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MOLECULAR APPROACH TO EARLY DIAGNOSIS OF COLONIZING OR INVASIVE CANDIDA IN CRITICALLY ILL VENTILATED PATIENTS

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ATTESTATION OF AUTHORSHIP

I hereby declare that this submission is my own work to the best of my knowledge and belief. It contains no material previously published or written by another person except for what appears in the citations and acknowledgements.

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"Nothing can succeed without the help of the teacher"

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LIST OF ABBREVIATION

A. baumannii	*Acinetobacter baumannii*
Als	Agglutinin-like sequence
APACHE II	Acute Physiology and Chronic Health Evaluation II
BAL	Broncho-alveolar lavage
BDG	β-D-Glucan
C. albicans	*Candida albicans*
C. nonalbicans	*Candida nonalbicans*
CDC	Centers for Disease Control and Prevention
CRP	C-reactive protein
ECE1	Extent of cell elongation 1
ELISA	Enzyme-linked immunosorbent assay
FiO_2	Fraction of inspired oxygen
HIV	Human Immunodeficiency Virus
HCH	Hue Central hospital
HUMP	Hue University of Medicine and Pharmacy
HWP1	Hyphae wall protein 1
HYR1	Hyphally regulated gene 1
IC	Invasive Candidiasis
ICU	Intensive Care Unit
IV	Intravenous
K. pneumoniae	*Klebsiella pneumoniae*
MALDI - TOF	Matrix assisted laser desortion ionization time-of-flight
Neg	Negative
PCR	Polymerase chain reaction
PEEP	Possitive end - expiratory pressure
P. aeruginosa	*Pseudomonas aeruginosa*
Pos	Positive
SAP	Secreted aspartic proteases

SDA	Sabouraud dextrose agar
SOFA	Sequential Organ Failure Assessment
S. aureus	*Staphylococcus aureus*
VAP	Ventilator-associated pneumonia
VARI	Ventilator-associated respiratory infection

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ABSTRACT

Candida colonization is a frequent event in respiratory tract of non-immunocompromised intensive care unit (ICU) ventilated patients. From 5 to 30% of *Candida* colonization patients will develop Invasive Candidiasis (IC), which is usually a late-onset ICU acquired infection. Until now, a lot of data highlight the necessity for new IC noninvasive diagnostic in high risk patients. IC is a serious complication in the ICU patients, around 35% mortality and up to 90% in patients with septic shock. How to diagnosis IC early and give appropriate antifungal therapy are the key for a remarkable reduction in mortality. The overall objective of this study was to identify the etiology of *Candida* and *bacteria species* in lower respiratory tract in the central of Vietnam, and to discriminate invasive or colonizing *Candida* by indirect ELISA (Enzyme-linked immunosorbent assay).

Ninety six critically ill ventilated patients from 2 hospital in Hue (central Vietnam) were followed in this study. The 3 main isolated fungal pathogens were *C. albicans* (42%), *C. tropicalis* (37%) and *C. glabrata* (16%). The fluconazole resistance of *Candida species* was 21.11% and caspofungin was 4.44%. *C. tropicalis,* that is becoming a predominant opportunistic in nosocomial fungal infections of ICU in developing country, showed highest fluconazole resistance (34.29%) and caspofungin resistance (5.71%). In ICU, 3 main bacteria resulted in ventilator-associated pneumonia (VAP) were *A. baumannii* (43.2%), *K. pneumoniae* (28.4%) and *S. aureus* (14.8%), with high levels of antimicrobial resistance. *A. baumannii* showed resistance to all cephalosporin 2, 3, 4 generation (100%) and carbapenem (94%). A 50% of *K. pneumoniae* was carbapenem-resistant while 100% *S. aureus* was resistant to methicillin.

To discriminate invasive or colonizing *Candida*, we chose 2 proteins, ECE1, present in *C. albicans* and *C. dubliniensis*, and HWP1, present in almost *Candida species*, selecting specific epitopes to develop indirect ELISA. ELISA results showed that 47.4% of patients with *C. albicans* had IC and 28.9% had invasive *C. albicans* pneumonia. In 19.23% of patients with *Candida species* had IC and 2.56% had invasive *Candida species* pneumonia. The sensitivity and specificity of ECE1 and HWP1 antibody detecting were 80% and 96% and 60% and 77% respectively, indicating the selected ECE1 epitope as a

good marker for IC due to *C. albicans* and *C. dubliniensis.* A correlation between the ELISA results and 4 clinical parameters (Candida score, procalcitonin, length of ICU stay, ventilation day) was also investigated, that should help physicians to decide early antifungal therapy waiting for a new IC test that include all *Candida species*.

1. INTRODUCTION

1.1. *Candida species*

1.1.1. History

The original name of *Candida* comes from the Latin term “candidus”. *Candida albicans* (*C. albicans*) was identified in the nineteenth century from three independent sources. First, in 1841, Fredrick Berg, a medical practitioner, discovered that thrush was caused by fungus with filaments that dispersed into epithelial cells. In 1842, David Gruby, a medical practitioner, 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 1923, Christine Berkhout, a mycologist, changed the name to *C. albicans* till now [1].

1.1.2. Taxonomy

The taxonomy of the genus *Candida* is increasing overtime because of the reclassification of certain species and the discovery of new species such as *Candida dubliniensis (C. dubliniensis), Candida orthopsilosis (C. orthopsilosis),* and *Candida metapsilosis (C. metapsilosis). C. orthopsilosis* and *C. metapsilosis* were previously classified as part of the *Candida parapsilosis (C. parapsilosis)* complex. More than 200 species of *Candida* have been described, most of which exist as saprophytes organisms. Approximately 20 species can infect humans and *C. albicans* is the most prevalent species [2]. *C. albicans, Candida glabrata (C. glabrata), C. parapsilosis, Candida tropicalis (C. tropicalis),* and *Candida krusei* (*C. krusei)* were results in 90 - 92% of all cases of candidiasis [3, 4]. These species are able to cause both superficial infections of the skin and mucosa as well as systemic infections. The dissemination of the fungus through the blood stream and subsequent organ colonization is life-threatening with high mortality rates.

1.1.3. Cell biology

Cell biology characteristics of *Candida species* are the same to those of eukaryotes and especially similar to *Saccharomyces cerevisiae* [5]. Polysaccharides are an indispensalbe compound in the cell walls of *Candida species* [5, 6]. *Candida* cell walls consist of mannans, glucans and a few of chitin (Figure 1.1) [7, 8]. These components are closely

bound to polypeptides and proteins found on the cell membrane [7]. Three types of adhesion molecules consist of glycoproteins, the protein moiety of glycoproteins, and the polysaccharide portion of a mannoprotein [5]. Moreover, the mannan polysaccharides structures found on the walls of *Candida* play an important role in its pathogenicity [5, 9]. *C. albicans* mannan is required for disruption of host processes that function to inactivate pathogens, leading to survival and escape of this fungal pathogen from host phagocytes [10].

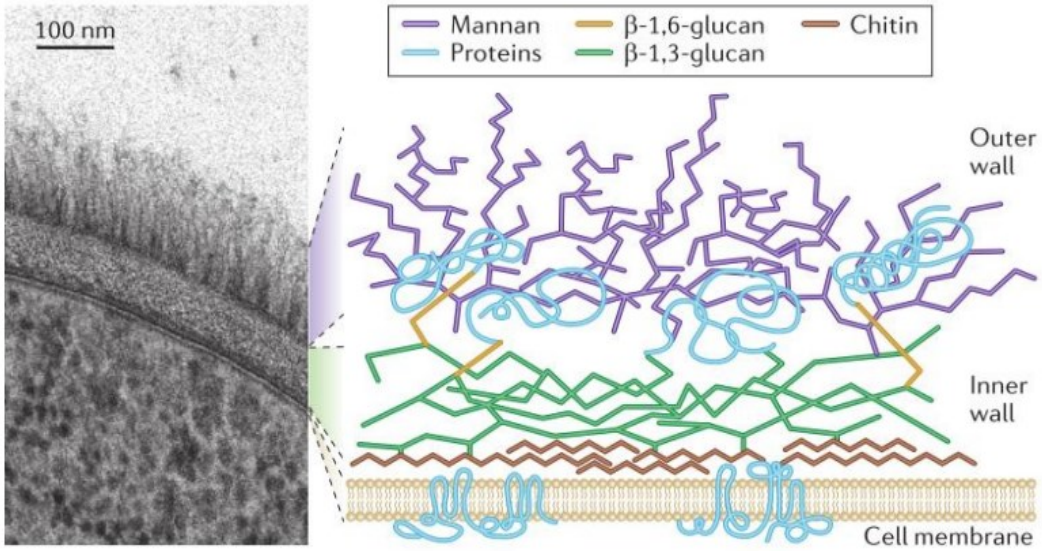


Figure 1.1. Structure of the C. albicans cell wall [9]

Phospholipids and sterols are dominant in lipid structure of *Candida species*. 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 [11].

1.1.4. Morphogenesis

Genus *Candida* constitutes a heterogeneous group of eukaryotic, dimorphic, or polymorphic organisms [5, 12, 13]. *Candida species* can grow in widely pH from below 2.0 to nearly 10.0 under microaerophilic and even anaerobic conditions as well as the more normal aerobic atmospheres of incubation [5, 14]. Normally, in the gut microbiota of humans or animals blastoconidia of *Candida species* exits in round and oval shape. *Candida species* grow as yeast cells or blastoconidia. Yeast cells are approximately 2 - 10

μm in the largest dimension, round to oval and reproduce by budding. They multiply principally by the blastoconidia production. However, *Candida species* can have different morphogenesis depending on the species and environmental triggers (Figure 1.2) [5, 15]. When the blastoconidia are produced from one another in a linear fashion without separating, a structure termed a pseudohypha is formed. Under certain circumstances, some yeast may produce true hyphae. True hyphae differ from pseudohyphae in forming long narrow filaments with parallel sides and no constrictions at the sites of septation, whereas pseudohyphae are generally shorter and wider with obvious constrictions showing at the septation sites [16]. Further morphological forms are opaque yeast cells and chlamydospores [17, 18]. The switch from yeast cells to opaque cells plays an important role in the mating process, whereas chlamydospores can only be observed in vitro when grown on nutrient-poor media and may therefore present a dormant growth form developing under harsh environmental conditions [18, 19].

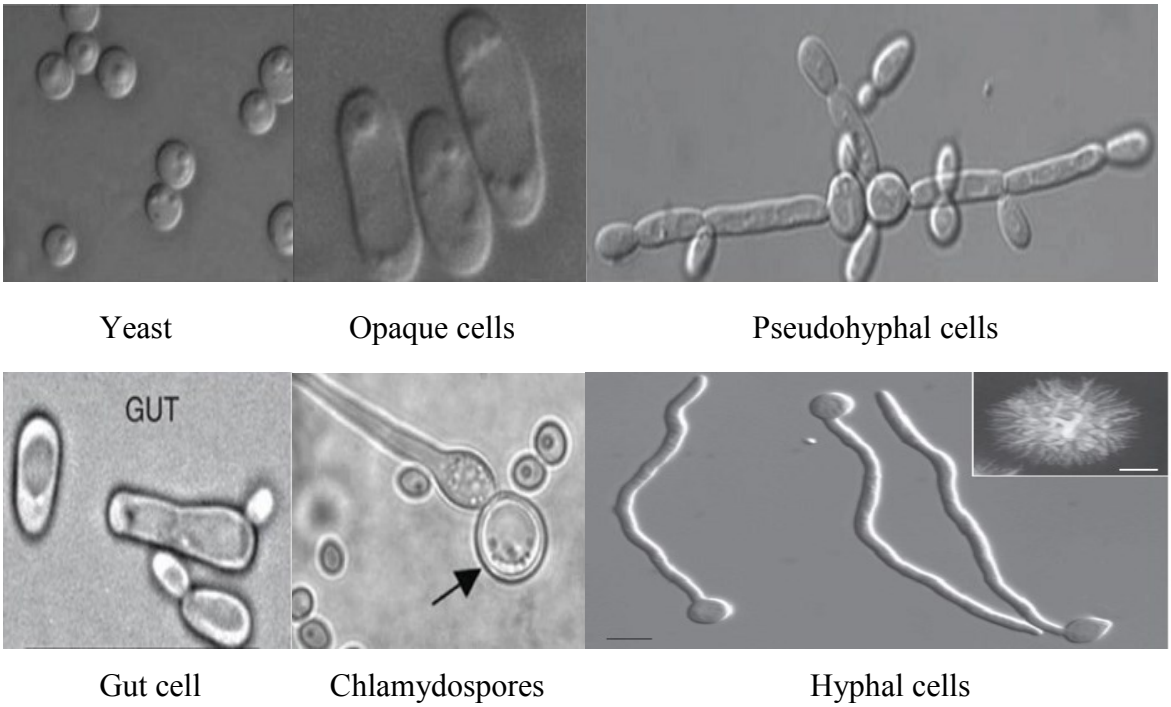


Figure 1.2. Candida species morphology [16, 20, 21]

The presence of budding yeasts, pseudohyphae, opaque cells, chlamydospores, and hyphae in the infected tissue are usually indicative of candidiasis [16, 22]. The

morphological flexibility in *Candida* pathogen play an important role in allowing *C. albicans* to penetrate and proliferate in a wide variety of host tissues [23].

1.1.5. Hyphal-specific proteins

In *C. albicans* and *C. nonalbicans species*, hypha formation is characterised by the expression of specific hypha-associated proteins, the most important hydrolytic enzymes are proteases and phospholipases [24] including the secreted aspartic proteases (SAP), agglutinin-like sequence (ALS), hyphally regulated gene (HYR1), hyphal wall protein 1 (HWP1), and extent of cell elongation 1 (ECEl) [16, 25].

Several studies have demonstrated a correlation between an increase in the synthesis and the activity of hydrolytic enzymes and an increase in clinical symptoms of severe candidiasis [26]. Ten SAP isoenzymes are responsible for the proteinase activity, SAP produce by *C. albicans, C. parapsilosis, C. tropicalis, C. dubliniensis, C. guilliermondii, C. kefyr, C. lusitaniae, and C. krusei* [27, 28]. These enzymes produce non-specific proteolysis of host proteins related the defence against the infection. Different kinds of SAP are associated with different locations within the yeasts and different pathogenicity [28].

C. albicans, *C. dubliniensis*, *C. tropicalis*, *C. glabrata*, *C. krusei*, *C. lusitaniae* and *C. parapsilosis* also produce phospholipases [5, 29]. These enzymes take in controlling of yeast growth, remodeling of fungal cell membranes and spreading in host tissues through the hydrolysis of phospholipids [29]. Seven phospholipase genes have been characterized. However, the role of the enzymes encoded by these genes remains unclear [30]. PLB1p is a glycoprotein present at hyphal tips during the tissue invasion and has hydrolase and lysophospholipase-transacylase activity [31]. The growth of hyphae, a virulence mechanism, plays an important function in the tissue invasion and the resistance to phagocytosis [32].

HYR1 is a *Candida* germ tube specific cell wall glycoprotein with a glycosylphosphatidylinositol motive. *HYR1* play a structural role in the *Candida* cell wall architecture. HYR1 protein shares significant structural homology to *A. baumannii* cell surface proteins, and becomes the receptor for *A. baumannii* binding to the fungus [33].

The *Als* genes encode cell surface glycoproteins implicated in adhesion of the organism

to host surfaces. Eight genes in the *Als* family have been discovered presently [34]. The expression of these proteins is correlated with *Candida species* infection. Among the *Als* family, *Als3* play main important role in epithelial adhesion [35, 36].

Hwp1 gene encodes for a fungal cell wall protein. HWP1 protein consists of 634 amino acids sequence. It's specific for *C. albicans, C. tropicalis, C. dubliniensis, C. africana* and play an important role in adhesin that is required for mating, hyphal development and biofilm formation [37]. HWP1 also promotes the binding of *Candida* to epithelial cells relating to oroesophageal candidiasis in mice [38].

Hypha formation of *Candida species* is essential for host tissue damage and immune activation and the extent of cell elongation 1 protein (ECE1), present in *C. albicans* and *C. dubliniensis* is one of the most early and abundantly expressed proteins during this process. ECE1 expression was not detected when *C. albicans* grew as a budding yeast cell but it was observed within 30 min after cells had been induced to the form of hyphae. Birse *et al* were able to show that ECE1 expression correlated with the extent of cell elongation, but the function of ECE1 remained unknown for a long time [39]. The characterization of this protein was determined since 1993 but only in 2016 a portion of ECE1 protein, called "Candidalysin", was indicated as the first cytolytic peptide toxin in a human fungal pathogen.

