

ORIGINAL ARTICLE

Rapid Detection of *Pseudomonas aeruginosa* Causing Ventilator Associated Pneumonia Using Multiple Cross Displacement Amplification Technique in Intensive Care Units in Tanta University Hospitals

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ABSTRACT

Key words:

Ventilator-associated pneumonia, multiple cross displacement amplification, *Pseudomonas aeruginosa*

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Background: Ventilator-associated pneumonia (VAP) stands as one of the critical distressing hospital infections. *Pseudomonas aeruginosa* (*P. aeruginosa*) represents a potentially fatal VAP causative agent that is contracted predominantly from intensive care units (ICUs). Prompt identification of this pathogen in the context of clinical specimens of VAP patients may open the gate for therapeutic optimization plans in that way stewarding antibiotic usage thus enhancing clinical outcomes as pathogen identification delay elicits forced empiric usage of less valuable or potentially toxic antimicrobials. **Objectives:** This study aimed to reveal the *P. aeruginosa* infection prevalence among VAP patients admitted to Tanta University Hospitals' ICUs via both multiple cross displacement amplification (MCDA) assay and phenotypic approach and to assess the sensitivity along with specificity of MCDA assay as a speedy alternative to the routine culture in favor of detection of *P. aeruginosa*. **Methodology:** Sixty respiratory specimens comprised endotracheal aspirate (ETA) in addition to broncho-alveolar lavage (BAL) fluid were retrieved from clinically diagnosed VAP patients. Specimens were investigated for the microbial content. Bacterial isolation and identification by phenotypic methods and detection of *P. aeruginosa* by MCDA assay were done. **Results:** Out of 60 samples, 50 samples showed either mono or polymicrobial growth. *P. aeruginosa* was the 2nd most frequently isolated microorganism after *Klebsiella pneumoniae* (*K. pneumoniae*). The MCDA assay exhibited 100% specificity, sensitivity, and accuracy in detecting *P. aeruginosa*. **Conclusions:** Instead of bacterial culture, the MCDA assay serves as straightforward, easy, quick, and practical technique aiming to "on-site" or point-of-care testing for the presence of *P. aeruginosa*.

INTRODUCTION

Ventilator-associated pneumonia has a worldwide prevalence of 15.6% so it represents noteworthy serious infections among critically ill admitted patients oscillating from 5 to 20 cases for each 1,000 mechanical ventilation days even with the implementation of VAP bundles of care¹.

In the developing countries, 30% of patients under mechanical ventilation suffer from VAP making it a foremost cause of demise with 16–94% mortality rate².

Pseudomonas aeruginosa is one of the most common causative pathogens of VAP with high mortality percentages (estimated to be around 13%), prolonged hospital stays in addition to extra hospital costs³.

The prime challenges allied to this bacterium are their biofilm producing power, the extreme ability to

acquire antimicrobial resistance (AMR) and consequently becoming one of the healthcare-associated nightmares worldwide and nationally⁴.

Prompt and precise diagnosis of *P. aeruginosa* diseases, remarkably at the early stages, is remarkably crucial to guarantee effective treatments. Even though the microbiological culture is the usual routine in most laboratories, numerous novel technologies to enhance speed, accurateness, as well as specificity of the detection approaches have been currently emerged, and in this manner advancing the surveillance and management of diseases⁵.

Multitude of detection approaches of *P. aeruginosa* have been developed, for instance immunological detection methods, flowcytometry, and molecular biology-centered detection methods⁶.

The most widely exercised amplification technique for tracing small quantities of nucleic acids is the polymerase chain reaction (PCR) that has been broadly

employed in innumerable fields, yet its drawbacks compromise the requirement for big, high-priced thermal cyclers, which mainly restraint the PCR application in resource- restricted locations and for point-of-care analysis⁷.

The conventional PCR shortcomings have been overcome by methods as isothermal amplification (IA) techniques. IA permits the rapid nucleic acids amplification at a steady temperature, which has not only abridged the equipment demand, but also has an elevated sensitivity⁶.

In a straightforward heating block or water bath, abundant IA techniques have been widely designated. Amazingly, the multiple cross displacement amplification assay, originally formulated via **Wang et al.**,⁸ permits nucleic acids amplification at a constant temperature (58– 69 °C) over 40 minutes serving as a quick and simple technique.

