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Original Article

Microbiological profile of ventilator-associated pneumonia among intensive care unit patients in tertiary Egyptian hospitals

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Abstract

Introduction: Ventilator-associated pneumonia (VAP) is one of the common serious infectious diseases encountered in the intensive care unit (ICU), which highly affects the healthcare cost and patient prognosis. VAP is caused by various antimicrobial-resistant aetiological agents and the clinical manifestations lack sensitivity and specificity, making the prompt treatment is a challenge. This study aimed to investigate the microbial profile of VAP causing microorganisms among ICU patients in Egypt, antimicrobial susceptibility patterns and the genetic diversity among the frequently isolated organisms.

Methodology: Throughout the period from August 2016 to August 2017, endotracheal aspirate (ETA) specimens were collected from ICU patients with clinically suspected VAP in two tertiary hospitals in Cairo. ETA specimens were investigated for the microbial content. The antimicrobial susceptibility was determined by the Kirby-Bauer method. ERIC-PCR was performed for genotyping.

Results: Fifty microbiologically confirmed VAP cases were identified. The most frequently isolated microorganisms were *Klebsiella* spp., followed by *Pseudomonas aeruginosa*, *Acinetobacter baumannii*. *Candida* spp. was the most isolated fungi. A single isolate of each *Cupriavidus pauculus* and *Aeromonas salmonicida* was isolated. Antimicrobial susceptibility profiles indicated 40% of isolates were multidrug-resistant (MDR). ERIC-PCR revealed no genetic relatedness among *K. pneumoniae* isolates, the most frequently isolated microorganism.

Conclusions: Gram-negative bacteria are the main causative agents of VAP cases, which mostly are MDR. Microorganisms like *C. pauculus* and *A. salmonicida* should be taken into consideration as VAP causative agents. There was no common source of infection suggesting likely endogenous sources of *K. pneumoniae*, the main causative agent of VAP in this study.

Key words: Ventilator-Associated Pneumonia; genotyping; ERIC; intensive care unit; multidrug-resistant.

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Introduction

The mechanical ventilator (MV) represents the most common organ support in the intensive care unit (ICU). One of the most common clinical complications caused by MV is ventilator-associated pneumonia (VAP) which carries substantial morbidity and mortality. VAP is an inflammation of lung parenchyma caused by microbial pathogens [1]. According to American Thoracic Society (ATS) guiding principle on nosocomial pneumonia, VAP is the main form of hospital-acquired pneumonia (HAP) which develops in a patient on MV for more than 48 hours, resulting in a burden on overall health care costs and extremely affects patient prognosis [2]. The predisposing factors for developing VAP include prior antibiotic therapy, pre-existing sinusitis, contaminated ventilator circuits or decreased gastric acidity in ventilated patients. VAP is difficult to diagnose because of the lack of respiratory symptoms in sedated patients, in addition to signs and symptoms that lack sensitivity and specificity. Thus, the detection of the causative organism is critical for the diagnosis of VAP. ATS guidelines recommended that endotracheal aspirate (ETA) or bronchoscopic aspiration from the infected lungs' segments should be sent for quantitative/semi-quantitative culture and antimicrobial susceptibility testing for the precise diagnosis and proper antimicrobial therapy [3].

Gram-negative bacteria have been found to predominate in VAP, particularly, *Pseudomonas aeruginosa* and *Enterobacteriaceae* members. In developing countries, VAP cases are usually caused by multidrug-resistant (MDR) microorganisms.

Unfortunately, the resistance of these bacteria to diverse classes of antimicrobial agents, including carbapenems, challenges the appropriateness of the empirical antibiotic therapy, hence making the therapeutic options are limited [4].

The incidence and mortality rate of VAP vary according to the population of patients in ICU, duration of hospital stay, time of onset, causative organisms and prior antimicrobial therapy [1]. Thus, regular monitoring of VAP causative organisms and their antimicrobial susceptibility patterns are essential for quick initiation of the appropriate antimicrobial treatment, thereby reducing the adverse effects on patients' prognosis [5]. Accordingly, this study aimed to determine the microbial profile of clinically suspected VAP cases in ICU patients, under MV, in two tertiary Egyptian hospitals in Cairo, determination of the antimicrobial susceptibility patterns of isolated microorganisms, and investigation of the genetic relatedness and/or diversity among the most frequent isolated microorganism.

