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Original Article

CD64 index on neutrophils can diagnose sepsis and predict 30-day survival in subjects after ventilator-associated pneumonia

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Abstract

Introduction: Sepsis associated with ventilator-associated pneumonia (VAP) causes mortality in intensive care unit (ICU) patients. The time of diagnosis is crucial, and microbiological cultures take time. In this study, the diagnostic accuracy of CD64 index to predict VAP-induced sepsis and survival time in subjects requiring mechanical ventilation were evaluated and compared to conventional biomarkers and culturing methods.
Methodology: A total of 32 subjects with VAP were included. Sepsis after VAP was diagnosed in 25 (78.1%) patients according to clinical signs, radiographic examination, and samples of blood and trachea taken for culturing. Simultaneously with cultures, CD64 index on neutrophils, C-reactive protein (CRP), procalcitonin (PCT), and count of leucocytes and neutrophils were determined.
Results: Biomarker values were evaluated in both groups of subjects (with and without sepsis after VAP). The values of CD64 index and CRP were significantly higher in the sepsis group. Receiver operating characteristic (ROC) curve analysis revealed an area under curve (AUC) of 0.929 for CD64 index in differentiating subjects with VAP-induced sepsis from those without sepsis. The biomarkers CRP and PCT showed comparable results (AUC of 0.869 and 0.909, respectively). Blood cultures were positive in 12 subjects, endotracheal aspirate in 19. CD64 index and isolation of pathogen with positive blood cultures or from endotracheal aspirate (positive in 24 cases) could predict survival time before application of more targeted antibiotic therapy.
Conclusions: CD64 index may be used as a useful diagnostic tool to recognize VAP-induced sepsis; moreover, accompanied with an identified pathogen, can predict survival for ICU patients.

Key words: ventilator-associated pneumonia; VAP-induced sepsis; CD64 index; endotracheal aspirate; survival time.

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Introduction

Intensive care unit (ICU) patients are at risk for dying of severe bacterial infection such as pneumonia or urinary tract infection that can lead to sepsis [1]. Nosocomial pneumonia is the second-most common infection, affecting 64% of all critically ill patients [2]; 86% of all nosocomial pneumonias are associated with mechanical ventilation as ventilator-associated pneumonia (VAP). The incidence rate of VAP is 5 to 10 cases per 1,000 patients admitted to the hospital [2,3] and the mortality has been reported to be between 0% and 50% [4]. Higher mortality rates were seen in infections caused by *Pseudomonas aeruginosa*, *Acinetobacter* spp., and *Stenotrophomonas maltophilia* [5].

Ventilator-associated pneumonia (VAP) is defined as pneumonia occurring 48 hours after patients have been intubated to receive mechanical ventilation. Diagnosis of VAP requires bedside and radiographic examinations, and microbiologic confirmation of a pathogen in respiratory secretions. It is well documented that early initiation of appropriate treatment has an impact on outcome; therefore, prompt initiation of empirical treatment to cover the potential pathogens is advised, and when the microbiologic results are available, treatment may be de-escalated to a targeted one where possible. Guided targeted antibiotic therapy is essential, due to the microorganisms' resistance in the ICU and in critically ill patients. Over the past decades, knowledge about VAP has grown significantly [6]. Hospitals that have implemented recommendations of the Surviving Sepsis Campaign from 2012 have seen a reduction in mortality rates in pneumonia and hospital-acquired sepsis [7,8]. The mortality rates of VAP may be further reduced by the new approaches to diagnosing bloodstream infection, especially at an early stage [9].

To prevent the worst outcome, we need a sensitive and specific diagnostic tool that can accurately identify patients at risk of developing sepsis after VAP and then

guide targeted therapy since clinical judgment is not always enough [10-12]. Septic patients have increased or low temperature (> 38°C or < 36°C) and leukocytes counts > 12×10^9/L or < 4×10^9/L. They can have tachycardia and rapid breathing [13,14]. Time to diagnosis and introduction of empirical antibiotic therapy are crucial [15]. Clinical examination is often combined with measurement of an acute-phase biomarker C-reactive protein (CRP), procalcitonin (PCT), and identification of the pathogen from blood cultures and endotracheal aspirate. Commonly used concentration of CRP in the blood is useful information for diagnosis, therapy, and monitoring the course of disease [16].

