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ORIGINAL ARTICLE

Potential Clinical Value of Quantitative Molecular Based Assay for Rapid Diagnosis of Ventilator-associated Pneumonia

[1]**Sahar M. Fayed*** **and** [2]**Mohammad H. Kamel**
[1]Assistant professor of Clinical and Chemical Pathology, Faculty of Medicine, Benha University, Egypt
[2]Assistant professor of Chest Diseases, Faculty of Medicine, Benha University, Egypt

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Corresponding Author:
Sahar M. Fayed. Clinical and Chemical Pathology Department, Faculty of Medicine, Benha University.
Tel: 01227154603
saharmohamadfayed@gmail.com

ABSTRACT

Background: *Ventilator-associated pneumonia (VAP) is the commonest infection in critically ill patients. The definite mortality rate of VAP is still controversial but may exceed 50% when the initial treatment is not appropriate.* ***Objective:*** *was to compare the performance of quantitative real-time PCR with semi-quantitative culture for diagnosis of VAP caused by P. aeruginosa using two sample types bronchoalveolar lavage and endotracheal aspirates (BAL and ETA) and to phenotypically determine some of P. aeruginosa virulence factors associated.* ***Methodology:*** *Two samples were collected from every patient on the day of suspected VAP, one is endotracheal aspirate sample and the other is bronchoalveolar lavage sample. Each sample (BAL and ETA) was divided into two aliquots, one for conventional microbiological analysis and the other was frozen at −80°C for molecular detection and quantification of P. aeruginosa. Isolates were phenotypically tested for the expression of some of the virulence factors frequently associated with human infections.* ***Results****: There was excellent concordance correlation between qPCR and conventional culture for detection of P. aeruginosa in BAL samples, between both methods for detection of P. aeruginosa in ETA samples and between the two types of samples (BAL and ETA) for the two investigated methods. The most frequently associated virulence factor was alkaline protease production in 81.25% of isolates, followed by biofilm forming capacity in 75% of the isolates.* ***Conclusion:*** *Our study revealed that qPCR can afford rapid and reliable quantitative microbiological data, with very high sensitivity and specificity for P. aeruginosa involved in VAP. It also tended to verify that the same pathogen can be detected and quantified from the two sample types using conventional or molecular method. These results decrease the utility of BAL sample, which is difficult to perform, more invasive, expensive and more time consuming than ETA.*

INTRODUCTION

Ventilator-associated pneumonia (VAP) is the commonest nosocomial infection in critically ill patients. Between 10% and 20% of the patients receiving more than 48 h of mechanical ventilation will develop VAP [1].

The definite mortality rate of VAP is still controversial but may exceed 50% when the initial treatment is not appropriate[2]. Obtaining the identification and also the quantification of bacteria accountable for VAP in few hours would allow an early effective and targeted antibiotic treatment [3].

The ability of *P. aeruginosa* to survive on minimal nutritional needs and to stand different environmental conditions has allowed this opportunistic pathogen to persist both in general population and hospital settings [4].

Colonization rates of this pathogen may exceed 50% during hospitalization; mainly among patients have fracture, trauma to the skin and mucosal barriers due to surgical interventions, ventilation systems, tracheostomy or indwelling catheters. Patients hospitalized in the ICU were the main susceptible group to *P. aeruginosa* infections [5].

A combination of numerous factors e.g. intrinsic antibiotic resistance, predominance in hospital environment, and a propensity to form biofilms on medical devices lead to the quite high colonization rates by *P. aeruginosa*. Previous studies have reported that a group of colonized patients develop clinical disease, such as ventilator-associated pneumonia. However, others do not progress to significant disease, suggesting that variability in virulence and/or host factors impact clinical outcome [5].

The aim of the study: was to compare the performance of quantitative real-time PCR with semi-quantitative culture for diagnosis of VAP caused by *P. aeruginosa* using two sample types (BAL and ETA) and to phenotypically determine some of *P. aeruginosa* virulence factors associated.

METHODOLOGY

Study design

This prospective study was conducted between January 2016 to November 2017 at the Clinical Pathology Department and Intensive Care Unit of Benha University Hospital. ICU patients who were intubated and had received mechanical ventilation for more than 48 h were enrolled in the study if they fulfilled the following criteria: (a) > than 18 years, (b) clinical doubt of VAP defined by a progressive or new infiltrate on chest radiography accompanied by two or more of the following criteria: leucocyte count ≥10 000/µL or ≤ 4500/µL, body temperature >38.3°C or <35.0°C and new onset of purulent tracheal secretions [6]. The study design was approved by the local Ethics Committee of Benha faculty of medicine.

