid Chromogenic Candida Agar (USA), HiCrome Candida agar (HiMedia, Mumbai, India). These media help to differentiate *Candida spp.* by combine substrates linked to chemical dyes in a solid medium to made different color depend on species: *C. tropicalis* (dark blue colonies), *C. albicans*/*C. dubliniensis* (green colonies) and *C. krusei* (dry, irregular, pink-brown colonies). This medium is very useful for the identification of common *Candida* pathogenic species [46-48].

Unfortunately, two major limitions of current test methods are test time and test sensitivity. It is known that current tests are time consuming and are not sensitive enough to give the accurate results. This results in the delay of necessary antifungal therapy. Additionally, current test methods may not be sensitive enough to identify strains from different tissue specimens due to low number of cells present in different internal organs especially in case of invasive candidiasis. In summary, the limitations of *Candida spp.* identification based on phenotype includes long testing time requirement and inability to differentiate between several species.

Another method to diagnose candidose is immunological test which is the detection of antigen or antibody. The detection of antibodies against different *Candida* antigens may help in the diagnosis, but may not be able to differentiate between species. Futhermore, the methods traditionally used for the detection of antibodies have been based on crude antigenic fungal extracts, which generally have low reproducibility and have problems with cross reactivity [49].

***Candida spp.* genotypic identification**

The limitations described above for phenotypic identification techniques have led to the development of technologies to quickly and accurately identify *Candida* strains that will result in early diagnosis, treatment and management of candidemia and other infections caused by *Candida* species. Currently, three are three advanced methods that have been developed to identify *Candida*. These are polymerase chain reaction based *Candida* detection, MALDI - TOF MS and DNA Microarray for *Candida* detection [34, 43].

Polymerase chain reaction (PCR). A large number of different protocols have been developed over the last five decades to identify different fungal strains present in clinical specimens by polymerase chain based *Candida* detection techniques. Various *Candida* DNA markers include 5.8S rRNA genes, 18S rRNA gene, small unit rRNA gene, noncoding internal transcribed spacer (ITS) of rRNA genes, and lanosterol demethylase gene have been used for detection of *Candida* species. This technique also help to identify species base on specific primers. In addition, real-time PCRs is more sensitive and less time consuming techniques for rapid and accurate identification of different *Candida* species [34].

Matrix assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS). This technique is very useful in medical diagnostic for the rapid identification of clinically important bacteria and yeasts. In recent years, this technology has been applied to *Candida* biology in save timme manner and accurate identification. MALDI-TOF MS has been useful for identifying *Candida* species that are not easy to differentiate in the phenotypic

identification. Base on this technology, it could be discrinimate closed species such as *C. parapsilosis*, *C. orthopsilosis*, and *C. metapsilosis* as well as closely related species like *C. dubliniensis* /*C. albicans* , *C. glabrata/C. bracarensis* [50]

DNA Microarray. This techniques has revolutionized the understanding of molecular functioning of different genes in all the organisms including humans. In the oligonucleotide microarray method, specific probes targeted to internal transcribed spacer 2 (ITS2) can be used for hybridization with fungal DNA amplified by PCR from different species. This method is sensitive enough to discriminate among different fungal pathogens at species level and can detect as low as 15 pg/ml of DNA [51]. The sensitivity of the assay for *C. albicans* is 10 cells/mL [52].

In conclusion, molecular techniques have higher efficiency, specificity and sensitivity to the identification of *Candida* from culture and samples.

1.2. *Candida spp.* virulent factors

Invasion of host cells by *Candida spp.* progresses in several phases. Initially, blastospores adhere to epithelial cells, then hyphae are formed which penetrate cells actively or by endocytosis, progressively causing damage over time to the tissue. Several agents called virulence factors, are responsible for this process, among which the potential ones are the ability to grow at 37°C and physiological pH, the size enabling invasion of the human body; the others are: formation of hyphae and pseudohyphae, the ability of phenotypic switching, adherence to epithelial and endothelial cells, biofilm formation, secretion of hydrolytic enzymes (proteases, phospholipases, lipases), and thigmotropisms [23, 53, 54]. Each of these attributes influences the other, and all are essential for full pathogenicity of fungi from the genus. There is a growing number of studies reporting virulence factors of *Candida spp.*, several virulence factors including adhesion molecules, hydrolytic enzymes, phenotypic switching, morphological dimorphism, and fitness attributes have been identified (Figure 1.6) [32].

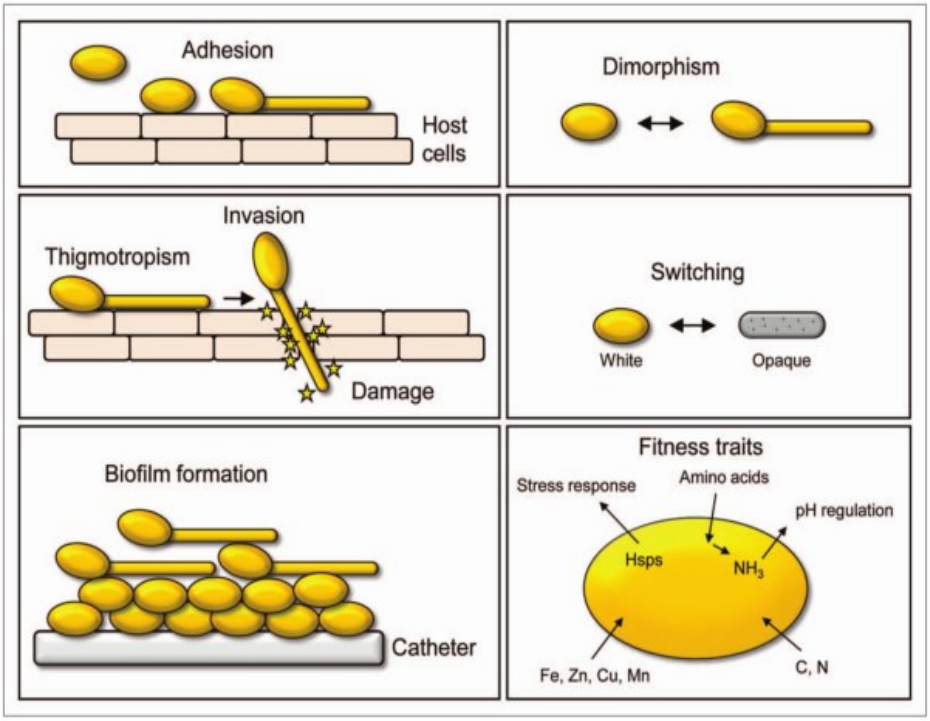

Figure 1.6. An overview of selected virulent factors distribute to *C.albicans* pathogenicity mechanism [32].

Adherence of *Candida spp.* to host tissues and cells is seen as an essential early step in the establishment of disease [23, 55]. The presence of specific compound in the fungal cell wall, most of which belong to the class of glycosylphosphatidylinositol cell wall proteins such as HWP, ALS, EPA, which promote adhesion to the proteins or carbohydrates in the host cell wall.

One of the most important genes responsible for the adhesion process, whose expression is induced by physical contact between the fungal and epithelial cells, is hyphal wall protein 1 (HWP1). It is a fungal cell wall mannoprotein specific for germ tubes and hyphal forms [16]. HWP1 adhesin is controled by Kex2 endoproteinase, which itself also relates the activity of *C. albicans* proteinases, indicating its play for providing virulence and drug resistance of *C. albicans*. Although HWP1 detecting both in carriers of *C. albicans* and patients with candidosis of the oral cavity or vagina, expression of this gene is higher among strains isolated from candidosis patients [55].

Agglutinin like sequence (ALS) are encoding a fungal cell surface glycoproteins, a type of adhesins that exhibit similarity to immunoglobulins, bind to peptide ligands of human cells and form aggregates with other microorganisms potentially pathogenic to human, which may lead to mixed infections. This family includes eight genes, although not all are present in each strain: *als1-7* and *als9* [53, 55], from which *als1-4* encode adhesins specific for germ tubes and hyphae [55, 56], while *als5-7* and *als9* are associated with blastospores [55]. The transcription of all family genes in vitro and during infection has been detected in *C. albicans*, but some genes (*als6* and *als7*) have been observed with only low levels of expression [16]. The genes most frequently reported to be involved in adherence for *C. albicans* are *als1*, *als3* and *als5*, which are characterized by their ability to adhere to a wide variety of substrates [16, 54]. In *C. tropicalis* and *C. dubliniensis* had at least 3 *als* genes, which have been identified by southern analysis and western blotting with an anti ALS antibody [23].

Another virulence factor is extracellular hydrolytic enzymes, including the secreted aspartyl proteinase (SAP) and phospholipase (PLB) gene [16]. These enzymes acting as virulence factors which contribute to host tissue invasion by digesting proteins (hemoglobin, keratin, collagen…). Comparing to *C. albicans*, *C. non albicans* produced at a lesser extent hydrolytic enzymes.

SAP are encoded by genes located on the same chromosomes as ALS, occur in similar numbers and and are regulated by similar mechanisms. *C. albicans* possesses at least 10 members of a *sap* gene family, all of which have been sequenced and extensively characterized. It was found that *sap1, sap3, sap4, sap7, sap8* expression was correlated with oral disease. Furthermore, *sap1*, *sap3*, and *sap8* were preferentially expressed in vaginal rather than oral disease. In vitro studies show that *sap1*, *sap2*, and *sap3* are expressed by yeast cells only, whereas *sap4-6* expression is confined to hyphae [23]. *Sap1-4* genes were identified in *C. tropicalis* and *sap1* is the predominant enzyme produced in vitro [23]. *Sap* genes also have a play in adhesion. The glycosylphosphatidylinositol-linked aspartyl proteases (Yps) is related to virulence of *C. glabrata* [53].

Four types of phospholipases have been revealed in *C. albicans*, including phospholipases A, B, C and D [23], but only the phospholipases B1 and phospholipases B2 products have been detected extracellularly [16, 23]. Although phospholipases B1 is thought to account for most of the secreted phospholipase B activity in *C. albicans*, phospholipases B2 contributes in a minor way, because a phospholipases B1 deficient strain still produces residual amounts of phospholipase B activity.

One contribution to *Candida spp.* virulence is hyphal formation, which makes a majority of the strains in this genus become dimorphism. Genes involved in these functions (*Als3*, *sap4-6*, *hwp1*, *hyr1*, and *ece1*) in *C. albicans* are differentially expressed [53]. The mitogen activated protein (MAP) kinase, cyclic AMP (cAMP), and pH sensing, Rim101 signal transduction pathways regulate cellular morphology and expression of hypha associated genes. The bud hypha transition may also contribute to virulence of other *Candida* species such as *C. glabrata*, even though *C. glabrata* strains do not exhibit germ tube formation in classical mycological assays [53].

C. albicans colonies can change among different phenotypes including smooth, rough, star, stippled, hat, irregular wrinkle, and fuzzy at high frequency (10^{-4} to 10^{-1}) [57, 58]. Smooth and white colonies with round ovoid cells (white) can switch to flat and gray colonies with elongated or bean shaped cells (opaque) [23]. The strains can revert from white colony to opaque colony and contribute to its virulence. White phase cells are more virulent in invasive infection, and opaque phase cells are better to colonize skin [23, 53]. Phenotypic switching also affects other virulence traits, including the bud hypha transition, sensitivity to neutrophils and oxidants, antigenicity, adhesion, secretion of proteinase, drug susceptibility, and phagocytosis by macrophages [53].

1.3. Molecular epidemiology of *Candida spp.*

Most *Candida* species belong to the *Candida* clade, exception of *C. glabrata*, which is more closely related to *S. cerevisiae* than to other *Candida*

species [59, 60]. *C. albicans* genome consists of eight pair of chromosomal homologs [61], ranging in size from 0.95 to 3.3 Mb in size and comprising 16 Mb in total. This species is predominantly diploid, however it exhibits a high degree of genome plasticity and exhibits frequent losses of heterozygosity as well as gross chromosomal rearrangements that may result in aneuploidy. Althought a reproduction is predominantly clonal, this species can also utilise a parasexual cycle involving the formation of tetraploid progeny from the mating of diploid parents, the former of which subsequently revert to diploidy by concerted chromosome loss [62]. The parasexual cycle occurs rarely in nature, possibly only under stressful conditions. The main function of the parasexual cycle is thought to enable diversification during times of stress, revealing new combinations of recessive traits by loss of heterozygosity (LOH), or resulting in aneuploidy and copy number variation enabling adaptation to adverse environmental conditions. Aneuploidy and revelation of recessive alleles may adversely alter the fitness of the organism, but in highly stressful conditions the parasexual cycle may be a significant source of diversity permitting adaptation and survival of the organism [63, 64].

Molecular epidemiology that combines traditional epidemiological investigation with molecular typing is useful for identifying community or nosocomial infections and tracing the source of transmission and outbreaks. Nowadays, several different molecular typing approaches including pulsed field gel electrophoresis (PFGE), restriction fragment length polymorphism(RFLP), random amplified fragment length polymorphism (RAPD), and multi locus sequence typing (MLST) [65]. Among these techniques, assays based on PFGE, RFLP or RAPD are labor intensive and time consuming. Furthermore, the results of these methods are difficult to compare among laboratories. By contrast, MLST, as a relatively new tool based on DNA sequencing, exhibits high discriminatory power and reproducibility, which overcome the flaws of more subjective methods, making it possible to compare results among laboratories. Therefore, nowadays MLST is a good tool to study and understand *Candida spp.* epidemiology molecular. Online global databases for many microorganisms are currently available at www.mlst.net,

including data from epidemiological studies carried out worldwide. This permits global epidemiological and population analysis [61, 65, 66]. MLST apply not only for *C. albicans* but also for others species like *C. glabrata*, *C. tropicalis*, *C. krusei*, *C. dubliniensi*, *C. parapsilopsis*.

A standard MLST protocol for molecular characterization of *C. albicans* has been proposed based on the sequences of seven housekeeping genes (AAT1a, ACC1, ADP1, MPI1b, SYA1, VPS13 and ZWF1b). This method has been widely used to study the population structure, transmission and microevolution of *C. albicans*. MLST studies have shown that this species'infections often arise from an endogenous source and persistent *C. albicans* strains are maintained by hosts over prolonged periods of time, occasionally undergoing minor genetic variations known as micro variation [66]. Strain replacement has also been observed, as has the transmission of *C. albicans* isolates between different individuals and microvariation of persistent isolates occurring in the same individual between recurrent infections [61, 66].

Based on MLST, a total of 18 clades have been identified worldwide and these clades are associated with the geographic distribution of *C. albicans* [61, 67]. Clades have been defined as clusters of at least 10 isolates with DSTs that have a p-distance below 0.04, while their p-distance with DSTs outside the clade is above 0.04. MLST clade 1 appears to have a global distribution, this clade represents 33% of UK isolates, 31% of isolates from elsewhere in Europe, 32% of isolates from southeast Asia and Japan, 28% of isolates from Australasia, 34% of isolates from the Middle East, 44% of isolates from South America and 49% of isolates from North America, 16% among isolates from Africa [61]. Other clades tend towards greater geographical specificity, though none are found exclusively within specific geographical limits: clade 2 is enriched with isolates recovered from the UK, clade 4 is enriched with isolates from the Middle East and Africa, clade 11 is enriched with isolates from continental Europe, and isolates recovered from the Pacific tend to cluster in clades 14 and 17 [61, 67]. Studies in several countries in Asia have demonstrated that the most common clade was clade1 [61, 68, 69]. Moreover, clade

6, clade 17 were the majority in China [69], while in South Korea were clade 4, clade 12 and hight rate (18.6%) belong to new clade [68]. Until now, there has not been any studies of *Candida spp.* MLST in Viet Nam, therefore one is necessary to understand the epidiomology of *Candida spp.* in Asia as well as all over the world.

