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Original Article

Microbiology Section

Clinical and Microbiological Study of Fungi as an Aetiological Agent in Patients of Ventilator Associated Pneumonia in Intensive Care Unit of a Tertiary Care Hospital

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ABSTRACT

Introduction: In Intensive Care Units (ICUs), Ventilator Associated Pneumonia (VAP) is the commonest nosocomial infection. Fungal infections especially *Candida* spp. have emerged as one of the commonest organisms in causing VAP. It increases morbidity and mortality to a great extent in critically ill patients, with risk factors like old age, co-morbidities like diabetes, Chronic Obstructive Pulmonary Disease (COPD), infectious or multisystem disease. Hence, it becomes necessary to isolate and speciate the fungi along with its Antifungal Susceptibility Testing (AFST) for early diagnosis and treatment. This is done by microbiological investigation where in samples (bronchoscopic/nonbronchoscopic) are obtained from the lower respiratory tract.

Aim: To study the fungal profile among clinically and radiologically diagnosed VAP cases.

Materials and Methods: This was a prospective study carried out in the Department of Microbiology, Dr Vaishampayan Memorial Govt Medical College, Solapur, Maharashtra, India, in which 120 clinically and radiologically diagnosed VAP patients were included. Endotracheal Aspirate (ETA) of these patients was collected aseptically. The sample was then subjected to Gram stain, Lacto Phenol Cotton Blue (LPCB), culture on Sabouraud's Dextrose Agar (SDA) and Chromogenic (CHROM) agar and AFST was performed by broth dilution technique. The data was statistically analysed.

Results: In this study, out of 120 patients, 52 patients were diagnosed as VAP on day 5. Late onset pneumonia was more common in 61.67%. Non-infective cases were 49.17% and infective cases were 28.33%. Patients belonged to different age groups with maximum between 31-40 years. Out of 120 cases of VAP, yeast was isolated from 26 cases. Most common pathogenic yeast isolated was *Candida albicans* (53.85%). All yeast isolates were susceptible to fluconazole and amphotericin B except one isolate of *Candida krusei*, *Candida tropicalis* and *Candida glabrata* each were resistant to both fluconazole and amphotericin B.

Conclusion: *Candida albicans* was the commonest yeast isolated. Resistance was noted among the non-*albicans* spp of *Candida*. The non-*albicans Candida* is growing as an emerging threat. Early diagnosis and AFST will help in reducing the morbidity and mortality.

Keywords: Amphotericin B, Antifungal susceptibility pattern, *Candida,* Chromogenic agar

INTRODUCTION

Respiratory infections are the second most common cause of infection among hospitalised patients. In ICUs, hospital acquired pneumonia particularly VAP, is the commonest [1-3]. It is an important form of hospital acquired pneumonia and refers to pneumonia developing in mechanically ventilated patients for more than 48 hours after tracheal intubation or tracheostomy [4]. It is also the second most common hospital acquired infection among neonatal and paediatric patients and even in adult ICU patients on ventilators [5,6]. The organisms commonly associated with VAP are extended spectrum beta-lactamase producing, AmpC producing and metallo-betalactamase producing *Enterobacteriaceae*, Methicillin Resistant *Staphylococcus aureus* (MRSA), *Pseudomonas aeruginosa* resistant to multiple antimicrobials including carbapenems, multidrug resistant *Acinetobacter* spp and fungi like *Aspergillus*, *Candida* spp and *Pneumocystis jiroveci* [1]. Despite major advances in techniques for the management of ventilator dependent patients and the routine use of effective procedures to disinfect respiratory equipment, VAP continues to complicate the course of 8 to 28% of patients receiving mechanical ventilation [7].

Detection of causative organism is thus crucial for management of VAP. This is done by microbiological investigation where in samples (bronchoscopic/nonbronchoscopic) obtained from the lower respiratory tract are cultured quantitatively and semi-quantitatively [8]. Diagnosis of VAP includes detection of organisms in specimens like ETA, Bronchoalveolar Lavage (BAL), bronchial biopsy, blood and urine cultures and radiological scans. Recently molecular methods like Polymerase Chain Reaction (PCR) are also being used to detect fungal pathogens.

Hence, this study aims to diagnose VAP cases combining both clinical and microbiological aspect and also to study the AFST pattern to reduce the emerging fungal resistance and thereby mortality among critical ICU patients.

