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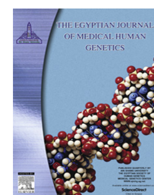

Original article

Multiplex polymerase chain reaction: Could change diagnosis of Ventilator-associated pneumonia in pediatric critical care units to the fast track?

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ABSTRACT

Background: Ventilator-associated pneumonia (VAP) is a frequent hospital-acquired infection in critically ill children. The increasing incidence of infections by antibiotic-resistant pathogens adds significantly to the cost of hospital care and to the length of hospital stays. Besides clinical prerequisites for presumptive diagnosis of VAP, rapid identification of the causative pathogen is essential for appropriate treatment.
Aim of study: To identify the causative bacterial pathogens of VAP by both conventional microbiological cultures and multiplex reverse transcriptase reaction (m-PCR) methods with assessment of turnaround time for both diagnostic modalities together with their diagnostic accuracy.
Methods: Patients were diagnosed to have VAP when their Clinical Pulmonary Infection Score (CPIS) index was more than 6. Endotracheal aspirate was subjected to both microbiological cultures and multiplex PCR for bacterial pathogens.
Results: Multiplex-PCR showed better sensitivity and positive predictive value than bacterial culture for etiological diagnosis of VAP. *Acinetobacter* and *Klebsiella pneumoniae* were the most common identified pathogens. Mean turnaround times were 6 h for multiplex PCR and 72 h for conventional microbiology. Significant shorter turnaround time was recorded with m PCR compared to microbiological culture.
Conclusion: Multiplex-PCR permits simultaneous detection of several bacterial pathogens in a single reaction with best turnaround time that permit optimization of emergency diagnosis of VAP and subsequently improve early management of selective bacterial pathogens.
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1. Introduction

Within twelve hours of endotracheal intubation; a biofilm is formed around the endotracheal tube which contains large amounts of bacteria that can be disseminated into the lungs by ventilator-induced breaths. This biofilm may become dislodged during suctioning, or repositioning of the endotracheal tube [1]. Impaired muco-ciliary clearance with mucosal injury and glottis dysfunction associated with prolonged intubation further aggravates the risk of VAP with re-intubation [2]. Daily interruption of sedative infusions in critically ill patients receiving mechanical ventilation decreases the duration of mechanical ventilation and reduces the length of intensive care unit (ICU) stay. Consequently, this practice can be considered worthy for reducing VAP risk and its occurrence [3,4].

Etiologic diagnosis of Ventilator-associated pneumonia is considered a microbiological emergency because of its impact on disease associated morbidity and mortality and antibiotic management, so rapid diagnostic information is clearly more beneficial to patients than more complete but delayed information. Multiplex-PCR is a universal technique making it possible to identify more than one micro-organism, from single patient' specimen [3,4].

The aim of this study was to identify the causative bacterial pathogens of VAP by both conventional microbiological cultures and multiplex reverse transcriptase reaction (m-PCR) methods with assessment of turnaround time for both diagnostic modalities together with their diagnostic accuracy.

2. Patients and methods

2.1. Study setting

This study was conducted in pediatric intensive care unit (PICU) of Ain Shams University Hospital, which is a multidisciplinary

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medical ICU with 10 beds and average occupancy rate of 100% during time of the study.

2.2. Patients' data acquisition

Demographic variables such as gender, age, underlying disease together with the patients' clinical data, and degree of critical illness by Pediatric Logistic Organ Dysfunction (PELOD) score [5], length of PICU stay, duration of mechanical ventilation, and antibiotic regimen were collected from patients' records after getting the care 'caregivers' consent and the approval of ethical committee of Ain Shams University. The work has been carried out in accordance with the Code of the World Medical Association (Declaration of Helsinki) for experiments in humans. Data collection began within 24 h from the time of admission to the PICU.