Molecular strain typing also demonstrate that certain types are more commonly associated with invasive disease than others. Analysis of the largest database from MLST research, did not demonstrate any statistically valid differential subset of strain types associated with disseminated infection [70]. However, there was a significantly greater proportion of isolates associated with superficial infections and commensal carriage in clade 1 versus other clades [70]. The obvious interpretation of this finding is that clade 1 isolates may be better adapted than others to colonize and invade epithelial surfaces, but have no inherent advantage over other types when it comes to traversing epithelia to cause deep tissue disease [61]. In addition, association between clade and the lengths of tandem repeats in some cell surface proteins (ALS) but not with virulence or type of infection, have been demonstrated [61]

Most individuals carry a single *C. albicans* strain type, but minor variations suggestive of microadaptation are commonly observed. Some evidence suggests many individuals harbor a mixture of strain types that includes a range of minor variants, typically differing in levels of genetic heterozygosity [61, 66].

Although most *Candida* infections appear to originate from an endogenous source, nosocomial transmission is not uncommon and may occur either by cross infection or by exposure to a common infecting source [61, 71, 72].

1.4. Molecular mechanism to resistant drugs in *Candida spp.*

The frequency of resistance to antifungal therapy continues to increase despite the introduction of new antifungal agents. Pathogen fungi have developed some mechanisms to survive in toxic environment. According to the literature, the following molecular mechanisms of drug resistance include: modification of the drug target affinity, overproduction of the enzymes that are targets to drugs,

development of alternative metabolic pathways, active efflux of the drug from the cell, impermeability of the cell membrane to drug molecules, active enzymes inactive the drug or degrading it outside the fungal cell [73-75]. Antifungal resistance is associated with elevated minimum inhibitory concentrations, poorer clinical outcomes, and breakthrough infections during antifungal treatment and prophylaxis.

Currently, there are two independent standards for broth microdilution (BMD) susceptibility testing of *Candida* and filamentous fungi: the Clinical and Laboratory Standards Institute (CLSI) method and the European Committee on Antimicrobial Susceptibility Testing (EUCAST) method. These methods are similar in that both use BMD, although there are some differences in inoculum size and MIC endpoint determination. These methods have been harmonized so that there is close agreement between MIC results obtained when testing azoles and echinocandins against *Candida*. The CLSI has also developed agar based, disk diffusion testing for yeasts. Compared with the BMD method, disk diffusion testing is convenient, simple, and economical, and is particularly well suited for water soluble antifungals such as 5-fluorocystosine, fluconazole, and voriconazole. Disk diffusion testing has been standardized for checking resistance of *Candida spp.* to fluconazole, voriconazole, caspofungine, micafungin, and break points have been provided for each of these agents [76].

Various mechanisms can lead to resistance of *Candida spp.* to antifungal compounds. For azole drugs, the most common mechanism include the efflux pumps encoded by the *mdr* or *cdr* genes, and point mutations in the gene encoding for the target enzyme ERG11 [76-81]. These mechanisms are shown in Figure 1.7. Resistance to amphotericine B is related to qualitative or quantitative changes in ergosterol by expression of mutations in *erg3, erg6, erg11* genes [82]. Resistance mechanism of *Candida sp.* to echinocandins is typically caused by the *fks1* genes encoding the major subunit of its target enzyme [75, 82, 83].

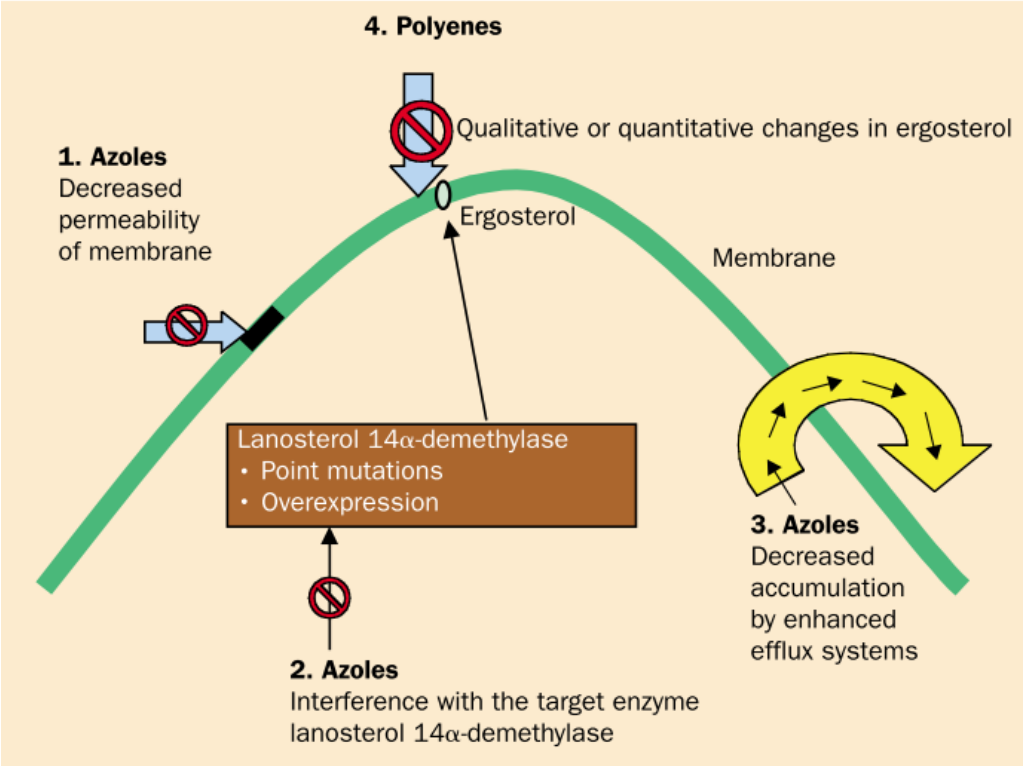

Figure 1.7. Main mechanisms of azole and polyens resistance [78]

Among antifungal drug, azoles are a class of antifungals that are widely used to treat both superficial mucosal and deep and disseminated fungal infections caused by *C. albicans* [77]. However, extensive use may lead to the development of resistance, resulting in therapeutic failure for *Candida spp*. [78]. In azole compounds, fluconazole is the antifungal agent of choice in the treatment and prophylaxis of infection by *C. albicans* [78, 84]. In addition, the emergence of fluconazole resistant *C. albicans* strains is a significant problem after long time use as the treatment for recurrent oropharyngeal candidiasis in acquired immunodeficiency syndrome (AIDS) patients [78, 80]. Physicans encounter a high prvelance of fluconazole-resistant *Candida*, which makes the treatment of candidemia a huge challenge [35]. Data from many coutries all over the world have reported higher levels of resistance from *C. non albicans* than *C. albicans* [7, 39, 85, 86].

1.5. *Candida spp.* and bacteria interaction

The origin of bacterial life predates the appearance of eukaryotic organisms by more than two billion years. Therefore for bacteria, symbiosis with eukaryotes regardless of whether the outcome is negative, positive, or neutral for the organisms united through the interaction is a derived lifestyle [87]. Research in the interaction between bacteria and fungi has developed significantly in both breadth and depth in recent years. Bacterial fungal interactions (BFIs) are antagonistic, cooperative, synergistic, commensal, and symbiotic [88]. It has been reported that the most common BFIs are ones where the bacterial partner exploits resources from the associated fungus through a parasitic or commensalism interaction, although there are intriguing examples where the fungus is able to take advantage of bacterial resources in mutualistic interactions [87].

Humans are naturally colonized by fungi and bacteria in a variety of niches, including the skin, the oral cavity, and the respiratory, digestive, and genital tracts [89]. In healthy individuals, these microorganisms are commensal and in some cases even beneficial to human health. By contrast, in immunocompromised patients, pathogenic fungi and bacteria represent a serious threat to their health. There are some studies reported that bacteria and fungi have been found together in infections of human burn wounds and keratitis [90-92]. Moreover, the forming of mixed communities between bacteria and fungi makes them more virulent and resistant to antibiotic therapies [93, 94]. Therefore, an understanding of the functioning of BFIs, particularily the development of strains related to resistance to drug therapies, in human health is an emerging and important challenge for medical researchers [95].

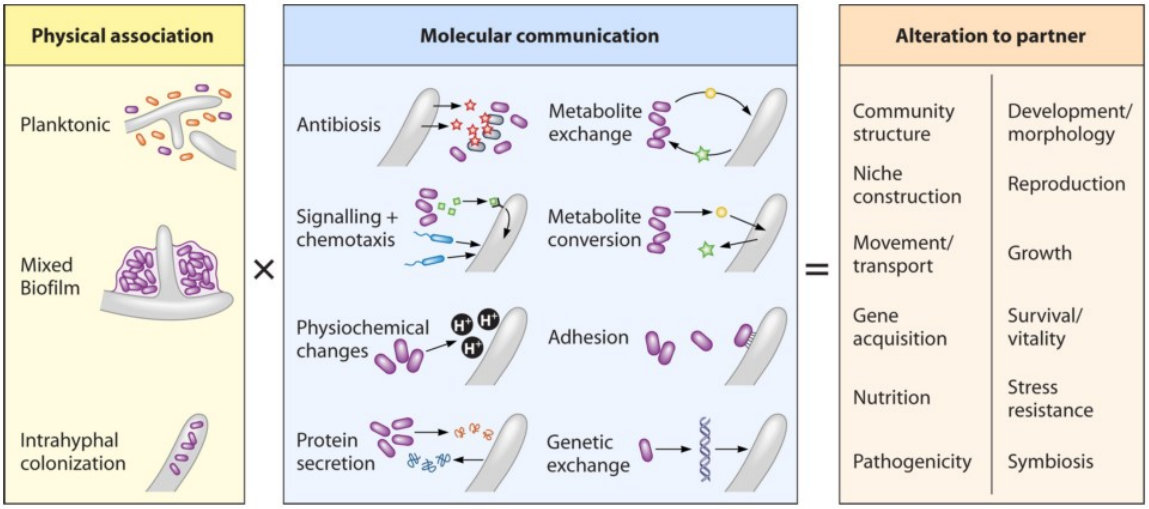

Figure 1.8. The bacteria fungal interaction: the combination of physical associations and molecular interactions [88]

Observation of the effects of fungi on bacterial development is difficult due to the small size and single cellular nature of bacteria. However, if consideration is given to bacterial fungal biofilms, it is clear that fungi can promote distinct differences in bacterial development by contributing to a distinctive ecological niche, within which bacteria exhibit physiological differences, such as resistance to antibiotics, stress, and an altered expression of virulence genes, compared to free living bacteria [95, 96].

Recently, several studies have described the association of *Candida spp.* and bacteria, such as *Staphylococcus aureus*, *Pseudomona aerugirosa*, *Staphylococcus epidermidis*, ect. [93, 94].

Helicobacter pylori (*H. pylori*) is a Gram negative, spiral shaped bacterium that infects more than 50% of the human population and can cause gastritis, peptic ulcer, or gastric malignancies [97]. It is necessary to understand *H. pylori* transmission to prevent this disease. The oral cavity has been hypothesized as a reservoir for gastric *H. pylori*, which has been detected by culture and with PCR in both dental plaque and saliva. Some researchers have proposed *H. pylori* in oral cavity may play an important role in its transmission and reinfection. Oral-oral or fecal-oral transmission are thought to be the most possible means of transmission [97].

H. pylori is generally considered an extracellular microorganism. However, there have been some evidences supporting a hypothesis that *H. pylori* microorganisms have an intracellular location [98, 99]. These findings support the hypothesis that this bacteria can invade fungi and survive from stressful environments. Normally, *Candida spp.* can also colonize in the gastric regions [91] and invade epithelium cells in certain conditions [100]. Recently, some studies focused on the relationshop between the microrganism. Evidence has been suggested to show that *Candida* vacuoles can be a niche for *H. pilory* and and *H. pylori* specific genes, such as *vacA*, *ureA* and peroxiredoxin were detected by PCR and Western blotting [101].

2. RESEARCH OBJECTIVES

Candida species are normally associated with human beings as harmless commensals. They are commonly found on the mucosal surfaces of gastrointestinal and genitourinary tracts and skin of humans [5]. *Candida spp.* infect billions of people every year all over the world, causing a broad spectrum of infections, ranging from mucosal or localized cutaneous infections to systemic or potentially fatal diseases [36, 40]. To date, the incidence of invasive fungal diseases by *Candida spp.* is rising as a result of immunocompromised patients, modern medical interventions and therapies. For example, AIDS patients or who have undergone transplants generally receive immunosuppressive drugs or aggressive regimens of chemotherapy in cancer, respectively. Patients under these conditions are prone to mycosis, a majority of which are caused by *Candida spp* [40]. Systemic mycosis often requires high associated costs, particulary mycosis caused by *Candida* species which are less senstive to antifungal therapy [76, 102]. Therefore, a better understanding of the epidemiologic features and etiology of candidose will enable physicians to provide better management strategies, preventitive measures, and treatment to infections.

For many years, fungal laboratory methods based on the detection of phenotypic characteristics, such as microscopy and in vitro culture, have played an essential role in fungal etiology identification. However, these procedures are generally slow or non-specific. Furthermore, phenotypic features can be easily influenced by external factors such as variations in temperature, medium, and chemotherapy, making species identification difficult. Recently, genotypic approaches have proven to be useful for fungal identification. In fact, genotypic differences are considered more stable and precise than phenotypic differences. Molecular biology techniques, such as PCR, amplicon sequencing, MLST and MALDI-TOF MS, are useful in the identification and investigation of pathogenic fungi [103], particularly *Candida* species [104, 105].

In Vietnam, *Candida* superficial infections are common. Recently, invasive fungal infections have become more frequent due to the growing number of immunocompromised and other susceptible individuals in the population [106-109]. Although physicians have been faced with managing more of these infections, there has been a lack of systematic epidemiological data in this field [108, 110, 111]. In addition, the current gold standard techniques for the indentification of mycoses are direct examination and in vitro culture [112]. The use of of molecular techniques in the study of etiology and epidemiology of *Candida spp.* infections in Central Vietnam will be essential, useful and realistic. Furthermore, molecular techniques will allow for a more accurate overview of *Candida* molecular epidemiology and *Candida* causatives in Vietnam.

For these reasons, the aims of this research are as follow:

2.1. To study candidiasis etiology

- Application of molecular techniques (e.g. Matrix Assisted Laser Desorption Ionization Time-of-Flight Mass Spectrometry and Internal transcribed spacer sequencing) in the identification of *Candida* species.

- Analysis of the distribution of *Candida* species in candida colonization and candidiasis.

2.2. To analyze the phenotypic and genotypic features of *Candida spp.* isolates

- Checking antifungal susceptibility tests by disk diffusion method.

- Detecting and analyzing polimorfism of resistance genes linked to fluconazole resistance in *Candida tropicalis* by PCR and sequencing (mutations in ERG11 gene).

- Detecting virulence genes by Multiplex PCR (*als1, hwp1, sap4*).

- Studying molecular typing of selected isolates of *Candida albicans* by Multi Locus Sequence Typing (MLST).

2.3. To analyze relationship between *Candida spp.* and *Helicobacter pylori*

Testing for the presence of *Helicobacter pylori* specific gene by nested PCR in *Candida* isolates

3. MATERIALS AND METHODS

3.1. Study site

This cross sectional study was carried out from October 2012 to August 2015 at two Vietnamese Hospitals, Hue University of Medicine and Pharmacy Hospital and Hue Central Hospital, and the Biomedical Science Department at the University of Sassari in Italy.

Samples were collected in 10 departments at Hue University of Medicine and Pharmacy Hospital (Dermatology, Endoscopy, Intensive Care, Internal Medicine, Obstetric, Oncology, Ophthalmology, Otorhinolaryngology, Pediatric and Surgery) and 2 departments at Hue Central Hospital (Hematology and Pediatric).

Direct examination, fungal cultivation, and fungal phenotype identification were carried out in Vietnam at the Parasitology laboratory at the Hue Medicine and Pharmacy University. Fungal genotype was conducted at the Carlo Urbani Centre in Hue, Vietnam and at the Microbiology laboratory, Department Biomedical Science in Sassari, Italy.

3.2. Study population

All patients were subsequently divided into four groups. The first group included patients with candida colonization. These patients had *Candida spp.* isolated from unsterile body sites by both direct examination and culture but that did not display any symptoms related to superficial candidiasis or invasive candidiasis. These patients, furthermore, recovered without assistant from antifungal therapy. The second group was mucosal candidiasis. These patients displayed symptoms of the disease (oral candidiasis, vulvovagiginite candidiasis) and were clinically identified by doctors. In this case, *Candida spp.* was isolated by both direct examination and culture. Cutaneous candidiasis was the third group of patients. These patients had symptoms including parochynia, onychomycosis, and skin candidiasis. *Candida spp.* in these patients were isolated by both direct examination and culture. The fourth group was systemic candidiasis (candidemia or invasive

candidiasis). These patients had clinical symptoms and *Candida spp.* was isolated from blood, tissue, sterile body sites by a combination of direct examination and culture or culture only.