Samples collection: Two samples were collected from every patient on the day of suspected VAP [7].

- An endotracheal aspirate sample (ETA): (under aseptic conditions, a catheter was introduced 30 cm into the endotracheal tube and the sample was suctioned).
- A bronchoalveolar lavage sample (BAL). A bronchoscope was introduced via an intratracheal tube in the breathing duct through an extension tube. BAL was done by sequential introduction of five aliquots of twenty milliliter of isotonic saline (37°C) in the segment or lobe in which the progressive or the new infiltration had developed. Each aliquot was suctioned carefully before administration of the subsequent one, the first being used for microbiological analysis.

Each sample (BAL and ETA) was divided into two aliquots, one for conventional microbiological analysis and the other was frozen at −80°C for molecular analysis.

Laboratory methods:

Conventional culture method

For ETA, one hundred microlitres were diluted with one mL of sterile broth and were mixed by vortex. 10 µL of this suspension were inoculated onto blood agar and cystine lactose electrolyte deficient (CLED) agar (Oxoid), incubated at 37°C for 24 h. Dilutions were made so as to achieve a detection threshold of 10^3 CFU/mL. 10 µL of raw BAL were directly inoculated onto the same agar plates with a detection threshold of 10^2 CFU/mL.

Bacterial identifications were performed using the Vitek2® compact system, (bioMérieux). A significant culture was defined as at least one pathogen load $\geq 10^4$ CFU/mL for BAL and $\geq 10^6$ CFU/mL for ETA [8].

Molecular biology-based assay

Using quantitative real time-PCR for detection and quantification of *P. aeruginosa* oprl gene.

DNA extraction: DNA extraction from BAL and ETA by QIAmp DNA Mini Kit (154011824) according to manufacturer's instructions.

Primer design:
For amplification of *P. aeruginosa* oprl gene,
Forward primer Ps-F
(5-CGAGTACAACATGGCTCTGG-3)
Reverse primer Ps-R
(5-ACCGGACGCTCTTTACCATA-3)
Probe (5-Hex-CCT GCA GCA CCA GGT AGC GC-Tamara-3).

DNA amplification: Enymatic amplification of the extracted DNA was performed by Applied Biosystem, Step One Plus TM Real -Time PCR System (Singapore), fax (025257210) telephone (0225262860) using oasigTM lyophilised 2x qPCR Mastermix (JN182004-55818). The condition of amplification was 5 minutes at 94 ℃ for initial denaturation, then 50 cycle of 1 minute at 94℃ for denaturation, 45 seconds at 63℃ for annealing, 45 seconds at 72 ℃ for extension and finally 15 minute at 72 ℃ for final extension. Quantifications were performed using qPCR standard curves, by converting cycle threshold (Ct) to CFU/mL. As for conventional culture, a significant sample was defined as at least pathogen load $\geq 10^4$ CFU/mL for BAL and $\geq 10^6$ CFU/mL for ETA [9, 10] as shown in Fig 1

Phenotypic characterization of virulence factors

P. aeruginosa isolates were phenotypically tested for the expression of some virulence factors frequently associated with human infections.

Detection of phospholipase activity using egg yolk agar (Himedia) medium. Positive result denoted by appearance of a milky white opaque halo around the colony [11, 12].

Determination of lipase activity: Isolates were tested for lipolytic activity on tween esterase agar (Himedia). The lipolytic activity was detected by the appearance of an opaque zone around the colony [11, 12].

Investigation of alkaline protease production was performed using skim milk agar (Himedia).The appearance of a transparent area around the colony indicating positive result [12].

The biofilm forming capacity has been determined by two qualitative determination methods, the microtiter plate method in which *P. aeruginosa* forms a biofilm which appears as a ring form at air-liquid interface of the microtitre plate wells and the tube method in which biofilm formation was considered positive when a visible film lined the bottom and wall of the tube appeared [13, 14].

