ORIGINAL ARTICLE

Molecular detection of *Candida* spp. and *Aspergillus fumigatus* in bronchoalveolar lavage fluid of patients with ventilator-associated pneumonia

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Background: Ventilator-associated pneumonia (VAP) is a common nosocomial infection in critically ill patients with high morbidity and mortality rates. The etiology of VAP is usually bacterial. Opportunistic fungi such as *Candida* and *Aspergillus* species (spp.) are found frequently in the respiratory track secretions of immunocompetent critically ill patients known as colonization. Contribution of fungi colonization to severe bacterial VAP and poor prognosis of these patients has been documented in several studies. The aim of this study was to detect *Candida* spp. and *Aspergillus fumigatus* colonization in patients with a clinical diagnosis of VAP as a marker of high risk pneumonia. **Materials and Methods:** Bronchoscopic alveolar lavage (BAL) fluids from patients with VAP in central intensive care unit (ICU) of a tertiary university hospital in Isfahan were examined by real time polymerase chain reaction (PCR) to detect *Candida* spp. or *A. fumigatus*. Rate of fungi colonization and its association with clinical criteria of the patients was determined. **Results:** BAL fluids from 38 patients were tested from which six samples (15.8%) were positive for *Candida* spp. and five (13.2%) for *A. fumigatus*. Fungi colonization was not associated with age, sex, or mortality rate of patients. Rate of *A. fumigatus* colonization was significantly more in traumatic patients ($P = 0.036$), and higher in patients ventilated more than 4 weeks ($P = 0.022$). **Conclusion:** High rate of *A. fumigatus* colonization in our ICU patients indicates that underlying causes such as unfavorable ICU conditions and other patient related factors such as unnecessary antibiotic therapy should be further evaluated.

Key words: *Aspergillus*, *Candida*, colonization, real time polymerase chain reaction, ventilator associated pneumonia

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INTRODUCTION

Hospital acquired pneumonia (HAP) is the second most prevalent cause of nosocomial infection, and it's mortality rate is more than any other hospital acquired infection world-wide. Most of the HAPs occur in intensive care units (ICUs).[1] It is estimated that intubation causes 6-21 folds increase in the risk of HAP, thus a subset of HAP is defined as ventilator-associated pneumonia (VAP), which is pneumonia in patients who have been on mechanical ventilation for more than 48 h.[2]

Overall prevalence of VAP is reported 9.3%. The mortality rate for VAP varies between 20 and 50% and up to 76% when high risk pathogens are the cause. Moreover VAP is associated with increased morbidity and hospital costs, due to prolonged ICU stay and prolonged need for mechanical ventilation.[2] Many risk factors have been attributed to VAP such as duration of mechanical ventilation, trauma, prior surgery, severity of an underlying illness, prior use of antibiotics, and chronic pulmonary diseases.[3]

It is generally accepted that the etiology of VAP is typically bacterial. Opportunistic fungi such as *Candida* and *Aspergillus* spp. are rare causes of VAP that mostly occur in immunocompromised patients.[4] However, many patients in ICUs have respiratory specimens positive for *Candida* without clinical and pathological evidence of invasive candidiasis known as *Candida* colonization.[5] Detection of *Aspergillus* in airway secretions is not common in comparison with *Candida* spp., and its significance depends on immune status of the patient. In intensive immune suppression such as neutropenia, transplantation, and corticosteroid therapy detection of *Aspergillus* in airways suggests invasive

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aspergillosis, but in immunocompetent patients, it typically represents colonization.[6] Although the clinical impact of airway colonization with these fungi is not clearly addressed, now it is obvious that in immunocompetent patients under mechanical ventilation, presence of *Candida* or *Aspergillus* in the respiratory track is associated with worse outcomes of bacterial VAP and lower survival of patients. This is greatly evidential in the case of *Candida* colonization.[6]