3.3. Sample collection and *Candida* strains isolates

Samples were collected from 163 patients admitted in 10 different hospital wards at Hue University of Medicine and Pharmacy Hospital and 2 wards at Hue Centrel Hospital (see Table 3.1)

Table 3.1 . Distribution of patients in hospital wards

Hospital	Ward	Number of patient isolate
Hue University of Medicine and Pharmacy Hospital	Internal medicine	26
	Obstetric	24
	Intensive care	17
	Dermatology	18
	Surgery	17
	Endoscopy	14
	Oncology	13
	Pediatric	5
	Otorhinolaryngology	11
	Ophthalmology	2
Hue Central Hospital	Hematology	10
	Pediatric	6
Total		**163**

Selected patients were required to not have received antifungal treatment with the last seven days in order to prevent false negative cases. One sample was collected from each patient, with the exception of the seven patients in the Intensive Care Unit (HUMP Hospital), three patients in the Hematology ward (HC Hospital), two patients in the Pediatrics ward (HC Hospital), and one patient in the Endoscopy ward (HUMP Hospital). In detail, for the seven patients in the Intensive Care Unit, two samples from two different body sites were collected for 6 patients and three samples from three different body sites for 1 patient. For the three patients in Hematology ward, the two patients in the Pediatrics ward, and the one patient in the Endoscopy ward, two samples isolated from two different body sites were collected. For the two patients in

the Pediatrics ward, two samples isolated from two different body sites. In total, 177 samples from 163 patetients were used in the study.

Specimens had been collected according to the patient's symptoms in primary candidiasis patients. By contrast, in patients without candidiasis, we detected *Candida* colonization status from oral, sputum, urine, gastric drainage fluid, gastric biopsy, bronchoalveolar lavage fluid, endotracheal aspiration fluid.

Samples were collected from patients based on type of disease. Samples from mucosal (oral, vaginal, nasal) and skin lesions were obtained using sterile swabs [113-115]. Gastric biopsy was collected by endoscopy. Sputum and stool were collected into wide-mouthed sterile container [10]. From lower respiratory tract, bronchoalveolar lavage, endotracheal aspiration fluid were collected in volumes of 1 -5 ml [116]. Urine was isolated from midstream with volume from 10 ml to 15 ml. For patients with catheters, urine was obtained by clamp of Foley's catheter distally [114]. Nail scrapings were collected in case of onychomycosis. Gastric fluid was removed from drainage in volumes of 10 -15 ml. A volume of 10 - 20 ml blood was collected from adult patients; for pediatric patients, the volume was 4-10 ml [41, 42, 114, 117, 118].

When necessary, oral, vaginal, skin lesion, and gastric biopsy samples were placed in distilled water and transferred to the laboratory. Urine and other body fluids were processed by centrifuging at 2500g for 10 minutes before microscopy.

Each sample was examined microscopically with 20% KOH to detect fungal morphological forms (budding yeast or/and hyphae) [10, 53]. The samples were then processed for fungal culture.

Each specimen was inoculated first on Sabouraud dextrose agar (SAD) with chloramphenicol for 24 - 48h at 35^0C [10, 114]. *Candida* colonies were sub cultured several times to purify strains. All isolates were stored at - 80^0C in 15% glycerol [119] for future antifungal susceptibility tests and identification using molecular biology methods.

3.4. *Candida spp.* identification

3.4.1. Phenotypic identification

All *Candida* strains isolated from colony in SAD medium were sub-cultured in Brilliance Candida agar (Oxoid) and incubated aerobically at 30°C. The plates were checked at 24, 48 and 72 hours. Brilliance Candida agar was used for differentiating among species of *Candida* since they produced different colored colonies [43]. The precense of two chromogens in this medium (5-bromo-4-chloro-3-indolyl N acetyl ß-D-glucosaminide and 5-bromo-6-chloro-3-indolyl phosphate p-toluidine salt) helped in identify for the presence of two target enzymes, the hexosaminidase and the alkaline phosphatase, respectively. The precense of either enzymes allows for the differentiation of *C. albicans* and *C. tropicalis* from other species of *Candida* within 48 hours. The green colour of *C. albicans* and *C. dubliniensis* is caused by the same chromogenic reaction as well as the dark blue color of *C. tropicalis*. For others species, it is quite difficult to differentiate based on color. For example, the colors of *C. glabrata*, *C. kefyr*, *C. parapsilosis*, *C. lusitanae* could be beige, yellow or brown. Moreover, this medium can help recognize mixed strains showing different color, that could not be achieved by culture on SAD medium [44]. Based on culturing using Brilliance *Candida* agar medium, we had 16 sample with mixed strains (15 samples having 2 strains and 2 samples having 3 strains). Therefore, in this study 196 *Candida* strains were isolated from 177 collected samples.

Figure 3.1. Colonies of *C. albicans* and *C. glabrata* on Brillant Candida medium (http://www.oxoid.com/UK)

(*C. albicans*: green colonies and *C. glabrata*: yellow colonies)

We also performed germ tube tests and chlamydospore assays to identify certain species, such as *C. albicans* and *C. dubliniensis* [10, 114]. For the germ tube assays, yeast was inoculated in 0.5ml-1 ml of human sera and incubated aerobically at 37^0C for 3-4 hours [10]. A drop of the yeast-human serum mixture was examined microscopically and the appearance of small filaments projecting from the yeast cell surface indicates that the germ tube test was positive. In the chlamydospore assays, yeast cells were plated onto Cornmeal agar under a glass coverslip to maintain a semi anaerobic condition and grown in the dark for 3 days at 25^0C [10].

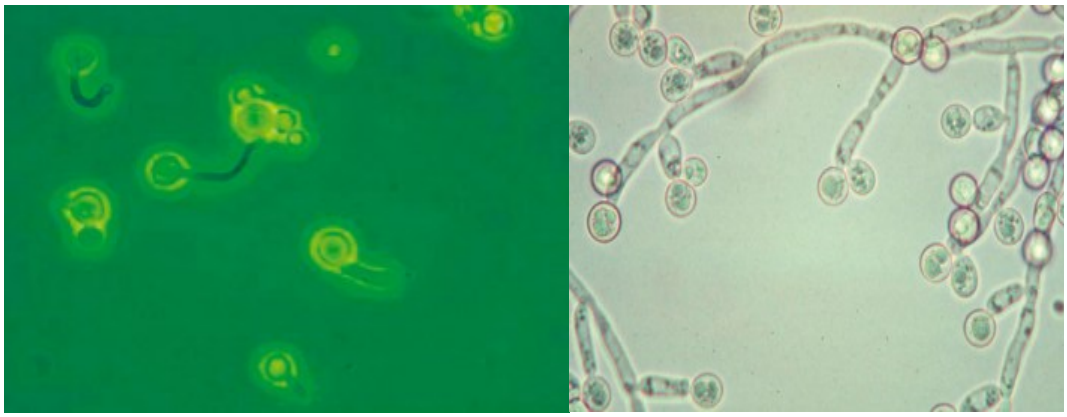

Figure 3.2. Possitive germ tube test and chlamydospore assay [10]

3.4.2. Genotypic identification

We applied Matrix Assisted Laser Desorption/Ionization Time of Flight Mass Spectrometry (MALDI-TOF MS) to identify *Candida* species. MALDI-TOF MS is a type of mass spectrometry that measures the time of travel of ionized peptides. This method is used for rapid and reliable microbial identification. For this technique, the sample is first absorbed onto a matrix then bombarded with a laser beam. After irradiation, the matrix vaporizes and the sample is left with an electrical charge (ionization). Ions are accelerated by an electric field of known intensity. The acceleration of the ions occurs within a drift tube at constant kinetic energy. Since the speed of the ionized particles is inversely related mass, it follows that lighter ions will reach the detector first. The output measurement is the time it takes for a particular particle to reach the detector at a known distance. The ions separate

according to their mass-charge ratio (m / z) and the amount of each ion is measured. The detection is performed at the end of the flight tube. The spectrum generated is analyzed as an individual proteomic profile with the molecular mass ranging from 2000 to 20000 Da. The Principle of MALDI-TOF Mass Spectrometry system is described in Figure 3.3.

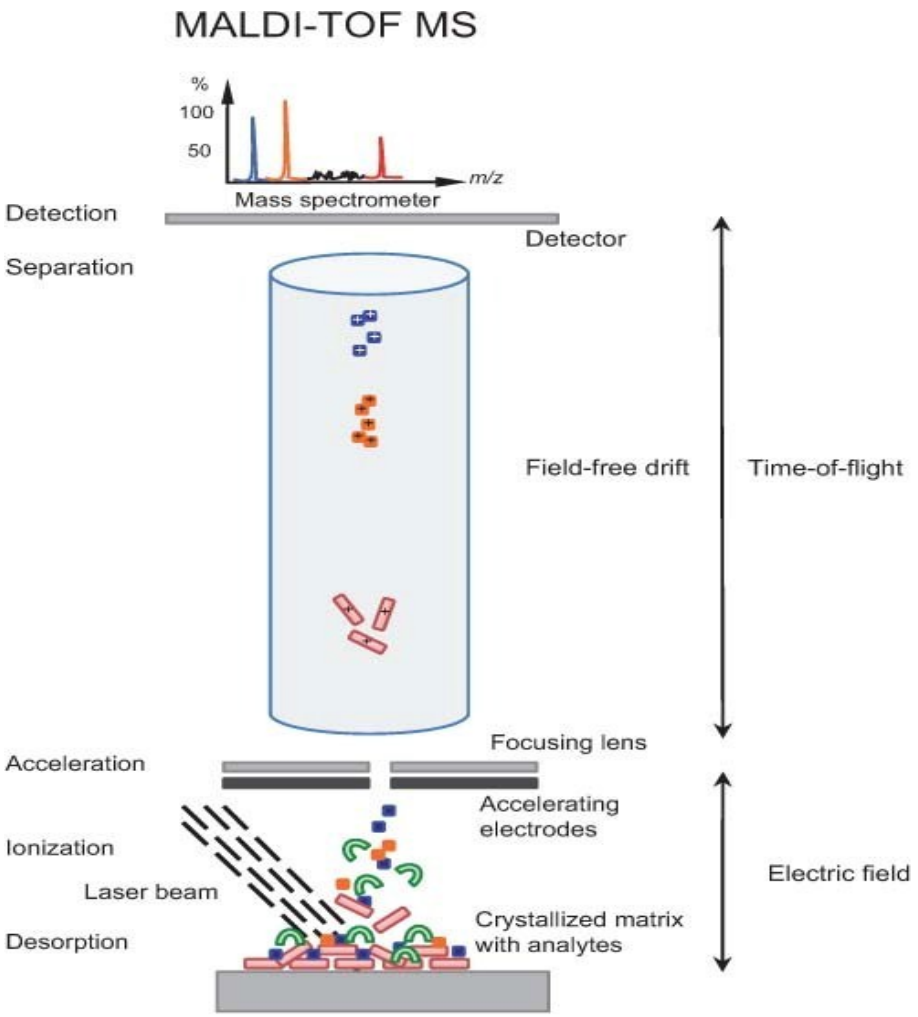

Figure 3.3. Principle of MALDI-TOF Mass Spectrometry system [120]

This technique is able to differentiate bewteen closely related species, such as *C. glabrata* from *C. bracarensis*, *C. albicans* from *C. dubliniensis*, and C. *metapsilosis* and *C. orthopsilosis* from *C. parapsilosis*.

Fungi were subcultured in SAD plates from strains stored in 15% glycerol at - 80^{0}C. Affter 24h incubation, they were used for identification with MALDI-TOF MS. A small amount of sample from a single colony was picked by a sterile tip and transferred directly and spotted in duplicate onto the MALDI target (MSP 96 target polished steel plate; Bruker Daltonik MALDI Biotyper) and air-dried at room temperature. Next, 1µl pure ethanol was added to each well to fix the sample. Aliquots of 1 µl of 70% formic acid were added and mixed gently with the yeasts. When the liquid medium was almost evaporated, each spot was overlaid with 1 µl of HCCA (a-cyano-4-hydroxy cinnamic acid) matrix solution and left at room temperature to dry completely before MALDI-TOF MS measurement. Next, the loaded plate was analyzed by MALDI Biotyper CA System. The spectrum of each isolate was compared with those in the database and the identification was provided by score of reliability. Identification was provided with accompanying scores as the manufacturer's schemes: score <1.7: no reliable identity; score from 1.7-<2.0: identity at genus level; score from 2.0 to upper: identity at species level. This procedure was conducted in Microbiology Department, Nuoro Hospital, Italy.

Strains that had a score under 1.7 by MALDI-TOF MS were further identified by PCR and sequence analysis using specific primers directed against the internal transcribed spacer (ITS) region.

DNA isolation

The total DNA was isolated using the thermolysis method according to the protocol of Zang et al [121]. All purified strains were removed from storage and placed on SDA plates at 25^{0}C and incubated for 24h to allow for growth of single colonies. Next, a 1µl loop was used to transfer a small amount of yeast cells from the colony into 100 µl of sterilized water in a 1.5ml micro centrifuge tube to wash the cells. The mixture was vortexed thoroughly and then centrifuged at 8.000-10.000g for 1 min. After carefully discarding the supernatant using a pipette tip, 100 µl of lysis solution was added to the pellet. The mixture was finally

incubated at 85 ^{0}C in a water bath for 30 min. The crude extract containing genomic DNA was stored at - 20 ^{0}C until use.

The ingredients of the lysis solution, which was referred to as the breaking buffer, contained 50 mmol l^{-1} sodium phosphate at pH 7.4, 1 mmol l^{-1}EDTA and 5% glycerol. Before use, the lysis solution was autoclaved at 121^{0}C for 20 min and then stored at 4^{0}C [121].

Internal Transcribed Spacer PCR assay

The internal transcribed spacer (ITS) regions have important play of ribosom RNA (rRNA) processing in Eukaryotype [122]. They form specific secondary structures they are needed for correct recognition of cleavage sites and provide the binding sites for nucleolar proteins and RNAs during ribosome maturation. Therefore, rDNA genes have been used to identify pathogenic fungal disease. ITS primers amplify a targeted region of the ribosomal DNA of fungi [123]. In this study, internal transcribed spacer regions were amplified by using the following primers:

ITS1 (5'-TCCGTAGGTGAACCTGCGG-3')

ITS2 (5'-GCTGCGTTCTTCATCGATGC-3')

All primers used in the present study were synthesized by Invitrogen (USA).

PCR reactions were performed in a 25 µl volume with 2.5 µl of 10x PCR reaction buffer, 0.2 mmol/l of each of the four dNTPs, 1.5 mmol/l MgCl2 , 0.2 µmol/l of each primer, 1 µl extracted DNA, 1.25 U Taq polymerase and sterile ultrapure water (all chemicals from Invitrogen, USA). Negative control was performed with sterile deionizer water in place of template DNA.

Amplification procedure was performed in a thermal cycler (Hybaid, UK) with an initial denaturation in 5 min at 95^{0}C, then followed 30 cycles: 1 minute at 95^{0}C for denaturation step, 1 minute at 56^{0}C for annealing step, 1 minute at 72^{0}C for extension step, and in 7 minute at 72^{0}C for final extension. The products were then separated in 1% agarose gel in TAE buffer 1x containing GelRed™ nucleic

acid gel stain, Biotium (1 µl /100 ml in TAE 1x), and viewed on a UV trans - illuminator. The lengths of *Candida spp*. amplified fragments were confirmed by the bands under 300bp, with the exception of *C. glabrata*.

The amplicons were then cut and purified by the DNA Clean and Concentrator ^{TM} -5 (ZYMORESEARCH, USA) columns, and quantified by bands comparison to low molecular mass ladder, and sent for sequence at Sequencing Service LMU Munich, Germany (http://www.gi.bio.lmu.de/sequencing). Sequencing results were analyzed by Geneious version 4.8.4 and were blast in GeneBank for species identification. A blast match result that is greater than 99% was considred to be a good match (http://blast.ncbi.nlm.nih.gov/Blast.cg).