Detection of proteolytic activity through gelatin liquefaction on nutrient gelatin medium (Himedia), DNase production using DNA agar medium (oxoid), detection of esculin hydrolysis on bile esculin agar (Oxoid) and detection of hemolysin on blood agar [15]

Statistical analysis

The collected data were tabulated and analyzed using SPSS version 16 software (SpssInc, Chicago, ILL Company). Categorical data were presented as number and percentages. Z test for independent proportions (ZProp.) was used to analyze categorical variables. Lin's concordance correlation coefficient (Pc) was used to assess degree of agreement between the studied techniques. ROC curve was constructed to assess the performance of PCR methods in diagnosis of VAP due to *P. aeruginosa*. The accepted level of significance in the present work was stated at 0.05 ($P < 0.05$ was considered significant).

RESULTS

Sixty-six patients were enrolled in the present study, they were 46 male and 20 female with median age of 64 years. Suspicion of VAP appeared 5 days or more (5–9 days) after intubation and 60.6% (40/66) of the patients received antibiotic therapy on the day of BAL and ETA collection. No significant statistical difference was noticed in patients with previous antibiotic therapy and those without antibiotics (data not shown).

BAL and ETA were collected form all patients, regarding BAL, 24.2% (16/66) and 27.3% (18/66) of clinically suspected VAP were confirmed for *P. aeruginosa* by conventional culture and by qPCR, respectively. Regarding ETA, 22.7% (15/66) and 27.3% (18/66) of clinically suspected VAP were confirmed to be caused by *P. aeruginosa* by conventional culture and by qPCR respectively.

There was excellent concordance correlation between qPCR and conventional culture for detection of *P. aeruginosa* in BAL samples (ρ_c =0.92, $P < 0.001$ (92%). Sensitivity of qPCR for detection of *P. aeruginosa* in BAL samples measured at the decision threshold (10^4 CFU/mL) was 100%. Specificity was 98%, PPV was 88.9 and the NPV was 100% as shown in Table 1, 2, Fig 2.

There was excellent concordance correlation between qPCR and conventional culture for detection of *P. aeruginosa* in ETA samples in patients with suspected VAP (ρ_c =0.879, $P < 0.001$ (87.9%). The qPCR sensitivity for ETA samples was 100%, specificity was 94%, PPV was 83.3 and the NPV was 100% as shown in Table 3, 4, Fig 3.

Concordance results for BAL and ETA samples were calculated for the two tested methods and we found that, there was excellent concordance correlation between both sample types for detection of *P. aeruginosa* { ρ_c =0.958, P = <0.001 (95.8%) and ρ_c =1.0, $P < 0.001$ (100%)} for conventional culture and qPCR respectively as shown in Tables 5, 6.

The overall percentage agreements (percent of patients with either positive or negative quantification in both specimens, using a threshold at 10^4 CFU/mL for BAL & 10^6 CFU/ mL for ETA was excellent (ρ_c = 0.90, $P < 0.001$ (90%) and we obtained 5 false positives out of all tests performed as shown in Table 7.

P. aeruginosa isolates (no=16) were phenotypically tested for the expression of some virulence factors frequently associated with human infections. The most frequently encountered virulence factor was alkaline protease production in 81.25% of isolates, followed by biofilm forming capacity in 75% of isolates. Phospholipase activity was detected in 68.75% of the isolates as shown in Table 8, Fig 4-8.

Table 1: Concordance between qPCR and conventional culture for detection of *P. aeruginosa* in BAL samples

			BAL Culture		Total
			Positive	**Negative**	
q PCR- BAL	**Positive**	Count	16	2	18
		% within CultureBAL	100.0%	4.0%	27.3%
	Negative	Count	0	48	48
		% within CultureBAL	.0%	96.0%	72.7%
Total		Count	16	50	66
		% within CultureBAL	100.0%	100.0%	100.0%

P = <0.001 (HS)
Concordance correlation coefficient (ρ_c) = 0.92
95% Confidence interval = 0.87 to 0.95
Pearson ρ (precision) = 0.92
Bias correction factor C_b (accuracy) = 0.99
Concordance correlation coefficient (ρ_c) ranges from 0 to ± 1

Table 2: Diagnostic performance for qPCR assay on BAL samples using culture as a reference method

Technique	Sensitivity %	Specificity %	PPV%	NPV%	Accuracy %	AUC	95%CI	P
qPCR BAL	100%	98%	88.9%	100%	97%	0.98	0.94-1.0	<0.001 (HS)