2.3. Patients' enrollment

All admitted patients were observed daily for the diagnosis of VAP. The method of establishing the diagnosis of VAP remains controversial and no method has emerged as the gold standard. For these reasons, clinical guidelines are available to aid in decision making about acquisition of ventilator-associated pneumonia [5,6]. We used the Clinical Pulmonary Infection Score (CPIS) to help quantify clinical findings and represents a "weighted approach" to the clinical diagnosis of VAP. This scoring system includes both clinical and radiological factors that increase the likelihood of the presence of VAP. Point values are assigned to each criteria and a sum is calculated. Traditionally, a threshold score of more than six has been used to diagnose VAP (Table 1) [5,6].

2.4. Microbiological assessment

2.4.1. Clinical specimens

Endotracheal aspirate (ETA) was screened from enrolled patients. These specimens were tested against important and common bacterial pathogens by both bacterial culture and multiplex Polymerase Chain Reaction (m-PCR).

2.4.2. Bacterial cultures

Specimens were obtained and sent to the laboratory within one hour of collection. They were decontaminated and centrifuged before inoculation. Inoculation was on Blood agar and Chocolate agar for blood specimens and further on McConkey agar for ETA. Specimens were incubated for one week and observed for colonies every day in ETA and every 48 h in blood specimens. Colonies were identified by gram stain and biochemical profile. Infection was defined by semi-quantitative count of more than 10^5 CFU/ml. Antibiotic sensitivity test was done for positive isolates using disc diffusion method according to National Committee for clinical Laboratory standards (NCCLS) [7]. Isolated bacterial agents are *Streptococcus pneumoniae*, Methicillin-resistant *Staphylococcus* (MRSA), *Klebsiella pneumoniae*, *Mycoplasma pneumoniae*, *Chlamydia pneumoniae*, *Pseudomonas aerogenosa*, *Legionella pneumophilia* and *Acinetobacter*.

2.4.3. Multiplex PCR

Collected specimens were assessed for seven bacterial agents; that are considered serious causative pathogens for VAP worldwide. These organisms are sharing some physical properties during their processing for PCR (*Acinetobacter* was not involved in the panel because it has different incubation temperature than the selected primers for the other 7 organisms). *DNA extraction:* DNA was extracted from the samples using MagNA pure Compact Nucleic Acid Isolation Kit I (Cat. No. 03730964001); supplied by (Roche, Germany). *Amplification by PCR:* This was done by Light Cycler-DNA Amplification Kit SYBR Green I (Cat. No. 2015137). The kit used Light Cycler 2.0 System (Roche, Germany). *Primers:* Primers were non-labeled forward primers and biotin-labeled reverse primers with horseradish peroxidase – labeled probes. Primers for *Mycoplasma pneumoniae*, *Chlamydia pneumoniae*, *Legionella pneumophilia*, *Staphylococcus aureus*, and *Streptococcus pneumoniae* were selected according to Kumar et al. [7]. Primers for *Klebsiella pneumoniae* were made according to Kurupati et al. [8]. Primers for *Pseudomonas aerogenosa* were chosen according to Qin et al. [9].

2.4.4. Turnaround time (TAT)

We calculated **brain to brain TAT (Total TAT)** as outlined by Lundberg including the total testing cycle as a series of nine steps: ordering, collection, identification, transportation, preparation, analysis, reporting, interpretation and taking a decision by the ordering physician. We used the term **Laboratory TAT** as the time elapsed from physician request of the laboratory test till a report is available by the laboratory [10].

2.5. Statistical analysis

Analysis of data was done by IBM computer using SPSS 12-USA. Description of quantitative variables is expressed as mean and standard deviation (SD). Description of qualitative variables is expressed as number and percentage. The Wilcoxon's signed-rank test compared the difference between culture and PCR in different study specimens. There is no gold standard for the diagnosis of VAP in pediatric patients; therefore, we used a Clinical Pulmonary Infection Score of NNIS (National Nosocomial Infections Surveillance) age-specific guidelines. Sensitivity and Specificity of m-PCR and bacterial culture were referred to CPIS as the clinical

Table 1
Modified Clinical Pulmonary Infection Score.