MATERIALS AND METHODS

This was a prospective study carried out in the Department of Microbiology, Dr Vaishampayan Memorial Govt Medical College, Solapur, Maharashtra, India, in collaboration with Department of Medicine for a period of one year and nine months from July 2011 to April 2013. Sample size of 120, was calculated using the formula.

$$N=\frac{Z^2\,(1-\alpha/2)p\,(1-P)}{D^2}$$

P=With anticipated population proportion-10%

D=Allowed error 5%.

Confidence level 95%

Inclusion criteria: Patients included in the study were those on ventilator for more than 48 hours, clinically diagnosed as VAP and with radiological evidence of infiltration suggestive of pneumonia.

Exclusion criteria: Patients who did not give consent and those who were on ventilator for less than 24 hrs.

The study was carried out after approval by Institutional Ethics Committee, Reference no. EC/18/2011 and an informed consent of all the included patients were obtained prior to the study.

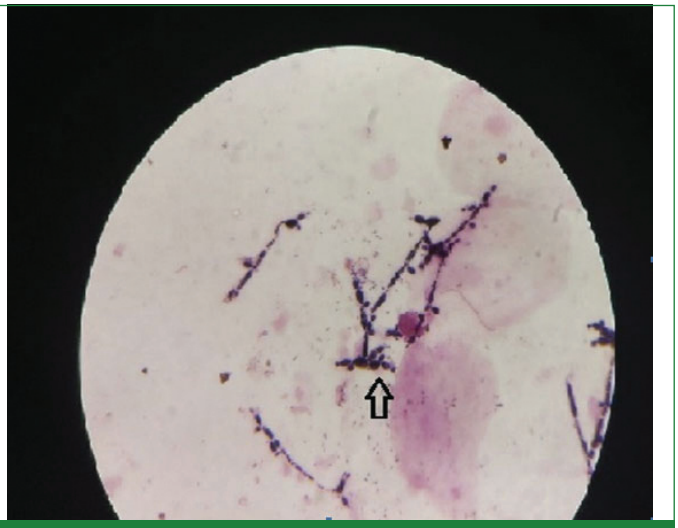
Collection of ETA: Suction catheter was introduced through the endotracheal tube and aspiration of the secretion was done. One sample was collected from each patient. The sample collected was immediately transported to Microbiology Department.

A detailed history of patients including the age, gender distribution, associated co-morbidities, infective or other aetiology, number of ventilator days was documented.

Microbiological processing for fungal isolation and examination was done by following methods:

a. Direct microscopic examination of gram stained smear for fungal elements
b. Inoculation of sample on SDA, both plain and with antibiotics
c. Germ tube test
d. Inoculation on CHROMagar
e. Identification on cornmeal agar by Dalmau plate method
f. AFST

Loop full of ETA was smeared on a clean, sterile glass slide and subjected to Gram staining. Only yeasts were included in present study. After observing gram smear slide for budding yeast cells and pseudohyphae [Table/Fig-1], culture was done on SDA and incubated at both 25°C and 37°C to check for the growth of dimorphic fungii. After a period of 48-72 hours, yeast colonies appeared. The isolates obtained were processed by standard mycological tests [9]. Germ tube test differentiated between *Candida albicans* and non-*albicans Candida* spp. The isolated yeast was then subjected to biochemical tests like sugar assimilation and sugar fermentation. For fermentation, the sugars tested were 2% conc. each of glucose, maltose, sucrose, lactose, galactose, trehalose and the sugars tested for assimilation were glucose, maltose, sucrose, lactose, galactose, trehalose, mellibiose, cellobiose, inositol, xylose, raffinose, dulcitol, 10% conc Disc for each test.

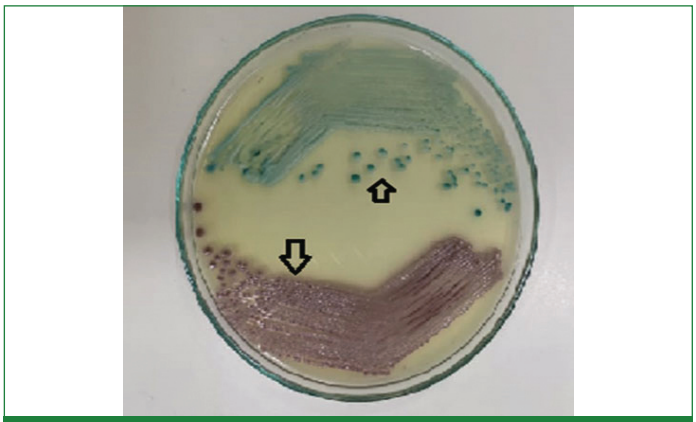


[Table/Fig-1]: Gram stain showing budding yeast cell with pseudohyphae at 100x (pointed by arrow).