It is reported that *Candida* colonization in airways is associated with increased risk of bacterial VAP, presence of multidrug-resistant bacteria, and increased mortality rate in patients with VAP.[7] Nseir *et al.* showed that antifungal therapy decreased the risk of *Pseudomonas aeruginosa* infection in these patients.[8] Furthermore, it is shown that isolation of *Aspergillus* in critically ill patients illustrates poor prognosis irrespective of invasion or colonization.[9] Because of air-born transmission of the *Aspergillus*, its detection in the respiratory track is usually related to environmental contamination such as polluted air ducts or unhygienic dusty ICU rooms.[6]

In the current study, we aimed to detect *Candida* spp. or *Aspergillus fumigatus* in bronchoscopic alveolar lavage (BAL) fluid of patients with VAP. Although routine detection and treatment of fungi colonization in VAP is yet under debate, however the rate of colonization for *Candida* spp. or *A. fumigatus* can specify high risk VAPs, and the *A. fumigatus* colonization can determine high risk environment for immunosuppressed ventilated patients in our ICU.

MATERIALS AND METHODS

Setting

This cross-sectional study was conducted in central ICU of Al Zahra Hospital, a Tertiary University Hospital in Isfahan, from April 2011 to March 2012. The hospital is an 800 bed referral hospital with a central ICU for adults. The ICU contains 20 divided patient rooms, and admits critically ill patients from both medical and surgical wards. The protocol was approved by the Research and Ethical Committees of Isfahan University of Medical Sciences, Isfahan, Iran (Research project numbers: 289146-7). Informed consent was obtained from closest relative of each patient.

Patients

All ICU patients who were intubated and mechanically ventilated were observed for signs and symptoms of VAP during the study period. A combination of clinical, laboratory, and radiologic signs were used to diagnose VAP according to the National Nosocomial Infection Surveillance (NNIS) system pneumonia definition [Table 1].[10] The NNIS has been developed by the Centers for Disease Control and Prevention as a tool to describe the epidemiology of nosocomial pneumonia, and the criteria showed acceptable sensitivity and specificity for diagnosis of VAP in some previous studies.[11]

Patients with a clinical diagnosis of VAP were undergone BAL if there was no contraindication for the procedure. The BAL fluids were used for both bacterial cultures and real time polymerase chain reaction (PCR) assays. Patients with any kind of preceding immunosuppression such as neutropenia, hematological or solid organ malignancy, bone marrow tran splantation, immunosuppressive therapy, systemic corticosteroid therapy, and human immunodeficiency virus infection were not included. A predesigned checklist was filled for each patient containing demographic and clinical data of the patient.

Deoxyribonucleic acid (DNA) extraction and real time PCR

For cell lysis, 200 µl of the BAL fluid was mixed with 200 µl of binding buffer (Roche Diagnostics) and 50 µl of Proteinase K (Roche Diagnostics). The mixture was incubated at 72°C for 20 min. Conventional phenol-chloroform method was used for DNA extraction as was explained elsewhere.[12] A predesigned TaqMan primer and the probe was used to identify *A. fumigatus* and the real time PCR was conducted as previously explained.[13] *Candida* spp. were detected by a predesigned general primer for *Candida* genus.[14] The QuantiFast SYBR Green PCR Kit (Qiagen) was employed

Table 1: NNIS system criteria for diagnosis of VAP

Radiology signs	Clinical and laboratory signs	
Two or more serial chest radiographs with at least 1 of the following	At least 1 of the following	Plus at least 2 of the following
New or progressive and persistent infiltrate	Fever (temperature >38 C)	New onset of purulent sputum, or change in character of sputum
Consolidation	Leukopenia (<4000 WBC) or leukocytosis (>12000 WBC)	Increased respiratory secretions, or increased suctioning requirements
Cavitation	Altered mental status, for adults 70 years or older, with no other recognized cause	New-onset or worsening cough, or dyspnea, or tachypnea
		Rales or bronchial sounds
		Worsening gas exchange
		Increased oxygen requirements

Adopted from Miller *et al*. 2006; NNIS = National Nosocomial Infection Surveillance; VAP = Ventilator associated pneumonia; WBC = White blood cell

according to the kit instructions to identify the *Candida* spp. All real time PCR reactions were performed on a Rotor-Gene 6000 system (Corbett, Australia) and standard positive and negative controls were run parallel with each real time PCR round.