3.5. Antifungal susceptibility testing

Antifungal susceptibility testing by disk diffusion methods was performed according to CLSI guidelines (CLSI document M44-A) [124] and manufacturer's instructions. The media and antifungal disks used in the testing were from Liofilchem Laboratories, Italy. The standard medium used for disk diffusion test was Mueller-Hinton agar supplemented with 2% dextrose and 0.5 µg/ml methylene blue. Incorporation of methylene blue in the medium has been found to improve the yeast growth and provide sharp zones of inhibition for the azole group of drugs.

The colonies were suspended in 5 ml of sterile 0.85% saline, and the turbidity was adjusted to yield 1×10^{5} - 1×10^{6} cells/ml (0.5 McFarland standard). Next, a sterile cotton swab was dipped into the suspension and rotated several times. Any excess fluid from the swab was removed by pressing firmly against the inside wall above the fluid level before dispensing suspension inoculated on the plate surface. The plates were dried for 3-5 min at room temperature in laminar hood. Then, antifungal disks were placed on the inoculated agar with a forceps, and the plates were incubated at 37^{0}C. The zone of inhibition was recorded after 24 hours and 48 hours. Nystatin 100 unit/disk, fluconazole 10 µg/disk, itraconazole 8µg/disk, ketoconazole 10µg/disk, amphotericinB 20µg/disk, flucytosine 10µg/disk, clotrimazole 10µg/disk, posaconazole 5µg/disk, and voriconazole 1µg/disk were

used (all antifungal disks from Liofilchem Laboratory, Italy). The *C. albicans* strain ATCC 90028, *C. parapsilosis* ATCC 22019 and *C. krusei* ATCC 6258 were used as standard strains.

Zone diameter interpretive standards for Antifungal Disk Diffusion Susceptibility Testing of *Candida spp.* are followed manufacturer' instructions (Liofilchem Laboratories) and are shown in detail in table below.

Table 3.2. Zone diameter interpretive follow Liofilchem Laboratory's instructions

Drug	Code	Potency	Zone in diameter (mm)		
			S	I	R
Amphotericin B	AMB	10 µg	≥15	10-14	No zone
Fluconazole	FLU	25 µg	≥19	15-18 (DD)	14
Flucytosine	AFY	1 µg	≥20	12-19	≤11
Itraconazole	ITC	8 µg	>16	10-15 (DD)	<9
Ketoconazole	KCA	15 µg	>30	23-29 (DD)	<22
Clotrimazole	CLO	50 µg	≥20	12-19	≤11
Voriconazole	VRI	1 µg	≥17	14-16	≤ 13
Nystatin	NY	100 UI	≥15	10-14	No zone
Caspofungin	CAS	5 µg	≥11		≤10

(S: Susceptible, DD: Dose dependent, I: intermediate, R: resistant)

3.6. Detection *Candida* virulence genes by multiplex PCR and sequencing

C. albicans and *C. non albicans* may have different genes according to virulent factors. As *C. albicans* is the most important pathogen in this genus, three significant virulent genes were investigated in this study. These included *hwp1* (*C. albicans* hyphal wall protein 1 HWP1), *sap4* (*C. albicans* secretory aspartyl proteinase SAP4), and *als1* (*C. albicans* agglutinin-like sequence ALS1). DNA for multiplex PCR was extracted as described above.

We designed primers for hwp1, als1, sap4 from reference genes, including U64206.1 *C. albicans* hyphal wall protein 1 (HWP1) gene-completed cds, XM_712961.1 *C .albicans* secretory aspartyl proteinase SAP4p (SAP4) mRNA-

completed cds and L25902 *C.albicans* agglutinin-like sequence (ALS1) gene – completed cds

Primers used for PCR were as follows:

hwp1 5'-TGCTCCAGCCACTGAAACACCA-3'

5'-GGTTTCACCGGCAGGCATGGAT-3'

sap4 5'-TGCCGATGGTTCTGTTGCACAAGG-3'

5 -GAGTCCTGGTGGCTTCGTTGCT-3'

als1 5'-ACCCAACTGTCACCACCACTGA- 3'

5'-TCACCAGGTGGAGCGGTAATGGT-3'

PCR was conducted as follow: 1 µl DNA was added to a 0.2 ml PCR tube containing 0.2 µmol each oligonucleotide primer mix, 0.2 µmol/l each of dNTPs, 1.5 mmol/l MgCl2, 1 U Taq DNA polymerase and 10x buffer in a final volume of 25 µL (all chemicals from Invitrogen, USA). Negative control was performed with sterile deionizer water in place of template DNA.

The mixtures were incubated for 5 min at 95^0C, followed by 30 cycles of amplification. Each cycle consisted of denaturation at 95^0C for 30 seconds, annealing primer at 55^0C for 40 seconds, and primer extension at 72°C for 30 seconds, and then the mixtures were kept at 72^0C for 10 minutes in a thermal cycler (Hybaid, UK). The products were then separated in 1% agarose gel in TAE buffer 1x containing GelRed™ nucleic acid gel stain, Biotium (1 µl /100 ml in TAE 1x) and viewed on a UV trans illuminator. The lengths of amplicons fragment for *hwp1*, *sap4* and *als1* were 274bp, 158bp and 400bp respectively. Amplicon bands were cut from the gel, purified using the Zymoclean™ Gel DNA Recovery Kit (ZYMORESEARCH, USA), quantified and sent for sequencing (http://www.gi.bio.lmu.de/sequencing) as describe above. Sequencing results were blast in GeneBank (https://blast.ncbi.nlm.nih.gov) to confirm the genes identity with match score >99%.

3.7. Detection of *erg11* gene mutations associated to fluconazole resistance in *C. tropicalis* by PCR and sequencing

Azoles are the most common drugs using to treat fungal diseases, and the indiscriminate use of these drugs, especially fluconazole, has leaded to the emergence of resistance in *Candida spp*. Mutations in ERG 11 gene coding for lanosterol 14α-demethylase [76, 125], is the main molecular resistance mechanism to azole in *Candida spp.*, so we decided to apply PCR and sequencing to detect possible polimorfism in ERG11 gene in 15 *C. tropicalis* resistant strains isolates. In addition, one sensitive strain isolate was include in the study and use as control (Strain code number 144).

We designed two pairs of primers on *erg11* reference gene (AY942645.1 *C. tropicalis* strain IHEM 21234-lanosterol 14 alpha demethylase- completed gene).

Primers were as follows:

*erg11*a Fwd 5'-TCTTTTGTCAACACAGTAATGGC-3

Rev -TGGATCAATATCACCGCTTTCTC-3

erg11 b Fwd 5'-GCGGTGATATTGATCCAAAGAG

Rev- GGGATTTTTCTAGCTACTCCATGG

Two separate PCR assays were performed to amplify the completed *erg11* gene. *C. tropicalis* DNA was extracted as previously described [121]. Each PCR reaction was done in a 25 µl volume with 12.5 µl of 2x PCR SuperMix, 0.2 µmol/l of each primer, and 1 µl extracted *C. tropicalis* DNA, and sterile ultrapure water (all chemicals from Invitrogen, USA). Negative controls were performed with sterile deionizer water in place of template DNA.

Target DNAs were amplified in a thermal cycler (Hybaid, UK). The amplication conditions were as follows: initial denaturation at 94^0C for 5 minutes, then followed by 30 cycles, each cycle consisting of 30 seconds at 94^0C for denaturation, 40 seconds at 50^0C for annealing, and 50 secondes at 72^0C for

elongation, and by a final elongation step of 10 minutes at 72^0C. The products were then separated in 1% agarose gel in TAE buffer 1x containing GelRed™ nucleic acid gel stain, Biotium (1 µl /100 ml in TAE 1x), and viewed on a UV trans illuminator. The bands for *erg11*a and *erg11*b were 834 bp and 816 bp respectively.

PCR products were purified, quantified and sent for sequencing as described above. Sequencing was performed in both directions using forward and reverse primers to ensure complete gene sequencing. The obtained nucleotide sequences were analyzed using Geneious Pro 4.8.4, translated into amino acid sequences, aligned and compared with a ERG11 reference sequence (GenBank accession: *C. tropicalis* AY942645). The similarity values between *Candida* reference strains and GenBank sequence was 99%- 100%. The nucleotide changes found in the ERG11 sequences were characterized using the yeast differential genetic code for the CUN codon [126].

3.8. *Candida albicans* Multi Locus Sequence Typing

Fifteen selected *C. albicans* strains isolates were typed by using an MLST. Strains were selected from different sources and different departments, including Internal medicine (n=2), Pediatric (n=2), ICU (n=2), Oncology (n=2), Hematology (n=2), Surgery (n=1), Dermatology (n=1), Obstetric (n=1), Ortohinolaryngology (n=1).

The internal regions of seven housekeeping genes (AAT1a, ACC1, ADP1, MPIb, SYA1, VPS13, and ZWF1b) were amplified using specific sets of primers included in the *C. albicans* MLST scheme (http://pubmlst.org/calbicans) and shown in table below.

Table 3.3. Primers of 7 housekeeping genes (http://calbicans.mlst.net/)

Primers	Housekeeping gene (enzyme)	Gene size (bp)
AAT1aF5'-CTCAAGCTAGATTTTTGGC-3' AAT1aR5'-CAGCAACATGATTAGCCC-3'	Aspartate aminotransferase	478
ACC1F-5'-GCAAGAGAAATTTTAATTCAATG-3' ACC1R5'-TTCATCAACATCATCCAAGTG-3'	Acetyl-coenzyme A carboxylase	519
ADP1F5'-GAGCCAAGTATGAATGATTTG-3' ADP1R5'-TTGATCAACAAACCCGATAAT-3'	ATP-dependent permease	537
MPIbF5'-ACCAGAAATGGCCATTGC-3' MPIbR5'-GCAGCCATGCATTCAATTAT-3'	Mannose phosphate isomerase	486
SYA1F5'-AGAAGAATTGTTGCTGTTACTG-3' SYA1R5'-GTTACCTTTACCACCAGCTTT-3'	Alanyl-RNA synthetase	543
SVP13F5'-TCGTTGAGAGATATTCGACTT-3' SVP13R5'-ACGGATGGATCTCCAGTCC-3'	Vacuolar protein sorting protein	741
ZWF1b F5'-GTTTCATTTGATCCTGAAGC-3' ZWF1bR5'-GCCATTGATAAGTACCTGGAT-3'	Glucose-6-phosphate dehydrogenase	702

PCR was carried out in 25 µl reaction volume containing 12.5 µl 2x PCR SuperMix (Invitrogen), 0.4µmol/l primer mix and 1µl DNA extracted. The mixture was run in a thermal cycler (Hybaid, UK), by following protocol: initial denaturation at 95^0C for 5 minutes, followed by 35 cycles consisting of 40 seconds at 95^0C for denaturation, 40 seconds at 52^0C for annealing, and 45 seconds at 72^0C for elongation; then the mixtures were kept at 72^0C for 10 minutes for final extension. The products were then separated in 1% agarose gel in TAE buffer 1x containing GelRed™ nucleic acid gel stain, Biotium (1 µl /100 ml in TAE 1x), and viewed on a UV trans illuminator. Purified amplicons (DNA Clean and Concentrator ^{TM}-5 (ZYMORESEARCH, USA) were sent for sequencing at LMU, Germany (http://www.gi.bio.lmu.de/sequencing).

The obtained nucleotide sequences were trimmed and analyzed using Geneious Pro 4.8.4, and DSTs were assigned according to the *C. albicans* MLST

database (http://pubmlst.org/calbicans/). Each DST resulted from the combination of the genotypes obtained at the seven loci.

The eBURSTv3 program (http://eburst.mlst.net/) was used to assign isolates to genetic complexes and to evaluate the genetic relationships of the DSTs. Isolates that did not cluster were labeled as singletons.

3.9. Detection *H. pylori* from *Candida spp.* by nested PCR and sequencing

Candida strains isolates from clinical samples were subculture five time on SAD agar with chloraphenicol and penicillin in order to leave out all *H.pylori* that can co-exist with *Candida spp*. from specimens before *Candida* DNA process as describe above.

We used nested PCR to detect *H. pylori* DNA from the DNA extracted from all *Candida spp.*

The specific H.pylori *UreA* gene was detected according with the protocol of Miyabayashi et al using primers HP 64F 5'-TCACCCCAAAAGAGTTAGAC-3' and HP 64R 5'-GAAGTGTGAACCGATTTGAA-3'(428bp)[127]. The second primers pair, HP UreA new F 5'-GTGGAAGCGGTACGTTTGAT- 3' and HP UreA new R 5'-TTCCTGATGGGACCAAACTC-3' (183 bp) were designed by this study using the first amplicons sequence as a template.

In the first nested PCR step, a mixture was prepared in 0.2 ml PCR tube by add 2.5 µl of 10x PCR reaction buffer, 0.2 mmol/l of each of the four dNTPs, 1.5 mmol/l MgCl2, 0.5 µmol/l of primer, 1 µl extracted DNA, 1.25 U Taq polymerase and sterile ultrapure water to have 25 µl final volume (all chemicals from Invitrogen, USA). DNA templates were replaced with sterile deionizer water in negative control and pure *H. pylori* DNA for the positive control. The first PCR conditions was as follows: at 96^0C for 2 minutes, 40 amplification cycles of at 95^0C for 15 seconds, at 53^0C for 15 seconds and primer extension at 72^0C for 30 seconds; and then at 72^0C for 10 minutes.

In the second nested PCR step, the first PCR product was amplified by HP UreA new primers in 0,2 PCR tube containing 2.5 µl of 10x PCR reaction buffer, 0.2 mmol/l of each of the four dNTPs, 1.5 mmol/l MgCl2, 0.4 µmol/l of primer, 1 µl of the first PCR product used as a template, 1.25 U Taq polymerase and sterile ultrapure water. Total volume was adjusted to 25 µl. The second amplification procedure was performed with an initial denaturation (5 minutes, 95^0C), 35 cycles of 15 seconds at 95^0C, 15 seconds at 55^0C for annealing temperatures of primer pair, 30 seconds at 72^0C of primer extension, and a final extension (7 minutes, 72^0C) in a thermal cycler (Hybaid, UK).

The UreA gene sizes of amplicons were determined using 1.5% agarose gel. Specific and nonspecific bands were cut from the gels and purified using a Gel DNA Recovery Kit (ZYMORESEARCH, USA). Purified bands from both first and second amplifications were sent for sequencing (LMU). Obtained sequences were blast with the GenBank database to confirm the precence of *H. pylori* DNA. A result that had greater than 99% similarity was considered to contain H. pylori.

With *Candida* strains presenting *H. pylori*, we also did experiments to culture *H. pylori* from fungal broken cells by selected *H. pylori* specific medium, Columbia blood agar in anaerobic condition. Nested PCR also performed to detect *H. pylori ureA* gene from *Candida* strains were subculture from storage at -80^0C.

3.10. Data analysis

Results were analyzed with Geneious 4.8.4, SPSS 15.0 and Medcal 13.1. A p-values less than 0.05 was considered to be statistically significant.

4. RESULTS

4.1. Study population and *Candida spp.* identification by MALDI-TOF MAS and ITS sequencing

4.1.1. Population distribution by wards, age and gender

Totally, 163 patients were enrolled during the study period October 2012 to August 2015. In Hue University of Medicine and Pharmacy Hospital, most of the patients came from the departments of Internal Medicine, Obstetric, Intensive Care, Dermatology, Surgery, Endoscopy, Oncology and Otorhinolaryngology. In addition, some rare candidiasis cases were investigated in and Pediatric and Ophthalmology wards. 18 patients from Hue Central Hospital conducted to this study came from Hematology and Pediatric wards. The age of the patients ranged from less than one month to 89 years. This study included more number of female patients than male (94 vs 69), and there was statistically significant difference in the distribution of patient isolates by gender (p=0.0078).

The number, rate of patients in each hospital ward, age and gender are shown in Table 4.1.