Measurement	Points		
	0	1	2
Temperature (°C)	36.5–38.4	38.5–38.9	≤36.4 or ≥39
Peripheral white blood cell count	4000–11,000	<4000 or >11,000 (>50% bands: add 1 extra point)	
Tracheal secretions	None	Nonpurulent	Purulent
Chest radiograph	No infiltrate	Diffuse or patchy infiltrate	Localized infiltrate
Progression of infiltrate from prior radiographs	None		Progression (acute respiratory distress syndrome or congestive heart failure thought unlikely)
Culture of endotracheal tube suction	No growth/light growth	Heavy growth (some bacteria on gram stain: add 1 extra point	
Oxygenation (Pao_2/fraction of inspired oxygen [Fio_2])	>240 or acute respiratory distress syndrome		≤240 and no acute respiratory distress syndrome

Adapted from Alicia et al. [5] and Swoboda et al. [6].

standard for diagnosis of VAP (CPIS $\geq$ 6 is the cut-off for diagnosis of VAP) [6].

Sensitivity of culture or m-PCR represents the proportion of patients with VAP who test positive. Specificity is the proportion of patients without HAP who test negative. Predictive value of a positive test (PPV) is the proportion of patients with positive tests who have disease. Predictive value of a negative test (NPV) is the proportion of patients with negative tests who do not have disease. Laboratory turnaround time and total turnaround times were calculated in hours and were compared two-sample *t*-test for Independent samples.

3. Results

3.1. Patients' cohort characteristics

Ninety-six patients were admitted to the PICU during the study period with mean age 21 ± 18.7 months, and males represented 53% of them. Their median PELOD score was 24 with predicted mortality rate of 34%. Almost half of admitted cases were admitted due to neurological emergency (coma and acute flaccid paralysis). Recorded frequency of VAP was 48% according to CIPS score during the study period with a rate of 23 per 1000 patients' days. Twenty-one patients developed early VAP while twenty-five patients had late onset VAP. Forty-four percent of patients with VAP were less than 12 months, and 84.5% of them were males. The distribution of the primary cause of PICU admission didn't differ significantly between patients who acquired VAP and those who didn't (Table 2).

3.2. Clinical outcomes of Ventilator-associated pneumonia

Length of PICU stay was doubled in patients who acquired VAP compared to patients without VAP ($p < 0.05$). Duration of mechanical ventilation was significantly longer in patients with VAP ($p < 0.01$). VAP lead to 10% increment in the actual mortality rate above the predicted rate for the affected patients with significant higher mortality than patients without VAP (Table 2).

3.3. Turnaround times for bacterial culture and multiplex PCR

Laboratory turnaround times for mPCR constitutes nearly one-sixth of positive culture ($p < 0.0001$) and one-tenth for mPCR compared to negative culture ($p < 0.0001$). A highly significant similar discrepancy of total TAT were found between mPCR and culture. On the other hand, the total cost of the molecular diagnosis by mPCR was significantly higher than microbiological culture (Table 3).

3.4. Etiologic diagnosis of VAP by multiplex-PCR and microbiological culture

In our study, multiplex-PCR had a better diagnostic yield for VAP than bacterial culture (sensitivity 76%, specificity 97%, PPV 90%, NPV 93% vs. 24%, 92%, 55%, 79% respectively for culture).

Acinetobacter was the most frequent organism isolated by culture followed by *Klebsiella pneumoniae* and *Pseudomonas aerogenosa* (Table 4). Multiplex-PCR significantly increased the diagnostic

Table 3
Turnaround time and cost of both endotracheal aspirate cultures and multiplex PCR.

	ETA Cultures	Multiplex PCR	*P*
Mean Turnaround time; hours (Range)			
Laboratory TAT for positive test	87 (48:186)	14 (9:24)	<0.0001
Laboratory TAT for negative test	147 (96:220)	14 (9:24)	<0.0001
Total TAT for positive test	107 (60:180)	20 (15:40)	<0.0001
Total TAT for negative test	190 (108:230)	20 (15:40)	<0.0001
Total Cost (Egyptian pounds)	9600	13,800	<0.0001

Table 4
Summary of m-PCR and microbiological culture results in endotracheal aspirate (ETA).