1.1.6. A first cytolytic peptide toxin in a human fungal pathogen "Candidalysin"

Cytolytic proteins and peptide toxins are virulence factors of bacterial pathogens which disrupt epithelial barrier function, damage cells and stimulate host immune responses [40, 41]. However, cytolytic peptide toxins in fungi pathogenic had not been identified for long time, until the discovery of the ECE1 toxin.

ECE1 is a protein consist of 271 amino acids, including a signal peptide for secretion (recognized by the signal peptidase) and seven dibasic lysine-arginine (KR) motifs which are recognized by the Golgi complex-associated endoproteinase Kex1p and Kex2p [42, 43]. These subtilisin or kexin-like proteases have been implicated in the activation of various bacterial toxins [44]. *C. albicans* Kex2p is a member of a family of eukaryotic proprotein protease enzymes including proprotein convertase 1, proprotein convertase 2 and furin, which possess catalytic domains homologous to the degradative serine proteases

of the subtilisin family [45]. *C. albicans* Kex1p is a protease with a carboxypeptidase B-like function involved in releasing the C-terminal processing of the lysine and arginine residues from protein precursors. Recent studies proved that ECE1 was cleavaged completely by Kex2 resulting in a signal peptide and eight other peptides, with seven of these peptides ending in KR. After Kex2p processed, these peptides were subsequently cleaved by Kex1p for removing the C-terminal R [46-48].

C. albicans ECE1 amino acid sequence

MKFSKIACATVFALSSQAAIIHHAPEFNMKRDVAPAAPAAPADQAPTVPAPQEFN
TAITKRSIIGIIMGILGNIPQVIQIIMSIVKAFKGNKREDIDSVVAGIIADMPFVVRAV
DTAMTSVASTKRDGANDDVANAVVRLPEIVARVATGVQQSIENAKRDGVPDVG
LNLVANAPRLISNVFDGVSETVQQAKRDGLEDFLDELLQRLPQLITRSAESALKDS
QPVKRDAGSVALSNLIKKSIETVGIENAAQIVSERDISSLIEEYFGA

SP	ECE1-I_{1-31}	ECE1-II_{32-61}	ECE1-III_{62-93}	ECE1-IV_{94-126}	ECE1-$V_{127-160}$	ECE1-$VI_{161-194}$	ECE1-$VII_{195-228}$	ECE1-$VIII_{229-271}$

+ ECE1-I_{1-31}: MKFSKIACATVFALSSQAAIIHHAPEFNMKR
+ ECE1-II_{32-61}: DVAPAAPAAPADQAPTVPAPQEFNTAITKR
+ ECE1-III_{62-93}: SIIGIIMGILGNIPQVIQIIMSIVKAFKGNKR
+ ECE1-IV_{94-126}: EDIDSVVAGIIADMPFVVRAVDTAMTSVASTKR
+ ECE1-$V_{127-160}$: DGANDDVANAVVRLPEIVARVATGVQQSIENAKR
+ ECE1-$VI_{161-194}$: DGVPDVGLNLVANAPRLISNVFDGVSETVQQAKR
+ ECE1-$VII_{195-228}$: DGLEDFLDELLQRLPQLITRSAESALKDSQPVKR
+ ECE1-$VIII_{229-271}$: DAGSVALSNLIKKSIETVGIENAAQIVSERDISSLIEEYFGA

Moyes *et al* discovered that among these eight peptides, only ECE1-III peptide was found in the presence of epithelial cells, indicating that the fungus secretes this toxin during mucosal infection [47]. In early infection progress, ECE1-III_{62-92K} (SIIGIIMGILGNIPQVIQIIMSIVKAFKGNK) levels accumulate into a membrane-bound 'invasion pocket' [49, 50] which affect direct tissue damage and stimulate the release of lactate dehydrogenase from the host epithelium (Figure 1.3).

During stages of infection, concentrations of ECE1-III_{62-92K} induce epithelial immunity by activating the 'danger response' pathway (MAPK, p-MKP1/c-Fos) resulting in the production of immune regulatory cytokines and alerting the host to the transition from

colonizing yeast to invasive, toxin-producing hyphae (Figure 1.4). As a result, *C. albicans* ECE1-III$_{62\text{-}92K}$ is proved as the first cytolytic peptide toxin in a human fungal pathogen and reveals the molecular mechanisms of epithelial damage and the host recognition of this clinically important fungus [46-48].

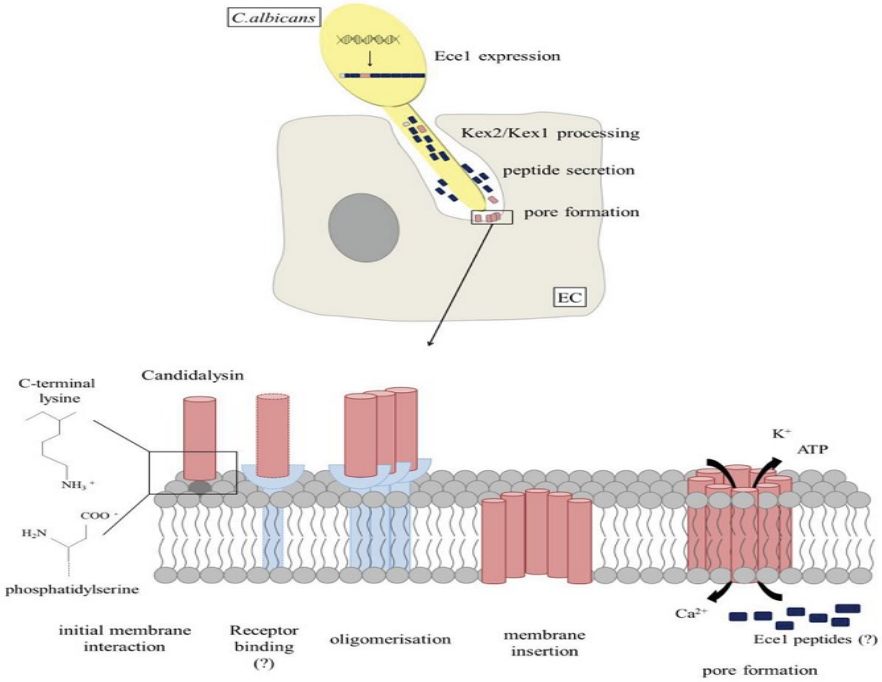


Figure 1.3. Model of Candidalysin-induced pore-formation [48]

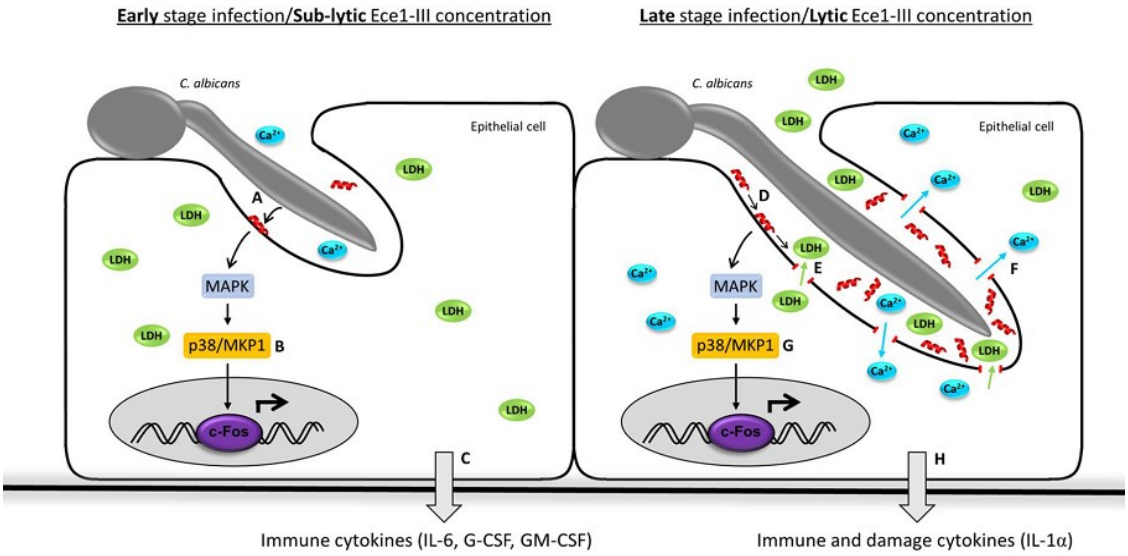


Figure 1.4. Schematic of the role of C. albicans ECE1-III in another stage infection of epithelial cells [47]

1.2. *Candida species* in ventilator-associated pneumonia patients

Nowadays, pneumonia is a leading cause of death worldwide. Ventilator-associated respiratory infection (VARI), which consists of ventilator-associated tracheobronchitis (VAT) and ventilator-associated pneumonia (VAP), is the commonest hospital-acquired infection in ICU [51]. According to the Centers for Disease Control and Prevention (CDC) definition, VAP is a pneumonia where the patient is on mechanical ventilation for > 2 calendar days on the date of event [52]. VAP is a significant problem in a resource-restricted ICU which remains important cause of morbidity (9% - 35%) and mortality (30% - 40%) despite the advances in prevention strategies and antimicrobial therapy [53, 54].

Previously study showed that the main microorganism pathogens causing VAP in United State of America are *Staphylococcus aureus (S. aureus)* 27.9%, *Pseudomonas species* 16.3%, *Klebsiella species* 13.3%, and *Candida species* 6.3% [54]. The incidence of VARI and VAP in Vietnam were 24.6% and 9.9% respectively, mainly caused by Gram-negative organisms *Acinetobacter baumannii (A. baumannii)* 43.8%, *Klebsiella pneumoniae* (*K. pneumoniae*) 35.6%, and *Pseudomonas aeruginosa (P. aeruginosa)* 32.9% [53]. In other studies, VAP with *Candida species* isolation was 8.8% - 19.5% [55, 56].

Nowadays, *Candida species* become more and more a predominant microrganism in healthcare-associated infections. CDC estimated that 46000 healthcare-associated *Candida* infections occur among hospitalized patients each year. *Candida species* is the fourth most common causes of healthcare-associated bloodstream infections in the United States [2]. *Candida species* are human commensals which commonly appear on the mucosal surfaces of gastrointestinal, respiratory tracts, urinary tracts, skin and under fingernails [5, 57]. Moreover, *Candida species* are also isolated from hospital sources, such as the floor, water, soil, food, medical equipments, medical staff, etc [10]. The prevalence of *Candida* colonization varies depending on site, population sampled, sampling equipment and sampling method. It's estimated that between 25 - 40 % of people are colonized by *C. albicans* [11], this rate is approximately 47% (13 - 76%) in hospitalized patients [5]. Almost 100% of humans carry one or more *Candida species* from the mouth to the colon. The numbers of yeasts carried at any point in the gut can critically increase in ill patients.

At ICU, the critically ill ventilated patients commonly have a lot of risk factors such as total parenteral nutrition, invasive procedures, a long time broad spectrum antibiotics, immunotherapy and hemodialysis, that contribute to *Candida species* infections increasingly [58]. Furthermore, the automatic protecting reflection of respiratory tract was reduced by the feeding tube, endotube and the main disease during mechanical ventilation. By this condition, *Candida species* have opportunity to penetrate and break the epithelial barriers and enter the blood stream (Figure 1.5). Therefore, infections can become life-threatening.

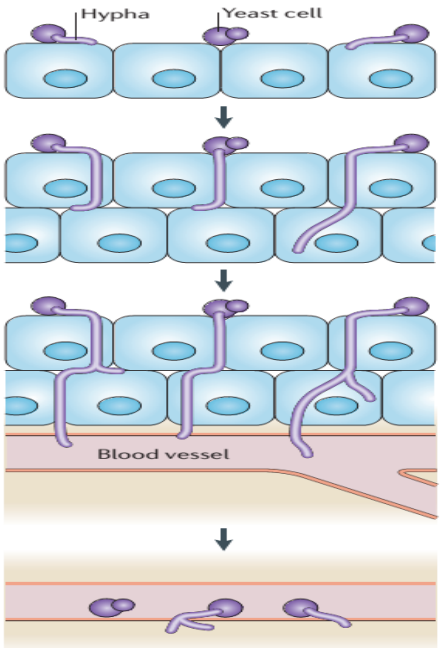


Figure 1.5. The steps of C. albicans tissue invasion [9]

The ventilated ICU patients can have *Candida species* infections due to haematogenous spread or pulmonary aspiration of the contents of colonies of oropharyngeal or gastric origin [24]. As a result, the colonization or even invasion to the respiratory tract by *Candida species* is common in patients receiving mechanical ventilation for a long time. However, distinguishing invasive *Candida* from colonizing *Candida* in respiratory tract has been a challenge until now. In the clinical practice guidelines for the management of candidiasis of Infectious Disease Society of America (IDSA) and European Society for Clinical Microbiology and Infectious Diseases (ESCMID) showed that *Candida species* from respiratory secretions usually indicate colonization and rarely requires any treatment

with the antifungal therapy [59, 60]. However, in a multiple *Candida* colonization patient with signs and symptoms of infection, it might prompt antifungal treatment [61]. Moreover, the isolation of *Candida species* from respiratory tract samples in a patient who is severely immunosuppressed should trigger a search for evidence of IC and requires antifungal treatment. Until now, some studies supported that *Candida* pneumonia and *Candida* lung abscess are very uncommon [62], only rarely after the aspiration of oropharyngeal material has primary *Candida* pneumonia or abscess [63, 64]. Because of the rarity of *Candida* pneumonia, the common *Candida* colonization in the respiratory tract and the lack of sensitivity and specificity of diagnostic test [65], a decision to initiate antifungal therapy should not be based only on the respiratory tract culture results for avoiding the overuse of antifungal therapy [59].

Recently, some studies indicated that the infection symptoms, the duration of mechanical ventilation and ICU stay as well as the mortality correlate to *Candida species* in the respiratory tract. Azoulay *et al* found an association between *Candida* colonization of the respiratory tract secretions and a prolonged period of mechanical ventilation, longer ICU stay, hospital stay and an increased risk for *Pseudomonas* VAP [66]. In other studies concluded that the colonization of the airway with *Candida species* is associated with the development of bacterial colonization and pneumonia. *Candida* airway colonization was also associated with worse clinical outcomes and higher mortality [67-70]. *Candida species* colonization not only affected the pulmonary epithelial, but also promoted the antibiotic-resistant and the biofilm formation with the bacteria in suspected VAP patients [66, 70]. *Candida species* become more and more microinvasive in this ventilated ICU patients who had a lot of risk factors of IC [71, 72] such as:

+ Hospitalisation in ICU

+ Acute or chronic organ dysfunction requiring intensive care or invasive procedures (e.g. mechanical ventilation, vasoactive drugs, renal substitution, extracorporeal circulation systems, high-volume fluid or haemocomponents infusions, tracheostomy)

+ Solid organ transplantation

+ Onco-haematological diseases and stem cell transplantation, especially with graft-versus-host disease (GVHD)

+ Surgery (especially abdominal surgery and surgical revision), trauma and burn patients

+ Pediatric and neonatal intensive care units

+ Multiple underlying medical conditions (e.g. elderly patients in medical wards)

+ Immunosuppressive therapy

+ Renal failure requiring haemodialysis or haemofiltration

+ Neutropaenia

+ APACHE II score > 20

+ Multiple site colonisations

+ Duration of hospital stay

+ Previous history of *Candida* infection

+ Disruption of physiological barriers in the digestive tract

+ Total parenteral nutrition and use of indwelling catheters

+ Diabetes mellitus

+ Previous prolonged antibiotic therapy

1.3. Invasive Candidiasis diagnostic test and treatment

IC ranges from 5 to 10 cases per 1000 ICU admissions and represents 5% to 10% of all ICU-acquired infections [73, 74]. *Candida species* are the fourth most common cause of candidemia in United State of America and the drugs resistant of *Candida* infections results in significant increasingly healthcare expenditures each year [2]. In Europe, the incidence of Candidemia ranges from 2 - 3% of the blood stream isolates, but Candidiasis stays among the top ten blood stream pathogens [75]. Although *C. albicans* is the most common cause of invasive fungal infections, the increasing number of infections from *C. nonalbicans species* is recently reported as a major source of infection [76, 77]. Pfaller *et al* determined that *C. albicans* was the most common cause of IC (63 - 70%) all worldwide, followed by *C. glabrata* (44%), *C. tropicalis* (6%), and *C. parapsilosis* (5%) [77]. However, the species distribution of *C. non albicans* is different. While in North America and Europe, *C. glabrata* is the second species, in South America *C. tropicalis* is the most commonly after *C. albicans* [78]. 5 - 30% of *Candida* colonization patients will develop IC, which is usually a late-onset ICU acquired infections [73, 79]. The increasing

of IC is related to the use of a broad spectrum of antibiotics, total parenteral nutrition, surgical procedures, indwelling invasive devices, intensive care support, hemodialysis, cytotoxic therapy and immune suppressive therapy [58, 74, 80], usually related to high rates of mortality, approximately 30%, as well as increases in cost and length of hospital stay [2].