Table 3: Concordance between qPCR and conventional culture for detection of *P. aeruginosa* in ETA samples

			Culture ETA		Total
			Positive	Negative	
qPCR - ETA	Positive	Count	15	3	18
		% within CultureETA	100.0%	5.9%	27.3%
	Negative	Count	0	48	48
		% within CultureETA	.0%	94.1%	72.7%
Total		Count	15	51	66
		% within CultureETA	100.0%	100.0%	100.0%

P = <0.001 (HS)
Concordance correlation coefficient (ρ_c) = 0.879
95% Confidence interval = 0.81 to 0.92
Pearson ρ (precision) = 0.885
Bias correction factor C_b (accuracy) = 0.992

Table 4: Diagnostic performance for qPCR assay on ETA samples using culture as a reference method

Technique	Sensitivity %	Specificity %	PPV%	NPV%	Accuracy %	AUC	95%CI	P
PCR ETA	100%	94%	83.3%	100%	95.5%	0.97	0.93-1.0	<0.001 (HS)

Table 5: Concordance results for BAL and ETA samples by culture method

			Culture- BAL		Total
			Positive	Negative	
Culture -ETA	Positive	Count	15	0	15
		% within CultureBAL	93.8%	.0%	22.7%
	Negative	Count	1	50	51
		% within CultureBAL	6.2%	100.0%	77.3%
Total		Count	16	50	66
		% within CultureBAL	100.0%	100.0%	100.0%

P = <0.001 (HS)
Concordance correlation coefficient (ρ_c) = 0.958
95% Confidence interval = 0.93 to 0.97
Pearson ρ (precision) = 0.958
Bias correction factor C_b (accuracy) = 0.999

Table 6: Concordance results for BAL and ETA samples by qPCR method

			PCR- BAL		Total
			Positive	Negative	
PCR -ETA	Positive	Count	18	0	18
		% within PCRBAL	100.0%	.0%	27.3%
	Negative	Count	0	48	48
		% within PCRBAL	.0%	100.0%	72.7%
Total		Count	18	48	66
		% within PCRBAL	100.0%	100.0%	100.0%

P = <0.001 (HS)
Concordance correlation coefficient (ρ_c) = 1.0
95% Confidence interval = -1.0 to -1.0
Pearson ρ (precision) = 1.0
Bias correction factor C_b (accuracy) = 1.0

Table 7: The overall percentage agreements between the two methods

			Culture		Total
			Positive	**Negative**	
PCR	**Positive**	Count	31	5	36
		% within CultureBAL	100.0%	5.0%	27.3%
	Negative	Count	0	96	96
		% within CultureBAL	.0%	95.0%	72.7%
Total		Count	31	101	132
		% within CultureBAL	100.0%	100.0%	100.0%

P = <0.001 (HS)
Concordance correlation coefficient = 0.90
95% Confidence interval = 0.86 to 0.93
Pearson ρ (precision) = 0.90
Bias correction factor C_b (accuracy) = 0.995

Table 8: Frequency of different virulence factors in *P. aeruginosa* isolates

Virulence factor		N=66 No	N=66 %
Phospholipase	Negative	5	31.25
	Positive	11	68.75
Lipase	Negative	6	37.5
	Positive	10	62.5
Protease	Negative	3	18.75
	Positive	13	81.25
Biofilm	Negative	4	25
	Positive	12	75
DNase	Negative	15	93.75
	Positive	1	6.25
Gelatinase	Negative	6	37.5
	Positive	10	62.5
bile esculin	Negative	5	31.25
	Positive	11	68.75
Hemolysin	Alpha	6	37.5
	Beta	6	37.5
	None	4	25

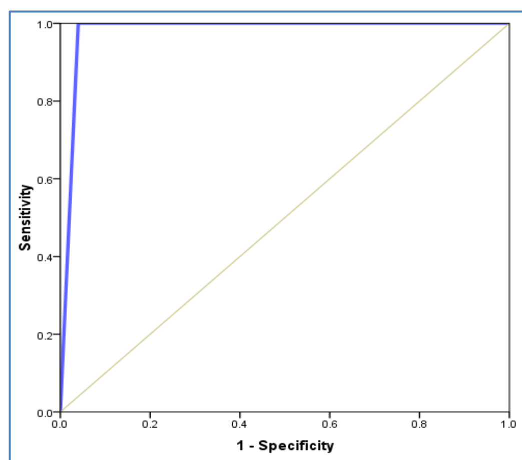

Figure 2: Diagnostic performance test for qPCR assay in detection of *P. aeruginosa* from BAL samples using culture as a reference method