During the amplification, MCDA utilizes an array of ten specific primers that are designed by target sequence's ten distinct areas thus MCDA assays attained high selectivity for target sequence detection. Only a simple isothermal instrument is required offering isothermal temperature all through amplification, and the yield is visually noticed by color changes. The MCDA assay abolishes the need of special laboratory facilities or skilled personnel making MCDA-based methods convenient for "on-site" diagnostic application in resource limited nations⁹.

Pseudomonas aeruginosa outer membrane protein "L- lipoprotein" which is involved in antimicrobials resistance is encoded by the *oprL* gene. Being unique in this organism, this protein or its gene could be characteristic target for rapid *P. aeruginosa* identification via molecular approaches¹⁰.

Therefore, this study aimed to reveal the *P. aeruginosa* infection prevalence among VAP patients admitted to Tanta University Hospitals' ICUs via both multiple cross displacement amplification (MCDA) assay and phenotypic approach and to assess the sensitivity along with specificity of MCDA assay as a speedy alternative to the routine culture in favor of detection of *P. aeruginosa*.

METHODOLOGY

Study design:

This research was descriptive cross-sectional study that was executed on 60 ICU patients who were admitted during the period of research from March 2021

to March 2023 at Tanta University Hospitals. They were intubated and suffered from nosocomial lower airway infection following at least 48 hours of invasive mechanical ventilation.

The protocol of this study was approved by the research ethical committee of Tanta University's faculty of medicine (approval code 34424/1/21). Informed written consent was obtained from all participants in this research or their caregivers (first degree relatives).

Collection and processing of samples

Samples were retrieved under complete aseptic precautions and included BAL fluid and endotracheal aspirates. Samples were taken by the physicians according to the standard techniques and collected in sterile bottles¹¹. Each sample was collected in two bottles; one bottle was processed for standard culture and the other one was stored at – 70 °C until the DNA extraction time.

Isolation and identification of the infecting organism:

- A quantitative culture via calibrated loops (1µl) was performed as done for urine culture¹². Colonies were counted and the number of colonies obtained was multiplied by 1000 to obtain the CFU/ml.
- Microbiologic confirmation of pneumonia was explained by the existence of at least one pathogen in respiratory samples above the predefined thresholds 10⁴ CFU/mL for BAL and 10⁵ CFU/mL for ETA¹³.
- All samples were cultured on blood, MacConkey, and nutrient agar plates (Oxoid, UK) and the entire plates were incubated at 37°C for 24 h then the yielded isolates were identified via colony morphology, Gram staining and diverse biochemical reactions.

Multiple cross displacement amplification (MCDA) genotypic detection of *P. aeruginosa*:

- **Primers used for multiple cross displacement amplification:**
- Multiple cross displacement amplification primers (table1) for *oprL* gene were designed according to the published nucleotide sequence of *oprL* gene (The GenBank accession no. Z50191) of *P. aeruginosa* by Primer Explorer V5 software (<http://primerexplorer.jp/lampv5e/index.html>).
- The primers were manufactured by Macrogen, South Korea.

Table 1: Primers consumed for multiple cross displacement amplification in the study.

Primer name	Sequences (5'-3')	Length
CP1	GCCGAATTTTCAGCATTTCCATCATG-CCTGAACTGACGGTCGCC	43mer
CP2	CGATGCTTCCGGTGAAGGTGC-AACGGCACCGCTGTTG	37mer
F1	GCCTTCCTGGTCCCCTTA	18 nt
F2	CGGCTTCGTCGCTCAG	16 nt
C1	GCCGAATTTTCAGCATTTCCATCATG	25 nt
C2	CGATGCTTCCGGTGAAGGTGC	21 nt
D1	ACTCCTAATGAACCCCAGT	19 nt
D2	ACCCGAACGCAGGCTATG	18 nt
R1	CAGAGCCAGCGCAGCA	16 nt
R2	GGCTGTGGCTGTGGGT	16 nt
P1	CCTGAACTGACGGTCGCC	18 nt
P2	AACGGCACCGCTGTTG	16 nt

DNA extraction:

Samples were subjected to DNA template extraction via DNA extraction kit (Qiagen-Germany) as stated by the manufacturer's instructions.