Distinguishing invasive *Candida* from *Candida* colonizing is very difficult till now and may guide to deciding a proper antifungal therapy. Clinical symptoms suggestiveness of IC did not differ from those of other nosocomial infections. *Candida* isolation from blood cultures has low sensitivity. Cultures turn round times of several days and turn positive late in the course of disease [81] whereas antifungal therapy delayed after blood sampling had been associated with an increase of hospital mortality [82, 83]. An important factor contributing to the outcome of an IC also lies in a timely diagnosis. Quickly IC diagnostic test help to decide early proper antifungal therapy leading to reducing the mortality in IC patients. The development of nonculture assays is critical to providing the occasion for earlier IC diagnosis. Untill now, the available IC diagnostic tests are:

- Role of the mannan antigen or antimannan antibody test: The mannan or antimannan detection test may be useful for the diagnosis of IC. Several standard serological tests detecting antibodies against *Candida* mannan have been invented. In a meta-analysis of 14 studies, the sensitivity/specificity of mannan and anti-mannan IgG were 58%/93% and 59%/83% [84]. However, the specific is low because anti-mannan antibodies are ubiquitous in human sera and the sensitiveness was extremely poor in severely immunosuppressed patients [85]. Consequently, the separate detection of either mannan or antimannan was not recommended on the guideline [71].

- Beta-D-glucan (BDG) test: BDG is a cell wall constituent of *Candida species* and others fungi, but not on mammalian and bacterial cells [86]. Therefore, BGD detection in blood or other bodily specimens may represent a marker of a fungal disease. The sensitivity and specificity of serum BDG testing for diagnosing IC have ranged from 57% to 97% and 56% to 93% [87]. The BDG test are recommended on guideline as a diagnostic test in a patient with signs and symptoms of IC infection [71]. However, the

inaccuracy for BDG detection is in specificity and false-positivity value. False-positive results are commonly in patients with gram-positive and gram-negative bacteremia and ICU residents [88]. True-positive results are not specific for IC and several other causes of false-positivity have been identified [81], such as:

False-positive Results	**Fungi that yield positive β-D-Glucan Results**
Human blood products (albumin, immunoglobulin, coagulation factors, plasma protein fractions)	Yeast: *Candida species, Trichosporon species, Saccharomyces cerevisiae*
Hemodialysis	Molds: *Acremonium*, *Aspergillus species*, *Fusarium species*
Surgical gauze or other materials containing glucan	Dimorphic fungi: *Coccidioides immitis, Histoplasma capsulatum, Sporothrix schenckii*
Antibiotics such as piperacillin-tazobactam and ampicillin-clavulanate	Others: *Pneumocystis jiroveci*
Systemic bacterial infections, severe mucositis	
Excess manipulation of sample	

Table 1.1. Bias of β-D-Glucan results for IC diagnosis

- Nucleic acid-based diagnostic techniques: Molecular-based diagnostic tests may potentially be sensitive in detecting an invasive fungal infection and can provide results more rapidly than culture, therefore enabling the possibility for earlier diagnosis and more timely initiation of antifungal therapy [89-91]. Nevertheless, molecular-based diagnostic techniques are not yet recommended on the guideline, because of the heterogeneity of the available results, the lack of reliable reference standards and differences in techniques [71]. β-D-glucan, mannan antigen or antimannan antibody and molecular-based diagnostic assay of blood samples are recommended as adjuncts to cultures for the diagnosis of IC. However, these assays do not provide high sensitive and specific data for IC diagnosis.

Currently, for management of IC infections and an early proper antifungal therapy decision, a physician should usually consider the combination of clinical symptoms such as individual risk factor of IC [71, 72], Candida colonization index [92, 93], Candida score [94], Ostrosky-Zeichner prediction rule [95, 96] and laboratory test result for deciding anearly proper antifungal therapy for their patients.

2. RESEARCH OBJECTIVES

Ventilator-associated pneumonia (VAP) becomes the most frequent ICU-acquired infection nowadays with significant mortality (35%) despite the advances in the understanding of contributing causes and prevention. *Candida species* is the most common opportunistic mycosis at intensive care unit and *Candida* colonization is a frequent microrganism in respiratory tract of mechanically ventilated non-immunocompromised ICU patients [70, 94]. CDC estimates that each case of *Candida species* infection causes 3 - 13 days of additional hospitalization and a total of $6,000 - $29,000 in direct healthcare costs per patient [2]. Therefore, a better knowledge of the epidemiologic features of *Candida* in ventilated patients will be critically, enable physicians to provide appropriate prevention and treatment strategy [58].

A 5 - 30% of *Candida* colonization patients will develop IC [79]. IC is a serious complication in the ICU patients, around 35% mortality [2, 59, 60] and up to 90% in patients with septic shock [97]. Early diagnosis IC still remains a major challenge especially in respiratory tract now. Blood cultures, which were considering the gold standard for diagnosis are positive in a minority (50%) of cases and often late in the course of infection [71, 81]. Deep-seated tissue sampling usually requires extremely invasive procedures at high risk of complications and has a low specific especially in ICU patients who have received empirical therapy, whereas the antifungal therapy delayed beyond 12 hours after the sampling of blood has been associated with an increase of in-hospital mortality from under 20% to 40% [82, 83]. Early appropriate antifungal therapy is the key for a remarkable reduction in mortality [59, 98]. Using antifungal therapy early for *Candida* colonization in ICU patients with fever despite broad-spectrum antibiotics was recommended in the guideline. However, 70% of critically ill patients are receiving systemic antifungal therapy although they have no documented invasive fungal infection, suggesting an urgent need for antifungal stewardship strategies [59, 98]. The lack of rapid and sensitive IC diagnostic tests has led to overuse of antifungal therapy resulting in increased costs, drugs resistance and drugs toxicity. Until now, all available data highlight the essential for new noninvasive diagnostic tools for IC in high risk patients. In Vietnam, the total price of one intravenous antifungal therapy is from 250€ with fluconazole to

3800€ with caspofungin, while GDP was 1950€ (2017). Furthermore, insurance did not accept intravenous antifungal payments in almost hospitals. “Treatment or not?” is still a big question to a physician and the relationship of patients in Vietnam. Therefore, this research has been carried out with the following objectives:

1. To identify the etiology of *Candida* and *Bacteria species* in lower respiratory tract infections in the central of Vietnam.

2. To discriminate between invasive or colonizing *Candida* by indirect ELISA test by selecting specific epitopes from invasive ECE1, HWP1 proteins.

3. MATERIALS AND METHODS

3.1. Study site

This study was conducted in the Hue University of Medicine and Pharmacy and University of Sassari from September 2016 to August 2018, in the following departments:

- ICU of Hue University of Medicine and Pharmacy (HUMP) Hospital
- ICU of Hue Central Hospital (HCH)
- Department of Parasitology, Hue University of Medicine and Pharmacy Hospital
- Carlo Ubani Center, Department of Microbiology, Hue University of Medicine and Pharmacy
- Microbiology laboratory, Department of Biomedical Sciences, University of Sassari.

3.2. Study population

Patients, admitted to the ICU of HUMP hospital and HCH, were included in the study following specific criteria:

3.2.1. Inclusion criteria

- Patients over 18 years old
- Admission intensive care unit within the last 48 hours
- Mechanical ventilation over 48 hours

3.2.2. Exclusion criteria

- VAP or suspected VAP before ICU admission
- End stage Human Immunodeficiency Virus (HIV) patients, neutropenic patients
- Refused permission to join on this study given by patient's relatives

3.2.3. VAP diagnosis according to CDC 2013

Deterioration in ventilation following a period of stability

PEEP: ≥ 2 days of stable or decreasing daily minimum PEEP followed by a rise in daily minimum PEEP of ≥ 2.5 cmH_2O, sustained for ≥ 2 calendar days

Or

FiO_2: ≥ 2 days of stable or decreasing daily minimum FiO_2 followed by a rise in daily minimum FiO_2 of ≥ 0.15 points, sustained for ≥ 2 calendar days

And systemic signs

Fever of > 38°C or < 36°C

Or

WBC > 12 x 10^9/L or < 4 x 10^9/L

And

Chest radiography: New and persistent infiltrate, consolidation, or cavitation as read by two study physicians

Or

Decision to replace new antibiotics, physician starts antibiotics within a window period of 2 days before the deterioration in ventilation to 2 days after.

And pulmonary secretions

Increased/new purulent tracheobronchial secretions

Or

≥ 25 neutrophils per low power field (10 objectives) on Gram stain of endotracheal aspirate [52, 99].

All ventilated patients were divided 5 groups based on VAP definition and the Broncho-alveolar lavage (BAL) culture results:

- Group 1: VAP with *Bacteria* and *Candida species* on BAL
- Group 2: VAP with only *Bacteria species* on BAL
- Group 3: VAP with only *Candida species* on BAL
- Group 4: NonVAP with *Candida species* on BAL
- Group 5: NonVAP without any agents on BAL

Baseline demographic, pertinent clinical data and medications were recorded on admission to the study. Necessary variables were recorded in order to calculate the Acute Physiological and Chronic Health Assessment II (APACHE II) and Sequential Organ Failure Assessment (SOFA) scores on the sampling day. The microbiology results, the length of mechanical ventilation, ICU stay and hospital outcome were recorded completely.

3.2.4. Protocol of broncho-alveolar lavage sampling

A. Preparation

A.1. Medical staff

- 01 specialized doctor who had training in bronchoscopy

- 01 nurse who had training in bronchoscopy

- Patients: The relatives of patient were explained about the procedure, the benefit and the side effect during the procedure. The procedure was performed when the relatives of patients accepted and signed on medical record. Patients had not been feeding for 4 hours before the procedure.

A.2. Instruments and drugs

- Instrument: Bronchoscopy tube (Pentax, Japan), light system (XD 320, Germany), suction machine (High-vacuum, Taiwan), sterile bottle sample, sterile gloves, sterile gauze, surgery clothes, ambu, oxygen, monitor, ventilation machine were used.

- Drugs utilized were atropin 0.25 mg, ephedrin 30 mg, lidocain 2%, propofol 200 mg, fentanyl 0.5 mg, methylprednisolon 40 mg, adrenalin 1 mg, salbutamol 0,5mg, midazolam 5 mg, natriclorua 0,9%.

- Medical records: X-ray, blood count cells, coagulation, HIV negative, blood gas and electrocardiography had already done.

A.3. Procedure

Propofol 2 - 3 µg/kg IV plus fentanyl 2 - 3 µg/kg IV was injected in patients before 5 minutes the procedure or midazolam 150 - 350 µg/kg IV was injected in unstable hemodynamic patients.

Endoscopy tube was slipped into endotube from mouth to tracheal. The lidocain 2% was used for local anesthesial before moving to bronchus. After connecting a steril bottle sample to endoscopy tube, the endoscopy tube was put at wedge broncho position which intends to clean. Normal saline was slowly instilled through the bronchoscope, the total volume was 150 - 200 ml and divided into three to four aliquots. By keeping the endoscopy tube and slightly suctionning (80 mmHg), the lavage fluid was flowing inside a sterile bottle sample. When the BAL was enough, the sterile bottle sample and endoscopy tube were taken out the patients. Patients were observed carefully during the procedure and 15 minutes after this procedure. It was 2 hours after the procedure that patients could eat.

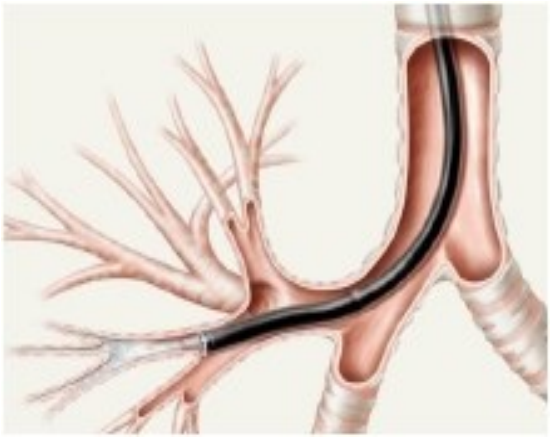


Image 3.1. Bronchoendoscopy to the wedge of bronchus

A.4. Handling of the BAL fluid

BAL fluids were put on ice after sampling and quickly transportated to laboratory within 30 minutes. In the laboratory, BAL fluid was centrifuged at 15000 rpm for 10 min at 4^0C. The supernatant was separated from the pellet. The supernatant was stores at -20^0C for detecting antibody by ELISA, the cellpellet was use for microbiological culture.

3.2.5. Protocol of blood sampling

2 ml nonheparinized blood of the patient was collected in sterilized vials. The blood was centrifuged at 3000 rpm for 2 minutes to collect serum. The sera were store at -20^0C until the the ELISA test was performed.

Samples / Place	BAL	Blood	BAL control		Blood control	
			Negative	Positive	Negative	Positive
HUMP hospital	30	30	5	0	30	0
HCH	66	66	10	0	0	0
Bach Mai hosppital	#	#	#	#	#	9
Viet Duc hospital	#	#	#	#	#	3
74 central hospital	#	#	#	#	#	2
Da Nang hospital	#	#	#	#	#	1
Nouro hospital	#	#	#	#	#	1

Table 3.1. Distribution of samples on study

3.2.6. *Bacteria species* identification and antimicrobial susceptibility

The microbiology was determined by isolating at least one pathogenic organism from

blood culture or from BAL culture. Microbiological techniques are varied by site in line with routine clinical microbiological work of HCH and HUMP hospital. BAL samples were subjected to Gram staining prior to incubation on rabbit blood in blood agar base, brain heart infusion, drigalski lactose agar, and chocolate blood agar.

Antimicrobial susceptibility test of bacteria strains was performed by Kirby-Bauer disk diffusion method according to CLSI 2015 guideline [100] and manufacturer's instructions. The standard medium used for disk diffusion test was Mueller-Hinton agar, tested antibiotics were cefotaxime, cotrimoxazol, ciprofloxacin, levofloxacin, gentamicin, ceftazidime, imipenem, meropenem, amikacin, augmentin, colistin. Antimicrobial agents were used at the concentrations indicated in table 3.2.

Drugs	Code	Potency	Zone in diameter (mm)		
			S	I	R
Cefotaxime	CTX	30 µg	≥ 23	-	≤ 14
Coxtrimoxazol	SXT	25 µg	≥ 16	-	≤ 10
Ciprofloxacin	CIP	5 µg	≥ 21	15 - 16	≤ 15
Levofloxacin	LEV	5 µg	≥ 17	15 - 16	≤ 13
Gentamicin	GM	10 µg	> 15	7 - 9	< 12
Ceftazidime	CAZ	30 µg	≥ 18	14 - 22	≤ 14
Imipenem	IMP	10 µg	≥ 16	15 - 18	≤ 13
Meropenem	MEM	10 µg	≥ 19	16 - 20	≤ 15
Amikacin	AMC	30 µg	≥ 18	13 - 17	≤ 12
Augmentin	AN	30 µg			
Colistin	COL	10 µg	≥ 11		< 10

(S: Susceptible, I: Intermediate, R: Resistant)

Table 3.2. Zone diameter interpretive instructions of antimicrobial susceptibility

The colonies were suspended in 5 ml of sterile 0.85% saline, and the turbidity was adjusted to yield 1×10^8 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. Streaked the swab all over the surface of the

medium three times, rotating the plate through an angle of 60^{o} after each application then passed the swab round the edge of the agar surface. Left the inoculum to dry for a few minutes at room temperature with the lid closed. Antimicrobial disks were placed on the inoculated agar with a forceps, and the plates were incubated at 37^{o}C. A maximum of seven discs can be placed on a 9 - 10 cm plate. Six discs may be spaced evenly, approximately 15 mm from the edge of the plate, and 1 disc placed in the center of the plate. Each disc was gently pressed down to ensure even contact with the medium. The plates were incubated at 37°C. The zone of inhibition was recorded after 24 hours and 48 hours.The diameter of each zone (including the diameter of the disc) was made with a ruler on the under-surface of the plate without opening the lid.