Table 4.1. Population distribution by wards, age and gender

Hospital ward	Number of patients	Percentage
Internal Medicine*	26	15.95
Obstetric*	24	14.72
Intensive care*	17	10.43
Dermatology*	18	11.04
Surgery*	17	10.43
Endoscopy*	14	8.59
Oncology*	13	7.98
Hematology**	10	6.13
Pediatric*	5	3.07
Pediatric**	6	3.68
Otorhinolaryngology*	11	6.75
Ophthalmology*	2	1.23
Total	**163**	**100**
Age	Mean:45 ± 25 yrs.	Median: 45 yrs.
Gender	Male: 69 (42.33%)	Female: 94 (57.67%)

* Hue University of Medicine and Pharmacy Hospital

** Hue Central Hospital

4.1.2. Distribution of samples source in hospital wards

A total of 177 clinical samples were collected from 163 patients admitted in Hue University of Medicine and Pharmacy Hospitals (145 patients) and Hue Central Hospital (18 patients). Distribution of samples source in 11 hospital wards is shown in Figure 4.1. From Internal Medicine ward, sputum (61.54%), stool (26.92%), bronchoalveolar lavage (3.85%), oral mucosa (3.85%), and urine (3.85 %) samples, were collected. All of 24 samples gathered from Obstetric ward were vaginal secretion (100%). At Dermatology ward, samples were nail (61.11%), skin lesions (22.22%) and oral mucosa (16.67%). At Intensive Care Unit, a high rate of endotracheal aspiration fluid samples (44%), the same rate of gastric drainage fluid and urine samples (20%), 8% of oral mucosa and 4% of both blood and sputum were collected. Specimens from Surgery ward were gastric drainage fluid (70.59%), peritoneal fluid (5.88%), skin wound (5.88%) and sputum (17.65%). From Endoscopy ward 80% were gastric biopsies and 20% were oral mucosa specimens. Oral mucosa and sputum represented 84.62% and 15.38%. of samples from Oncology unit. There were 38.46% oral mucosa and 61.54% urine samples from Hematology unit. Specimens from Pediatric ward were 53.85% for oral mucosa, 30.77% for stool and 15.38% for urine at. At Otorhinolaryngology, source samples were external ear (54.55%), nasal mucosa (9.09%), and sinus (36.36%). We collected two cornea ulcer samples at Ophthalmology ward.

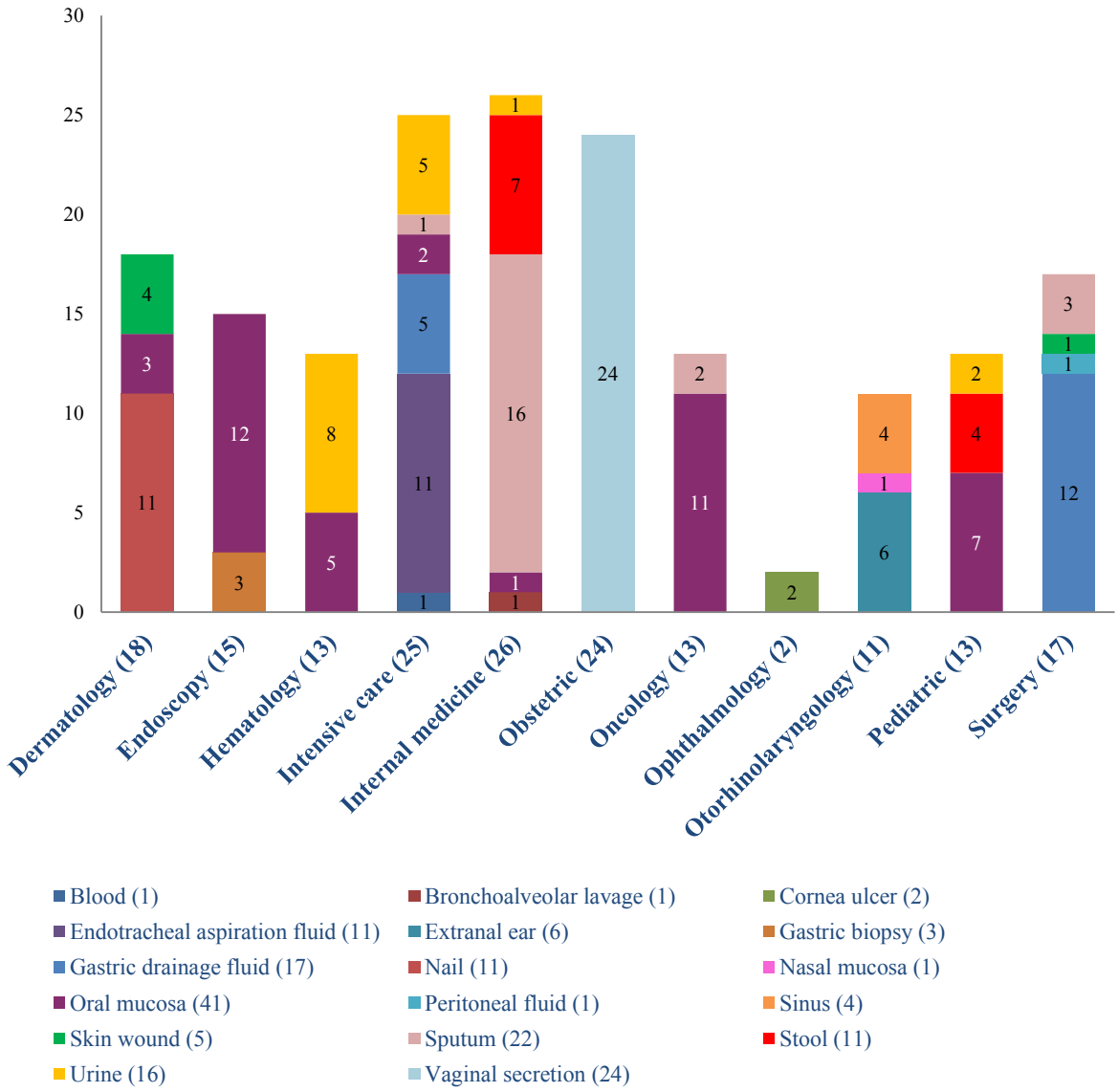

Figure 4.1. Distribution of samples sources by hospital ward

(Absolute number of samples isolated is shown in each column)

4.1.3. *Candida* species identification by MALDI TOF MS and ITS sequencing

In total 196 *Candida* strains were isolated from 177 clinical samples, 94.9% of them were identified by MALDI-TOF (Matrix Assisted Laser Desorption Ionization-Time of Flight) Mass Spectrometry with score ≥1.7.

Ten *Candida spp* having score<1.7 were identidied by ITS sequencing as *C. non albicans*, including *C. parapsilopsis* (2 strains), *C. orthosilopsis* (1 strains), *C. digboiensis* (3 strains), *C. famata* (2 strains), *C. mesorugosa* (1 strains) and *C. blankii* (1 strains). Molecular identification details are shown in Figure 4.2 and Table 4.2.

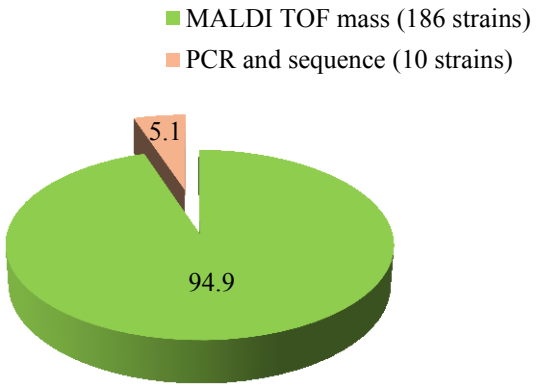

Figure 4.2. *Candida* identification result by MALDI -TOF MS and ITS sequence

Table 4.2. *Candida spp.* identification

Candida spp.	**Number of strain isolates**	**Percentage**
Candida albicans	94	47.96
Candida non albicans	102	52.04
C.tropicalis	32	16.33
C.glabrata	17	8.67
C.parapsilosis	21	10.71
C.orthopsilopsis	11	5.61
C.metapsilosis	3	1.53
C.krusei	7	3.57
C.guilliermondii	3	1.53
C.digboiensis	3	1.53
C.famata	2	1.02
C.mesorugosa	1	0.51
C.norgvebsis	1	0.51
C.blankii	1	0.51

Although *C. non albicans* had high rate than *C. albicans* (52.04% vs 47.96%), the incidence rate difference was not significant (p=0.567).

4.1.4. Distribution of *Candida spp.* in hospital wards

There was diversity in *Candida* species distribution in the wards, in particular in Internal Medicine, Dermatology, and Pediatric. Overall, *C. albicans* was the dominant species in almost all hospital wards, with the exception of Ophthalmology and Otorhinolaryngology

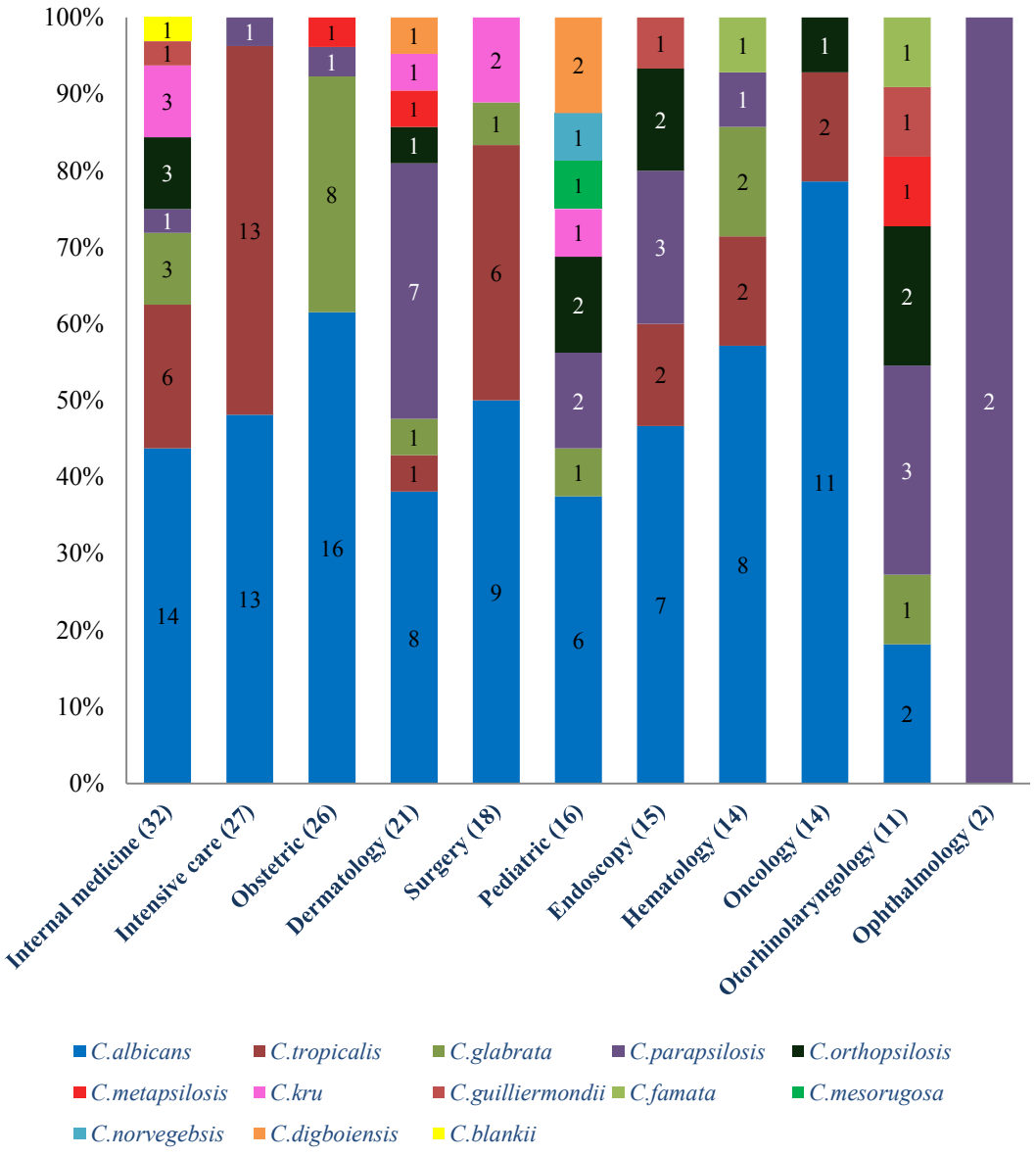

Figure 4.3. Distribution *Candida spp.* in hospital wards

(Absolute number of *Candida* strains isolated is shown in each column)

4.1.5. Distribution of *C. albicans* and *C. non albicans* in candida colonization and candidiasis

Patients status was classified in four groups depending on whether they showed candida colonization, cutaneous candidiasis, mucosal candidiasis, or system candidiasis. Patients with candida colonization (85 patients) were defined if *Candida spp.* were isolated from unsterile body sites (urine, sputum, stool, gastric, endotracheal aspiration, external ear, bronchoalveolar), they didn't have any symptom of candidiasis and they recovered without antifungal therapy. Mucosal candidiasis patients (73 patients) included oral candidiasis, vaginal candidiasis, nasal candidiasis, sinus candidiasis, cornea candidiasis. Onychomycosis and skin candidiasis patients belong to cutaneous candidiasis (20 patients). Systemic candidiasis (18 patients) included candidemia, peritoneal candidiasis, rhinosinusitis, pulmonary candidiasis. There was a different significant presence of *C. albicans* and *C. non albicans* in colonization and mucosal candidiasis, with high proportion of *C. non albicans* in candida colonization (p value was 0.0186), and contrast of *C. albicans* in mucosal candidiasis (p value was 0.0019). The result is shown in figure below.

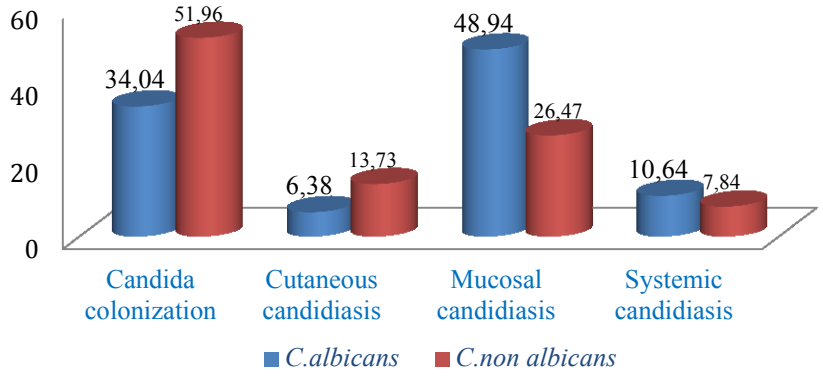

Figure 4.4. Distribution of *Candida spp.* in colonization and candidiasis

4.2. Phenotypic and genotypic characterization of *Candida spp.* isolates

4.2.1.In vitro antifungal susceptibility testing

Antifungal susceptibility of *Candida spp.* by CLSI disk diffusion method (n=178)

Nine drugs testing by CLSI disk diffusion method were amphotericine B, nystatin, fluconazole, ketoconazole, clotrimazole, voriconazole, itraconazole, 5-fluorocystocine, and caspofungin. The percentage of resistance for each drug by *Candida spp* is described in Table 4.3

Table 4.3. Antifungal susceptibility test by disk diffusion

Drugs	Resistant (%)	Intermediate (%)	Susceptible (%)
AmphotericinB	0	8.99	91.01
Nystatin (100UI/ml)	0	0	100
Fluconazole (25µg/ml)	23.60	5.05	71.35
Itraconazole (8µg/ml)	24.16	15.73	60.11
Voriconazole (1µg/ml)	23.03	3.94	73.03
Clotrimazole (50µg/ml)	2.25	3.37	94.38
Ketoconazole (15µg/ml)	1.12	1.69	97.19
5-Fluorocystocine	19.66	5.06	75.28
Caspofungin (5µg/ml)	10.67		89.33

Trend of Polyene drugs resistance

There wasn't any resistance to polyene group drugs in this study. 100% *Candida* strain isolates were sensitive to nystatin. The susceptible rate to amphotericine B was 91.09%. Some strains were intermediate for amphotericine B, including *C. tropicalis* (13.33%), *C.parapsilosis* 4.67%, *C. krusei* (50%), *C. digboiensis* (33.33%), *C. mesorugosa* (100%).