Differentiation of spp was done on the basis of colour on CHROMagar [Table/Fig-2,3] and Dalmau plate was used to differentiate on the basis of different morphological characters for various spp.

M27-A2 reference method was followed for AFST of the isolated *Candida* species [10]. *Candida albicans* ATCC 60193 was used as control strain. Interpretive susceptibility criteria for fluconazole and amphotericin B were those recommended by the Clinical and Laboratory Standards Institute (CLSI) M27-A2 [10]. Isolates showing

[Table/Fig-2]: *Candida tropicalis* on CHROMagar.



[Table/Fig-3]: *Candida albicans* (light green) and *Candida glabrata* (pink) on CHROMagar (pointed by arrow).

MICs ≤8.0 µg/mL were considered as susceptible to fluconazole, 16-32 µg/mL considered as dose dependent susceptible and ≥64 µg/mL as resistant. For amphotericin B isolates, MIC of ≤1.0 µg/mL were taken as susceptible and those with MIC >1 µg/mL were considered as resistant.

STATISTICAL ANALYSIS

Descriptive analysis was done and percentages were calculated using Microsoft Excel.

RESULTS

A total of 120 clinically and radiologically diagnosed VAP patients were enrolled for the study. The major age group affected was between 31-40 years followed by 21-30 years with male preponderance as shown in [Table/Fig-4].

		Numbers	Percentage
Age (years)	0-10	-	-
	11-20	1	0.83
	21-30	23	19.16
	31-40	45	37.5
	41-50	22	18.33
	51-60	13	10.83
	61-70	11	9.16
	71-80	3	2.5
	81-90	2	1.7
	Total	120	
Gender	**Sex**	**No. of cases**	**Percentage**
	Male	89	74.17
	Female	31	25.83

[Table/Fig-4]: Baseline data of ICU patients with VAP.

Predominant clinical diagnosis were of non-infective cases (49.17%), followed by infective cases (28.33%) while multisystem involvement was seen in (22.5%) as depicted in following [Table/Fig-5].

Sr. No.	Clinical diagnosis	No. of cases	Percentage
A	**Infective**		
1	Central nervous system (viral encephalitis)	6	17.64
2	COPD	16	47.05
3	Leptospirosis with renal failure	2	5.88
4	Acute gastroenteritis with renal failure	2	5.88
5	Cerebral malaria	8	23.52
	Total	34	28.33
B	**Non-infective**		
1	Central nervous system (head injury)	25	42.37
2	Central nervous system (hypertension with CVA)	8	13.55
3	Blunt chest trauma with flail chest	15	25.42
4	Sedative overdose	3	5.08
5	Organophosphorus poisoning	4	6.77
6	Snakebite	2	3.38
7	Drowning	1	1.69
8	Malignancy (Carcinoma larynx)	1	1.69
	Total	59	49.17
C	**Others-multisystem involvement**		
1	Bronchopneumonia with ARDS	11	40.74
2	Pulmonary tuberculosis with ARDS	8	29.62
3	Diabetes mellitus with septicaemia with ARDS	6	22.22
4	Pre-eclamptic toxaemia with ARDS	2	7.40
	Total	27	22.5
	Grand total	120	

[Table/Fig-5]: Clinical diagnosis of cases under study.
COPD: Chronic obstructive pulmonary disease; CVA: Cerebro vascular accident; ARDS: Acute respiratory distress syndrome

Use of antibiotics, antacids or H2 blockers and feeding through nasogastric tube as risk factors were present in all 120 patients as shown in the following [Table/Fig-6].

Sr. No.	Risk factor	Number	Percentage
1	Chronic illness	52	43.33
2	Severe illness	68	56.66
3	CNS dysfunction/coma	21	17.5
4	Metabolic acidosis	47	39.16
5	Underlying lung condition (COPD/ARDS)	82	68.33
6	Septicaemia	21	17.5
7	Diabetes mellitus	66	55
8	Hypertension	91	75.83
9	Alcoholism	33	27.5
10	History of smoking	27	22.5
11	Use of antibiotics	120	100
12	Use of corticosteroids	59	49.16
13	Use of antacids/H2 blockers	120	100
14	Nasogastric tube	120	100
15	Reintubation	68	56.66

[Table/Fig-6]: Risk factors associated with development of pneumonia in patients on ventilator.
CNS: Central Nervous System; COPD: Chronic obstuctive pulmonary disease; ARDS: Acute respiratory distress syndrome

Late onset VAP was observed in maximum number of patients. Development of VAP was highest on 5th day followed by day 4 and day 7 as shown below in [Table/Fig-7].