Methodology

Study population

The study population included 78 patients with clinically suspected VAP at ICU in two Al-Azhar University hospitals in Cairo, El-Hussein hospital and Sayed Galal hospital, over a one-year period from August 2016 to August 2017. Enrolled ICU patients in this study were of different ages on MV for more than 48 hours with clinically suspected VAP as reported by the responsible health professionals and patient medical record of observed and clinical parameters, in addition to informed consent. The clinical suspicion of VAP was established based on the clinical pulmonary infection score (CPIS) of greater than six. Inclusion criteria of patients also included patients with two out of the following three findings: fever ≥ 38°C, purulent bronchial secretions and leucocytosis (WBC more than 10,000/mm³) or leukopenia (WBC < 3500/mm³) [3]. For a definite diagnosis of VAP cases, the microbial cause was identified as follows: 10^5 CFU/mL was considered as a threshold while the growth of any microorganism below the threshold was assumed to be due to colonization or contamination [6]. Patients with clinical and radiological signs suggestive of pneumonia at the time of admission to hospital were excluded from this study. VAP cases in this study were classified as early-onset VAP (occurring within the first 4 days of MV) or late-onset VAP (occurring 5 days of MV or more) [2]. The reasons for admission to ICU were medical problems in 64% of patients (such as a disturbance in conscious level due to chronic liver disease (such as positive HBV) or surgical problems (such as post-caesarean section arrest) in 36% of patients.

Collection and processing of ETA specimens

A total of 78 non-duplicate endotracheal aspirate (ETA) specimens were collected from patients in this study (one specimen from each patient) upon the approval of the review board in the two hospitals and the ethics committee of Faculty of Pharmacy for girls, Al-Azhar university. These specimens were collected in the study hospitals with a dedicated team through the medical care of each patient. ETA specimens were taken by the non-bronchoscopic bronchoalveolar lavage method as follows [6]. The ETA was collected from each patient using a 22-inch suction catheter, which was gently introduced through the endotracheal tube for a distance of approximately 25 - 26 cm. Gentle aspiration was then performed without instilling saline, and the catheter was withdrawn from the endotracheal tube. After the catheter was withdrawn, the part of the catheters containing the aspirate was cut and placed in a sterile container and transported to the microbiology laboratory immediately for processing. To flush the exudates, 2 mL of sterile 0.9% normal saline was injected into the catheter with a sterile syringe. Specimens were mechanically liquefied and homogenized by vortex for 1 minute and centrifuged at 3000 rpm for 10 minutes. The semi-quantitative scoring of Gram staining was calculated. Semi-quantitative scoring of Gram staining was used. The semi-quantitative scoring of Gram stain was based on the number of bacteria per high-power (×1000) oil immersion field: 0 = no bacteria per field; 1+ = less than one bacterium per field; 2+ = 1–5 bacteria per field; 3+ = 6 – 30 bacteria per field; and 4+ = more than 30 bacteria per field. The semi-quantitative Gram stain score of 0 indicates a low probability of VAP, and the semi-quantitative Gram stain score of ≥ 3+ indicates a high probability of VAP, defined as quantitative cultures with ≥ 10^6 CFU/mL [7].

Identification of microbial isolates

For primary isolation, a volume of 0.01 mL of each sample solution was plated on nutrient agar (Oxoid, Hampshire, UK), manually prepared blood agar and chocolate (heated blood) agar, MacConkey agar (Oxoid, Hampshire, UK), and sabouraud dextrose agar (Biolife Italiana, Milano, Italy). All plates were incubated aerobically overnight at 37°C and checked for growth after incubation. In addition, chocolate agar

plates were incubated in a carbon dioxide enriched atmosphere for up to 48 hours using Gas generating kits (carbon dioxide system) (Oxoid, Hampshire, UK) [8], and the sabouraud dextrose at room temperature (25°C) for 4 weeks. All isolated microorganisms were identified to the genus and species level based on microscopy, conventional biochemical testing and cultural characteristics on differential microbiological media including Uri selective media (Bio-Rad, France) for bacterial isolates and CHROM agar (CHROMagar, Paris, France) for *Candida* fungal isolates. VITEK® 2 automated system (bioMérieux, Craponne, France) was used to confirm the identification of both bacterial and fungal isolates. Germ tube test was performed to differentiate *albicans* from non-*albicans Candida* species [8].