Statistical analysis

Data were analyzed using SPSS software version 16 (SPSS Inc., Chicago, IL, USA). Chi-squared test was used to determine the significance of differences between groups. $P < 0.05$ was considered as significant.

RESULTS

During the study 38 patients developed signs and symptoms of VAP whose BAL fluid samples were examined. The characteristics of patients are summarized in Table 2. Twenty one patients (55.3%) were males, and 17 (44.7%) were females. Age of the patients ranged from 20 to 86. Underlying diseases, which led to ICU hospitalization, were diverse. We arranged the underlying diseases in four groups:

1. Internal diseases such as organ failures and infectious diseases,
2. Post-surgical when a surgical procedure led to ICU admission,
3. Brain events such as cerebro-vascular accident and intra-cerebral hemorrhage, and
4. Trauma.

Underlying disease for most of the patients was post-surgical (12 patients) followed by internal diseases (10 patients).

BAL fluid sample of six patients (15.8%) were positive for *Candida* spp. and five (13.2%) for *A. fumigatus*. Patients with positive results for *A. fumigatus* were further assessed according to the criteria from the European Organization for Research and Treatment of Cancer/Invasive Fungal Infections Cooperative Group and the National Institute of Allergy and Infectious Diseases Mycoses Study Group.[15] None of the patients were classified as proven or probable invasive pulmonary aspergillosis (IPA) and were considered colonization.

Colonization with *Candida* spp. or *A. fumigatus* was not associated with age or sex of the patients. Mortality was not more in colonized patients, and also underlying disease and duration of mechanical ventilation was not related to *Candida* colonization. However rate of *A. fumigatus* colonization was significantly different in subsets of different underlying diseases ($P = 0.036$), with the most colonization observed in traumatic patients (three of six traumatic patients (50%) were positive for *A. fumigatus*). The proportion of *A. fumigatus* colonization was significantly higher in patients ventilated more than 4 weeks ($P = 0.022$).

DISCUSSION

In the current study, two important opportunistic fungi, *Candida* and *A. fumigatus* were detected by real time PCR in BAL fluid sample of patients with a clinical diagnosis of VAP, and association of clinical data of patients with the rate of colonization was evaluated.

In this study, 15.8% of BAL fluids were positive for *Candida* spp. Rate of *Candida* colonization is comparable with

Table 2: Demographic and clinical characteristics of patients

Patients' characteristics	*Candida* spp.	*P* value*	*A. fumigatus*	*P* value*	Total (38)
Sex					
Male	3 (14.3)	0.77	3 (14.3)	0.81	21 (55.3)
Female	3 (17.6)		2 (11.8)		17 (44.7)
Age					
20-39	4 (30.8)	0.20	2 (15.4)	0.67	13 (34.2)
40-59	0		2 (22.2)		9 (23.7)
60-79	2 (16.7)		1 (8.3)		12 (31.6)
80≥	0		0		4 (10.5)
Underlying disease					
Internal	2 (20.0)	0.82	0	0.036	10 (26.3)
Surgical	2 (16.7)		1 (8.3)		12 (31.6)
Brain event	1 (25.0)		1 (25.0)		4 (10.5)
Trauma	1 (16.7)		3 (50.0)		6 (15.8)
Undetermined	0		0		6 (15.8)
Duration of ventilation					
≤4 weeks	5 (16.7)	0.77	2 (6.7)	0.022	30 (78.9)
>4 weeks	1 (12.5)		3 (37.5)		8 (21.1)
Mortality	1 (20.0)	0.78	1 (20.0)	0.62	5 (13.2)