The results of culture and antimicrobial susceptibility were usually available after 3 days.

3.2.7. *Candida species* identification

A. Microbiological media

- Sabouraud dextrose agar (SDA) medium and Brilliance *Candida* agar medium were used.

- 5 ml of BAL was centrifuged 15000 rpm for 10 minutes at 4^{0}C. The cellpellet was dispensed on the plate surface containing Sabouraud dextrose agar (SDA) medium and chloramphenicol to incubate for 24 - 48 hours at 37^{0}C. All *Candida* strains isolated from colony in SDA medium were sub-cultured in Brilliance *Candida* agar medium and incubated aerobically at 37°C. The plates were checked at 24, 48 and 72 hours. Brilliance *Candida* agar was used to identify the *Candida species* following the guide line of the manufacturer. The *species of Candida* were also selected basing on different colour of colonies. This media contains two chromogens (5-bromo-4-chloro-3-indolyl N acetyl ß-D-glucosaminide and 5 bromo-6-chloro-3-indolyl phosphate p-toluidine salt), which help identify the presence of the two target enzymes, the hexosaminidase and the alkaline phosphatase. The presense of either enzymes allows the differentiation of *C. albicans* and *C. tropicalis* from other *species of Candida* within 48 hours. The green colour of *C. albicans* comes by the chromogenic reaction as well as the dark blue color of *C. tropicalis*. Other species were very difficult to differentiate based on the colour. The isolated *Candida* strains were stored for matrix assisted laser desortion ionization time-of-flight

(MALDI-TOF) identification.

B. MALDI-TOF MS

Candida species was subcultured in SDA plates. After 24 hour incubation at 37^0C, single colony was transferred directly and spotted in duplicate into the MALDI target. 1µl pure ethanol was added to each well to fix the sample. Next, 1µl of 70% formic acid was added and mixed gently. When the liquid medium was evaporated completely, each spot was overlaid with 1 µl of HCCA matrix solution and dried at room temperature. The loaded plate was analyzed by MALDI Biotyper CA System. The spectrum obtained was compared with the Maldi database. Identification was provided with accompanying scores as the manufacture 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

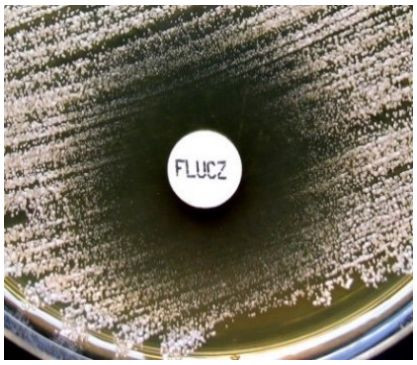
In our study, one hundread fungal strains were isolated and identified from ninety six BAL samples.

3.2.8. Antifungal susceptibility testing

- Mueller-Hinton medium supplemented with 2% dextrose and 0.5 µg/ml methylene blue (Liofilchem Laboratories, Italy) and antifungal disk (Liofilchem Laboratories, Italy) were used.

- *Candida species* were suspended in 5 ml of sterile 0.9% normal saline, shaked 15 seconds and the turbidity was adjusted to yield 1×10^5 - 1×10^6 cells/ml (0.5 McFarland standard). A sterile cotton swab was soaked into the suspension and pressed firmly against the inside wall above the fluid level. The *Candida species* was spread on media by moving the swab 3 times arround the plates. After that, the plates were dried at room temperature for 3 - 5 min. Antifungal disks were put on the inoculated agar with forceps, and the plates were incubated at 37°C. The antifungal tested included fluconazole 10 µg/disk, intraconazole 8 µg/disk, amphotericin B 20 µg/disk, nystatin 100 unit/disk, flucytosine 10 µg/disk, caspofungin 5 µg/disk. The zone of inhibition was recorded after 24 hours and 48 hours. 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 species* following

manufacture instructions.



Sensitive of *C. albicans*

Resistant of *C. albicans*

Image 3.2. Antifungal susceptibility of *C. albicans* to fluconazole

Drugs	Code	Potency	Zone in diameter (mm)		
			S	I	R
Fluconazole	FLU	25 µg	≥ 19	15 - 18 (DD)	≤ 14
Intraconazole	ITC	8 µg	> 16	10 - 15 (DD)	< 9
Amphotericin B	AMB	10 µg	≥ 15	10 - 14	< 10
Flucytosine	AFY	1 µg	≥ 20	12 - 19	≤ 11
Nystatin	NY	100 UI	≥ 15	10 - 14	< 9
Caspofungin	CAS	5 µg	≥ 16	13 - 15	≤ 12

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

Table 3.3. Zone diameter interpretive instructions of antifungal susceptibility

3.2.9. Indirect ELISA protocol

A. Epitopes selection

ECE1, HWP1 proteins were chosen as antigen. ECE1-III$_{62\text{-}93K}$ Candidalysin sequence [46, 47] and HWP1 protein sequence (CP017626.1) were used to select two epitopes by using BepiPred 2.0 software. Peptides from 14 to 16 amino acids were produced with a C-terminal cysteine residue to allow cross-linking with maleimide activated carrier proteins

Peptide	Sequence	Molecular mass (Da)
ECE1	H-CIQIIMSIVKAFKGNK-OH	1793.26
HWP1	H-CDNPPQPDQPDDNP-OH	1551.56

Table 3.4. Amino acidic sequences of synthetic peptides in this study

B. Procedure

B1. ELISA plates preparation

Microwell plates (NUNC^{TM}, Denmark) were washed in distilled water 3 times before using. 0.5 µg BSA-M (Sigma Aldrich, USA) per well were dispensed into microwell plates and incubated at 4°C overnight. The plates were then firmly washed in distilled water. Firstly, the two selected peptides (ECE1 and HWP1, GenScript, Hong Kong) were dissolved in maleimide conjugation buffer (appendix 2). This solution was diluted with distilled water at 1:2 ratios to a final concentration 10 µg/ml. Secondly, 50 µl of each dilution was dispensed into BSA-M precoated plates. Microwell plates were incubated for 2 hours at room temperature to allow cross-linking, then washed with distilled water. Thirdly, 300 µl per well PBS - Tween 0.05% - BSA 3% was added for saturation and incubate for 1 hour at room temperature. Finally, the microwell plates were washed with distilled water. After drying in air for 1 - 2 hours, the microwell plates were stored at 4^0C.

B.2. ELISA

Each ELISA test included a positive sera control, negative sera control and one well without sera. Positive sera control was collected from patients positive for *Candida species* in blood culture. Negative sera control was collected from healthy men from 18 - 50 years old. Sera were diluted 1:50 in PBS - Tween 0.05% - BSA 3% solution, and 100 µl were added to each well. The microwell plates were incubated for 2 hours at room temperature and washing the well for 3 times by PBS - Tween 0.05%. Anti-human IgG specific alkaline phosphatase conjugate (No. A9544 of Sigma Aldrich, USA) was diluted 1:20000 in PBS - Tween 0.05% - BSA 3% immediately before adding 100 µl into each well and incubated for 2 hours at room temperature. After incubation, the microwell plates were washed 3 times by PBS - Tween 0.05%. Two pills of ELISA substrate (Nitrophenyl phosphate tablets, No. 2770 of Sigma Aldrich, USA) were dissolved in 20 ml distilled water. After adding 100 µl/well of substrate solution and covering the plate without bright light, the optical density of the microwells were read at 405 nm in 15 - 30 minutes by ELISA reader (Multiskan go, Thermo scientific).

3.3. Ethical issue

Study protocols were approved by Hue University of Medicine and Pharmacy Institutional Review and Ethical Board.

3.4. Analyze and handling data

- Statistical analysis was performed using Microsoft Excel and R software.

- Comparison of proportions between two rates were calculated by Chi-square test.

- Comparison of two means were calculated by Independent sample T-test for evaluation ELISA.

- Levene's test for equality of variances, ANOVA test for evaluating the relationship. All reported confidence intervals were two-sided 95% confidence intervals and P-values < 0.05 were regarded as statistically significant.

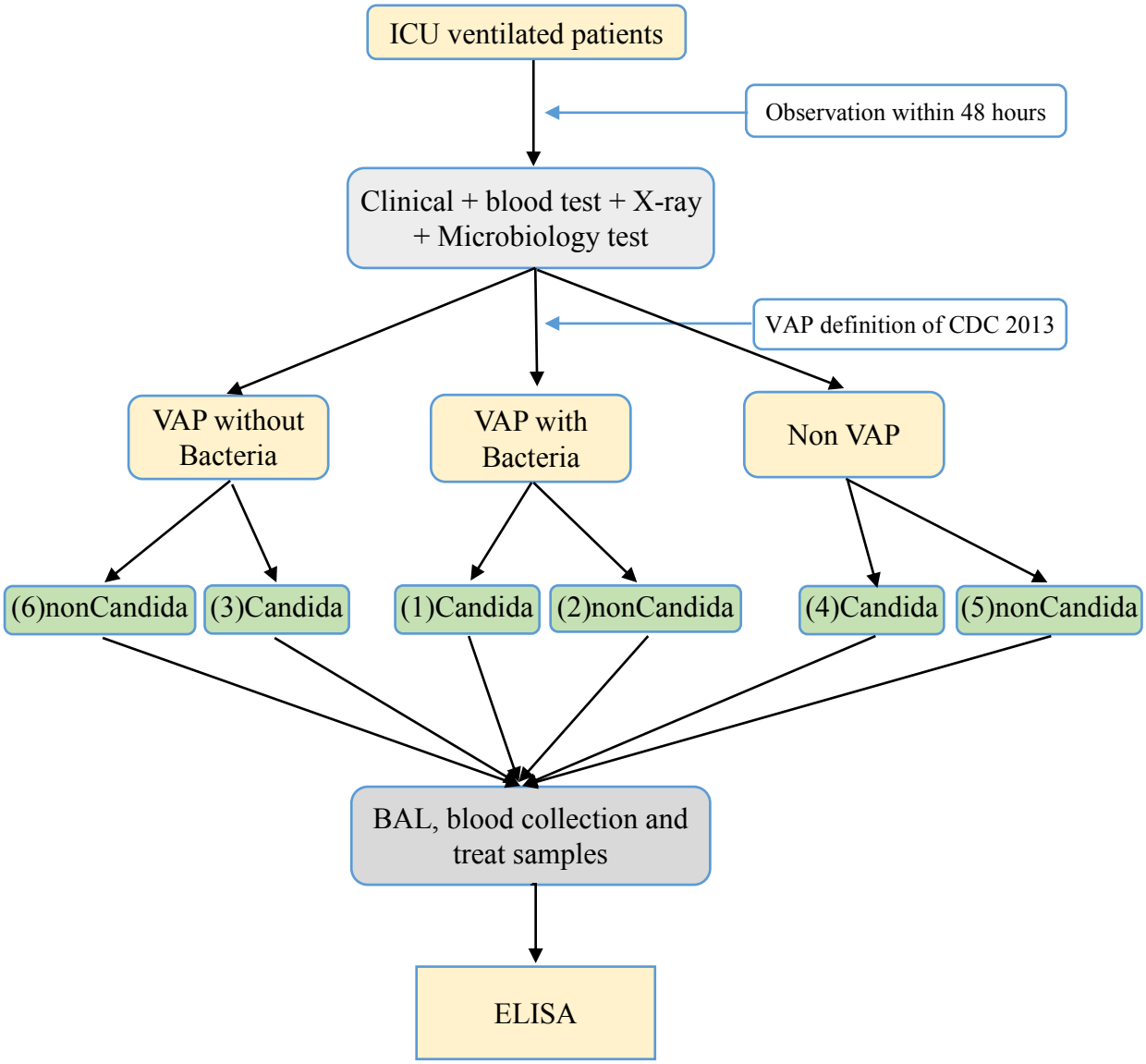


Figure 3.1. Scheme of study

4. RESULTS

4.1. General clinical and laboratory characteristics

4.1.1. Clinical symptoms

Totally, 96 patients were enrolled during the study period from October 2015 to September 2018. 30 patients from HUMP hospital and 66 patients from HCH were observed in this study. The primary admission diagnosis is shown on figure 4.1.

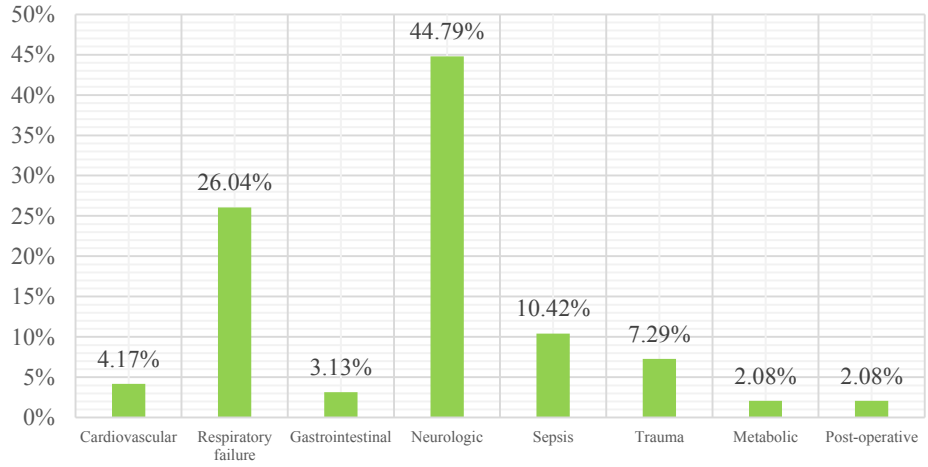


Figure 4.1. Primary admission diagnosis of patients

The neurologic injury (44.79%), respiratory failure (26.04%) and sepsis (10.42%) were prevalent reasons leading to ICU admission. The mean age was 63 years old, the length of mechanical ventilation and ICU stay was around 18 days and 30 days. All patients in this study were in bad condition with high SOFA score (5 points) and APACHE II score (15 points) respectively, associated with high ICU mortality (52.08%).

Age (year)	SOFA	APACHE II	Ventilation (day)	ICU stay (day)	ICU mortality
63.12±20.83	4.69±2.99	15.1±6.89	18.06±17.92	29.77±24.23	52.08%

Table 4.1. Clinical symptoms of patients

4.1.2. Inflammatory symptoms of infection

The table 4.2 shows that the procalcitonin (22.97 ng/ml) and CRP (90.52 mg/l) index was increased strongly in this infection. The response of the patients to infection was weak with slightly increasing in temperature and leukocytes. The Candida score, which was

used for deciding early antifungal treatment in nonneutropenic critical ill patients with *Candida* colonization, was 1.7 point.

Temperature (^{0}C)	Leukocytes (4-10 G/L)	Procalcitonin (0-0.05 ng/ml)	CRP (0-5 mg/l)	Candida score
37.8±0.6	12.3±5.84	22.97±60.61	90.52±86.24	1.7±1.19

Table 4.2. Inflammatory symptoms of patients

4.1.3. Group classification based on VAP definition and BAL culture results

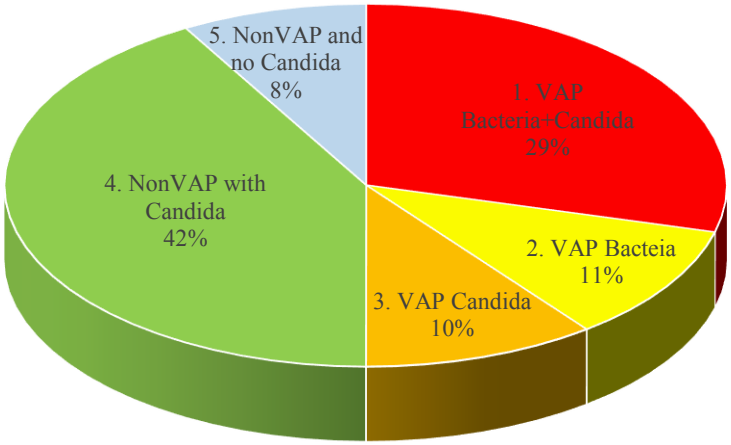


Figure 4.2. Group classification of all patients

According to VAP definition of CDC 2013 and BAL culture results, the patients were divided into five groups. Group 1 (VAP with *Bacteria* and *Candida species*) was 29%, group 2 (VAP with only *Bacteria species*) was 11%, group 3 (VAP with only *Candida species*) was 10%, group 4 (nonVAP with *Candida species*) was 42% and group 5 (nonVAP without pathogens) was 8%. Totally, the ratio of VAP group (goup 1 + group 2 + group 3) and nonVAP group (group 4 + group 5) were 50%.