Determination of the antimicrobial susceptibility profiles

Susceptibilities of microbial isolates to different antimicrobial agents representing diverse classes of antimicrobials were determined using the Kirby-Bauer disc diffusion method [9] on Mueller-Hinton (MH) agar (Oxoid, Hampshire, UK) following Clinical Laboratory Standard Institute (CLSI) guidelines [10]. Antimicrobial discs (Bioanalyse, Ankara, Turkey) were stored at 4°C and allowed to reach room temperature before being used. Antimicrobials used in this study, against different recovered bacterial and fungal species, represented diverse classes of both antibacterial and antifungal agents. Antibacterial discs included amikacin (30 µg), amoxicillin (30 µg), amoxicillin-clavulanate (20/10 µg), azithromycin (15 µg), aztreonam (30 µg), cefazolin (30 µg), cefepime (30 µg), cefotaxime (30 µg), ceftazidime (30 µg), cefuroxime (30 µg), cefoxitin (30 µg), ceftriaxone (30 µg), ciprofloxacin (5 µg), chloramphenicol (30 µg), clarithromycin (15 µg), clindamycin (2 µg), colistin (10 µg), doxycycline (30 µg), gentamicin (10 µg), linezolid (30 µg), levofloxacin (5 µg), meropenem (10 µg), novobiocin (30 µg), oxacillin (1 µg), piperacillin (100 µg), piperacillin/tazobactam (100/10 µg), teicoplanin (30 µg), tobramycin (10 µg), trimethoprim-sulfamethoxazole (25 µg) and vancomycin (30 µg). Antifungal discs used in this study included amphotericin B (100 µg), nystatin (100 µg), clotrimazole (50 µg), ketoconazole (50 µg), fluconazole (25 µg), griseofulvin (10 µg), itraconazole (50 µg) and terbinafine (30 µg). Isolates that showed resistance to at least three different classes of antimicrobial agents were considered as MDR.

Molecular typing by ERIC-PCR amplification

ERIC-PCR fingerprinting was carried out to determine the genetic diversity among *K. pneumoniae* isolates. For ERIC-PCR amplification, genomic DNA was extracted from examined *K. pneumoniae* isolates using Gene JET Genomic DNA Purification Kit (Thermo Scientific, Waltham, Massachusetts, USA-K0721) according to manufacturer's instructions. The PCR reactions were prepared in total volumes of 25 µL, contained ~ 10 ng of template DNA, 10 pmol of ERIC-1 primer (5′-ATGTAAGCTCCTGGGGATTCAC-3′), 12.5 µL of Dream Taq Green PCR Master Mix (2X) (Promega, Madison, Wisconsin, USA) and the volume was completed to 25 µL by adding nuclease-free water. The PCR amplifications were carried out with Veriti 96-Well Thermal Cycler (Applied Biosystems, Foster City, California,, USA) programmed for an initial denaturation at 95°C for 5 minutes and 40 cycles of denaturation at 95°C for 1 minute, primer annealing at 45°C for 1 minute and extension at 72°C for 8 minutes, followed by final extension at 72°C for 10 minutes [11].

Detection of amplified PCR products by TAE (Tris-acetate-EDTA) agarose gel electrophoresis

PCR products were resolved through TAE agarose gel (0.8 %) electrophoresis prepared using molecular biology grade agarose (Bioline, London, UK) in 1X TAE buffer. DNA fragments were electrophoresed (0.5X TAE buffer (Thermo Scientific, Waltham, Massachusetts, USA) at 100 V and 90 mA for 30 minutes) in the horizontal gel electrophoresis apparatus (Bio-Rad, Munich, Germany), stained with ethidium bromide (Alliance Bio, Bothell, Washington, USA), and visualized by placing on a UV transilluminator (UVP, LLC, Upland, California, USA,) and photographed directly. For the sizing of the separated DNA fragments, GeneRuler 1 Kb DNA ladder (Thermo Scientific, Waltham, Massachusetts, USA) was used.

ERIC profiles analyses

The obtained ERIC patterns were clustered by dendrogram generated with the Dice similarity coefficient and the UPGMA clustering method using DendroUPGMA software (http://insilico.ehu.es/dice_upgma/).

Statistical analysis

Results were analyzed using GraphPad Prism software version 8.3 (GraphPad Software, San Diego, USA). The frequencies and percentages were used to designate demographic and antimicrobial resistance-

related characteristics. The P-value < 0.05 was considered as statistically significant.