CD64 receptor on neutrophils is a Fcγ receptor 1 for immunoglobulines G (IgG) and has been found to be one of the most useful markers for diagnosis of infection or sepsis beside common CRP and PCT [17-21]. CD64 expression in patients with sepsis is normally induced within two to four hours. Their number in the membrane of neutrophils significantly increases in infections or systemic inflammatory response (SIRS) and tissue damage. The expression of CD64 on monocytes does not elevate, whereas the diagnostic assessment of higher specific expression of CD64 on neutrophils in adults and neonates with sepsis or bacterial infection has been proven. Meanwhile, negative CD64 index was observed in SIRS patients without bacterial infection [18,19-21].

In several studies, CD64 index has proved to be a good identification marker for sepsis [18-21]. The aim of our pilot study was to establish if it could predict clinically hard-to-recognize sepsis and mortality in patients after VAP.

Methodology

Subject evaluation

This was an observational pilot study that included subjects from the ICU who acquired VAP after mechanical ventilation. The relatives of participants were informed and gave consent in written form. In addition, the national ethics committee approved the study design. The subjects for the study were chosen on the clinical grounds at the bedside, after radiographic examination of the lungs, which showed pneumonia. Microbiologic confirmation of a pathogen in respiratory secretions was determined later. Sepsis was diagnosed clinically, following the guidelines for the Surviving Sepsis Campaign from 2012. Only subjects who were older than 18 years of age, were reported to have fever ≥ 38°C during the last 24 hours, and had at least two out of four signs for SIRS were included. All the subjects in the study were admitted to the hospital through the emergency care unit because they had respiratory failure and furthermore needed mechanical ventilation. Subjects who had taken antibiotics during the last 24 hours of hospitalization were excluded. Patients were then divided into two groups: those with sepsis and those without sepsis after VAP.

The physician and infectious disease specialist at the ICU evaluated the subjects; according to mentioned strict criteria for sepsis, 32 subjects were accepted in the final analysis. SIRS criteria for evaluation were body temperature > 38°C or < 36°C, heart rate > 90 per minute, breathing rate > 20 per minute, and low blood pressure. A final clinical diagnosis was based on clinical, laboratory, and microbiological data together as the golden standard for sepsis, along with health improvement after administration of antibiotic therapy.

Confirmation of infection with microbiological methods of culturing

To confirm the bacterial infection, the necessary cultures were performed. The following samples for culture were retrieved: urine, respiratory tract samples (endotracheal aspirate), and blood in two pairs of hemoculture bottles per subject (BacT/ALERT 3D, BioMerieux, Marcy-l'Étoile, France). Non-bacterial causes of infection were excluded. Standard microbiology methods on agar plates identified pathogens from samples of endotracheal aspirate or urine samples when the growth was positive.

Analysis of laboratory biomarkers

In the study, the following biomarkers were included: the count of white cells (leucocytes) and neutrophils, CRP (Siemens Healthcare Diagnostics, Erlangen, Germany), PCT (Brahms, Hennigsdorf, Germany) and CD64 expression on neutrophils (Trillium Diagnostics LCC, Bangor, USA). Blood for biomarkers was taken when the body temperature was rising. CD64 expression was measured using a diagnostic kit, Leuko64 (Trillium Diagnostics LCC,) accompanied by instructions, performed on a BD FACSCanto II (Becton Dickinson, New York, USA) flow cytometer. Antibodies conjugated with FITC (fluorescein isothiocyanate) to CD64, PE (R-phycoerythrin) conjugated to CD163, and fluorescence bead suspensions with fluorescence signals to FITC, PE, and PerCP-Cy5-5 (peridinin-chlorophyll proteins) (Trillium Diagnostics LCC) were used. The flow cytometer settings and samples were prepared according to the manufacturer's instructions. Neutrophils, monocytes, and lymphocytes were

identified on dot-plot profile and gated (Figure 1). The intensity of CD64-expressed fluorescence was measured as mean fluorescence intensity as a linearized value of log scale. Additionally, index of expression of CD64 was calculated by automated software package Leuko64 QuantiCalc (Trillium Diagnostics LCC, Meine, USA).

Limits of positivity of biomarkers were used according to manufacturers' instructions. The limit of positive sepsis for white cell count was set at > 12×10^9 cells/L and > 80% for the neutrophils. For the CRP, values > 50 mg/L meant that the bacterial infection was very likely. The limit for PCT, suggested by the manufacturer, was > 0.5 µg/L, and for CD64 index was > 1.2. The values above this limit were considered possible for bacterial infection.