*Pearson Chi-square test is used to detect significance of differences between groups; *A. fumigatus* = *Aspergillus fumigatus*

results of a large study in Canada in which totally 17.8% of patients with clinical suspicion of VAP were colonized with *Candida*.[16] Culture of either BAL fluid or endotracheal aspirate (ETA) was used to detect *Candida* in the respiratory track and the rate of colonization was insignificantly more in BAL arm of the study (20%) than in ETA (15.6%).[16] The proportion of positive samples for *Candida* is higher in some other studies, for example in an investigation 26.6% of all immunocompetent critically ill patients apart from having VAP or not, had positive culture in their BAL fluid or other distal airway samples.[17] Furthermore, rate of colonization in patients suspicious of VAP was 56% in ETA culture[18] and 53% in ETA or BAL culture[5] in other surveys. Thus far, real time PCR has been used mostly to detect *Candida* in blood samples[19] and rarely in the respiratory track secretions,[20] We used real time PCR to detect fungi and only included the patients who were undergone BAL. While the sensitivity of real time PCR is high and the rate of false positive possibly is more than culture, rate of *Candida* colonization was lower than most of the previous reports. Different patient populations and different sampling methods might stand for this inconsistency. Furthermore, prior use of antibiotics can increase the rate of colonization which was not recorded in this study and made the comparison impossible.

No association between *Candida* Colonization and rate of mortality or length of mechanical ventilation was found, contrary to previous reports, which found an increase in median hospital stay,[16,17,21] length of mechanical ventilation[17] and hospital mortality.[16,18,21] The reason is perhaps low number of patients in this study.

Finally, 13.2% of samples were positive for *A. fumigatus*. Isolation of *Aspergillus* from respiratory samples represents either colonization or true infection. In contrast to *Candida*, a significant proportion of positive cases suffer from IPA that in ICU admitted patients ranges from 25% to 75% depending on the type of patients.[6] Immunosuppression is the main risk factor for development of IPA and the most frequent comorbidity in IPA patients is sever chronic obstructive pulmonary disease (COPD) and corticosteroid therapy.[9] None of our patients with positive *Aspergillus* sample were immunocompromised and none of them were diagnosed as proven or probable IPA. Interestingly, two of participants were admitted to ICU because of exacerbated COPD, but *A. fumigatus* was not identified in BAL fluid of any of them. *A. fumigatus* colonization was not associated with age or mortality of the patients. Although, in a survey isolation of *Aspergillus* in critically ill patients was higher in older patients and was attributed to worse outcomes.[22] Interestingly, half of traumatic patients in this study (three from six) were colonized with *A. fumigatus*. The reason for high colonization of traumatic patients is unknown and it is not reported before.

In an international survey rate of *Aspergillus* colonization in ICU patients was 1.4%.[23] Most of studies on *Aspergillus* are conducted in immunocompromised patients and investigated the incidence of IPA. Nasal colonization of *Aspergillus* has been examined and attributed to hospital constructions and air duct pollutions, with rates of 6% in a nephrology ward[24] and 1.5% in none ICU immunocompromised patients.[25] We examined the samples only for *A. fumigatus*. Although, it is established that about 80-90% of isolated *Aspergillus* around the world are *A. fumigatus*,[6] but recently an increase in incidence of other no-*fumigatus* species, especially *A. flavus* and *A. terreus* is noticed. For example, Zarrinfar *et al.* identified nine positive samples out of 30 BAL fluids by nested PCR from ICU patients from which seven were *A. flavus*.[12]

We would better detect other species of *Aspergillus* and identify *Candida* spp. which is a limitation for this study. Moreover, to evaluate the real time PCR technique, comparison with culture and microscopic techniques is suggested.

CONCLUSION

The rate of *A. fumigatus* colonization was high in our setting in comparison with other similar studies with the identical patient types. As the *Aspergillus* is an airborne easily transmissible infection and it causes severe invasion with high mortality rates in ICUs, specific measures should be carried out to decrease environmental pollutions. Air conditioners should be checked and regular cleaning of the ICUs must be considered. International guidelines for prevention of nosocomial pneumonia can provide useful instructions in this regard.[26] Furthermore, overall high rate of fungi colonization in our ICU might be due to unnecessary use of antibiotics which is documented in previous studies[17] and needs further evaluation.

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AUTHORS' CONTRIBUTION

All authors have contributed in designing and conducting the study. SA, MY, FA, FF, NA, MP, and FH collected the data and FKh, BA, and SGH did the analysis. All authors have assisted in preparation of the first draft of the manuscript or revising it critically for important intellectual content. All authors have read and approved the content of the manuscript and are accountable for all aspects of the work.

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