Results

VAP cases were caused by diverse microbial species

Microbiological investigations revealed that 50 specimens out of the 78 ETA specimens showed significant growth of microorganisms following the criteria mentioned before. These 50 cases were the microbiologically confirmed VAP cases in the current study. From these 50 ETA specimens' positive cultures, a total of 53 microorganisms were isolated as three specimens (6%) showed polymicrobial growth (positive culture of more than one microbial species), while the remaining 47 (94%) specimens showed monomicrobial growth (positive culture of only one microbial species). The causative microbial agents of VAP cases were diverse species of aerobic bacteria (Gram-positive and Gram-negative) and fungi with the predominance of Gram-negative bacteria (Table 1). *K. pneumoniae* was the most predominant bacterial species among all isolates (17/53, 32%). The polymicrobial specimens included one specimen harbored *Staphylococcus* species and *Candida* species, and two specimens harbored *Klebsiella* species with *Pseudomonas* species.

The 50 VAP-confirmed cases, based on clinical and microbiological records, included 18 cases (or 19 isolates) from Sayed Galal hospital and 32 cases (or 34 isolates) from El-Hussein hospital. VAP cases were distributed among males more than females with a percentage of 70% (35/50) of all cases. Upon grouping patients into four groups according to age: < 20 – 40 years, 41 – 60 years, 61 – 80 years and > 80 years, VAP cases were more frequent in the age group between 61 and 80 years old (20/50, 40%) with a mean age of 55 years old, followed by patients aged 41 – 60 years (17/50, 34%), then patients aged < 20 – 40 years (11/50, 22%) and lastly the age group > 80 years (2/50, 4%).

In this study, VAP cases were categorized based on the duration of MV into early-onset VAP with a frequency of 44% (22/50) and late-onset VAP with a frequency of 56% (28/50). The total number of microbial isolates recovered from the late-onset VAP cases was significantly higher than the early-onset ones (P < 0.05) (Table 1).

Antimicrobial resistance profiles of microbial species causing VAP cases in this study

Overall, the antimicrobial susceptibly profiles indicated high resistance profiles among isolated bacterial and fungal species against different antimicrobial classes examined (Table 2). In this study, 39.6% (19/48) of bacterial isolates were MDR, while 40% (2/5) fungal isolates were MDR. Concerning Gram-negative bacteria, all isolates were sensitive to colistin except *A. salmonicida* and *C. pauculus. Klebsiella* spp. isolates that showed sensitivity frequency of 73% to each of amikacin, meropenem and azithromycin. *P. aeruginosa* isolates showed high

Table 1. Types and frequencies of bacterial and fungal species recovered from ETA specimens.

Microbial species	Early-onset VAP isolates	Late-onset VAP isolates	Total number of isolates
Gram-negative bacterial species			
Klebsiella spp. (18 isolates)			
Klebsiella pneumoniae (17)	10[1] (43.4)[2]	8 (26.7)[2]	18 (34)[3]
Klebsiella oxytoca (1)			
Pseudomonas aeruginosa	3 (13)	8 (26.7)	11 (20.8)
Acinetobacter baumannii	4 (17.4)	2 (6.7)	6 (11.3)
Escherichia coli	0	3 (10)	3 (5.6)
Burkholderia cepacia	0	2 (6.7)	2 (3.8)
Stenotrophomonas maltophilia	2 (8.7)	0	2 (3.8)
Aeromonas salmonicida	0	1 (3.3)	1 (1.9)
Cupriavidus pauculus	0	1 (3.3)	1 (1.9)
Gram-positive bacterial species			
Staphylococcus aureus	1 (4.3)	2 (6.7)	3 (5.6)
Enterococcus faecalis	1 (4.3)	0	1 (1.9)
Fungal species			
Candida spp. (4 isolates)			
Candida tropicalis (1)	2 (8.7)	2 (6.7)	4 (7.5)
Candida glabrata (3)			
Cryptococcus laurentii	0	1 (3.3)	1 (1.9)
Total	23 (43.4)[3]	30 (56.6)[3]	53 (100)