Trend of azole resistance

Table 4.4 show the frequency of *Candida spp* resistance to azole group drugs.

Table 4.4. Frequency *Candida* species resistance to azole group drugs (%)

Candida species (no of strains testing)	Fluconazole	Itraconazole	Voriconazole	Clotrimazole	Ketoconazole
C.albicans (83)	2.41	3.61	4.82	1.20	0
C.tropicalias (30)	56.67	56.67	70	10.00	3.33
C.glabrata (15)	6.67	53.33	26.67	0	6.67
C.parapsilosis (21)	4.76	4.76	4.76	0	0
C.orthosilosis (11)	90.91	81.82	63.64	0	0
C.metasilosis (2)	50	0	0	0	0
C.krusei (6)	100	66.67	66.67	0	0
C.famata (2)	0	0	0	0	0
C.norgvebsis (1)	0	0	0	0	0
C.digboienis (3)	66.67	33.33	0	0	0
C.blankii (1)	0	0	0	0	0
C.guilliermondii (2)	50	0	0	0	0
C.mesorugosa (1)	100	0	0	0	0
Total (178)	**42**	**43**	**41**	**4**	**2**

Higher rate resistance to triazole (Fluconazole, itraconazole and voriconazole) than imidazole (Ketoconazole, clotrimazole) were found in *C. tropicalis*, *C. orthopsilosis*, *C. krusei*, *C. digboiensis*, *C. guilliermondii* and *C. mesorugosa.*

There was also different level of resistance to at least one type of azole drug in *C. albicans*, *C. tropicalis*, *C. glabrata*, *C. parapsilosis*, *C. orthopsilosis*, *C. metapsilosis*, *C. krusei*, *C. digboiensis*, *C. guilliermondii*, *C. mesorugosa.*

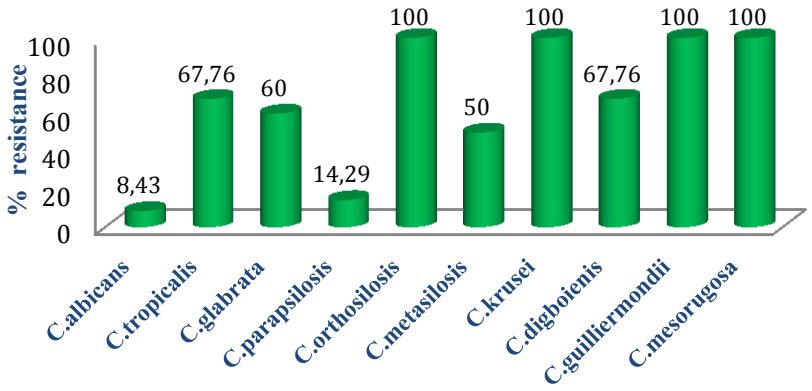

Figure 4.5. Resistance to at least one type of azole drug

Table 4.5. Cross - resistance to multiple azole drugs

Candida sp.	Fluconazole vs Itraconazole (%)	Fluconazole vs Voriconazole (%)	Fluconazole vs Itraconazole vs Voriconazole (%)
C.glabrata	6.67	6.67	6.67
C.krusei	66.67	66.67	66.67
C.orthopsilosis	72.72	63.63	54.54
C.tropicalis	50	46.66	53.33

A significant resistance to the two out or the three azoles tested was found in *C. tropicalis, C. krusei*, *C. orthopsilosis*.

Trend of 5-Fluorocytosine resistance

In this study 5-Fluorocytosine resistance occurred in *C. albicans* (19.28%), *C. tropicalis* (16.67%), *C. parapsilosis* (19.05%) (Figure 4.6). Although *C. krusei*, *C. norgvebsis* and *C. blankii* showed 100% resistance to 5-fluorocytosine, the small number of strains analyzed, to confirm the high resistance in these species, further study requires.

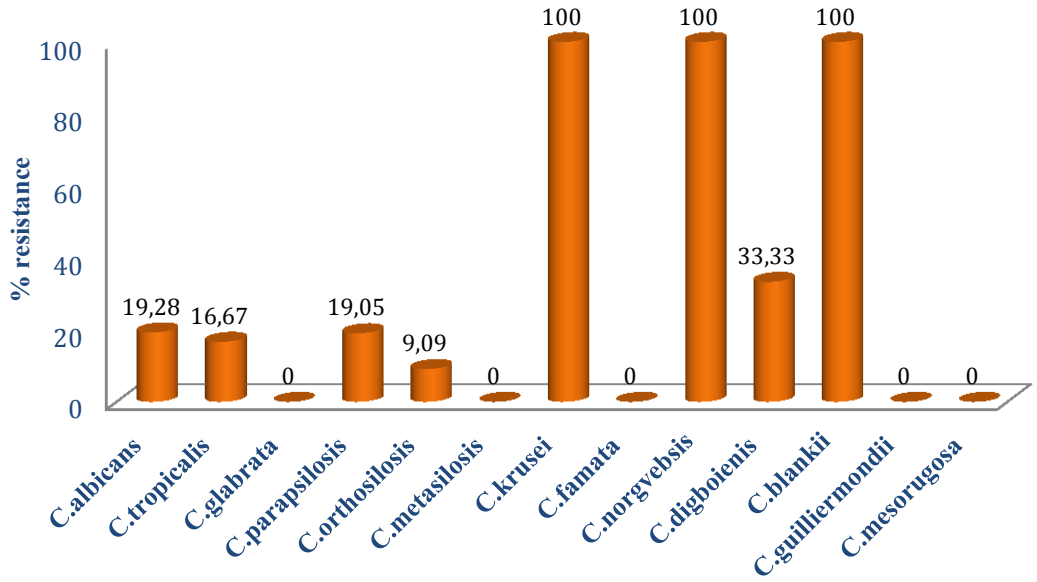

Figure 4.6. Frequency *Candida spp.* resistance to 5-Fluorocystocine

Trend of echinocandine resistance

Among *Candida* species isolates, *C. albicans* had higher percentage resistance to caspofungine than *C. tropicalis*, *C. parapsilosis*, but the difference is not statistically significant (p>0.05).

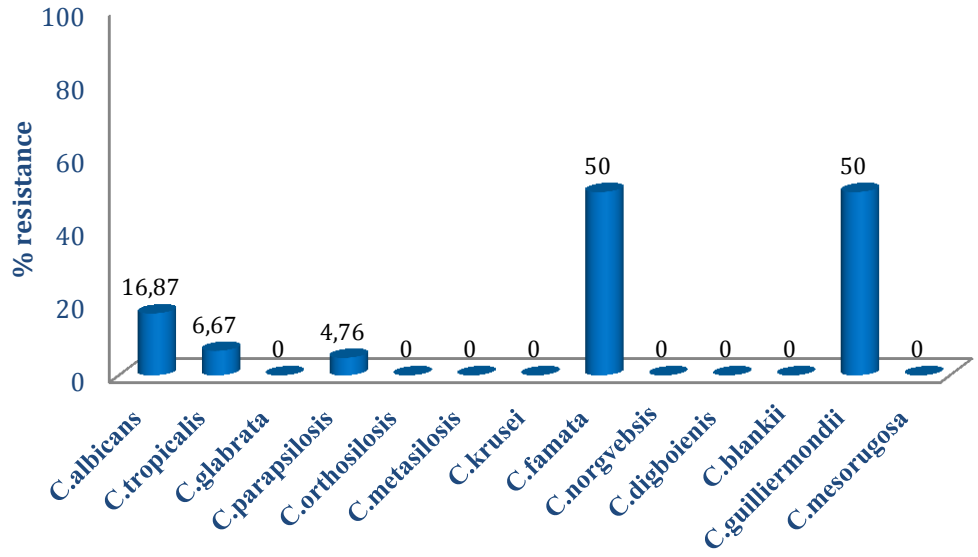

Figure 4.7. Frequency *Candida spp.* resistance to caspofungin

Multiple drugs resistance (MDR)

In 178 strains checked for antifungal testing, one *C. tropicalis* strain was MDR. This strain was detected from urine of female patient 91 years old hospitalized at Intensive Care Unit by urine infection. Antifungal testing shown that this strain was resistant to fluconazole, itraconazole, voriconazole, 5-fluorocytosine and caspofungine.

4.2.2. Mutations in ERG11 protein associated to fluconazole resistance of *C. tropicalis*

We detected two mutations in the *erg11* gene (A395T and C461T), that correspond to Y132F and S154F aminoacids substitution in the ERG11 protein

(Table 4.6 and Figure 4.8). The rate of these mutations was 26.67% among all *C. tropicalis* fluconazole resistant isolates.

Table 4.6. *erg11* gene mutations in 15 *C.tropicalis* strains

Strain code	Nucleotide mutations								Meaning
M23673	867	906	1281	1037	1103	1423	G2004	T2196	Reference Strain
144 (sensitive strain)							G1362R	T2196Y	Silent mutations
5A	T225Y	G264R	C639Y				G1362A	T1554C	Silent mutations
38A	T225Y	G264R	C639Y				G1362A	T1554C	Silent mutations
39							G1365R	T1554C	Silent mutations
41	T225C	G264A		A395T (Y132F)	C461T (S154F)		G1362A	T1554C	Missense Mutations
58							G1362A	T1554C	Silent mutations
66						T781A	G1362R	T2196Y	Silent mutations
189	T225C	G264A		A395T (Y132F)	C461T (S154F)		G1362A	T1554C	Missense mutations
70	T225Y	G264R					G1362A	T1554C	Silent mutations
88	T225Y	G264R	C639Y				G1362A	T1554C	Silent mutations
95							G1362R		Silent mutations
112	T225C	G264A		A395T (Y132F)	C461T (S154F)		G1362A	T1554C	Missense mutations
114							G1362R	T2196Y	Silent mutations
150							G1362R	T2196Y	Silent mutations
152	T225Y	G264R	C639Y				G1362A	T1554C	Silent mutations
193	T225Y	G264R		A395W (Y132F)	C461Y (S154F)		G1362A	T1554C	Missense mutations

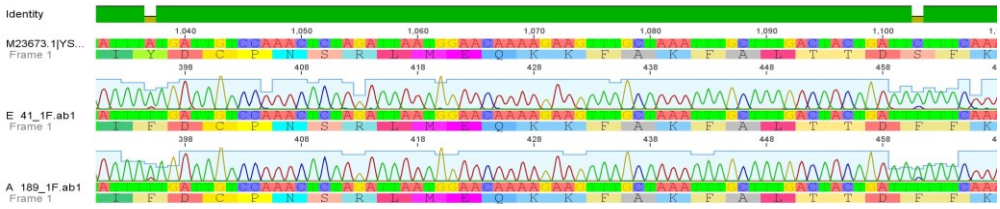

Figure 4.8. ERG11 missense mutations in *C. tropicalis* strain no. 41 and no.189

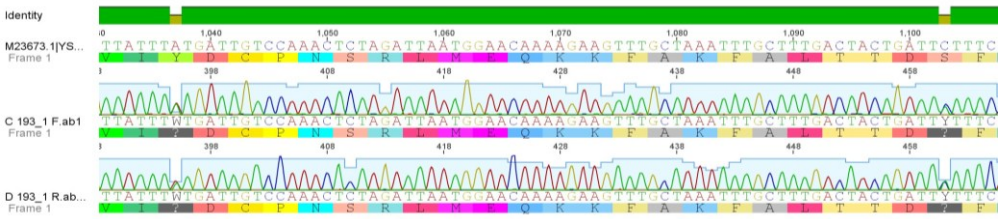

Figure 4.9. ERG11 silent mutations in *C. tropicalis* strain no. 193

4.2.3. *Candida* virulence genes (*als1, hwp1, sap4*)

We performed multiplex PCR to detect the *als1, hwp1, sap4* virulence genes presence in all *Candida* isolates. Overall, 96.8 % of C. albicans isolates carried all 3 genes (Figure 4.10), regardless of the sample source. Some C. albicans isolated from vaginal secretion and endoscopic aspiration fluid didn't carry the *als1* gene. None of these genes was amplified in other *Candida spp*. and none of the unspecific amplicons from other *Candida spp*. checked by sequencing, had any homology whith the virulence genes.

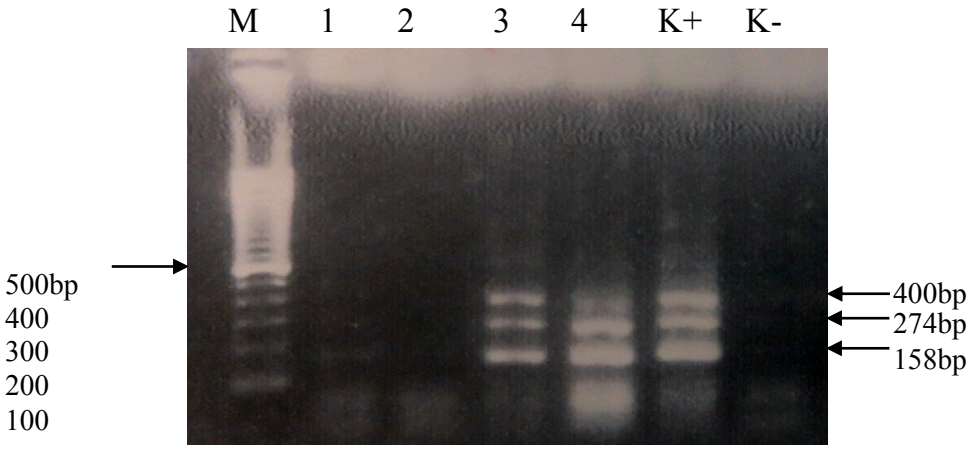

Figure 4.10. *C. albicans* virulent genes: *sap4*, *hwp1* and *als1* gene amplification products in positive samples

(*sap4*:158bp, *hwp1*: 274bp, *als1*: 400bp, M: molecular size markers)

Virulence genes percentage in 94 *C.albicans* strains isolated from different source are shown below in Table 4.7

Table 4.7. Source of *C. albicans* strains detecting virulent genes

Source	n	ALS1 (%)	HWP1 (%)	SAP4 (%)
Oral	30	100	100	100
Vaginal secretion	16	93.75	100	100
Sputum	12	100	100	100
Gastric drainage fluid	9	100	100	100
End. aspiration fluid	6	83.33	100	100
Urine	5	100	100	100
Stool	5	100	100	100
Nail	3	100	100	100
Skin wound	3	100	100	100
Gastric biopsy	1	100	100	100
External ear	1	0	0	100
Bronchoalveolar lavage	1	100	100	100
Peritoneal fluid	1	100	100	100
Sinus secretion	1	100	100	100
Total	**94**	**96.81**	**98.94**	**100**

4.2.4. *Candida albicans* Multi Locus Sequence Typing

Table 4.8 shows DTSs assigned according to the *C. albicans* MLST database (http://pubmlst.org/calbicans/)

Table 4.8. The DST assignments at each of seven MLST loci for all 15 *C. albicans* isolates

Strain code	Unit	Source	AAT1a	ACC1	ADP1	MPIb	SYA1	VPS13	ZWP1b	DST	CC
104	Oto	Sinus	4	4	6	6	96	111	15	2932**	
26	Obs	vaginal	4	4	6	6	96	111	15	2932**	
49	ICU	Oral	5	4	6	3	93	189	22	2933**	
69	Hem	Oral	5	5	5	27	2	6	146	2934**	
108	Onc	Oral	5	5	5	4	2	6	5	2445	69
89	ICU	intertrachea	59	5	21	21	80	108	15	2937**	
60	Ped	Oral	59	5	21	21	80	108	15	2937**	
17	Ped	Stool	1	7	15	6	61	105	112	693	693
19A	Int med	Sputum	4	17	21	19	27	83	22	299	299
91	Int med	Stool	4	17	21	19	27	13	22	459	299
179	Sur	Gastric	8	29	4	4	207*	279	266*	2936***	
5	Onc	Oral	8	29	4	4	207*	279	266*	2936***	
75	Hem	Oral	5	32	21	34	7	55	5	732	
120	End	gastric	47	35	4	21	74	118	105	2477	1698
32	Der	skin wound	5	78	5	9	2	6	5	2935**	

(Oto: Otorhinolaryngology, Obs: Obstetric, ICU: Intensive Care Unit, Hem: Hematology, Onc: Oncology, Ped: Pediatric, Int med: Internal Medicine, Sur:

Surgery, End: Endoscopy, Der: Dermatology)

* New allele detected from this study

** New combination detected from this study

*** New DSTs identified in this study.

A total 54 variable loci were identified among 15 *C. albicans* clinical isolates, representing 1.83% (54/2.937) of the total nucleotides. A total of 12 diploid sequence types (DSTs) were identified from 10 different wards. Six were new DSTs, 5 of which (DTSs 2932, 2933, 2934, 2935 and DTS 2937) derived from new combination of alleles already present in database and 1 (DTS 2936) with two new alleles assigned us from MLST database (SYA1=207, ZWP1b=266).