Variable	ETA (*n* = 46)		
	Culture	m-PCR	p
Positive cases, *n* (%)	35 (76%)	11 (24%)	<0.001
Streptococcus pneumoniae	4 (8.6%)	–	<0.16
Methicillin-resistant *Staphylococcus* (MRSA)	12 (26%)	–	<0.01
Klebsiella pneumoniae	18 (39%)	9 (19.5%)	0.03
Mycoplasma pneumoniae	11 (24%)	–	<0.01
Chlamydia pneumoniae	2 (4%)	–	0.3
Pseudomonas aerogenosa	–	4 (8.6%)	0.16
Legionella pneumophilia	–	–	
Acinetobacter#	–	12 (28%)	–
Acinetobacter# & MRSA	–	–	–
Klebsiella pneumoniae & *Pseudomonas aerogenosa*	–	1 (2%)	–

Acinetobacter# is not involved in the m-PCR panel.

Table 2
Comparative data of patients' clinical characteristics.

Characteristics	All Patients (*n* = 96)	Patients with VAP (*n* = 46)	Patients without VAP (*n* = 50)	*P* value
Patients-related Characteristic				
Mean age ± SD, months	21 ± 18.7	22.5 ± 19.8	23.6 ± 18	0.61
Male sex, n (%)	51 (53%)	39 (84.5%)	12 (24%)	<0.01
Median PELOD* Score (interquartile range)	24 (9–39)	25 (10–42)	23 (9–39)	0.71
Admission Diagnosis, n (%)				
Pulmonary Diseases	12 (12.5%)	5 (12%)	7 (13%)	0.96
Cardiac Diseases	13 (14%)	5 (12%)	8 (15%)	0.69
Neurologic Diseases	46 (48%)	23 (52%)	23 (46%)	0.61
Acute Gastrointestinal Infections	11 (11%)	6 (12%)	5 (10%)	0.69
Postoperative	16 (16%)	7 (12%)	9 (17%)	0.67
Outcome				
Length of PICU* Stay, Days; mean ± SD	34 ± 47	44 ± 45	22 ± 34	<0.05
Length of MV*, Days; mean ± SD	30 ± 35	39 ± 43	22 ± 28	<0.01
Predicted Mortality rate%, (range)	34% (1–99)	34% (1–90)	36% (2–98)	–
Actual Mortality, n (%)	31 (32%)	20 (44%)	11 (23%)	0.04
Rate of VAP, n/1000 patient days	–	23	–	–

PELOD* Pediatric Logistic Organ Dysfunction; PICU* Pediatric intensive care; MV*, Mechanical ventilation; VAP*, Ventilator-associated pneumonia.

yield of MRSA, *Klebsiella pneumonia*, *Streptococcus pneumoniae* and *Mycoplasma pneumoniae*. Polymicrobial Pneumonia was diagnosed in two patients, where a combined growth of MRSA and *Acinetobacter* was present in one patient and combined growth of *Pseudomonas aerogenosa* and *Klebsiella pneumonia* in another patient.

4. Discussion

Both developed and resource-poor countries are faced with the burden of health care-associated infections. Ventilator-associated pneumonia (VAP) is a complication in patients on mechanical ventilation [11]. The frequency of registered VAP in our PICU during this study reached 44% of patients on mechanical ventilation with a rate of 23 per 1000 patients admission days with associated mortality rate of 44% with predominance of gram-negative pathogens; *Klebsiella pneumoniae* was diagnosed in 41%, *Acinetobacter* in 28% with MRSA in 24%. For a resource-limited country with inappropriate infection control measures together with relatively high occupancy rate and high patients nurse ratio; we unfortunately could encounter this high prevalence and so forth the mortality burden. Patra et al. reported that VAP constituted 76% of patients with hospital acquired pneumonia and represented the most frequent nosocomial infection in intensive care units (80%) with an overall mortality reaching 60%; *Pseudomonas* contributing to 57.1% of deaths followed by *Klebsiella*, *E. coli* and *Acinetobacter* [1,12]. The increasing incidence of infections caused by antibiotic-resistant pathogens contributes to the emerging seriousness of these infections with expected higher mortality rate [13].