MCDA reactions:

- 1- Reactions were set up using WarmStart Colorimetric LAMP 2X Master Mix Typical LAMP (M1800, New England, BioLabs).
- 2- Reaction mix of MCDA Assay was prepared as the following:

Single -step reaction was set in a 25- μ l mixture comprising 12.5 μ l WarmStart Colorimetric LAMP 2X Master Mix, 0.1 μ l of each of the displacement primers F1 and F2, 0.2 μ l of every amplification primers C1, C2, R1, R2, D1, and D2, 0.4 μ l of each of the cross primers CP1 and CP2, 1 μ l of the DNA template in addition to 9.3 μ l nuclease free water. *P. aeruginosa* (ATCC 27853) control strain was used as positive control whereas *S. aureus* (ATCC 25923) was used as negative control. The tubes were

incubated at 63°C in water bath for 45 minutes then at 85°C for 5 minutes.

Detection of MCDA products

- a- The tubes were removed from the water bath and examined by naked eye.
 - Positive reactions changed into yellow whereas negative reactions persisted as pink (PH indicator) (Figure 3).
- b- The samples were loaded in 2.5% agarose gel electrophoresis for confirmation. Positive reactions appear as ladder-like pattern bands (Figure 4).

MCDA Optimization (Optimum Temperature):

- The optimum amplification temperature of the MCDA primer set was assessed using *P. aeruginosa* (ATCC 27853) control strain at fixed temperatures from 58 to 65°C.
- The color changed to yellow with 63 °C and 65°C but at 63 °C the color was more intense (Figure 1) and results confirmed by 2.5% Agarose gel electrophoresis (Figure 2).

**Fig. 1:** Optimization of MCDA reaction

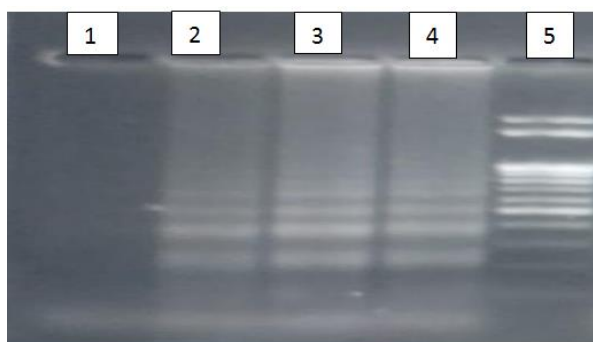


Fig. 2: 2.5% Agarose gel electrophoresis of MCDA optimization

Lane 1; amplification at 58°C, lane 2; amplification at 60°C, lane 3; amplification at 63°C, lane 4; amplification at 65°C and lane 5; 100bp DNA ladder.

Statistical analysis of the data

Data analysis was performed using IBM SPSS Statistics for Windows, Version 25.0. (IBM Corp, 2017). Categorical data were expressed as number and percentage of total while numerical data were expressed as mean±SD. To compare qualitative data when expected count is less than 5, Fischer exact test was used. Student t test was operated to compare continuous data. Pvalue ≤0.05 was reflected statistically significant.

RESULTS

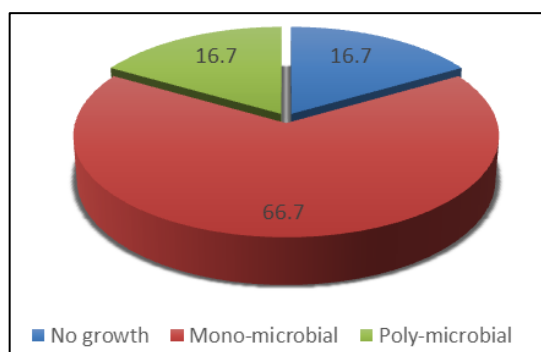


Fig. 3: Types of growth in the isolated samples

The majority of samples (40 out of 60; 66.7%) yielded single pathogen growth. In 10 samples (16.7%), two pathogens were detected. Yet, no pathogens were detected in the remaining 10 samples (16.7%) as shown in Figure 1.

Table 2: Types of organisms isolated from the samples.

Type of organism	No. of Organism	% *
1. Gram-negative	48	80
▪ <i>K. pneumonia</i>	18	30
▪ <i>P. aeruginosa</i>	15	25
▪ <i>E. coli</i>	7	11.7
▪ <i>Acinetobacter baumannii</i>	5	8.3
▪ <i>Proteus</i>	3	5
2. Gram-positive	12	20
▪ <i>S. aureus</i>	12	20
Total	60	100

*The percentage is calculated from the total number of isolated organisms (60).