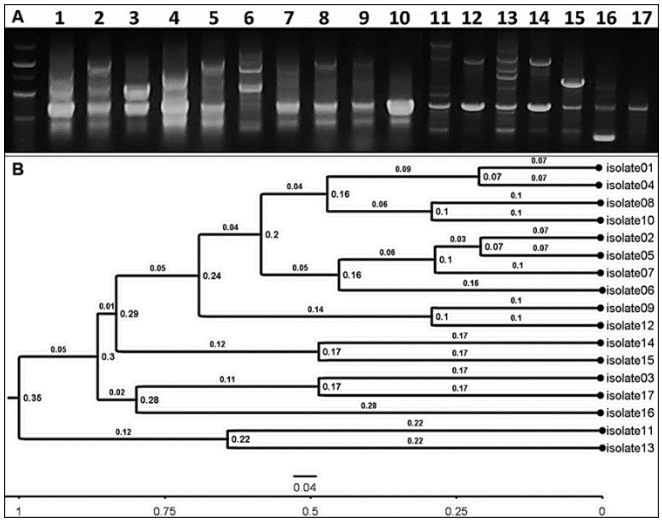
[1]number of isolates; [2]percentage correlated to the total number of isolates of each type of VAP cases; [3]percentage correlated to the total number of isolates in this study (53 isolates).

sensitivities to azithromycin and clarithromycin of 100% and 91%, respectively. The Gram-positive isolates showed sensitivities to teicoplanin, doxycycline and novobiocin. Studying the antifungal susceptibility revealed that all isolates were resistant to tested antifungal drugs, while *Candida* spp. were susceptible to itraconazole.

K. pneumoniae causing VAP cases were polyclonal

ERIC-PCR-based genotyping of the 17 *K. pneumoniae* isolates revealed a significant molecular heterogeneity of *K. pneumoniae* isolated within the same hospital or among all isolates from the two hospitals of study, which is indicated by 17 different ERIC-based patterns or fingerprints (Figure 1).

Figure 1. PCR-based ERIC patterns of K. pneumoniae isolates.



(A) Agarose gel (0.8 %) electrophoresis of ERIC-based PCR patterns; NEB 100 bp DNA size marker (most left lane). (B) Corresponding dendrogram generated with the Dice coefficient and the UPGMA clustering method.

Table 2. Antimicrobial resistance profiles of microorganisms causing VAP in this study.

Microbial species	Antimicrobial resistance pattern
Gram-negative bacterial species	
Klebsiella spp. (n = 18)	AK (28)[1], AM (100), AMC (72), AZM (28), ATM (72), CZ (100), FEP (67), CTX (72), CAZ (72), CXM (83), FOX (89), CRO (89), CIP (67), CLR (78), DA (94), CT (0), DO (67), CN (61), LEV (67), MEM (28), PRL (100), TPZ (67), TOB (61), SXT (78)
P. aeruginosa (n = 11)	AK (46), AM (100), AMC (100), AZM (0), ATM (82), CZ (100), FEP(100), CTX(100), CAZ (100), CXM (100), FOX (100), CRO (100), CIP (67), CLR (78), DA (94), CT (0), DO (18), CN (73), LEV (73), MEM (55), PRL (100), TPZ (73), TOB (73), SXT (82)
A. baumannii (n = 6)	AK (33), AM (100), AMC (100), AZM (17), ATM (100), CZ (100), FEP(100), CTX(100), CAZ (100), CXM (100), FOX (100), CRO (100), CIP (67), CLR (67), DA (100), CT (0), DO (17), LEV (83), MEM (83), PRL (100), TPZ (83), TOB (67), SXT (83)
E. coli (n = 3)	AK (33), AM (100), AMC (100), AZM (33), ATM (100), CZ (100), FEP(100), CTX (100), CAZ (100), CXM (100), FOX (100), CRO (100), CIP (67), CLR (67), DA (100), CT (0), DO (33), LEV (67), MEM (33), PRL (100), TPZ (67), TOB (67), SXT (100)
B. cepacian (n = 2)	AK (0), AM (100), AMC (100), AZM (0), ATM (100), CZ (100), FEP(100), CTX(100), CAZ (100), CXM (100), FOX (100), CRO (100), CIP (100), CLR (0), DA (100), CT (0), DO (100), CN (100), LEV (100), MEM (100), PRL (100), TPZ (100), TOB (100), SXT (100)
S. maltophilia (n = 2)	AK (0), AM (100), AMC (50), ATM (100), CZ (100), FEP (100), CAZ (100), FOX (100), CRO (100), CIP (50), CT (0), DO (0), LEV (0), MEM (0), PRL (100), TOB (0), SXT (0)
A. salmonicida (n = 1)	AK (0), AM (100), AMC (100), AZM (100), ATM (0), CZ (100), CXM (100), FOX (100), CRO (100), CIP (100), CLR (100), DA (100), CT (100), DO (0), CN (100), LEV (100), MEM (100), PRL (100), TOB (100), SXT (100)
C. pauculus (n = 1)	AK (100), AM (0), AMC (0), AZM (0), ATM (0), CZ (100), FEP (0), CTX (100), CAZ (0), CXM (100), FOX (100), CRO (100), CIP (100), CLR (0), DA (100), CT (100), DO (100), CN (100), LEV (0), MEM (0), PRL (0), TPZ (0), TOB (100), SXT (100)
Gram-positive bacterial species	
S. aureus (n = 3)	AM (100), AMC (67), AK (33), AZM (67), CRO (100), CRT(67), FOX (67), FEP (67), CIP (67), CLR (67), DA (67), DO (0), LEV (67), LNZ (33), NV (0), OX (67), PRL (100), TPZ (67), TEC (0), SXT (67), VA (33)
E. faecalis (n = 1)	AM (0), AMC (0), AK (100), AZM (100), CRO (0), CIP (100), DO (0), LEV (100), LNZ (100), TPZ (0), TEC (0), TOB (100), SXT (0), VA (0)
Fungal species	
Candida spp.	AB (100), NY (100), CTR (100), KET (50), FCA (100), GRS (100), ITR (0), TER (100)
C. laurentii	AB (100), NY (100), CTR (100), KET (100), FCA (100), GRS (100), ITR (100), TER (100)