4.2. Clinical and laboratory data among five groups

Most of patients in this study were in severe stage of diseases. All clinical and laboratory data among five groups were showed clearly on table 4.3. SOFA score was range from 3 - 5 points indicated that at least one organ of patient had malfunction. APACHE II score was range from 12 - 16 points indicated that predicting hospital mortality is from 14.6% to

23.5%. However, the length of ICU stays and ventilation day, SOFA score, APACHE II score, Candida score of patients in group 1 were higher than in other groups. The temperature and Candida score were significantly different among five groups.

Group	1	2	3	4	5	p
Age (year)	64.57±21.97	64.3±21.67	59.3±23.83	62.98±19.83	62.12±21.65	>0.05
ICU stay (day)	39.36±32.36	34±18.54	24.8±21.12	24.87±17.46	21.63±23.95	
Ventilation (day)	23.22±26.8	20.8±8.9	17.4±17.08	15.49±12.6	10.62±6.76	
Temp (^{0}C)	38.1±0.5	38.02±0.73	37.86±0.39	37.69±0.65	37.26±0.33	<0.05
Leukocyte (4-10 G/L)	13.41±4.61	14.58±13.15	11.83±4.27	11.75±4.19	9.02±2.64	>0.05
Procalcitonin (0-0.05ng/ml)	20.19±46.81	0.68±0.59	95.72±142.09	6.43±6.66	0.24±0.0	
CRP (0-5 mg/l)	87.38±71.45	98.1±68.7	118.22±98.88	80.17±105.19	108.0±0.0	
SOFA	5.11+2.97	4.3±1.77	5.2±1.69	4.55±3.67	3.75±1.67	
APACHE II	16.14±7.5	15.1±4.46	12.6±3.72	15.63±7.62	12.12±4.16	
Candida score	2.46±1.26	0.8±0.63	2.2±0.92	1.5±1.01	0.5±0.54	<0.001
Mortality	57.14(16/28)	50%(5/10)	60%(6/10)	47.5%(19/40)	50%(4/8)	>0.05

Table 4.3. Clinical data among 5 groups

4.3. Clinical and laboratory data between VAP and nonVAP group

The clinical symptoms and inflammatory maker in VAP group were higher than in nonVAP group. The table 4.4 shows that the length of ICU stay, mechanical ventilation, temperature and Candida score of VAP group are longer and higher than nonVAP group. The length of ICU stays, ventilation day, procalcitonin index, SOFA score, APACHE II score of ICU ventilated patients with *Candida species* group were longer and higher, but there was not significantly difference between the two groups.

Group	NonVAP	VAP	p	NonCandida	Candida	P
Age (year)	62.83±19.9	63.42±21.92	>0.05	63.33±21.04	63.08±20.92	
ICU stay (day)	24.33±18.44	35.21±28.04		28.5±21.41	30.06±24.95	
Ventilation (day)	14.66±11.9	21.47±21.99	<0.05	16.28±9.37	18.49±19.42	
Temp (^{0}C)	37.62±0.62	38.02±0.54		37.68±0.69	37.85±0.59	
Leukocytes (4-10 G/L)	11.3±4.08	13.32±7.05		12.11±10.13	12.36±4.37	>0.05
Procalcitonin (0-0.05 ng/ml)	5.74±6.56	35.82±78.9		0.53±0.49	26.66±64.95	
CRP (0-5 mg/l)	81.62±102.43	96.35±75.13	>0.05	99.34±63.7	88.75±90.61	
SOFA	4.42±3.41	4.96±2.52		4.06±1.7	4.83±3.21	
APACHE II	15.04±7.24	15.19±6.38		13.78±4.47	15.42±7.3	
Candida score	0.8±0.63	2.46±1.26	<0.05	0.67±0.59	1.94±1.78	<0.05
Mortality	23/48(47.9%)	27/48(56.3%)	>0.05	(9/18)50%	(41/78)52.56%	>0.05

Table 4.4. Clinical symptoms between VAP group and nonVAP group

4.4. Microbiology results

4.4.1. Frequencies of *bacteria species* isolation from BAL

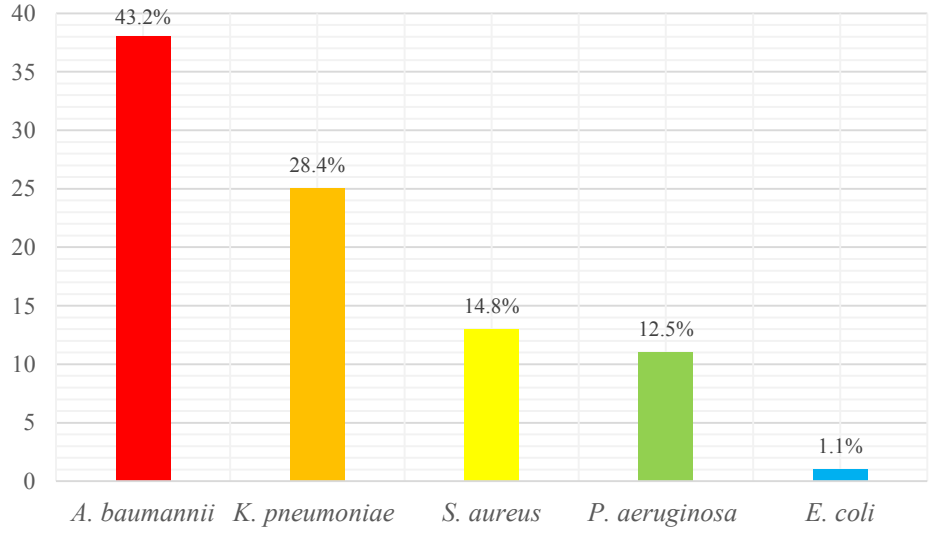


Figure 4.3. Bacteria species isolation from BAL

A total of 89 *bacteria species* were isolated from 96 ICU ventilated patients in this study. The 3 main *bacteria species* in VAP were *A. baumannii* (43.2%), *K. pneumoniae* (28.4%) and *S. aureus* (14.8%).

4.4.2. Frequencies *bacteria species* isolation between two hospitals

The bacteria pathogens in VAP patients were different from HCH and HUMP hospital. *A. baumannii* was the most frequent pathogen in both hospitals while *P. aeruginosa* (in HUMP hospital) and *K. pneumonia* (HCH) were the second agent caused by VAP.

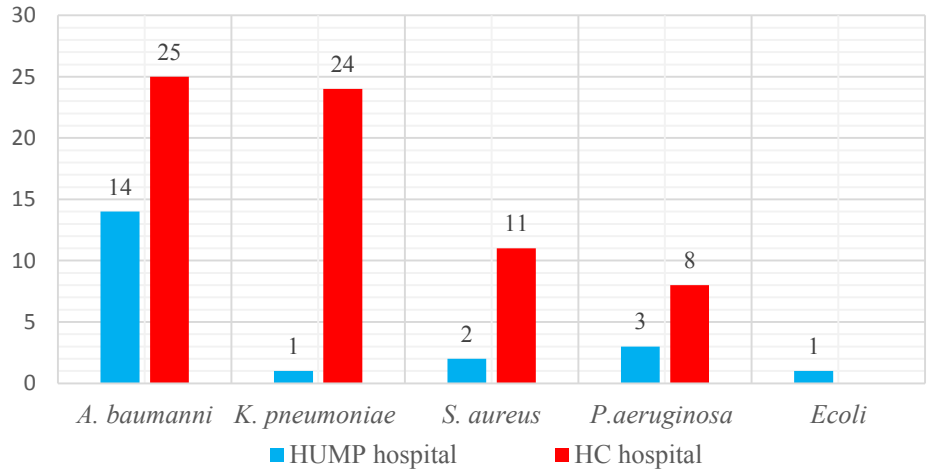


Figure 4.4. Bacteria species isolation from BAL in the two hospitals

4.4.3. Antimicrobial susceptibility of *A. baumannii*

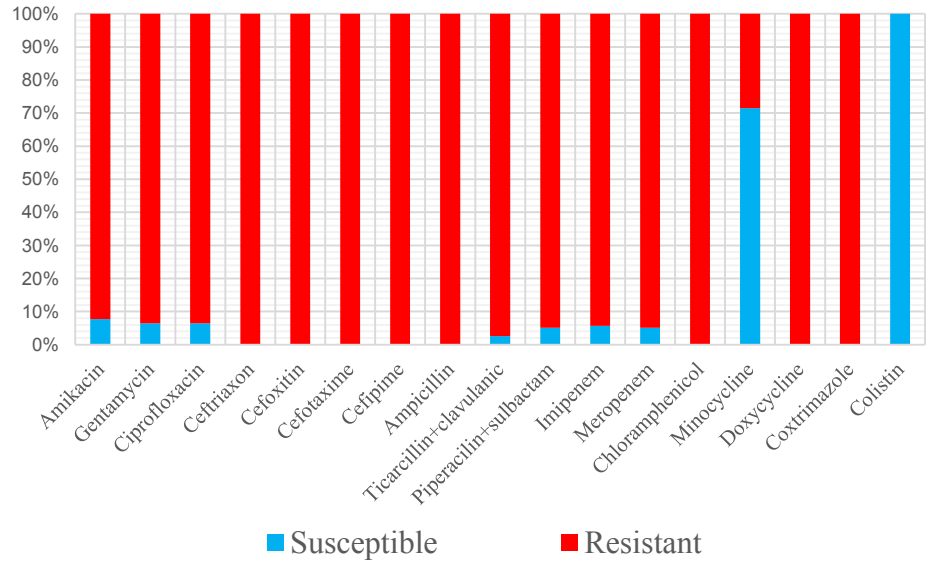


Figure 4.5. Antimicrobial susceptibility of A. baumannii

A. baumannii was the first pathogen caused VAP for multidrug resistance characteristic. In this study, *A. baumannii* was resistant to cephalosporin 2, 3, 4 generation (100%), carbapenem and quinolone group (>94%). However, all of them were still sensitive to colistin. The antimicrobial resistance of *A. baumannii* in HCH was higher than in HUMP hospital.

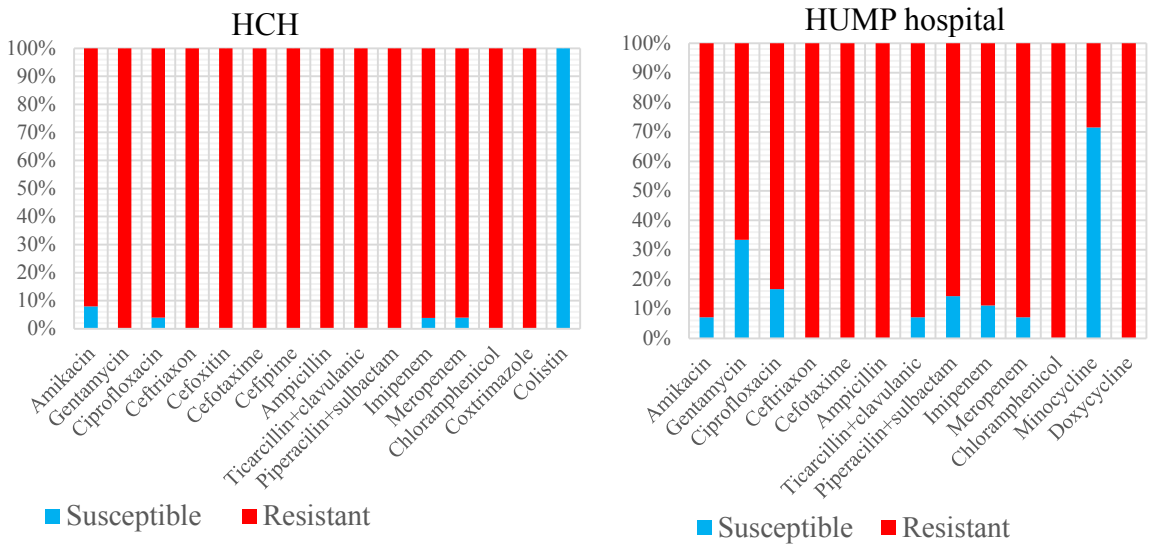


Figure 4.6. Antimicrobial susceptibility of A. baumannii in the two hospitals

In HUMP hospital, *A. baumannii* was still showed some sensitive to minocycline, penicillin + sulbactam, quinolone and aminoglycoside group.

4.4.4. Antimicrobial susceptibility of *K. pneumoniae*

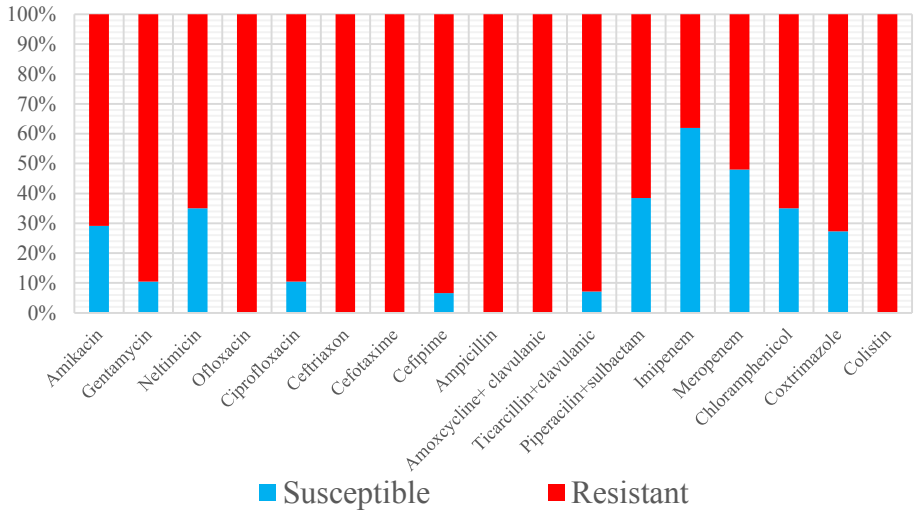


Figure 4.7. Antimicrobial susceptibility of K. pneumoniae

K. pneumoniae is more and more becoming a predominant pathogen in VAP with high multidrug resistance percentage. 50% *K. pneumoniae* was resistant to carbapenem group. One isolated was resistant to all antibiotics tested.

4.4.5. Antimicrobial susceptibility of *S. aureus*

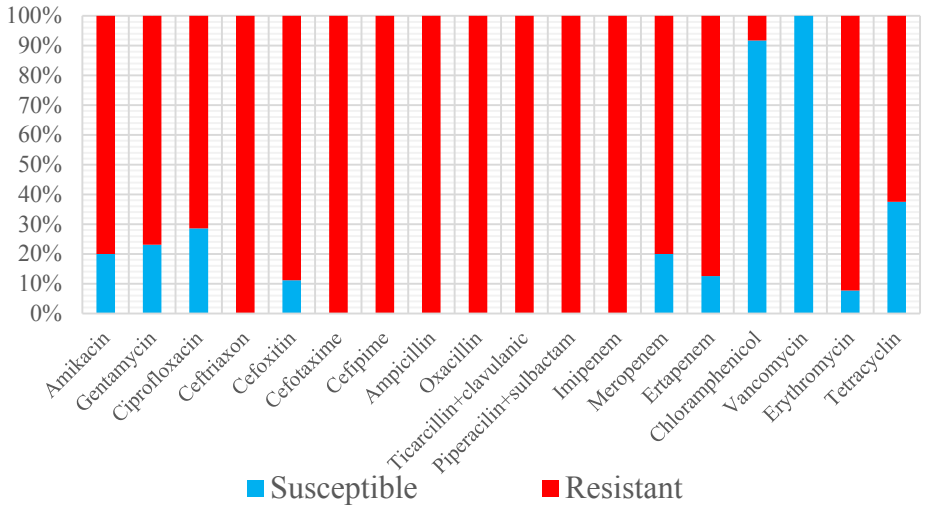


Figure 4.8. Antimicrobial susceptibility of S. aureus

All *S. aureus* were resistant to methicillin, penicillin + sulbactam group, cephalosporin 2, 3, 4 generation (100%) and sensitive to vancomycin (100%).

4.4.6. Fungal isolation from BAL

One hundred fungal strains isolated from ninety six BAL samples were identified by culture and MALDI-TOF Mass Spectrometry.