Furthermore, *P. aeruginosa* was the second most commonly isolated bacteria (25%) after *K. pneumoniae* (30%). Of notice, *S. aureus* constituted 20% of the isolated strains (Table 2).

Table 3: Comparison between MCDA test and culture-based assay in the detection of *P. aeruginosa*

Gold standard detection of <i>P. aeruginosa</i> by culture	Detection of <i>P. aeruginosa</i> by MCDA	
	+ ve	- ve
+ ve (n=15)	15(100%)	0(0%)
- ve (n=35)	0(0%)	35(100%)
Total	15(100%)	35(100%)

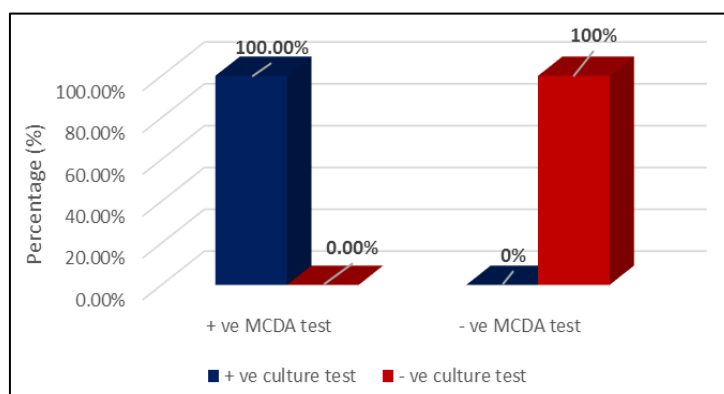


Fig.4: Comparison between MCDA and culture-based assay in the detection of *P. aeruginosa*

Table (3) and Fig (4) show that all *P. aeruginosa* culture positive strains (n=15) also had positive result via MCDA test. Also, all samples that did not reveal *P. aeruginosa* in culture were negative via MCDA test. So, sensitivity and specificity of MCDA test were 100%. Thus, the accuracy of this test was 100% when compared with the results of culture.

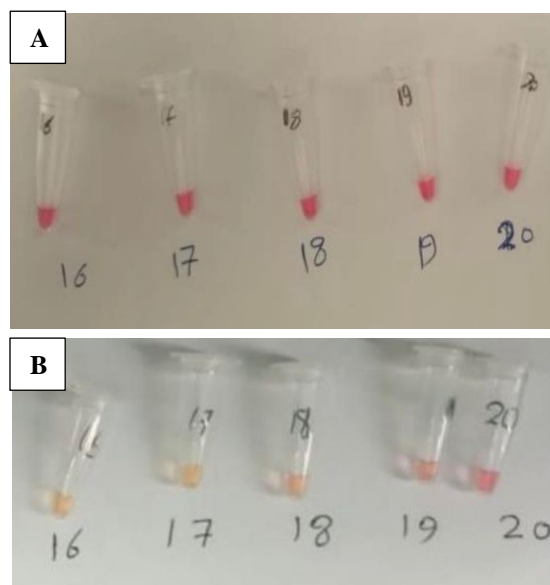


Fig. 5: Detection of *P. aeruginosa* by MCDA before (A) and after amplification (B) showing that positive samples turned to yellow whereas negative ones remained pink (tube 17: positive sample, tubes 17 and 18: negative samples, tube 16: positive control, tube 20: negative control)

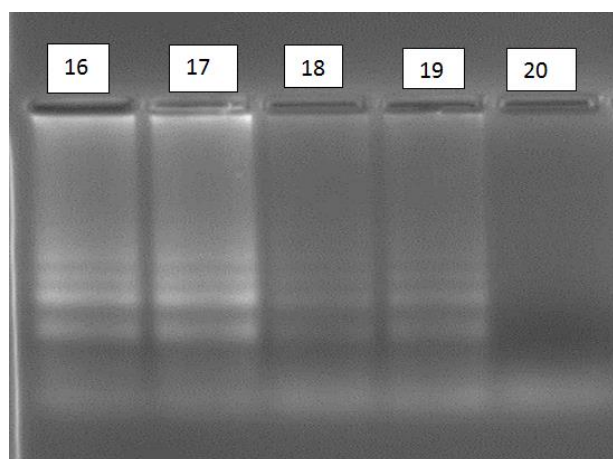


Fig. 6: *P. aeruginosa* MCDA products detection via 2.5% agarose gel electrophoresis. Lane 17; positive sample, lanes 18,19; negative samples, lane 16; positive control, lane 20; negative control

DISCUSSION

Ventilator-associated pneumonia is a troublesome issue worldwide and is classified as one of leading triggers of death among ICUs patients¹⁴.