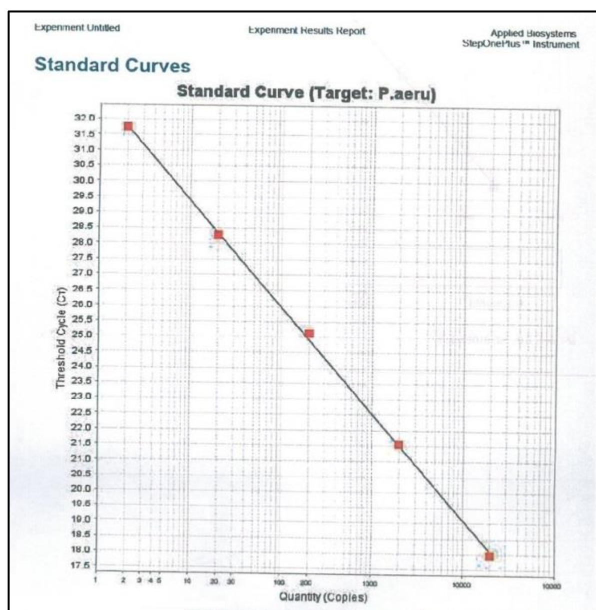

Figure 1: Quantitative PCR standard curve

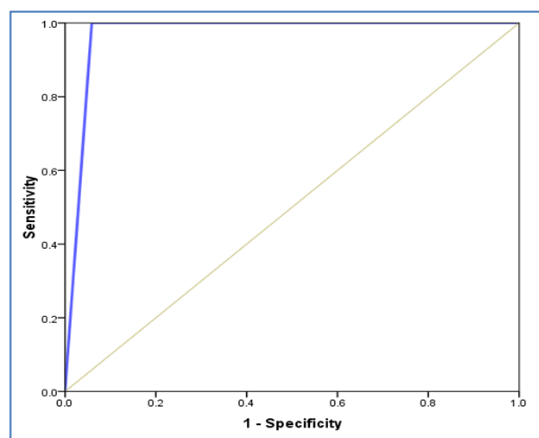

Figure 3: Diagnostic performance test for qPCR in detection of *P. aeruginosa* from ETA samples using culture as a reference method

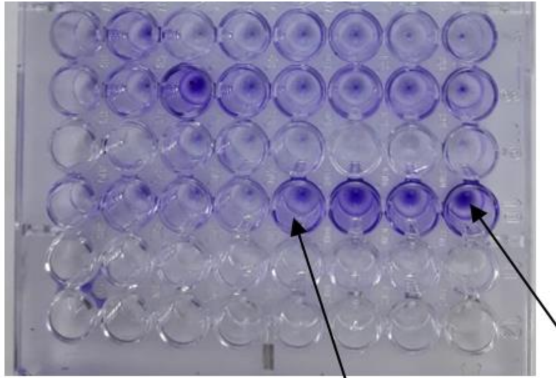

Figure 4: A top view of the biofilm formed by P. aeruginosa which appears as ring form at air-liquid interface of the micrptitre plate wells

Figure 5: Biofilm formed by *P. aeruginosa* as determined by tube method (Rt: positive and Lt: negative)

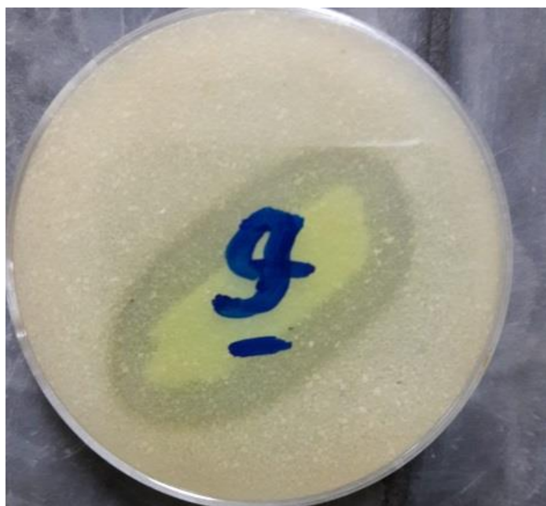

Figure 6: A clear zone around the spotted inoculum indicating positive protease activity on Skim milk agar