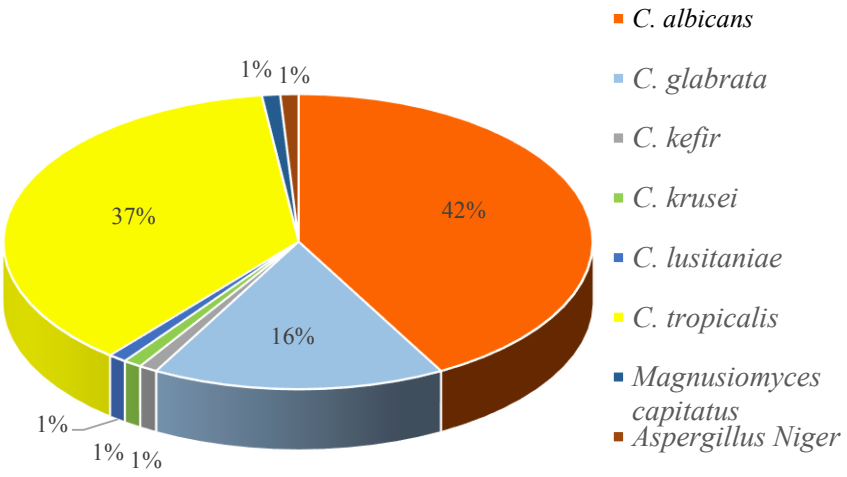


Figure 4.9. Fungal isolation from BAL

The 3 main fungal pathogens *C. albicans* (42%), *C. tropicalis* (37%) and *C. glabrata* (16%) were commonly isolated from BAL of ICU ventilated patients.

4.4.7. Antifungal susceptibility of *Candida species*

The percentage of *Candida species* resistance for each drug is described in figure 4.10. The resistance of total *Candida species* to fluconazole occurred in 21.11% and caspofungin was 4.44%. There was not cross - resistance to multiple azole drugs. All *Candida species* were sensitive to intraconazole.

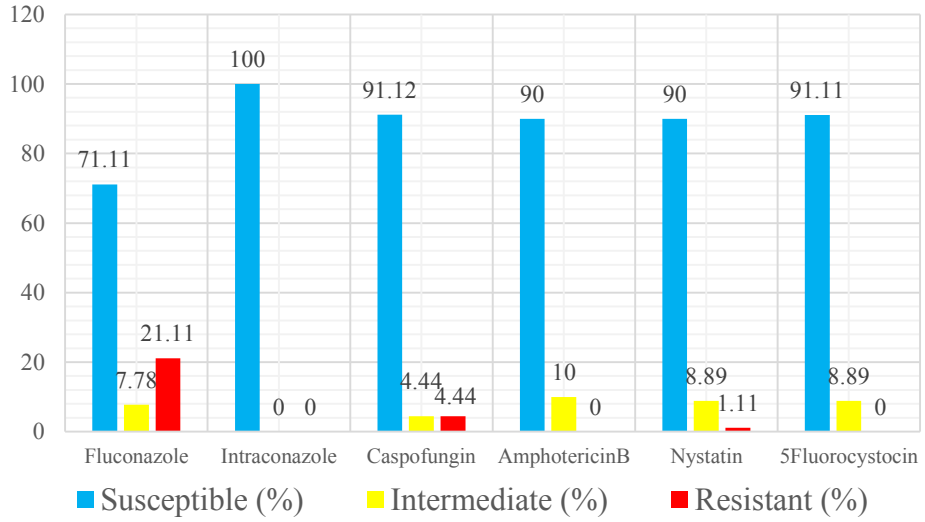


Figure 4.10. Antifungal susceptibility of Candida species

4.4.8. Antifungal susceptibility of *C. albicans*

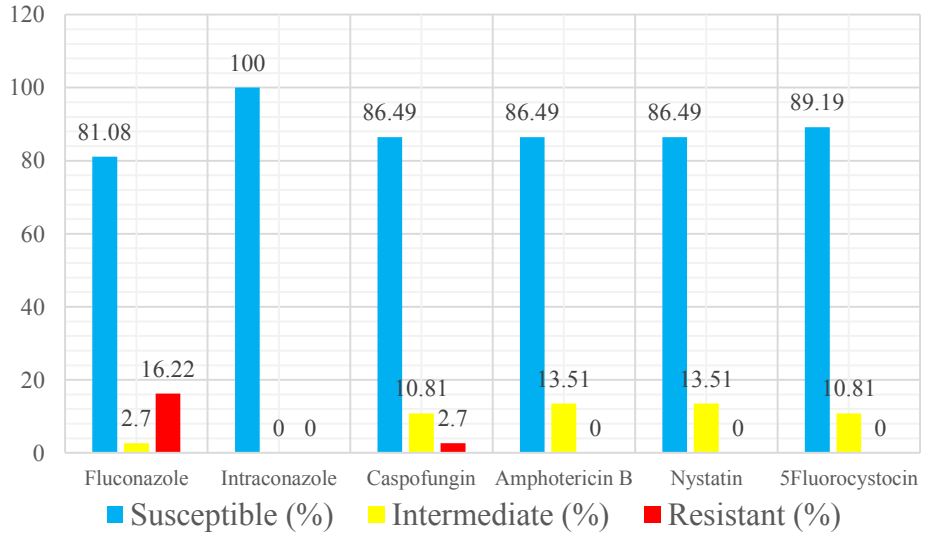


Figure 4.11. Antifungal susceptibility of C. albicans

In this study, among *Candida species*, the main pathogen in ICU ventilated patients was *C. albicans.* The percentage of fluconazole resistance was 16%, caspofungin resistance was 2.7%. All of *C. albicans* was sensitive to intraconazole, amphotericin B, nystatin and 5 fluorocystocin. However, the antifungal susceptibility results were different between the two hospitals. In HUMP hospital, *C. albicans* was higher resistant than in HCH. While the fluconazole resistance in HUMP hospital was up to 37% and caspofungin resistance was 6%, all *C. albicans* in HCH were sensitive to all antifungal drugs tested. The results are shown in figure 4.12.

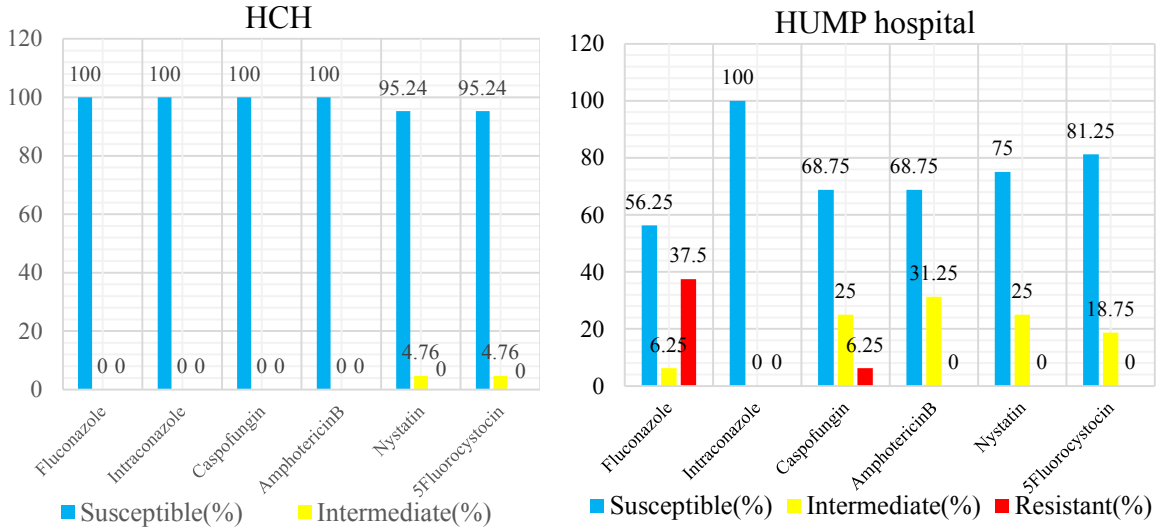


Figure 4.12. Antifungal susceptibility of C. albicans between two hospitals

4.4.9. Antifungal susceptibility of *C. tropicalis*

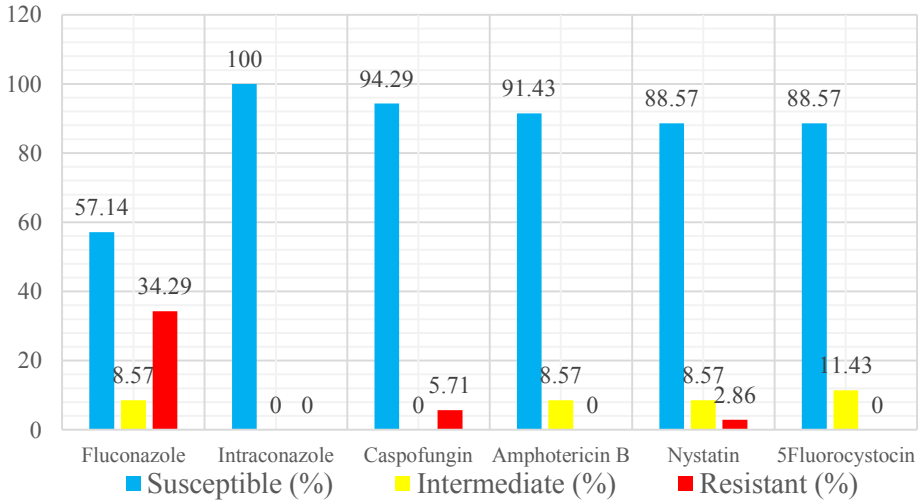


Figure 4.13. Antifungal susceptibility of C. tropicalis

C. tropicalis is becoming a predominant pathogen in developing country nosocomial infections in ICU with high rate of antifungal resistance. In this study, the *C. tropicalis* fluconazole resistance was 34.29%, caspofungin resistance was 5.71% and nystatin was 2.86%. Only 57% *C. tropicalis* was sensitive to fluconazole. The fluconazole resistance of *C. tropicalis* in HUMP hospital was higher than HCH. In HUMP hospital, the percentage of fluconazole resistance was up to 56.2% instead in HCH was 15.79%. However, all *C. tropicalis* in both hospitals was sensitive to intraconazole (100%). In this study, *C. tropicalis* resistant to amphotericin B and 5 fhuorocystocin was not found.

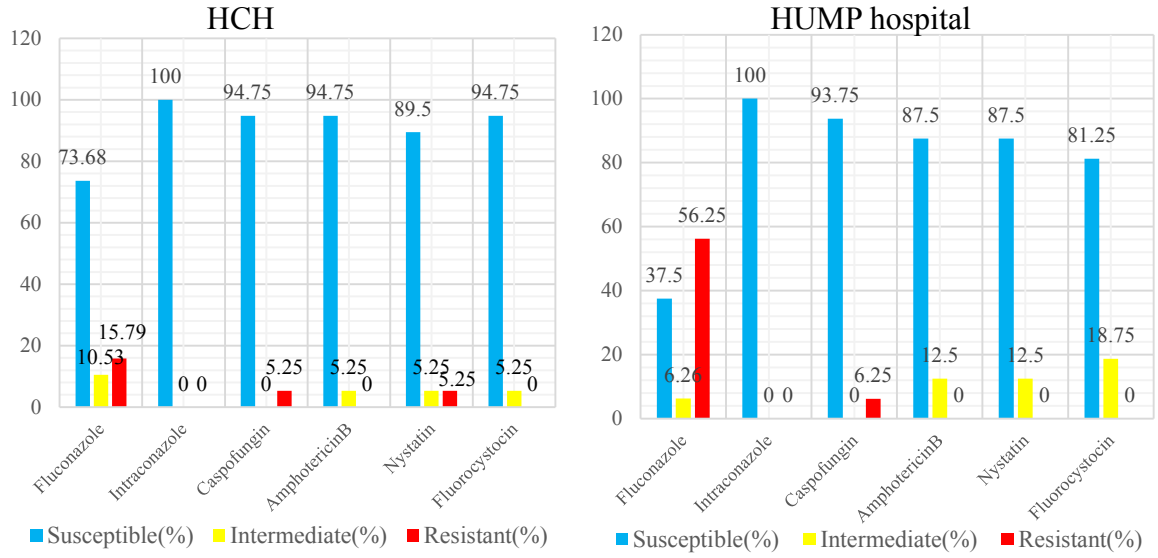


Figure 4.14. Antifungal susceptibility of C. tropicalis between two hospitals

4.4.10. Antifungal susceptibility of *C. glabrata*

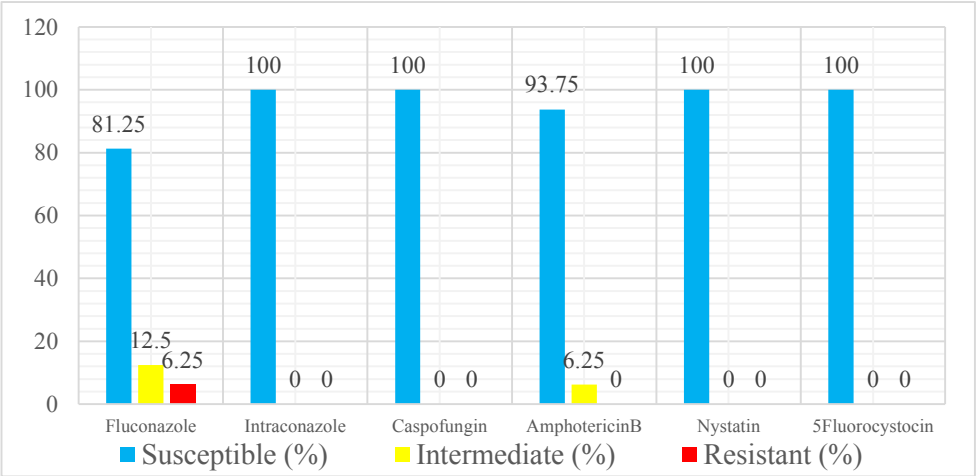


Figure 4.15. Antifungal susceptibility of C. glabrata

The fluconazole resistance of *C. glabrata* was 6.25% while 100% of *C. glabrata* were susceptible to intraconazole, caspofungin, amphotericin B, nystatin and 5 fluorocystocin.

4.5. Treatment

4.5.1. Firstline antibiotics prescription

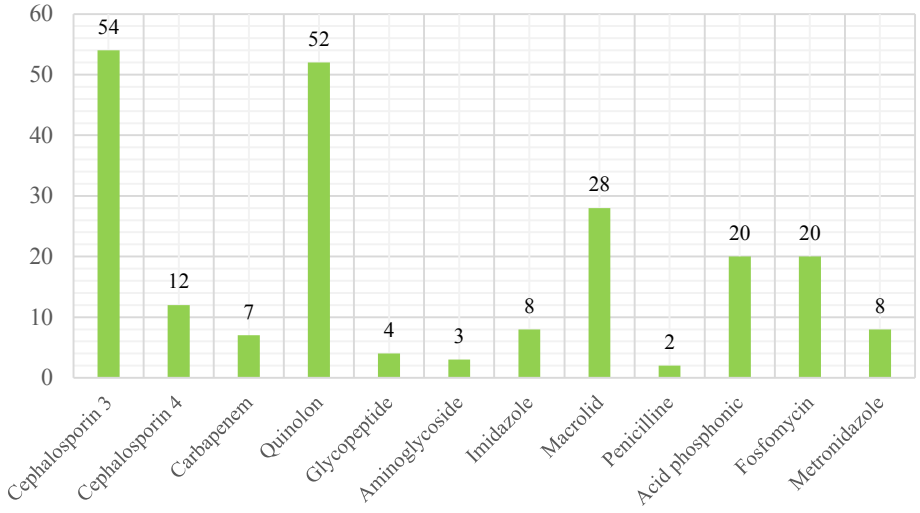


Figure 4.16. Frequencies of firstline antibiotics indication

Firstline antibiotics were prescribed following the international guidelines and based on the doctor's experience. The physicians often combine third-generation cephalosporin + quinolone group. The percentage of antibiotic combination was 76.04%, as described in figure 4.17.