Pseudomonas aeruginosa, of the most widespread VAP triggering pathogens, is synchronous with substantial morbidity and mortality, extended hospital stays besides added financial burden. Moreover, when the initial empiric antibiotic therapy of VAP is either inadequate or inappropriate, the mortality allied to *P. aeruginosa* is further increased owing to its multi or even extended drug resistance nature¹⁵⁻¹⁷.

Thus, the best approaches to combat these infections are prompt diagnosis coupled with suitable medical therapies. It can take more than two calendar days to fully identify *P. aeruginosa* from clinical samples by means of routine culture-biochemical methods. Therefore, there is utmost need for accelerated, sensitive, plus precise assays to distinguish this pathogen¹⁸.

Polymerase chain reaction being a fast molecular method to detect the microorganisms' nucleic acids, nevertheless, this assay is somewhat composite, time consuming, and necessitates dedicated, costly instruments as well as expertise. Furthermore, MCDA assays were validated as probable, effortless, quick, very sensitive and specific replacement for PCR-based assays isothermally (at 58°C–69°C) demanding a simple heater merely^{8,19}.

The current study showed that VAP is caused mainly by Gram-negative organisms (80%) whereas Gram-positive organisms represented (20%) of the VAP offending pathogens.

Our finding that VAP was mainly triggered by Gram-negative bacteria was in line with numerous researches in Egypt. As, study performed by Elkolaly et al.²⁰ at Tanta university hospitals reported that Gram-negative bacteria were isolated from 62.5 % of VAP cases Also, a study carried out by Adel et al.²¹ at Zagazig University hospitals concluded that the most frequently isolated microorganisms from VAP patients (84.2%) were Gram-negative bacteria.

In addition, a study performed by Farag et al.²² at Al-Azhar University hospitals in Cairo reported that 83% from ETA specimens of VAP patients yielded Gram-negative pathogens. Also, a study conducted at Zagazig University by Maewed et al.²³ found that VAP was mainly triggered by Gram-negative bacteria (90.8%).

Furthermore, at Upper Egypt, a study conducted by Maebed et al.¹¹ at Beni-Suef University reported that the isolated organisms from VAP patients were only Gram-negative bacteria. Moreover, a study conducted by El-Shinnawy et al.²⁴ at Assiut University found that the

most common organisms isolated from VAP patients were Gram-negative organisms (86%).

At the global level and in agreement with our study, an Indian study conducted by Kelkar et al.⁽²⁵⁾ found that 94.3% of VAP cases were instigated by Gram-negative organisms and only 5.68% were Gram-positive. Our result more or less correlates with a study done in Hail Saudi Arabia by Saleem et al.²⁶ who reported that VAP is predominantly caused by Gram-negative organisms (98.5%).

On the other hand, a study conducted by Quartin et al.²⁷ from New York reported that of the yielded organisms from VAP specimens, Gram-positives represented 63.4% of them 42.7% were MRSA. Yet the Gram-negative pathogens only represented 36.6%.

Another American study performed by Risa et al.²⁸ recounted that the paramount isolated pathogens from VAP patients was *S. aureus* (57%).

The Gram-negative organisms' predominance can be allied with two factors: gut colonization and antimicrobials exposure. The critically ill patients contract hospital flora either exogenously or endogenously within 24–48 hours of hospitalization resulting in colonization of the oral cavity by hospital Gram-negative pathogens. As an outcome, the risk of infection increases while aspirating these oropharyngeal secretions²⁵. Additionally, the establishment of resistant Gram-negative pathogens by selection pressures on these colonizers occurs when broad-spectrum empirical antibiotics are administered to those vulnerable patients²⁵.

The current study revealed that amid the isolated Gram-negative bugs, *K. pneumoniae* ranked first (30%) while, *P. aeruginosa* came second (25%) followed by *E. coli* (11.7%), lastly *Acinetobacter* (8.3%) and *Proteus* (5%) were the least.