Total yeast isolates were 26. Among these, *C.albicans* was the commonest yeast found in the study followed by *C.* non-*albicans* as depicted in [Table/Fig-8].

Day of development of VAP					
VAP	**Early (<5 days)**		**Late (>5 days)**		
Days	Day 3	Day 4	Day 5	Day 7	Day 10
No. of cases	8	38	52	20	2
(N=120) percentage	(46) 38.33%		(74) 61.67%		

[Table/Fig-7]: Classification of early and late onset of Ventilator Associated Pneumonia (VAP).

Yeast Isolates	Number	Percentage
C. albicans	14	53.85
C. non-albicans	12	46.15

[Table/Fig-8]: Total yeast isolates found in the study (n=26).

Among the non-*albicans* isolates, *C.krusei* (41.67%) was predominant followed by *C.dublinensis* (33.33%) as shown in [Table/Fig-9].

Most common combinations isolated were *Candida albicans* with *Pseudomonas* (14.2%) and *Candida krusei* with *Pseudomonas* (28.5%) as shown in the [Table/Fig-10].

Sr. No.	Non-albicans isolates	Number	Percentage
1	*C. krusei*	5	41.66
2	*C. dublinensis*	4	33.33
3	*C. parapsilosis*	1	8.33
4	*C. tropicalis*	1	8.33
5	*C. glabrata*	1	8.33

[Table/Fig-9]: Distribution of Non-albicans isolates (n=12).

Sr. no.	Yeast+Bacteria	Number	Percentage
1	*C. albicans+Klebsiella*	1	14.2
2	*C. albicans+Pseudomonas*	2	28.5
3	*C. dublinensis+Pseudomonas*	1	14.2
4	*C. krusei+Pseudomonas*	2	28.5
5	*C. glabrata+Pseudomonas*	1	14.2
Total		7	

[Table/Fig-10]: Distribution of polymicrobial yeast isolates.

All *Candida albicans* were susceptible to fluconazole, isolates of *Candida krusei* (20%), *Candida tropicalis* (100%) and *Candida glabrata* (100%) were resistant to fluconazole and one isolate of *Candida krusei* (20%) and *Candida parapsilosis* (100%) were dose dependent susceptible to fluconazole as shown in the following [Table/Fig-11].

Organisms	Fluconazole				
		0.8-64 µg/mL			
	<0.8 µg/mL	0.8-16 µg/mL	16-32 µg/mL	32-64 µg/mL	≥64 µg/mL
C. albicans (n=14)	12 (85.7%)	2 (14.2%)	-	-	-
C. krusei (n=5)	1 (20%)	2 (40%)	-	1 (20%)	1 (20%)
C. dublinensis (n=4)	3 (75%)	1 (25%)	-	-	-
C. tropicalis (n=1)	-	-	-	-	1 (100%)
C. parapsilosis (n=1)	-	-	-	1 (100%)	-
C. glabrata (n=1)	-	-	-	-	1 (100%)
Total=26	16 (61.5%)	5 (19.2%)	-	2 (7.6%)	3 (11.5%)

[Table/Fig-11]: Susceptibility testing of *Candida* spp. for fluconazole.

All *Candida albicans* isolates were susceptible to amphotericin B. Among non-*albicans*, all the isolates of *Candida tropicalis* and *Candida glabrata* were resistant to amphotericin B, whereas 20% *Candida krusei* were resistant to Amphotericin B as shown in [Table/Fig-12].

DISCUSSION

A prospective study which included 120 clinically and radiologically diagnosed cases of VAP for microbiological profile was done.

Candida spp.	Amphotericin B	
	<1.0 µg/mL	>1.0 µg/mL
C. albicans (n=14)	14 (100%)	-
C. krusei (n=5)	4 (80%)	1 (20%)
C. dublinensis (n=4)	4 (100%)	-
C. tropicalis (n=1)	-	1 (100%)
C. parapsilosis (n=1)	1 (100%)	-
C. glabrata (n=1)	-	1 (100%)
Total=26	23 (88.4)	3 (11.5)

[Table/Fig-12]: Susceptibility testing of *Candida* spp to Amphotericin B.