[1]percentage of resistant isolates correlated to the number of isolates within each species. AK, amikacin; AM, amoxicillin; AMC, amoxicillin-clavulanate; AZM, azithromycin; ATM, aztreonam; CZ, cefazolin; FEB, cefepime; CTX, cefotaxime; CAZ, ceftazidime; CXM, cefuroxime; FOX, cefoxitin; CRO, ceftriaxone; CIP, ciprofloxacin; C, chloramphenicol; CLR, clarithromycin; DA, clindamycin; CT, colistin; DO, doxycycline; CN, gentamicin, LNZ, linezolid, LEV, levofloxacin; MEM, meropenem; NV, novobiocin; OX, oxacillin; PRL, piperacillin; TPZ, piperacillin/tazobactam; TEC, teicoplanin, TOB, tobramycin; SXT, trimethoprim-sulfamethoxazole; VA, vancomycin; AB, amphotericin B; NY, nystatin; CTR, clotrimazole; KET, ketoconazole; FCA, fluconazole; GRS, griseofulvin; ITR, itraconazole; TER, terbinafine.

Discussion

VAP is a common cause of increased morbidity and mortality in hospitalized patients, particularly in ICU patients, despite advances in diagnostic techniques and management [12]. In developing countries, the total costs because of VAP infections are nearly five-fold higher than those of other diseases [13]. The clinical signs and symptoms of VAP lack sensitivity and specificity [12,13], thus this study aimed to investigate the microbial profile of VAP causing microorganisms among ICU patients in tertiary care hospitals in Cairo, El-Hussein hospital and Sayed Galal hospital. Of 78 VAP clinically suspected ICU patients included in the current study, 50 patients were definitely diagnosed as VAP cases based on both the clinical and microbiological criteria [14]. This 50-patients group was sub-grouped into four groups based on the patients' ages, with the highest incidence of VAP cases was in patients aged 61 – 80 years (40% of all cases) with a mean age of 55 years old. The study of Eida *et al*. [15] has recorded a comparable age distribution with a mean age of 63.8 while Curcio *et al*. [16] has recorded almost the same age average of 56 years old. However, based on our records, it seems that the age has not a notable contribution to acquiring VAP disease as well as the infection and progression process as the disease was developed in patients in different age groups. Regarding gender, the incidence of VAP cases in the current study was found to be highly significant among male patients than in females. This observation might indicate that males are more susceptible to VAP acquisition than females. Other studies showed also the majority of males among VAP-suspected patients [13,17,18,19]. In this study, the reason for admission of the majority of VAP cases was medical problems like other previous studies [4,20]. That may indicate that medical prolonged diseases and/or extended treatment strategies could play a role in the development of VAP [20].