These results were reinforced by multitude of research that entirely concluded high predominance of *Klebsiella* in VAP specimens. For instance, Azzab et al.²⁹ found that *Klebsiella* and *P. aeruginosa* represented 43% & 15.1% respectively. This was also the case in Farag et al.²² and Mohamed et al.³⁰ with *K. pneumoniae*, *P. aeruginosa* then *E. coli* represented 42.6%, 14.6% & 6.6 % of the isolated pathogens in the latter study. In contrary the study conducted by Hamed et al.³¹ who reported that the total number of *P. aeruginosa* cases was one hundred forty-five out of two hundred and fifty clinical specimens with a percentage of 58%.

El-Shinnawy et al.²⁴ highlighted similar results as *Klebsiella*, followed by *Pseudomonas* and *E. coli* were yielded from 44%, 22 and 16% from BAL specimens respectively. Contrary to our study, the Egyptian study conducted by El-Halim et al.³² who detected that the most prominent isolates were *Pseudomonas aeruginosa*.

A study conducted by Maewed et al.²³ more or less correlates with our study as they reported that the most

frequently isolated organisms were *K. pneumoniae*, after that *Acinetobacter baumannii* then *P. aeruginosa* in 41.1%, 27.4% & 20.8% respectively. Maebed et al.¹¹ found also that *K. pneumoniae* (45%) was the most widespread isolated pathogen then *Acinetobacter baumannii* (12%). Another study which was done on 30 isolates of *P. aeruginosa* by Elsaid et al.³³ who reported that the same results and reported that *P. aeruginosa* prevalence depends on other various factors like nature of geographical locations, degree of contamination, immune status of patients, virulence of strains and degree of implementation of measures of infection control in hospitals.

On the other hand, a study conducted by Elkolaly et al.²⁰ found that *P. aeruginosa* was ranked as the first isolated organism (37.5%) followed by *K. pneumoniae* (25%). In addition, American study by MacVane et al.³⁴ is inconsistent with our result as they found *S. aureus* (10.5%) and *P. aeruginosa* (6.4%) followed by *Klebsiella* spp. (3.1%) were the most common isolated organisms.

Also, Kelkar et al.²⁵ findings in India were inconsistent with our results and found that *Acinetobacter baumannii* (38.7%) were isolated most frequently followed by *P. aeruginosa* (17.5%) and *K. pneumoniae* came then (16.6%). So as, Saleem and his colleagues,²⁶ where *Acinetobacter baumannii* were found in 25.7% followed by *K. pneumoniae* (21.4%) then *Proteus* (20%) and *P. aeruginosa* (12.9%).

Variations among studies can be elucidated by that the organisms causing VAP to differ in line with patient characteristics, geographic locations, the case mix, clinical circumstances, prior antibiotic exposure, and length of mechanical ventilation & ICU stay⁽¹¹⁾.

The current study shows that MCDA targeting *P. aeruginosa oprL* gene showed remarkable (100%) specificity, sensitivity as well as accuracy when compared with the results of culture as this assay yielded positive results for the entire *P. aeruginosa* strains harboring clinical samples, and negative results for the whole other bacterial strains and negative control. Thus, the MCDA method is highly selective for detecting *P. aeruginosa*.

This comes in agreement with the study of Zhao et al.¹⁸ who reported that MCDA approach displayed undistinguishable sensitivity and specificity to distinguish *P. aeruginosa* in respiratory specimens as the reference assay (named culture-biochemical tests).

Moreover, Li et al.¹⁹ reported that the MCDA assay could distinguish *P. aeruginosa* in diarrheal stool specimens with the same sensitivity and specificity and accuracy (reached 100%) versus the conventional culture-based assay.

Furthermore, a study by Wang et al.³⁵ found that MCDA assay could detect *P. aeruginosa* in BAL specimens from VAP suspected patients with specificity 100%. In that study, the MCDA assay

showed 92.31% sensitivity with a superior *P. aeruginosa* detective rate than bacteria culture in patients scheduled on anti-pseudomonal therapy. The cost was about \$8 per sample compared to standard culture which is \$10 per sample.

To the best of our knowledge, this study is the first in Egypt reporting the application of detecting *P. aeruginosa* in endotracheal aspirate and BAL specimens from suspected VAP patients via MCDA technique.

CONCLUSION

Instead of requiring a particular bacterial culture, the multiple cross displacement amplification assay targeting the *oprL* gene serves as straightforward, easy, quick, and practical technique aiming to "on-site" or point-of-care testing for the presence of *P. aeruginosa* with excellent specificity and sensitivity.

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