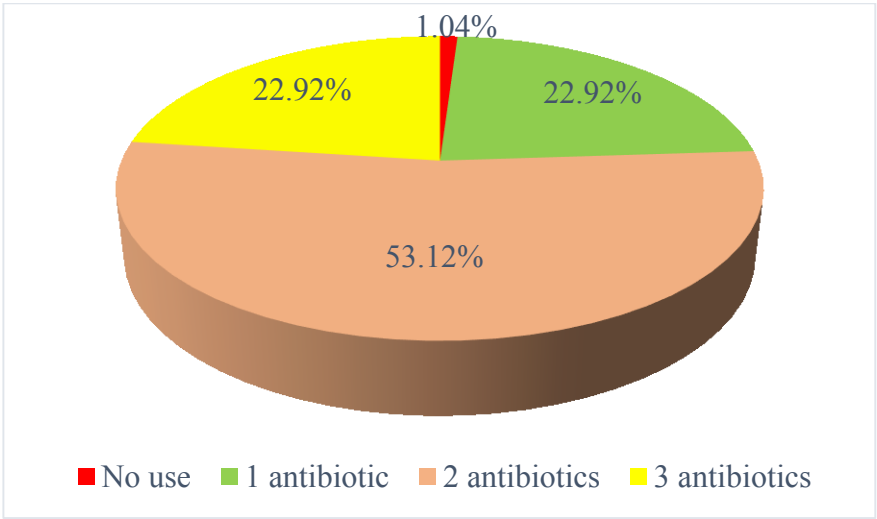


Figure 4.17. Characteristic of firstline antibiotic therapy

4.5.2. Suitable of firstline antibiotics treatment

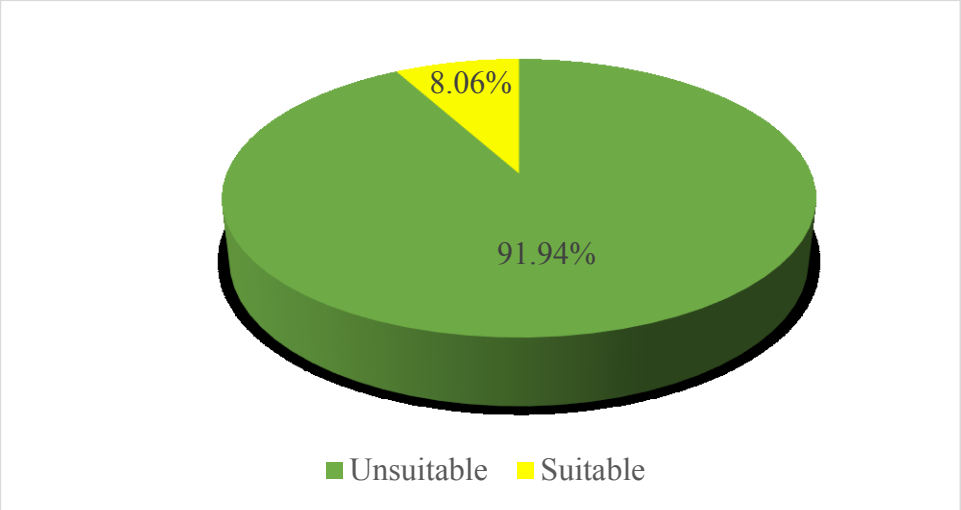


Figure 4.18. Suitable of firstline antibiotics treatment

92% of firstline antibiotic could not kill the pathogens causing VAP. When antimicrobial susceptibility resulted, the physicians changed the antibiotic therapy. However, only 6/39 patients with *A. baumanni* infection were treated with colistin in HCH.

4.6. Indirect ELISA results

4.6.1. Positive and negative control

In the standard antibody detection ELISA, a cutoff value was determined by calculating an average optical density for 30 normal serum samples and 15 normal BAL samples of healthy men plus 3 standard deviations. The cutoff value was shown in table 4.5. Sera from Candidemia patients were used as ELISA positive control.

Cutoff value	**ECE1sera**	**HWP1sera**	**ECE1bal**	**HWP1bal**
Neg (mean ± 3SD)	0.274	0.234	0.079	0.085

Table 4.5. Cutoff value of indirect ELISA results

4.6.2. Indirect ELISA results in 5 groups

By comparing the patient's optical density with positive control and cutoff value, the indirect ELISA results in 5 groups are shown in the below table 4.6. The results of ECE1 antibody detecting in blood and BAL were significant different among the 5 groups. With ECE1 antibody detecting, 14 cases of group 1 were positive and 4 cases were positive

belonging to group 3 in blood while 7 cases were positive belonging to group 1 and 4 cases were positive belonging to group 3 in BAL. With HWP1 antibody detecting, 6 cases of group 1 were positive, 3 cases of group 3 were positive, 5 cases of group 4 were positive in blood and only 2 cases of group 1 were positive in BAL, but the difference is not statistically significant.

Value / Group	ECE1sera		HWP1sera		ECE1bal		HWP1bal	
	Pos	**Neg**	**Pos**	**Neg**	**Pos**	**Neg**	**Pos**	**Neg**
1	14	13	6	21	7	20	2	25
2	0	10	1	4	0	5	0	5
3	4	6	3	7	4	6	0	10
4	0	40	5	35	0	40	0	40
5	0	8	0	5	0	5	0	5
F	34.41		3.35		17.26		4.53	
p	<0.001		>0.05		<0.001		>0.05	

Table 4.6. Indirect ELISA results among 5 groups

4.6.3. Indirect ELISA results between VAP group and nonVAP group

With ECE1 antibody detecting, all 18 cases resulted positive in blood and 11 cases positive in BAL belonged to the VAP group. There was not any positive case belonged to nonVAP group. Significant difference between the two groups was found in blood as well as in BAL. With HWP1 antibody detecting, 10 cases in group VAP and 5 cases in group nonVAP were positive in blood while in BAL, 2 cases were positive in VAP group. Here, in both blood and BAL the difference among the VAP and nonVAP were not statistically significant.

Value / Group	ECE1sera		HWP1sera		ECE1bal		HWP1bal	
	Pos	**Neg**	**Pos**	**Neg**	**Pos**	**Neg**	**Pos**	**Neg**
NonVAP	0	48	5	40	0	45	0	45
VAP	18	29	10	32	11	31	2	40
F	22.68		2.46		13.49		2.17	
p	<0.001		>0.05		<0.001		>0.05	

Table 4.7. Indirect ELISA results between VAP group and nonVAP group

4.6.4. Mean of ECE1, HWP1 optical density in serum and BAL of 5 groups

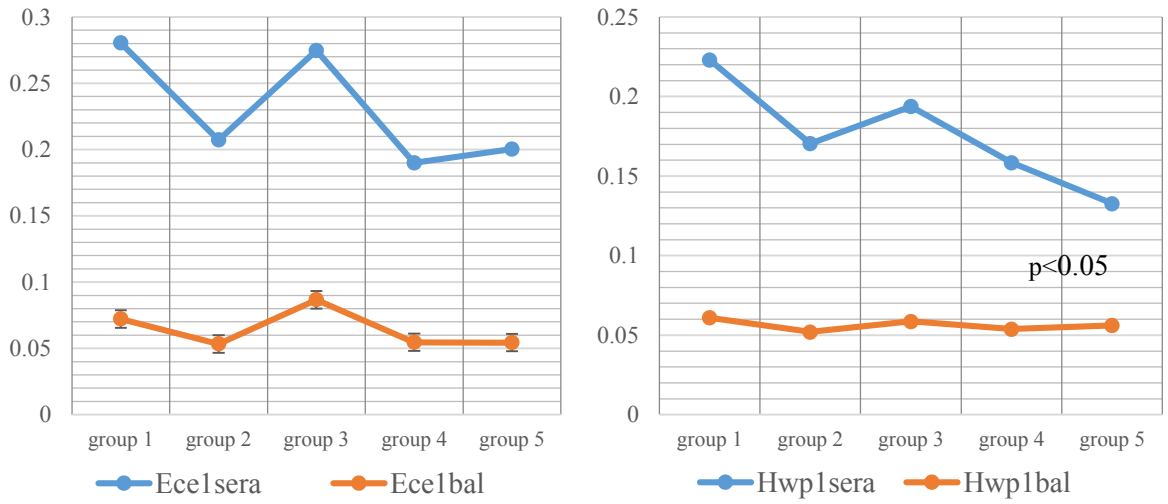


Figure 4.19. Mean of ECE1, HWP1 optical density of 5 groups

Among 5 groups, the optical density of group 1 and group 3 is always higher than group 2, group 4 and group 5. There was significant difference among 5 groups with ECE1 antibody detecting.

4.6.5. Mean of ECE1, HWP1 optical density of VAP group and nonVAP group

Comparing the group of VAP and of nonVAP in figure 4.20, the ECE1, HWP1 optical density in VAP group was always higher than in nonVAP group.

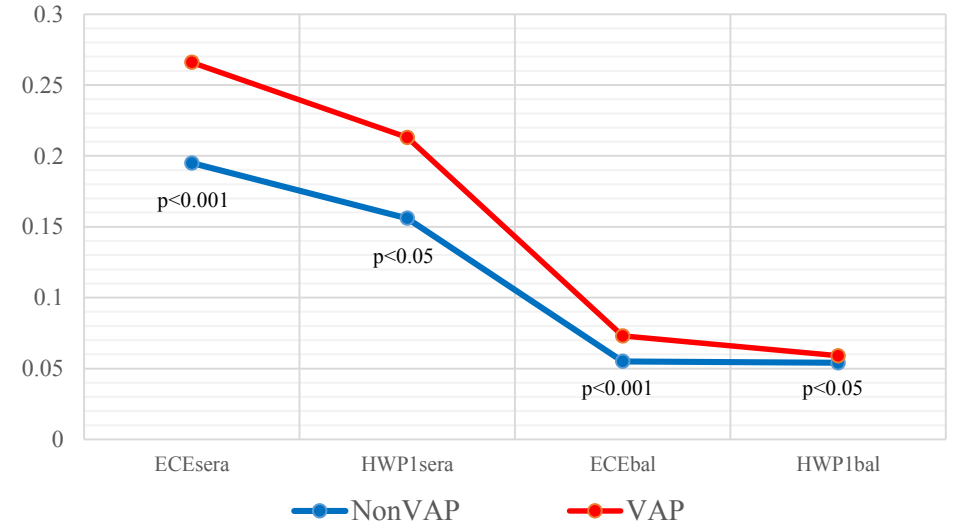


Figure 4.20. Mean of ECE1, HWP1 optical density of VAP and nonVAP group

4.6.6. Sensitivity and specificity of indirect ELISA test

In this study, from 15 serum samples from patients with candidemia and 30 normal serum samples from healthy men, the sensitivity and specificity of indirect ELISA test was calculated. The sensitivity/specificity of ECE1 antibody detecting and HWP1 antibody detecting were 80%/96% and 60%/77%, respectively.

4.6.7. Association between indirect ELISA results and clinical data

Group 1 (ECE1sera)	**Positive (14)**	**Negative (13)**	**p**
ICU stay (day)	49.93±41.22	29.23±15.83	>0.05
Ventilation (day)	29.71±36.04	16.33±7.1	
Leukocyte (4-10 G/L)	13.46±5.34	12.85±3.61	
Procalcitonin (0-0.05 ng/ml)	34.29±61.34	1.37±0.93	
Candida score	3.00±1.11	1.92±1.26	<0.05

Table 4.8. Association between clinical data and indirect ELISA results

In Vietnam, the physicians decide the antifungal treatment basing on clinical data and culture results. Therefore, all patients in group 1 here should be treated with antimycotic drugs. However, following these ELISA results, only 50% of the patients in group 1 should be treated with proper antifungal therapy. The rest of the patients should not be treated with any antifungal therapy for avoiding the onset of antifungal resistance and unnecessary costs for patients. In group 1, the length of ICU stays and ventilation day, leukocyte, procalcitonin index and Candida score of 14 positive cases were higher than 13 negative cases.

4.6.8. Correlation between indirect ELISA results and clinical data

There was a correlation between ELISA results and clinical data, a strong correlation between Candida score and ECE1 optical density in BAL and in blood was found while a weak correlation between ECE1 optical density in blood and ICU stay, ventilation days

was found. These correlations were all statistically significant.

Correlation	R	p
ECE1sera - Candida score	0.51	<0.001
ECE1bal - Candida score	0.42	
ECE1sera - ICU stay	0.31	
ECE1sera - Ventilation day	0.39	

Table 4.9. Correlation between clinical data and indirect ELISA results

5. DISCUSSION

We conducted a prospective analysis of data from critically ill ventilated patients who were treated at ICU of HCH and HUMP hospital. Our main findings are that *Candida species* is colonizing or invading in critically ill ventilated patients and the relationship between *Candida species* and *bacteria species* in pathogenicity.

5.1. Clinical characteristics of ICU ventilated patients

In our study, neurologic injury (44.79%), respiratory failure (26.04%) and sepsis (10.42%) were prevalent reasons leading to ICU admission. This is in agreement with main cause for ICU admission in a lot of prevoius studies [66, 67, 101] and the variuos rate depending on ICU characteristics.

In our study, ventilated patients with *Candida species* (Candida group) isolated from BAL have levels of CRP and procalcitonin higher than other patients, but the difference among groups was not statistically significant. Several studies proved the important role of procalcitonin in IC diagnosis during the last decade. Recently, Williamson *et al* showed that CRP and procalcitonin levels were significantly increased in the *Candida* group compared to the *nonCandida* group in patients with a clinical suspicion of VAP [67]. In our study, the strength in inflammatory response resulted in increasing length of mechanical ventilation, ICU and hospital stay. A lot of other studies had shown an association between *Candida species* colonization and prolonged mechanical ventilation, ICU and hospital stay [66, 67].

Most patients in our study were in the severe stage of diseases with high SOFA score (5 point), APACHE II (15 point) score and high mortality rate (52.08%). Almost all patients had at least one organ failure. The recorded SOFA score and APACHE II score for evaluating our treatment processing. The SOFA and APACHE II score were not higher than other studies, however, the mortality was higher than other studies in Europe and America [66, 70] probably because of the limitation in the resource-restricted ICU, the high rate of nosocomial infections with multidrug resistance [53] and low income of patients. A lot of patients in developing countries do not access to appropriate treatment, crucial equipment and high technology of health care system because of high hospital fees. This is probably reasons why almost patients were not be prescripted colistin for *A.*

baumannii infections and caspofungin or intraconazole intravenous for *Candida species* infections according to antimicrobial susceptibility results. Consequently, the mortality rates of patients is increased because of unspecific treatments.

5.2. *Bacteria species* characteristics from ICU ventilated patients

In our study, 89 *bacteria species* were isolated from BAL. The 3 main bacteria species causing VAP in Hue hospitals were *A. baumannii* (43.2%), *K. pneumoniae* (28.4%) and *S. aureus* (14.8%) with high level of antimicrobial resistance, while in the north and the south of Vietnam, *P. aeruginosa* (32.9%) was the third pathogen instead of *S. aureus* results in VAP [53]. Globally, several previous studies have concluded that Gram-negative bacteria were the main pathogens causing VAP in ICU [102]. The 3 principally agents which we identified in our study, were listed in a global priority organisms of antibiotic resistant bacteria to help prioritize the research and development of new and effective antibiotic treatments [103].

We report high levels of antimicrobial resistance for *A. baumannii* to cephalosporin 2, 3, 4 generation (100%), and carbapenem-resistant was > 94%. The carbapenem-resistant of *K. pneumoniae* was 50% and 100% *S. aureus* was resistant to methicillin. This is an alarming incidence of high level of antimicrobial resistance in central of Vietnam. The main reasons most probably came from the inconsequential antibitotic stewardship and the antibiotic abusing behavior of physicians. About 92% of pathogens resulting in VAP were resistant to firstline antibiotic therapy. From this results, antibiotic stewardship and nosocomial prevention bundle care must be improved at the ICU of HCH and HUMP hospitals.

5.3. *Candida species* characteristics from ICU ventilated patients

Candida infection in ICU ventilated patients may be endogenous (aspiration from gastro-intestinal flora or mucocutaneous colonisation) and exogenous (hands of healthworkers, contaminated equipments) [104].