Loss of heterozygosity was observed in VPS13 sequences (Figure 4.11) obtained from two sequential isolates from two patients from Internal medicine wards (strain no.19A and no. 91).

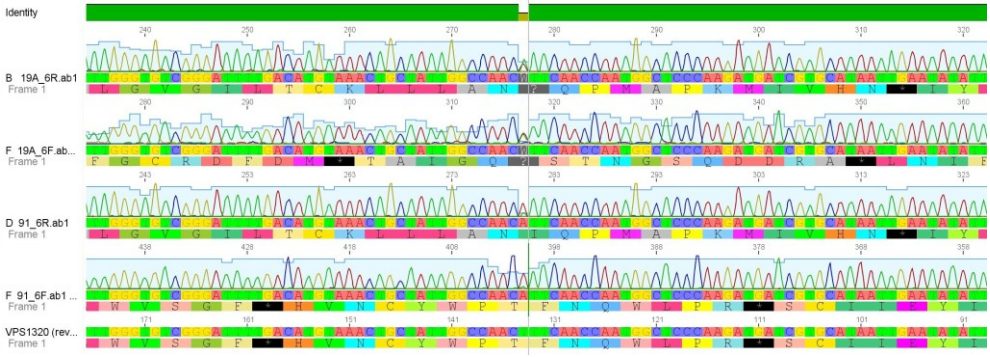

Figure 4.11. Loss of heterozygosity in loci VPS13 (W to A) in strain no. 19A gave origin to strain no. 91

We detected the same DTS clone from different wards: DTS 2933 (Obstetric and Otorhinophagology), DTS 2936 (Surgery and Oncology), DTS 2937 (Intensive Care unit and Pediatric).

E.Burst results

MLST results were analyzed by eBURSTv.3 to assign isolates to genetic complexes and to evaluate the genetic relationships of the DSTs. Comparative

eBURST analysis of the 12 DTSs identified in this study, compared with the 2937 *C. albicans* distinct DSTs presented in the MLST database (www.calbicans.mlst.net at the 30. 09. 2015) are showed in Figure 4.12. Results showed that 2 clones (DST 2445 and DST 2935) belong to the largest Clonal Cluster 1 (C1), a common CC reported from worldwide [61], based on DST 69 as the putative predictor founder. The DTS299 and DST459, from which it derives for loss of homozygosis in the VPS13 gene, belong to C6, and DTS459 was previously isolated in China [69]. DST 723 belong to the C12 previously found in South Korea [68]. DST 693 belonging to the C21 was previously reported in Taiwan and South Korea [128]. New potential nosocomial clones DST 2937, DST2932 belong to the C14, C22, respectively with the respectively predicted founders DST 443 and DST 768 isolated previously in China and Japan, while the DST 2936 is a singleton together with DTSs 2933, 2934 and 2477.

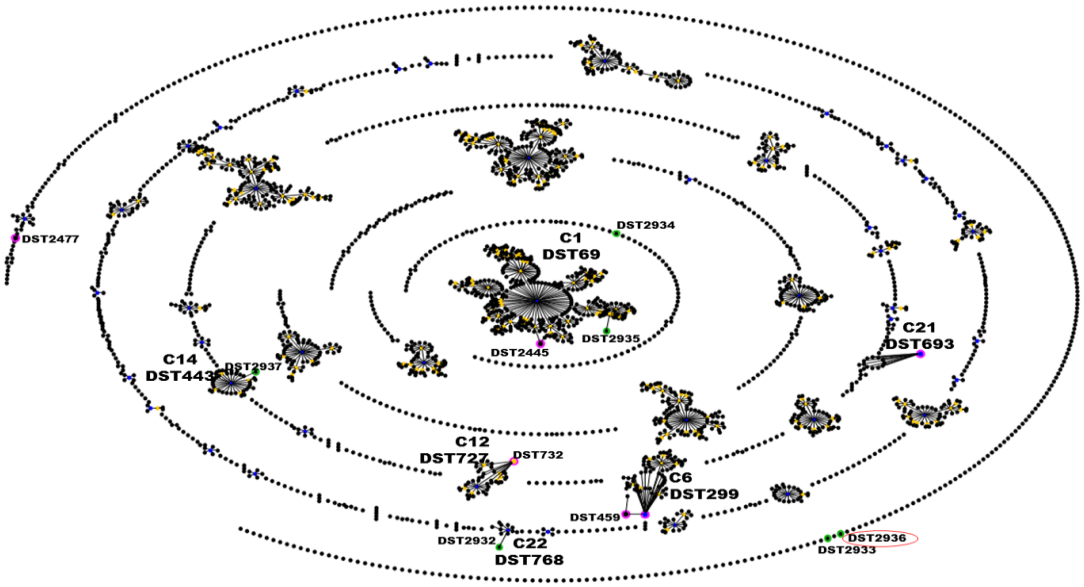

Figure 4.12. Population snapshot of the 2973 C. albicans distinct DSTs currently in the MLST database (www.calbicans.mlst.net) defined using eBURSTv3.

In the snapshot a single line joins DSTs that differ by only one of the seven loci. The putative founding DST of the clones founded in our hospital and the MLST clade (C) (defined by Odds et al., 2007; Shin et al., 2011) to which these

DST belongs are indicated beside each. Predicted Founder DSTs for all CC are indicated in blue, and founders of sub-groups are indicated in yellow. Pink circle represent DTS founded in our hospital and already present in database, green circle represent new DTS derived from new allele combination while the encircled one is a new DTS with 2 new alleles(SYA1=207, ZWP1b=266). Single black dots represent singletons.

Other Phylogenetic analysis, unweighted-pair group method with arithmetic mean (UPGMA), from concatenated sequences of the 7 MLST alleles of the whole DSTs database clusterized DST into specific Clades, Table 4.9 show the Clades (deduced from Odds et al 2007, Wang et al 2015 Shin et al 2011) to which correspond the specific Clonal Cluster obtained by E.burst in this study

Table 4.9. Clades corresponding to the clonal Cluster by E.burst and UPGMA

Strain Code	Unit	DST	Eburst	Clade
32	Der	2935	C1	1
108	Onc	2445	C1	1
17	Ped	693	C21	3
26	Obs	2932	C22	5
104	Oto	2932	C22	5
120	End	2477	SINGLETON	8
19A	Int med	299	C6	12
91	Int med	459	C6	12
89	ICU	2937	C14	17
60	Ped	2937	C14	17
49	ICU	2933	SINGLETON	New
5	Onc	2936	SINGLETON	New
179	Sur	2936	SINGLETON	New
69	Hem	2934	SINGLETON	New
75	Hem	732	C12	New Korea clade

4.3. Detection *H. pylori* from *Candida spp.* by nested PCR and sequencing

4.3.1. Presence of *H. pylori* in *Candida spp.*

Almost 15.27% of the *Candida spp.* tested for the presence of *H.pylori ureaA* specific gene showed positivity at first or second step of nested PCR. Sequencing of specific bands obtained from the first and second PCR amplifications, and also of unspecific bands obtained from the second PCR amplifications (Figure 4.13 and 4.14) were identified as *H. pylori* by http://blast.ncbi.nlm.nih.gov/Blast.cgi (99% identity with *H. pylori* urease alpha subunit (*ureA*) strain G27 Genebank AM997161)

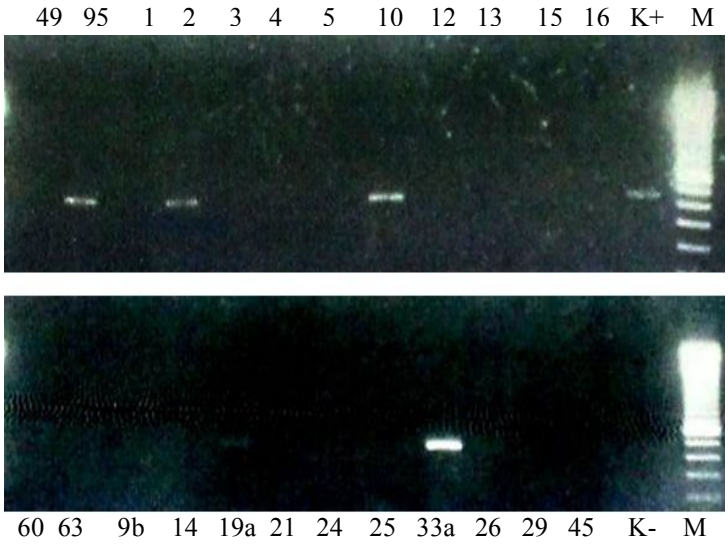

Figure 4.13. Detection of *ureA* gene (408bp) in first amplicons

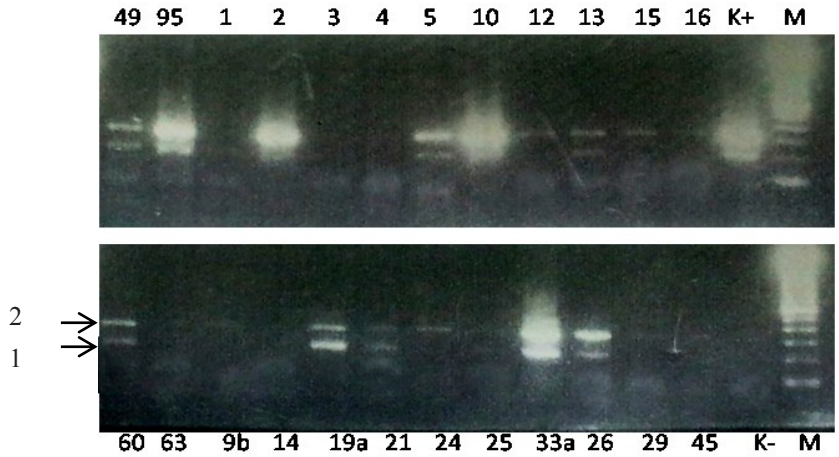

Figure 4.14. (1) Specific band (183bp) and (2) unspecific band (300 bp) from second amplicons

4.3.2. Distribution of *Candida spp.* in *H. pylori* DNA (+)

In particular *H. pylori* DNA was amplified from three *Candida* species, including *C. albicans*, *C. tropicalis* and *C. orthopsilosis*. (Figure 4.15).

Despite the higher *H. pylori* frequency, in *C. albicans* compared the others species, this difference was not significant (p>0.05). In addition, in this study we didn't find any correlation between *H. pylori* presence and *C. albicans* virulence genes presence *H. pylori* was detected in *Candida spp.* isolated from gastric drainage fluid, oral mucosa and vaginal secretion.

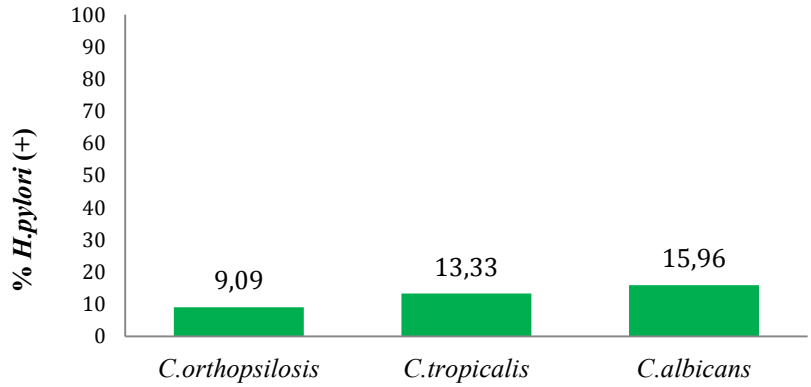

Figure 4.15. *Candida spp*. distribution in *H. pylori* presence

Table 4.10. Sources of samples used to detect *H. pylori* DNA

Sample sources (number of strain)	*H. pylori (+)*	%
Endotracheal aspiration fluid (11)	0	0
Gastric biopsy (3)	0	0
Gastric drainage fluid (17)	2	11.76
Oral mucosa (43)	11	25.58
Sputum (22)	5	22.73
Stool (11)	0	0
Vaginal secretion (24)	2	8.33
Total (131)	**20**	**15.27**

Regarding the two *C. albicans* nosocomial strains (DST 2936 as detected by MLST) only one positive for *H. pylori* (strain no.5 from Oncology, while strain no.179 from Surgery did not).

Additionally, our experiments showed that *H. pylori* could not be cultured from *Candida* broken cell by cultivation on Columbia blood agar in anaerobic condition and its Dna was degradable, expecially after storage period of *Candida* strains in 15%glycerol at -80^0C.

5. DISCUSSION

During the three year study period, 196 *Candida spp.* strains were isolated from 163 patients that attended Hue University of Medicine and Pharmacy Hospital and Hue Central Hospital.

5.1. *Candida* species identification by MALDI - TOF MS and ITS sequencing

In our study, 186 *Candida* strains isolates (94.9%) were identified by MALDI -TOF MAS. This result demonstrated reliability of this technique to identify yeasts from plate culture. In addition, this method was also able to discriminate between closed species (e.g. species in *C. parapsilosis complex* group or species in *C. rugosa complex* group), which cannot be differentiated by phenotypic identification [129]. Several previous studies have concluded that MALDI - TOF MS is a rapid and reliable technique to identify yeast species [105, 130-132].

Although MALDI-TOF MS has been shown to discriminate between closely related *Candida* species by certain studies [130], such as *C. parapsilosis*, *C. orthopsilosis* and *C. metapsilosis*, other studies have shown that this method is not always reliable. For example, De Carolis et al showed that this method can not distinguish beetwen *C. parapsilosis* and *C. orthopsilosis* [133]. Pulcaro et al showed that MALDI-TOF MS was unrealiable for the identification *C. glabrata* and *C. parapsilosis* from bood culture [105]. The inability of this method to identify cetain species may be due to the limitation of the database. In other words, the database may not account for the biological variability of these yeasts, even for a single species. A more extensive database would help solve this problem [130]. By MALDI-TOF MS, a number of species had an indentification score that was less than 1.7 in the present study. These included *C. parapsilosis*, *C. orthopsilosis*, *C. digboiensis*, *C. famata*, *C. mesorugosa*, *C. blankii*. Follow-up identification was achieved by PCR and sequencing. In study by Mancini N. et al demonstrated that

MALDI TOF MS identification of *Candida spp.* isolates in human by Bruker Biotyper system could not identify *C. blankii* and *C. orthopsilosis* [134]

Regarding the distribution of *Candida* species in the present study, *C. albicans* was the dominant species (47.96%), followed by *C. tropicalis* (16.33%), *C. parapsilosis* (10.71%), *C. glabrata* (8.67%), *C. orthopsilosis* (5.61%), *C. krusei* (3.57%), *C. guilliermondii* (1.53%), *C. digboiensis* (1.53%), *C. mesorugosa* (0.51%). *C. norgvebsis* (0.51%), and *C. blankii* (0.51%). Generally, the percentage of *C. non albicans* (52.04%) was higher than *C. albicans* (47.96%), but this difference was not significant ($p = 0.567$). The five common pathogenic species in this study was similar to results from other studies [7, 135, 136]. Globally, the most frequently *C. non albicans* species in Northern Europe and the USA was *C. glabrata*[9], in Italy, Spain and Brazil was *C. parapsilosis* [9, 135], and in Asian countries was *C. tropicalis* [137]. Althought the proportion of *C. tropicalis* was higher than others *C. non albicans* species in Asian surveillance study, this species was more likely to be isolated from tropical countries than other Asian countries [137]. For example, an investigation by Mahmoudi R. et al in Iran about candidiasis, revealed that the percentage if *C. albicans*, *C. glabrata*, *C. tropicalis*, *C. krusei*, *C. parapsilosis* are 67%, 18.3%, 6.8%, 5.8%, 1.6%, respectively [136]. A study by Zang L. et al in China showed five common *Candida* species were *C. albicans* (65.7%), *C. glabrata* (20.3%), C. tropicalis (8.8%), (3%) and (1.4%) [85]. In the meanwhile, Boonyasiri A. et al reported four popular *Candida* species isolated from candidemia in Thailand including *C. albicans* (39%), *C. tropicalis* (28%), *C.glabrata* (22%) and *C. parapsilosis* (6%) [138]. Ding CH. et al determined that the four common species from candiurine in Malaysia were *C. albicans* (59.4%), *C. tropicalis* (28.1%), *C. glabrata* (9.4%) and *C. parapsilosis* (3.1%) [139]. A study by Tran et al in Vietnam (2010) indicated *C. tropicalis* was more popular than others species in *C. non albicans* group [106] Therefore, our result above suggested that *Candida* distribution in Vietnam is similar to Southeast Asian Nations with higher rate of *C. tropicalis* isolates than *C. glabrata* or *C. parapsilosis*.