The onset of developing VAP may be attributed to many factors including patient condition when starting MV and how it progresses. In addition, it may be related to the nature of microorganisms aspirated, their ability and virulence to colonize, forming biofilm or even producing a disease [21]. Furthermore, developing MDR bacterial strains in immunocompromised patients usually occur which may also affect the time of disease acquisition [22]. In this study, the frequency of late onset-VAP cases (56%) among ICU patients, usually immunocompromised, was significantly higher than early-onset ones. Other studies reported also the high frequency of late-onset VAP cases more than those occurs in the early days of ventilation with almost comparable percentages to the present study; with the main route for acquiring VAP was the aspiration of oropharyngeal organisms into the distal bronchi [23,24].

The aetiological agents of VAP may extensively differ according to several factors including the population of the patients in ICU, duration of hospital stay and prior antimicrobial therapy [23] and co-morbid conditions [17]. In addition, previous extensive exposure to antimicrobials increases the risk of developing MDR pathogens [25]. It was reported that the most common causative pathogens of VAP, especially in patients with underlying serious diseases, include aerobic Gram-negative bacilli such as *P. aeruginosa*, *E. coli*, *K. pneumoniae* and *Acinetobacter* species, besides Gram-positive bacteria such as *S. aureus* [26]. Consistent with these previous data, Gram-negative bacteria represented the most frequently isolated microorganisms from ETA specimens in the current study with a percentage of 83% (44/53), followed by fungal isolates and Gram-positive bacterial isolates with percentages of 9.5% (5/53) and 7.5% (5/53), respectively. The most frequent Gram-negative bacterial species in this study were *Klebsiella* spp. (34%), *P. aeruginosa* (20.8%) and *A. baumannii* (11.3%). Similarly, in an Indian study, the most frequent microorganisms isolated from VAP patients were, in order, *Klebsiella* spp. and *P. aeruginosa* [27]. Additionally, previous studies in India and the same geographical country of our study Egypt on VAP reported *Klebsiella* spp. as the most frequent causative agent like ours [28-30]. Other isolated Gram-negative bacteria, *E. coli* (5.6%) and *S. maltophilia* (3.8%), were isolated with nearly the same frequencies in the study of Selina *et al*. [2]. *B. cepacia* was also isolated in the presented study with a percentage of 3.8%, which is similar to the percentage recorded by the study of Ahmed *et al*. [31].

C. pauculus is a gram-negative, aerobic, non-spore forming, non-fermentative motile bacillus. It can be isolated from water and ultrafiltration systems of water in hospitals. *C. pauculus* rarely causes human infections, however, it may be an infectious agent in immunocompromised individuals causing bacteremia and peritonitis [32]. In this study, a single case positive culture showed the growth of *C. pauculus*; identified by Vitek 2 system. In a previous study from Turkey, a VAP case was recorded to be caused by *C. pauculus* in a patient with breast cancer [32]. Thus, *C. pauculus* should be considered as a potential pathogen causing VAP infections, particularly in immunocompromised patients. Similarly, in the present study, only one case

of VAP was caused by *A. salmonicida* which was identified by Vitek 2 system. *A. salmonicida* is considered to be a fish pathogen and non-pathogenic for humans as it cannot grow at 37ºC. However, in a previous study in India [33], the laboratory culture plates and broths of human blood were incubated twice at 37ºC, and at each time, the same type of colonies was grown which were identified as *A. salmonicida* by Vitek 2 system [33]. That could be explained by *A. salmonicida* perhaps adapted or genetically modified to be more able to withstand different conditions and to be able to infect humans. However, more investigations are needed for the conditions and reasons for infections and to determine to what extent it represents a hazard to humans.

Gram-positive bacteria represented only 7.5% (4 isolates) of total isolates in the current study, three of them were *S. aureus*. *S. aureus* was isolated with almost the same percentage in another study of Júnior *et al*. [34] which was conducted on VAP patients in Brazil. Only one *E. feacalis* was isolated in this study which is consistent with the study of Girish and Rajendran [28].