Figure 7: Positive phospholipase test on egg yolk agar medium, positive result denoted by appearance of a milky white opaque halo around the colony

Figure 8: Positive lipase test on tween esterase agar shows, white precipitate around the spotted inoculum due to precipitation of calcium salts around the released fatty acids

DISCUSSION

Ventilator-associated pneumonia is a frequent problem in intensive care units, with a major impact on morbidity and mortality. The increasing incidence of infections by antibiotic-resistant pathogens prolongs the length of hospital stays and the cost of hospital care. Therefore, clinical need for presumptive diagnosis of VAP and rapid identification of the causative pathogen is necessary for proper treatment [16].

Initial antibiotic therapy is commonly broad spectrum, which enhances antibiotic resistance so new techniques are under research to get early microbiological identification and quantification [17].

Molecular methods adjusted to detect bacterial DNA in respiratory samples were formerly reported but they often lacked accurate quantification features for multiple bacterial species. Moreover, the use of highly manual procedures for nucleic acid extraction are

important sources of variability that prevented authors from showing a good correlation with conventional methods [18].

The importance of a faster molecular technique for microbiological diagnosis of VAP is to reduce the risk of inappropriate antibiotic therapy. Another importance of quantitative molecular based method is that; preceding antimicrobial therapy does not impact PCR diagnostic accuracy [17].

The aim this study was to compare the performance of quantitative real-time PCR with semi-quantitative culture for diagnosis of VAP caused by *P. aeruginosa* using two sample types (BAL and ETA) and to phenotypically determine some of *P. aeruginosa* virulence factors associated.

In the present study, there was excellent concordance correlation between qPCR and conventional culture for detection of *P. aeruginosa* in BAL samples {ρ_c =0.92, P <0.001 (92%)}, and in ETA samples ({ρ_c =0.879, P <0.001 (87.9%)}. On the other hand, Jamal *et al* [19] and Kunze et *al* [20] evaluated the accuracy of PCR for the microbiological diagnosis of health care-associated pneumonia (HAP) and reported lower percentages of agreement (63.3% & 50% respectively) than that detected in our study. This may be explained by the higher positivity rate of PCR method as it could detect more organisms than culture based method.

Good concordance between qPCR and conventional culture suggests that qPCR could provide reliable results to obtain a first-line microbiological diagnosis in less time than conventional culture method [18].

In the present study, the sensitivity, the specificity, the PPV, the NPV of qPCR for BAL samples were 100%, 98%, 88.9 and 100% respectively and for ETA samples were 100%, 94%, 83.3 and 100% respectively. Performance of our study method was really impacted by definition of thresholds as already observed by Schulte [9]. In this study, the same threshold values were applied for the reference and tested methods (10^4 CFU/mL for BAL and 10^6 CFU/mL for ETA).

Our results are in agreement with Clavel et al [6] who reported for BAL samples, a qPCR sensitivity, specificity, PPV and NPV values of 100%, 97%, 85.7 and 100% respectively and 100%, 92.2%, 65% (40.8–84.6) and 100% for ETA samples. These results recommend the accuracy of our PCR method in confirming the absence of targeted pathogen.

In our study, qPCR assay is innovative as it is a proof of concept that molecular technique can offer a rapid method to detect bacteria involved in VAP and in addition, it quantifies bacterial DNA to better assess the pathogenicity of the identified bacteria. Differentiation between patients with high versus low risk of infection could improve delivery of effective anti-pseudomonal therapy to infected patients, but decrease inappropriate antibiotic use in colonized patients.

Concordance was excellent between both sample types for detection of *P. aeruginosa* { ρ_c =0.958, P = <0.001 (95.8%) and ρ_c =1.0, P < 0.001 (100%)} for conventional culture and qPCR respectively. On the other hand, for *P. aeruginosa* qPCR had better concordance results for both sample types than conventional culture method [6].

The novelty of the present work is to compare the results of qPCR with conventional culture for both sample (BAL and ETA) types in the same patient. Although the results were obtained on a small number of patients (66), they tended to prove that the same pathogens might be identified and quantified from the two types of samples, using the conventional or the molecular method. These results decrease the utility of BAL, which is difficult to perform, more invasive, expensive and requires more time than ETA.