Patients were included as per the predefined criteria. Knowledge of organisms likely to be present and the local resistance pattern in respective hospital ICU helps in the rational use of antibiotics. It reduces colonisation and subsequent development of VAP with multidrug resistant pathogens.

Age and sex: In present study, age had a significant role in the development of VAP which co-relates with Dey A and Bairy I, study which showed patients of ages >30 years were more prone to get VAP [11]. Frequency was less in extremes of age (0.83% in less than 20 years and 1.6% in those above 80 years). Rello J et al., also observed VAP in younger patients aged under 35 years [12]. On the other hand, Dennesen PJ et al., found involvement of the patients in older age group [13]. Age >60 years has been considered as an independent risk factor for VAP [4]. Advanced age was probably associated with increased risk of pneumonia, primarily because of increased frequency of co-morbidity among the elderly. Age associated immune changes could also play a role in contributing to VAP development in elderly patients [13]. In the present study, 74.17% of the cases diagnosed were males while only 25.83% were females. Similar findings were encountered in a study done by Kollef MH et al., showing male preponderance of 60% [15]. Huang Y et al., also showed male preponderance (77.27%) in VAP patients in their study [16]. Male preponderance could possibly be attributed to their more outdoor exposure [15].

Nature of primary illness in VAP patients: Mechanical ventilation is indicated in cases of respiratory failure, due to various factors the most important being neurological, intoxication and ARDS. In the present study, non-infective aetiology was seen in 49.17% cases and infective primary illness was present in 28.33% cases. Multisystem involvement was observed in 22.5% cases.

However, in a study conducted by Rello J et al., 18.4% of cases presented with neurologic emergency and 31.6% of cases presented with ARDS [17].

In a study conducted by De Latorre FJ et al., patients with cerebral hemorrhage constituted 32.5% of the cases studied [18]. Delclaux C et al., found a very high incidence of 60% of VAP in patients suffering from ARDS [19]. Similarly, Markowicz P et al., found nosocomial pneumonia as a frequent complication of ARDS [20].

Management of acute respiratory failure is essential in these patients to deliver oxygen at the tissue level, improved lung mechanics and the work of the cardiopulmonary system. This frequently necessitates introduction of ETT or tracheostomy. In addition, an ETT also helps to perform tracheobronchial toilet, especially when secretions are thick, weak cough reflexes in the patient and to prevent aspiration. However, mechanical ventilation is not a normal physiological process and induces subtle forms of lung injury. The resultant nosocomial pneumonia may play a key role by worsening hypoxemia and causing sepsis, multiple organ failure and death [20].

Risk factors contributing to VAP: Risk factors that predispose a patient on ventilator to pneumonia are those increasing frequency of aspiration, quantity and pathogenicity of microorganisms inoculated, impair local respiratory tract defences and impair systemic immunity. Most commonly found reason for pneumonia in present study was mechanical ventilation or intubation. Study of George DL et al., records 3 to 21 fold increase in risk of developing pneumonia following intubations [21]. ETT are often covered by bacteria forming biofilm on inner surface quickly after intubation and can easily get dislodged by suction catheter use, leading to inoculation of lower respiratory tract thus avoiding effect of antibiotics [22]. In the present study use of antibiotics, antacids and feeding through nasogastric tube were factors that were present in all patients. These factors have also been found by Gaynes R in their study [23]. Use of antibiotics help in the selection of resistant pathogens leading to super infections [24]. Stress ulcer prophylaxis raises the gastric pH and help increase the incidence of pneumonia [25]. Underlying lung condition was seen in 68.33% patients in present study. Similarly Fagon JY et al., placed patients with Chronic Obstuctive Pulmonary Disease (COPD) in higher risk population [24]. COPD is recognised as important risk factor because of increased age of patients, high prevalence of pre-existing colonisation of lower airways, inhibition of mucociliary function and inability to generate effective cough because of airflow obstruction [25].

Altered state of consciousness as a risk factor for VAP was seen in 17% in present study. Gadani H et al., noticed incidence of VAP in stuporous (62.5%) and comatose (50%) patients was high compared to conscious (35.75%) and drowsy (18.42%) patients. This may be due to higher chances of aspiration in comatose patients [26]. Huang Y et al., observed Hypertension (29.55%) and Diabetes mellitus (22.73%) as important co-morbid factors in VAP patients [16]. An improved understanding of risk factors is needed for development of better approach for prevention of nosocomial pneumonia in critically ill patients. Airway management and respiratory care also play a crucial role in preventing pneumonia in ventilated patients.