In our study; the mean length of stay was doubled following VAP together with 50% increment in the mean duration of mechanical ventilation days. In another study, VAP lengthens the hospital stay by 7–9 days and is associated with a higher cost of medical care [13]. Fifty-two percent of enrolled patients with VAP in our study were admitted primarily to the PICU due to neurological emergencies (e.g. acute flaccid paralysis with rapid progression to involve respiratory muscles, status epileptics and coma). This group of patients had major risks of aspirations [14,15].

The microbial etiology of VAP varies according to patients' medical condition, the duration of ICU stay, the antibiotic policy and the prior exposure to antimicrobials. All these factors significantly influence the distribution patterns of etiologic agents from one ICU to another [15,16]. The most frequent organisms detected in our study were *Klebsiella pneumoniae* (40%), *Acinetobacter* (28%) and followed by *Mycoplasma pneumoniae* (24%) and MRSA (24%), while the least common isolates were *Streptococcus pneumoniae* (8%) and *Chlamydia pneumoniae* (4%).

Acinetobacter baumannii is now recognized to be capable of causing life-threatening infections including pneumonia [17]. This appears to be due to their ability to survive on health-care workers' hands and environmental surfaces and their intrinsic resistance to many common antibiotics, rather than any intrinsic virulence factors [3,17]. Antibiotic therapy and critical illness can suppress the normal bacterial flora and lead to an overgrowth of Enterobacteriaceae like *Klebsiella pneumoniae* in the respiratory tracts. The most concerning is the acquisition of extended-spectrum β-lactamases that render the bacteria resistant to penicillin and cephalosporin antibiotics [18]. Extended spectrum β-lactamase (ESBL) producing *Klebsiella pneumoniae* may cause serious nosocomial infections especially in critically ill patients. Numerous outbreaks have been described with ICU-acquired ESBL-producing *Klebsiella pneumoniae* [17].

MRSA was detected as a causative agent for HAP in 24% of cases in our study by m-PCR. *Staphylococcus aureus* (both Methicillin sensitive and resistant strains), constitutes the most frequently isolated pathogen in the ICU. The incidence of MRSA as a cause of VAP was 12–15%, but increased to approximately 30% in patients with prolonged mechanical ventilation and prior antibiotic therapy [19,20]. *Mycoplasma pneumoniae* was not detected by bacteriological cultures but was detected in 24% of patients and diagnosed only by m-PCR. Altogether with VAP task forces, the emergence of resistance is a concern for intensive care specialists worldwide thus it is imperative for investigators from different countries and regions to exchange precise and updated epidemiological data on the encountered HAP and VAP.

The use of PCR leads to an increase in diagnostic sensitivity, especially in micro-organisms that can't be easily cultured and in case of a low burden of micro-organisms with previous antibiotic therapy [21]. In our study, m-PCR optimized the diagnosis and management of VAP over bacterial culture. Both the laboratory and total turnaround times were remarkably shorter compared to microbial culture, hence substantial reduction of unnecessary antibiotic prescriptions seemed possible. This is in agreement with Strålin et al. [22], who recommended multiplex PCR to be a useful etiological diagnostic tool in lower respiratory tract infection patients, particularly in those treated with antibiotics. Inspite of the well-known high costs of real time PCR, multiplex reverse transcriptase PCR still had a lower cost and the advantage of the rapid and simultaneous detection rate of many bacterial pathogens in upper and lower respiratory tract infections significantly compared to that of the conventional culture method. The same was suggested early by Hendolin et al. [23,24] where multiplex PCR method improved the diagnosis and management of hospital and community-acquired pneumonia.

5. Conclusion

Ventilator associated pneumonia negatively impacts clinical outcome and resource consumption in studied critically ill pediatric patients by prolonging the length of mechanical ventilation and ICU stay and increase associated hospital mortality rate. Multiplex PCR optimized the emergency diagnosis of VAP over bacterial culture, especially in micro-organisms that can't be easily cultured. It is considered a rapid, reliable and efficient diagnostic service that has been delivered at a relatively accepted cost.

Conflict of interest

We declare that no conflict of interest between authors with any organization.

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