In critically ill ventilated patients, the continuous and prolonged support of failing organs such as intravascular catheters, endotracheal tubes, naso-gastric tubes, foley catheters and the longterm using of a broad-spectrum antibiotic constitute key risk factors for *Candida* infections. Azoulay *et al* showed that *Candida* colonization in the respiratory

tract was 19.5% on day 5, 31.5% on day 15, and 33.7% on day 30 [66]. Regarding the distribution of *Candida species* in our study, *C. albicans* was the predominant species (42%), followed by *C. tropicalis* (37%) and *C. glabrata* (16%). Each of these three species was usually the only pathogen retrieved from the respiratory tract of ICU ventilated patients, whereas *C. krusei*, *C. kefir* and *C. lusitaniae* were generally found in combination with another *Candida species*. Totally, the percentage of *C. nonalbicans* (56%) was higher than *C. albicans* (42%). The study of Pfaller *et al* in 41 countries around the world showed that 90% of IC infections between 1997 and 2007 were caused by 5 species consisting of *C. albicans, C. glabrata, C. parapsilosis*, *C. tropicalis and C. krusei* [77]. Our results were similar to other studies in the Asian area, *C. albicans* was the most frequently isolated species, followed by *C. tropicalis, C. krusei* or *C. glabrata* [105-107]. However, the distribution of *Candida species* was different in ICU of European countries. In the study of Azoulay *et al*, *C. albicans* was the most common species (68.7%), followed by *C. glabrata* (20.1%) and *C. tropicalis* (13.1%). The study by Hamet *et al* from 2006 to 2010 indicated that *C. albicans* was the main species (56%), followed by *C. glabrata* (15%) and *C. krusei* (7%) [78].

Although *C. albicans* is the most common species in this genus, a shift towards *C. nonalbicans* in critically ill patients throughout the world has been reported [24, 73]. The ARTEMIS DISK Global Antifungal Surveillance Study showed a decreasing rate of isolation of *C. albicans* (from 70.9% to 65.0%), while the number of isolates containing *C. nonalbicans* were increasing: *C. glabrata* (from 10.2% to 11.7%), *C. tropicalis* (from 5.4% to 8.0%), and *C. parapsilosis* (from 4.8% to 5.6%) in the period 1997 - 2000 compared to the period 2005 - 2007 [77]. The most frequently isolated *C. nonalbicans* species in the north of Europe and USA was *C. glabrata* [4], in Italy and Spain was *C. parapsilosis* [4, 108] and in Asian countries was *C. tropicalis* [109, 110].

Several risk factors of ICU ventilated patients have been associated with the increasingly of *C. nonalbicans* species. The study of Chowet *et al* indicated that the presence of a central venous catheter, fluconazole exposure and the number of antibiotics therapy were associated with an increased risk of bloodstream infections caused by *C. nonalbicans* compared to *C. albicans* [111]. Dimopoulos *et al* showed that the use of

medical devices, steroids and pre-existing candiduria were the risk factors associated with the presence of *C. nonalbicans species* [112]. The shift to *nonalbicans Candida species* is very important because some of these *C. nonalbicans species* are related to increasing the likelihood of resistance to fluconazole or other antifungal agents [113].

About the antifungal susceptibility results of ICU ventilated patients in our study, the resistance of *Candida species* to fluconazole and caspofungin occurred between 21.11% and 4.44%. All of them were still sensitive to intraconazole. Nevertheless, the antifungal resistance characteristics differ from the location and the species. The fluconazole resistance of *C. nonalbicans* was higher than *C. albicans* (34.3% to 16.2%) and HUMP hospital had the fluconazole resistance higher than HCH (37.5% to 0% towards *C. albicans*, 56.3% to 15.8% towards *C. nonalbicans*). The percentage of fluconazole resistance was similar to the percentage described in the study of Chau *et al*. However, there was not any cross - resistance to azole group drugs in our study. Chau *et al* point out that 50% *C. tropicalis* is resistant to both fluconazole and intraconazole [110]. The variuos results on antifungal susceptibility between two studies were dues to the difference on populations. In the results of a multicenter Italian survey at 18 ICU (AURORA Project), between 94.6 and 95.5 % of yeasts were sensitive to amphotericin B and caspofungin, 100% *C. albicans* was sensitive to fluconazole, 6/9 *C. glabrata* and 3/9 *C. tropicalis* resulted sensitive to fluconazole [114]. In other studies, 100% *C. albicans* was still sensitive to azole group drugs, high fluconazole resistance was recognized in *C. glabrata (16%) and C. krusei (78%)* [74, 113, 115].

In the 1990s, the percentage of *C. albicans* resistance to triazoles was extremely low, ranging from 0 - 0.5% and all cases were reported in previously exposed or in immunocompromised patients [116, 117]. Recent European studies have demonstrated increasing rates of decreased susceptibility to fluconazole in some but not all settings [73, 118, 119]. A recent French multicentre ICU cohort study reported that 17% of *Candida* isolates were less susceptible to fluconazole [73] and the high rate of fluconazole resistance of *C. nonalbicans*, especially *C. glabrata (47%) and C. tropicalis (14.3%).* Prolonged hospitalization is associated with a shift towards *Candida species* rather than *C. albicans* [120], particularly after fluconazole prophylaxis [121]. In our study, most of

patients stayed at ICU for a long time, but only patients at HUMP hospital (17/78 patients) were treated with fluconazole as a prophylaxis or pre-emptive therapy. That could be the reason why the resistance of *Candida species* in HUMP hospital was higher than HCH.

5.4. IC diagnosis by indirect ELISA results

The outcome of patients with IC has been associated with the timing of antifungal treatment initiation [122, 123]. An untargeted antifungal treatment is frequently used in the clinic with high associated costs and potential risk of drugs resistance [124].

An ideal IC diagnosis test should be minimally invasive with low volume samples, high sensitivity/specificity, minimal labor requiring [81], so that it can identify the early stage of IC and rapid turn-around time to physicians. Until now, the main methods for IC diagnosis are mannan and anti-mannan test, *C. albicans* germ tube antibody detecting, BDG detecting, T2Candida panel and PCR assays. The sensitivity/specificity of mannan and anti-mannan IgG antibody tests for Candidemia were around 58%/93% and 59%/86%, respectively. The sensitivity and specificity for a combined mannan/anti-mannan assay were 83% and 86% [84]. However, there a poor sensitivity and specificity of mannan and anti-mannan IgG antibody was detected in others deep-seated IC. In meta-analyses, the sensitivity and specificity of BDG for IC were around 75 - 80% and 80% [87, 125, 126]. The major limitation of BDG detection is specificity and false-positivity. T2Candida nanodiagnostic panel and PCR assay are available widely for clinical use in United State of America. The sensitivity/specificity of T2Candida panel and PCR are around 90%/98% and 90%/90%, respectively [127]. PCR assay and T2Candida panel offer potential advantages over mannan/anti-mannan and BDG in time of results. At present, there are no data in which T2Candida panel and PCR assay performance has been validated for IC diagnosis. In our study, we designed a new marker for IC diagnosis from the main virulence factors of *Candida albicans, a* "Candidalysin", that was proved as the first cytolytic peptide toxin in a human fungal pathogen and reveals the molecular mechanisms of epithelial damage and the host recognition. In an indiredct ELISA test, we showed that optical density in the severe patients (group 1 and group 3) who had lung injury (VAP) were higher than other nonVAP patients (group 4 and group 5). Based on clinical data, all patients in group 1 and 3 should be treated with prophylaxis or pre-emptive antifungal

drugs. However, there were only 50% of the patients in group 1 and group 3 positive in indirect ELISA. This means that 50% of patients could be overused antifungal drugs without ELISA test. The side effect of antifungal drugs and hospital cost will be decreased by reducing 50% antifungal treatment according to our ELISA results.

In our study, the new selected ECE1-III$_{62\text{-}92K}$ fungal toxin epitope from *C. albicans* showed high sensitivity/specificity (80%/96%) compared with another ECE1 epitope previously used (55.6%/80%) [128] as well as for the HWP1 epitope (60%/77%), demostrating to be the best candidate for IC indirect ELISA test.

In our study, we used small peptides from 14 to 16 amino acids, which were produced with a C-terminal cysteine residue to allow cross-linking with maleimide activated carrier proteins and direct coupling of peptides to polystyrene surfaces. Therefore, our ELISA test is simple, fast and inexpensive alternative protocol allowing to avoid the time-consuming and cost. This technique can be easy apply in ICU of resource-restricted countries for early IC diagnosis in high risk patients.

Moreover we also found a correlation between ELISA results and patient's clinical data including *Candida* score, procalcitonin, the length of ICU stay and number of ventilation days. These clinical data can be used for early antifungal treatment decision in the highrisk patients waiting blood culture result or other IC diagnosis test results. Clinical symptoms plus IC diagnosis test results will support clinicians in deciding antifungal therapy early and properly.

6. CONCLUSION

Three main fungal pathogens from ICU ventilated patients in Hue hospitals were *C. albicans* (42%), *C. tropicalis* (37%) and *C. glabrata* (16%). The fluconazole resistance of *Candida species* was 21.11% and caspofungin was 4.44%. There was not any cross - resistance to multiple azole drugs. However, *C. tropicalis* showed higher fluconazole resistance (34.29%) and caspofungin resistance (5.71%) compared with *C. albicans*. Three main bacteria identified in VAP at ICU were *A. baumannii* (43.2%), *K. pneumoniae* (28.4%) *and S. aureus* (14.8%) with high levels of antimicrobial resistance. *A. baumannii* was resistant to cephalosporin 2, 3, 4 generation (100%) and carbapenem-resistant was > 94%. The carbapenem-resistant of *K. pneumoniae* was 50% and 100% *S. aureus* was resistant to methicillin. Antibiotic and antifungal stewardship as well as nosocomial prevention bundle care must be improved in the future at ICU of HCH and HUMP hospitals from this results.

47.4% patients with *C. albicans* had invasive candidiasis. 28.9% had invasive *C. albicans* pneumonia.

19.23% patients with *Candida species* had invasive candidiasis and 2.56% had invasive *Candida species* pneumonia.

The ELISA sensitivity and specificity of ECE1 antibody detecting and HWP1 antibody detecting were 80%/96% and 60%/77%. Our study suggest to use ECE1 epitope as a good marker for IC diagnostic due to *C. albicans* and *C. dubliniensis.*

There was a correlation between ECE1 ELISA results and clinical data of patients (Candida score, procalcitonin and the length of ICU stay, ventilation day). These clinical data should be immediately use for the early diagnosis of IC helping physicians to decide early antifungal therapy while is waiting for a new IC test that include all *Candida species*.

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APPENDIX 1: List of scores for clinical assessment

Acute Physiology and Chronic Health Evaluation II (APACHE II) Score

Variables	4	3	2	1	0	1	2	3	4
(A) Acute physiological score									
Temperature	≥ 41	39-40,9		38,5-38,9	36-38,4	34-35,9	32-33,9	30-31,9	≤ 39,9
Mean Arterial BP	≥ 160	130-159	110-129		70-109		50-69		≤ 49
Heart Rate	≥ 180	140-179	110-139		70-109		55-69	40-54	≤
Respiratory Rate	≥ 50	35-49		25-34	12-24	10-11	6-9		≤ 5
A-aPO_2 (If FiO_2> 50%) PaO_2 (If FiO_2< 50%)	≥ 500	350-499	200-349		< 200 > 70	61-70		55-60	< 55
Arterial pH	≥ 7,7	7,6-7,69		7,5-7,59	7,33-7,59		7,25-7,32	7,15-7,24	< 7,15
Serum Na^+	≥ 180	160-179	155-159	150-154	130-149		120-129	111-119	≤ 110
Serum K^+	≥ 7	6-6,9		5,5-5,9	3,5-5,4	3-3,4	2,5-2,9		< 2,5
Serum Creatinine	≥ 310	176-299	132-167		52,8-123		< 52,8		
Hematocrit	≥ 60		50-59,9	46-49,9	30-45,9		20-29,9		< 20
WBC	≥ 40		20-39,9	15-19,9	3-14,9		1-2,9		< 1
Glasgow					13-15	10-12	7-9	4-6	3
(B) Age	< 44: 0	45-54: 2		55-64: 3		65-74: 5> 75: 6			
(C) Chronic disease adjustment	Points can be added if the patient has a history of the following + Biopsy proven cirrhosis + New York Heart Association Class IV + Severe COPD (hypercapnia, home O_2, pulmonary hypertension) + Chronic dialysis + Immune compromised If any of above are present, Add 2 points for elective surgery or for nonsurgical patients. 5 points for emergency surgery.								
Total APACHE II = (A) + (B) + (C)									

The Sequential Organ Failure Assessment (SOFA) Score

Variables	SOFA Score				
	0	**1**	**2**	**3**	**4**
Respiratory (PaO_2/FiO_2, mmHg)	> 400	≤ 400	≤ 300	≤ 200	≤ 100
Coagulation (Platelets×10^3/mm^3)	> 150	≤ 150	≤ 100	≤ 50	≤ 20
Bilirubin (mmol/l)	< 20	20 - 32	33 - 101	102 - 104	> 204
Cardiovascular (Hypotension)	No hypotension	Mean arterial pressure < 70mmHg	Dopamin < 5µg/kg/min or Dobutamin (any dose) at least 1 hour	Dopamin > 5µg/kg/min, adrenalin ≤0,1µg/kg/ph or noradrenalin ≤ 0,1µg/kg/min at least 1 hour	Dopamin > 10µg/kg/min adrenalin> 0,1µg/kg/min or noradrenalin > 0,1µg/kg/min at least 1 hour
Central nervous system (Glassgow Score)	15	13 - 14	10 - 12	6 - 9	< 6
Renal (Creatinin, µmol/l)	< 110	110 - 170	171 - 299	300 - 440	> 440

Candida score

Variables	**Points**
Multifocal *Candida species* colonization	1
Surgery on ICU admission	1
Severe sepsis	2
Total parenteral nutrition	1

APPENDIX 2: Buffers and media

1. Sodium phosphate buffer stock 1M (Appendix 2)
   - A solution: 1.38g Na_2HPO_4 $12H_2O$ in 10ml H_2O pH 7
   - B solution: 1.42g Na_2HPO_4 in 10ml H_2O pH 7

   Dilute 4.23ml A solution in 5.77ml B solution → 10ml Sodium phosphate buffer stock 1M
2. Maleimide conjugation buffer (13.96 ml)
   - 83mM sodium phosphate buffer
   - 0.1M EDTA
   - 0.9M NaCl
   - 0.02% sodium azide
3. Wash buffer
   - PBS (1L, 1X, pH 7.4)

     NaCl 8g

     KCl 0.2g

     Na_2HPO_4 $12H_2O$ 2.9g or Na_2HPO_4 1.44g

     KH_2PO_4 0.3g
   - Tween20: 250µl
4. Blocking buffer: 500ml PBS + 250µl Tween + 15g BSA

APPENDIX 3

PATIENTS INFORMATION COLLECTION FORM

"Molecular approach to early diagnosis of colonizing or invasive Candida in critically ill ventilated patients"

Name:…….Study code..……..

Gender: ……...

Age: …….....

Address:……Phone number…….....

Admission code:……..

Admission day:…….....

Discharging day:……..

Reason of ICU admission:…….....

Diagnosis: ……...

Date sampling:…….....

DAILY ASSESSMENT

Day (mm/dd/yy) / Varian	/ /	/ /	/ /	/ /
Intubated				
Ventilated				
Max temperature				
Min temperature				
Pulse (rate/min)				
Blood pressure (mmHg)				
SpO_2 (%)				
Glasgow score				
Fluid balance				
Mode				
PEEP (mmHg)				
FiO_2 (%)				

Vt or Pc				
I/E				
F (rate/min)				
Hemoglobin/Hematocrit				
Platelet (G/L)				
Natrium/Postasium				
Total bilirubin (mg/dL)				
Protein/albumin (g/l)				
Creatinin (μmol/l)				
pH				
PaO_2/FiO_2				
$PaCO_2$ (mmHg)				
HCO_3^-/BE (mmol/l)				
CRP (mg/L)				
Procalcitonin (ng/ml)				
X-ray	Infiltrate □ Yes □ No Consolidation □ Yes □ No Cavitation □ Yes □ No			
SOFA				
APACHE II				
Candida score				
Antimicrobial susceptibility				
Antifungal susceptibility				
First antibiotics therapy				
Alter antibiotics therapy				
First antifungal therapy				
Alter antifungal therapy				
Vasopressure drugs				

Inotrop drugs				
Total ventilation day				
ICU duration				
Outcome				

MICROBIOLOGY TEST

1. Blood cultures

1.1. Code samples: ……..

1.2. Date of sampling *(dd/mm/yy)*…….....

1.3. Had used antifungal before sampling? □ Yes □ No

Blood culture results (attach antimicrobial susceptibility results)……...

1.4. ELISA results……...

1.5. PCR results……...

2. BAL cultures

2.1. Code sample:……...

2.2. Date of sampling *(dd/mm/yy):* …….

2.3. Had used antifungal before sampling? □ Yes □ No

BAL culture results (attach antimicrobial susceptibility results)……..…….

2.4. Direct microscopy results…….

2.5. MALDI TOF results…….

2.6. ELISA results……...

2.7. PCR results……...

Hue, date…../……/20….

Interviewer