Period of onset of VAP: In present study 38.33% patients had early onset pneumonia (<5 days of intubation) and 61.67% had late onset pneumonia (≥5 days). Similar findings were observed by Violan J et al., and Abukhabar H et al., with early onset in 34% and 23.3% and late onset 65.8% and 76.6% patients, respectively [27,28]. De Latorre FJ et al., considered 7 days as the cut-off between early and late onset VAP and observed that duration of intubation and antimicrobial treatment predicted the isolation of drug resistant bacteria [18].

Yeast isolates in VAP: In present study, total yeast isolated were 26 from 120 cases. Of 26 isolates, 53.85% were *Candida albicans* and 46.15% were non-*albicans Candida*. Among the non-*albicans Candida* 41.67% were *Candida krusei* and 33.33% were *Candida dublinensis* while *Candida parapsilosis*, *Candida tropicalis* and *Candida glabrata* were 8.33% each. Similarly in a study conducted by Serban RI et al., *Candida albicans* were 16.97%, while *Candida glabrata*, *Candida sake*, *Candida krusei*, amounted 1.89% each, and *Pneumocystis jiroveci* was 3.77% in patients of VAP [29]. Also, in a study conducted by Adiguzel N et al., *Candida* species were isolated in 30% of mechanically ventilated patients [30]. A study from Turkey done by Turgut H et al., *Candida* spp. was the most frequent pathogen of device associated infection in ICU [31]. Hamet M et al., confirmed that *Candida* spp. colonisation is frequent in mechanically ventilated patients, as 56% of their patients harboured *Candida* in their airways, similar to 53% reported by Meersseman W et al., [32,33]. Olaechea PM et al., have reported the presence of *Candida* species in the lungs of immunocompetent ventilated patients [34]. Delisle MS et al., isolated 15.6% of positive *Candida* cultures from patients of VAP [35].

However, there are several limitations to this analysis, assuming that isolation of *Candida* from respiratory tract specimens indicates colonisation and not infection. However, this is in concordance with several similar studies [34,36]. Candidal invasion of lung parenchyma after haematogenous dissemination would be frequently associated with pneumonia but unlikely to have happened in the course of the trial as only two patients developed documented candidemia [34,37-39].

In the present study, polymicrobial aetiology was seen in 27% of cases, commonest combination isolated was *Candida albicans* with *Pseudomonas* and *Candida krusei* with *Pseudomonas*. Azoulay E et al., showed that *Candida* colonisation was associated with increased risk of *P.aeruginosa* VAP [36]. Another example was given by Nseir S et al., depicting that antifungal treatment decreased risk for *P.aeruginosa* infection in colonised patients. Farnesol which is *Candida albicans* main quorum sensing molecule is able to influence the motility and expression of virulence factors of *P.aeruginosa* [40]. In this study all *Candida albicans* were sensitive to fluconazole and amphotericin B. Among the non-*albicans*, one isolate each of *Candida krusei*, *Candida tropicalis* and *Candida glabrata* were resistant to fluconazole and amphotericin B. One isolate each of *Candida krusei*, *Candida parapsilosis* were dose dependent susceptible to fluconazole.

In study conducted by Delisle MS et al., commonest antifungal agent used was fluconazole in VAP patients [35]. Recent survey of blood stream isolates show that 10-15% of *C.glabrata* isolates are resistant to fluconazole. On the basis of a combination of invitro and in vivo data, it appears that significant proportion of isolates of *C.glabrata* and *C.krusei* has reduced susceptibility to amphotericin B [41].

To conclude, VAP is increasingly associated with MDR pathogens. Production of ESBL, AmpC β-lactamases and metallo β-lactamases are responsible for the multidrug resistance of these pathogens. Knowledge of the susceptibility pattern of the local pathogens should guide the choice of antibiotics in addition to the likelihood of organisms.

Limitation(s)

Antifungal susceptibility of other drugs could not be included in the study due to time constraint.

CONCLUSION(S)

Fungus being a dangerous aetiologic agent for ICU patients on ventilator, early diagnosis will help in better management and reducing mortality. As fluconazole and amphotericin B are commonly used antifungal drugs on patients of ventilator pneumonia, AFST helps in emergence of antifungal resistance to different emerging fungal species. This will also help the hospital to develop evidence based policy for chemoprophylaxis of VAP.

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