In the present study, fungal pathogens represented 9.5% of the total isolates. Four *Candida* spp. isolates (7.5 %) were isolated during this study. The almost same percentage was isolated in the study of Jampala *et al*. [5] in India. In addition, only one *Cryptococcus laurentii* was isolated. *C. laurentii* is one of several non-neoformans cryptococci that have rarely been associated with human infection. The spectrum of clinical infections due to non-neoformans species ranges from skin lesions to fungemia. Most cases of non-neoformans fungemia have been hospital-acquired and have been associated with indwelling intravascular catheters and neutropenia [35,36]. Notably, *C. laurentii* has recently been reported as a cause of pulmonary and cutaneous infections in humans [37].

VAP caused by MDR pathogens was significantly associated with high mortality [4]. In this study, the high resistance profile to cefazolin (100%), piperacillin (98%), cefoxitin (96%), ceftriaxone (96%) and clindamycin (93%) among Gram-negative isolates suggests that these antimicrobial agents are not suitable for initiation of empirical therapy for VAP cases. While the susceptibility rates to meropenem (54.5%) amikacin (68%) and doxycycline (57%) make them likely alternatives. Moreover, in critically complicated cases, we can consider other drugs like colistin and azithromycin that showed superior efficacy against *Klebsiella* spp., *P. aeruginosa*, *B. capecia* and *A. baumannii*. Thus, these drugs may be taken into consideration when determining the empirical therapy. Besides, teicoplanin, doxycycline and vancomycin can be considered as possible choices in the treatment of Gram-positive bacteria. Among fungal isolates, itraconazole was the most effective drug. Based on the definition of multidrug resistance as acquiring non-susceptibility to at least one agent in three or more antimicrobial categories, in this study, MDR pathogens were 18 isolates (39.6%). This ratio nearly resembles that recorded in another study which was 41.7% [33]. While it is higher and lower than other studies of Charles *et al*. [21] (29.2%) and Joseph *et al*. [20] (78.7%), respectively. The high incidence of MDR bacteria, particularly in developing countries, is due to the extensive and/or misuse of antimicrobial drugs in these countries which results in very limited therapeutic options.

The sources of VAP disease could be exogenous, or mostly endogenous source related to each patient itself [38]. The health and hygiene rules and clinical guidelines followed in ICU may play a role in determining the source of infection and the progression of the disease [39]. Regarding endogenous sources, it has been known for decades that the microbial flora of hospitalized and critically ill patients becomes drastically altered within days after admission, especially when antibiotics have been administered. The usual anaerobic flora of the colon and mixed flora of the oropharynx have typically low virulence. However, in critically ill patients these organisms become overgrown by endogenous aerobic Gram-negative bacilli, which can then colonize the airway causing lung infection [22]. In this study, the ERIC-PCR was performed to study the genetic relatedness of the 17 *K. pneumoniae* isolates as it was the most frequently isolated microorganism from the confirmed VAP cases. That may help in VAP epidemiological investigation and understanding more about the disease acquisition and transmission, in addition to studying if there was patient to patient transmission or a common source of infection as an exogenous source or it mostly was of the endogenous source. Seventeen different banding patterns were distinguished for the 17 *K. pneumoniae* isolates. The ERIC data obtained indicated that these bacteria were not transmitted between patients in ICU, since the banding patterns of the isolates from different patients showed no similarity, thus suggesting the possibility of endogenous sources of VAP cases. These results agreed with the study of Heo *et al*. [40] in which all patterns were unique to each patient in the ICU.

Conclusion

Gram-negative bacterial species *K. pneumoniae*, followed by *P. aeruginosa* were the most common causative organisms among VAP cases in immunocompromised patients in ICU, while *Candida* spp. were the common fungal isolates. Although, other potential unusual causes of VAP must be considered by clinicians in the immunocompromised host. In this study, colistin and azithromycin could be considered as empirical therapy for Gram-negative bacteria and other useful antimicrobials include meropenem, amikacin and doxycycline. For Gram-positive bacteria, linezolid and doxycycline showed good activity against isolated bacteria, thus they can be considered good alternatives and/or in combination with other antimicrobials. As antifungal therapy, itraconazole appeared to be a suitable choice. The high discriminatory power of ERIC-PCR indicated that there was no occurrence of bacterial spread among patients. In addition, pathogenic *K. pneumoniae* isolated from both hospitals comprise a genetically variable group of organisms. However, for VAP disease control, more studies should be performed periodically, in specific, for each health care center and/or country to determine the epidemiology of the disease, their antimicrobial susceptibility patterns, and how to control it. More specimens are needed to be investigated including samples from medical staff to determine the strains relatedness and study all possible causes of infection.

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