Detection of bacterial DNA does not always mean infection and the main challenge for a new qPCR method is to reduce the number of false-positive results. We obtained 2 and 3 false positives for BAL and ETA respectively. False-positive result is one of the main limitations of all PCR assays currently available; these results prove the importance of quantification that can decrease the number of false results. This is of major clinical importance as false positive results can lead to unnecessary prescription of antibiotics. Several theories can explain these results, DNA amplification is an essential step of qPCR but does not allow distinction between viable and non-viable organism. This may also be explained by the difficulty of distinguishing pathogenic specimens from asymptomatic carriage for these bacteria [18].

Regarding the time to result, molecular technique makes it possible to obtain rapid (6 h) and reliable microbiological results in patients with VAP in comparison to 72 h for conventional culture. To be of major clinical advantage, PCR assay has to be available 24 h per day, the complete automation of the method is needed to reduce hands on time, time to result and be appropriate for routine setting [6].

Limitations of our molecular method must be noted. First, our qPCR design was restricted to VAP caused by *P. aeruginosa*, detection and quantification of more pathogens (bacteria, fungi, and viruses) would be required. Second, samples for molecular analyses were frozen and this might affect the sensitivity and specificity of the assay. Third, the use of this method in the daily practices of a microbiology laboratory may be limited by the large number of manual steps. Fourth, this study is one of the first trying to include a quantitative molecular approach in the microbiological diagnosis of VAP and must be considered as a proof-of-concept study taking *in vitro* data to the *in vivo* field. Fifth, another additional problem with molecular assays is that they do not provide susceptibility profiles of bacteria. As bacterial resistance is a significant issue in

antibiotic treatment, detection of resistance markers is important to be added to our tool.

Other studies are needed to evaluate the influence of this molecular assay on treatment and outcomes of the patients. Thus, a medico-economic analysis must be carried out before its application in routine practice to balance the cost of the assay with the costs of broad-spectrum antibiotic therapies and those resulting from the use of inappropriate antibiotic therapies.

In this study, we hypothesized that certain *P. aeruginosa* virulence factors may be associated with greater risk of infection versus colonization in hospitalized patients with positive *P. aeruginosa* cultures. The ability of the isolates to produce virulence factors like elastase, protease, gelatinase, DNAse and biofilm leads to tissue damage and protect *P. aeruginosa* from recognition by the immune system and action of antibiotics [4].

In our study, P. aeruginosa isolates (no=16) were phenotypically tested for the expression of some virulence factors frequently associated with human infections. The most frequently detected virulence factor in our study was alkaline protease production in 81.25% of isolates, followed by biofilm forming capacity in 75% of isolates. These results are in agreement with Ledizet et al who found the presence of *P. aeruginosa* in conjunction with evidence of host tissue damage was indicative of infection, while the presence of bacteria with no or minimal tissue damage was taken to represent colonization [21].

It is thought that micro-aspiration of detached fragments of biofilm containing harmful organisms might cause infection in the lower respiratory tract, and are supposed to significantly contribute to the occurrence of VAP [16]. Thus Regular screening of endotracheal aspirates enables early diagnosis of pathogens linked to VAP and has been shown to influence patient treatment and survival [22].

The limitation of our work is that, all methods used for phenotypically test the expression of some virulence factors need culture and isolation of *P. aeruginosa* from primary clinical specimens, and all virulence factors were detected in vitro. We only determined whether an isolate could express a particular virulence factor under defined in vitro conditions. Thus our assays measured the potential for an isolate to express a virulence factor in the human host, but did not determine whether those virulence factors were actually expressed in the host.

CONCLUSION

Our study revealed that qPCR can afford rapid and reliable quantitative microbiological data, with very high sensitivity and specificity for *P. aeruginosa* involved in VAP. Automation of the process must currently be studied to enable, in the near future, a bedside quantitative analysis 24 h per day with available results in few hours. It also tended to prove that the same pathogens might be identified and quantified from the two sample types using conventional or molecular method. These results decrease the utility of BAL, which is difficult to perform, more invasive, expensive and more time consuming than ETA.

Conflicts of interest: The authors declare that they have no financial or non financial conflicts of interest related to the work done in the manuscript.

- Each author listed in the manuscript had seen and approved the submission of this version of the manuscript and takes full responsibility for it.
- This article had not been published anywhere and is not currently under consideration by another journal or a publisher.

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