Although *C. albicans* is the most common species in this genus, the rate of infections caused by *C. non albicans* species is increasing [140]. Results from the ARTEMIS DISK Global Antifungal Surveillance Study, occuring in 41 countries, showed a decreasing rate of isolation of *C. albicans* (70.9% to 65.0%), while the number of isolates containg *C. non albicans* were increasing: *C. glabrata* (10.2% to 11.7%), *C. tropicalis* (5.4% to 8.0%), and *C. parapsilosis* (4.8% to 5.6%) in time period 1997 - 2000 compared to 2005 - 2007 [7].

Our study also showed a variety in *Candida* species distribution in the wards. For example, Internal Medicine, Dermatology, and Pediatric wards, each contain 8 different species in total 11 species isolated. Overall, *C. albicans* was the predominant species in almost all hospital wards, with the exception of Ophthalmology and Otorhinolaryngology wards.

In candida colonization and cutaneous candidiassis the percentage of *C. non albicans* were higher than *C. albicans*. In contrast, in mucosal and systemic diseases, more *C. albicans* were isolated than *C. non albicans*. It seems that *C. albicans* has more virulent and damaging in mucosal candidiassis and are more invasive than other species. Batista G.C et al indicated that *C. albicans* was the most common species in oral and could be the causitive agent of invasive candidiasis in neonatal patients [141]. In study by Deorukhkar S. C et al (2014), it was demonstrated that *C. albicans* expressed more biofilm formation, coagulase production, hemolysin production, phospholipase production, and proteinase production than *C. non albicans* [142], meanwhile an investigation by Udayalaxmi J. S and D'Souza D indicated that phospholipase production was better in *C. albicans* than compared to other species of *Candid*a, and the difference in hemolysin and biofilm production between *C. albicans* and *C. non albicans* was not statistically significant [143].

5.2. Phenotypic and genotypic characterization of *Candida spp.* isolates

5.2.1. In vitro susceptibility testing

Antifungal susceptibility testing is required in antifungal therapy as well as in monitoring antifungal resistance. Many studies have reported an increasing number of resistant *Candida spp.* [7, 144, 145]. In the present study we followed CLSI disk diffusion method to testing antifungal resistance. Our results show that the percentage of *Candida spp.* that showed resistance to fluconazole, itraconazole, voriconazole, and 5-fluorocystocine were 23.60%, 24.16%, 23.03%, and 19.66%, respectively.

When examing resistant trends to azole drugs by species in this study, there was a higher rate in *C. non albicans* than *C. albicans* (Table 4.4). This result is similar to other studies [146-148]. We recognized that *C. non albicans* was more resistant to triazole compounds (Fluconazole, itraconazole and voriconazole) than to imidazole (Ketoconazole and clotrimazole), which could be explained by the widespread use of fluconazole in prophylaxis for immunocompromised patients or for critical patients in Vietnam. Our study also observed a high rate in cross resistance for triazole group, especially in *C. tropicalis*, *C. krusei* and *C. orthosilopsis*.

5.2.2. Mutations in ERG11 protein associated to fluconazole resistance of *C. tropicalis*

C. tropicalis has increased dramatically in emerging pathogenic yeast, due to its developed resistance to fluconazole [149]. Overall, the rate of *C. tropicalis* is third to fourth highest among the most common species in *Candida* genus [85, 150] Meanwhile, some reports in the Asia-Pacific region concluded that it is actually the first or second most common isolated species [40, 149]. In addition, *C. tropicalis* is associated with the development of resistance to antifungal therapy, resulting in higher mortality compared to other *Candida* species [151]

In our study, antifungal susceptibility testing for *C. tropicalis* showed strong resistance to fluconazole (56.67%). One mechanism of acquired resistance to azole

in *C. tropicalis* is caused by a mutation in the *erg11* gene. Two mutations in the *erg11* gene (A395T and C461T) were determined in this present study, which corresponded to Y132F and S154F aminoacids substitution in the ERG11 protein. These missense mutations are associated to fluconazole resistance in *C. tropicalis* as previously described by other authors [152, 153]

In total 15 strains of *C. tropicalis*, resistance to fluconazole were checked by indentifying mutations in ERG11 protein. The rate of missense mutations was found to be 26.67%. Mechanisms related to fluconazole resistance in other strains without protein mutations, could be associated with other mechanisms, such as overpression of this gene combine with mutations [125], or the upregulation of two multidrug efflux transporter genes, MDR1 and CDR1[153]

5.2.3. *Candida spp.* virulence genes (*als1, hwp1, sap4*)

Three virulent genes (*asl1, hwp1* and *sap4*) were checked for *Candida* isolates. The result showed that only *C. albicans* expressed these genes, and high proportion of *C. albicans* isolates carried all 3 genes (96.8%). A study conducted by Nas T. et al (2008) showed that the frequence of *asl1, hwp1* and *sap4* in *C.albicans* strains isolated from vaginitis in Turkey was detected as 69%, 62%, and 38%, respectively [154]. Monroy-Perez E. et al determined the frequency of *als1* and *hwp1* in *C.albicans* isolated from Mexican vaginal candidosis and showed that the percent of strains having *als1* and *hwp1* were 100% ad 92%, respectively [155].

Various studies indicated *C. albicans* is often more virulent than other species [156-158]. As a consequence, higher rate of virulence factors in *C. albicans* than other species indicates that the virulence factors play a crucial role in the pathogenesis. This is help to explain why *C. albicans* was more isolates from mucosal candidiasis and systemic candidiasis in the current study as mentioned above.

An initial ability adheren to host tissue is one of the most mechamism to infection. *Hwp1* and *als* genes play an important to adhenre host cells [23, 55]. The

als gene family encodes a cell surface protein that mediates adherence of *C. albicans* to endothelial cells [159]. The *als* gene family have different level expression in clinical samples. *Als1, als2, als3*, and *als9* are most commonly expressed, followed by *als6* and *als7*. The expression of *als4* and *als5* are the least common and usualy in clinical vaginal specimens [160]. Secreted aspartyl proteinase (SAP), an extracellular hydrolytic enzymes, is an another *Candida spp.* virulent factor. This enzymes contribute to host tissue invasion by digesting proteins, and it also plays in adhesion process. *SAPs* genes has been suggested to contribute to tissue invasion of *C. albicans*, particular *sap 4-6* [161]. Furthermore, *sap4-6* and *hwp1* are combine expression in hyphae form as these genes are specific genes of this form [161], and *ALS*s genes in *C. albicans* tend to be located on chromosomes that also encode genes from the *SAPs* family [162]. However, *als1* gen likely relates to transform of germ tube to hyphae. In summary, *C. albicans* isolates in colonization or in candidiasis in this study possesed high proportion three virulent genes (*als1, hwp1, sap4*) reflecting that this species has strong virulence factors to become pathogen.

5.2.4. *Candida albicans* MLST

Multilocus sequence typing was used to characterize the genetic diversity and population structure of *C. albicans* isolated from two hospital in Central Vietnam. Multilocus sequence typing exhibiting high discriminatory power and reproducibility, is a useful tool to identify community or nosocomial infections, tracing the source of transmission and outbreaks. Moreover, it is essential to have global surveillance in *C. albicans* MLST and for the comparison of genotypes in a central database accessed via the Internet http://pubmlst.org/calbicans/ enhancing global epidemiological knowledge.

There was diversity in clonal comlpex in 15 *C. albicans* strains isolates with a total of 12 diploid sequence types (DSTs) identified from 10 different wards. Clade 1 (C1), recognized as the most prevalent *C.albicans* clade globally, is presented 13,3% of our isolate (DST2445 and DST2935), DST 693 (C21)

belonging to the Clade 3 was previously reported in Taiwan and South Korea, DTS459(C6) from Clade 12 was previously isolated in China, DST 723 (C12) belonged to the new clade NCKorea, precisely previously found in South Korea,. New potential nosocomial clones DST 2937 (C14), DST2932 (C22) belong to the Clade 17 and Clade 5 respectively with their predicted founders DST 443 and DST 768 previously isolated in China and Japan. DST 2936 is a singleton together with DTSs 2933, 2934 and 2477. Summarizing 66.67% isolates clustered with previously known clades in global or Asian data, and 33.33% isolates were singleton.

Some studies in several Asian countries have indicated that Clade 1 was the most common [61, 68, 69], with the dominant clade varying by country (clades 6 and 17 in China [69], clade 4 and 12 in Korea [68]. An analizing *C. albicans* MLST in period time from 2003 to 2011 showed that the major ones were clades 1, 3, and 17 were found in Taiwan from 2003 -2011 [128]. Thus, *C. albicans* MLST in the current study showed the exsisting of "asian clades" circulation (clades 3, 5, 12 and 17 and new Korea clade). MLST also suggested a potential nosocomial transmissions of *C. albicans* in HUMP Hospital, since the same DTS clones were isolated from different wards, which were DTS 2933 in Obstetric and Otorhinophagology wards, DTS 2936 in Surgery and Oncology wards, and DTS 2937 in Intensive Care unit and Pediatric ward.

By analyzing MLST, loss of heterozygosity (LOH) was observed in VPS13 sequences obtained from two sequential clones (DST299/DST459) belonging to the clade 12 isolated from two patients from Internal medicine ward, in which DST 459 was later isolated clone. Loss of heterozygosity occurred by change W to A in loci VPS13 in DTS 299 (strain 19A) to become DTS 459 (strain 91). Forche A. et al showed that LOH rates are elevated during in vitro exposure to oxidative stress, heat stress, and antifungal drugs in *C. albicans* [163] and stress-indu ced LOH could speed evolution of *Candida* specifically when it is poorly adapted to its environment [164]. LOH observed in this study reflected the *C.*

albicans stress adaptation in hospital enviromental. In fact, antifungal susceptibility test showing DTS 459 was resistant to caspofungine while DTS 299 was not.

5.3. *H. pylori* detection from *Candida spp.*

Candida spp. colonize the gastric organs [91], and they are invasive to epithelium cells in some circumstances [100]. *Candida spp.* could supply a niche for *H. pylori* to survive inside as described by Siavoshi and coworkers. They gave evidence that *Candida* vacuoles can be a niche for *H. pylori*, and suggeted that ergosterol source at fungal membrane supply nutrition for *H. pylori* [101]. *H. pylori* is normally found in gastric and can be a intracellular microorganism in certain conditions [98, 99]. The interaction between fungi and bacteria can lead to the increase of antimicrobial resistance [95]. A resistant *H. pylori* for antibiotic therapy has become a major concern all over the world. Therefore, a symbiosis between *H. pylori* and *Candida spp.* could relate to resistant abilily of this bacteria. Recently, a study from Vietnam showed that the frequency of resistant *H. pylori* to clarithromycin, levofloxacin, metronidazole and amoxicillin were 42.4%, 41.3%, 76.1% and 1.15% respectively [165].

In order to excluding all *H. pylori* from yeast surface, we subcultured *Candida* isolates strains five time on Sabouraud dextrose agar with antibiotic (chloramphenicol and penicillin) before *Candida* DNA extraction process.

This study indicated that nearly 15% of the *Candida spp* tested was positive for the *H. pylori ureaA* gene at first or second step of nested PCR. *H. pylori* was detected from different *Candida spp.* including *C. albicans*, *C. tropicalis* and *C. orthopsilosis* isolated from gastric drainage fluid, oral, sputum and vaginal secrection. Previously other authors detected *H. pylori* specific genes from the whole DNA of *Candida spp.* isolated from oral and gastric [166], or from mothers' vaginal yeasts, mothers' oral yeasts and neonates' oral yeasts [167].

Our result suggest that only *Candida* species having hyphae (*C. albicans*, *C. tropicalis*) or pseudohyphae (*C. orthopsilosis*) could contain *H. pylori* because the

process related to invasive epithelium cell of both fungi and bacteria. In addition, the increasing of using multiple antibiotic or broad spectrum antibiotic in case having failure in antibiotic therapy for *H. pylori* trigger for an increasing of *Candida* in the stomach [168] or changing of the morphology by expression hyphae [169]. As a result, *H. pylori* escaped inside the fungi could be protected by the antibiotic action. Moreover, the presence of co-infection of *Candida* and bacteria in human diseases are complicated, and has important role for management, especially in treatment incase antimicrobial resistance [93]. In our study, we could not detect again *H. pylori* after storage of *Candida* strains at -80^{0}C, and our experiments showed that it could not culture *H. pylori* from *Candida* broken cell by cultivate on Columbia blood agar in anaerobic condition. It suggest that *H. pylori* could not live inside *Candida spp* in hospital enviromental since it has just been identify inside one of the nosocomial clones circulating between wards. It is not clear to confirm which conditions triggered the invasion of *H. pylori* into *Candida* vacuoles and release of this bacteria from fungi. Therefore further studies are necessary to better understand the symbiosis between H. *pylori* and *Candida spp.*

In conclusion, our results gave an overview of *Candida spp.* causatives and epidemiology in Central Vietnam, it is useful to provide a better management strategies, preventive measures, and treatment to *Candida spp.* infections in Vietnam. This study is also primary showing an evidence of *H. pylori* presence only in *Candida* isolates forming hyphae or pseudohyphae. Additional and extensive studies are necessary to clarify the symbiosis between these two microorganism.

6. CONCLUSION

A diverse number of *Candida* species were isolated from samples obtained from Hue University of Medicine and Pharmacy Hospital and Hue Central Hospital. The dominant species was *C. albicans* (47.96%) and *C. tropicalis* was the second most frequently isolated (16.33%). Different percentage of *C. albicans* and *C. non albicans* were recovered in candida colonization and candidiasis, with *C. non albicans* found in higher proportions in candida colonization, and *C. albicans* in mucosal candidiasis.

A significantly high rate of resistance to triazole (Fluconazole, itraconazole and voriconazole) was found in *C. tropicalis*, *C. orthopsilosis*, *C. krusei*, *C. digboiensis*, *C. guilliermondii* and *C. mesorugosa*. Two mutations in the *erg11* gene (A395T and C461T) that correspond to Y132F and S154F amino acid substitutions in the ERG11 protein were detected from 26.67% of fluconazole resistant *C. tropicalis* isolates.

A high percentage of *C. albicans* carried all 3 virulent genes (*als1, hwp1* and *sap4*) regardless of the sample source, which confirmed their capacity to be a causative in fungal diseases. *C.albicans* MLST results suggested the presence of "asiatic clades" (clades 3, 5, 12 and 17 and new Korea clade) in Central Vietnam, and provided an evidence for potential nosocomial transmissions of *C. albicans* in hospital wards.

After excluding the presence of *H. pylori* in *Candida* surface, a *H.pylori* specific *ureA* gene was found in 15.27% of *Candida spp*, including *C. albicans, C. tropicalis, C. orthopsilosis*, and from different sample sources, such as oral mucosa, sputum, vaginal secretion, gastric fluid. These species could form hyphae and pseudohyphae suggesting their possible involvement in *H.pylori* entrance into yeast, and they might supply a good niche that preserve *H.pylory* from antimicrobial therapy. Further studies are necessary to better understand the symbiosis between *H. pylori* and *Candida spp.* and if also bacteria is able to take advantage of fungal resources in mutualistic interactions.

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