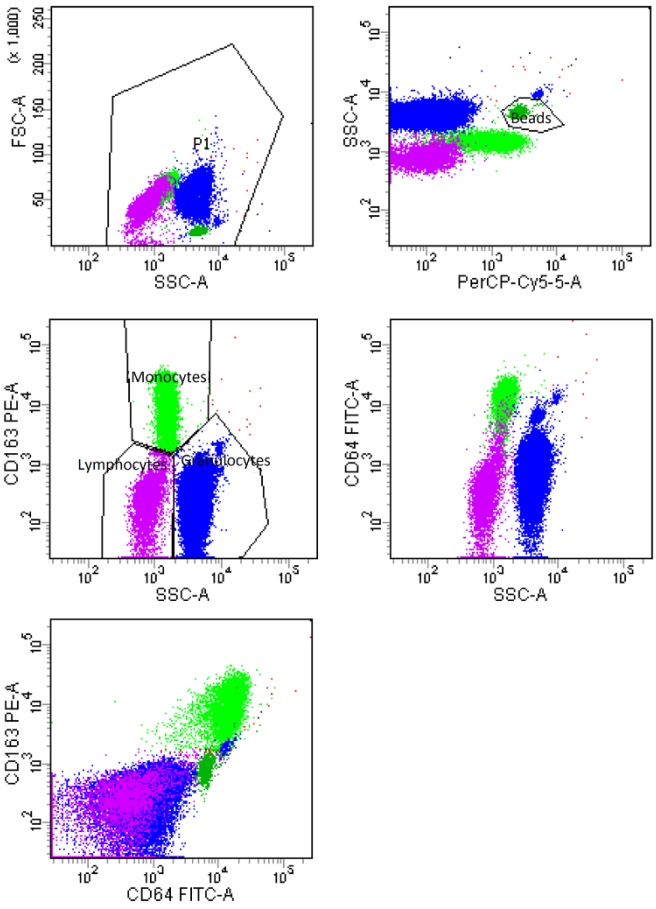
Statistical analysis

The statistical analysis was performed using Statistical Package for the Social Sciences (SPSS) version 21.0 (IBM, New York, USA). To compare quantitative variables between the VAP group and the VAP with sepsis group, an independent two-tailed t-test and a non-parametric Kruskal-Wallis test were used. Pearson's Chi-square test was used to find qualitative statistical significance between positive sepsis after VAP and positive results of biomarkers, and to calculate the positive and negative predictive values (PPV, NPV). A receiver operating characteristic (ROC) analysis with area under curve (AUC), sensitivity and specificity and cut-off values was performed for each biomarker, and their diagnostic accuracy for sepsis was calculated. Statistical significance was set at $p < 0.05$. Predictive values for sepsis, pneumonia, outcome, and survival were performed using univariate logistic regression. Additionally, a Kaplan-Meier curve for survival for statistically significant factors was constructed.

Results

A total of 32 subjects, presenting with VAP and SIRS, were included in the observational study analysis. Basic characteristics of those subjects are presented in Table 1. Seven subjects were diagnosed with VAP, and at the end did not develop sepsis that could be clinically or microbiologically proven. Twenty-five subjects were diagnosed with VAP and concomitant sepsis, which was proven clinically and with positive blood cultures or samples of endotracheal aspirate. Death was recorded in 15 out of 32 (46.9%) subjects (Table 1).

Figure 1. FACS diagrams of gated cells (neutrophils, monocytes, lymphocytes, and beads) created on BD FACSCanto II flow cytometer. The results were further imported in the Leuco64 software for calculation of CD64 index on neutrophils.



Levels of biomarkers were higher in subjects with VAP-induced sepsis compared to subjects without sepsis except for the percent of neutrophils, which were observed to be higher in the group of subjects with VAP and no sepsis. The differences were statistically significant for CD64 index ($p = 0.016$), CRP ($p = 0.002$), neutrophils ($p = 0.014$), and PCT ($p = 0.026$). Leucocyte count did not show any significance (Table 2).

Analysis of ROC curves performed for all biomarkers and positive microbiological samples taken from single subjects (blood cultures, urine cultures, cultures of endotracheal aspirate) for prediction of VAP-induced sepsis were performed and are displayed in Table 3. CD64 index on neutrophils and PCT showed the highest accuracy to predict sepsis with AUC of 0.929 and 0.909, respectively. Sensitivity and specificity for CD64 index were 100.0% and 85.7%, respectively, and for PCT were 81.8% and 100.0%, respectively. Moreover, CRP values also showed quite

good accuracy with AUC = 0.869 and sensitivity and specificity of 83.3% and 85.7%, respectively. However, only CD64 index showed good (> 80%) PNV and NPV for positive sepsis (p = 0.006). Leucocytes, neutrophils, PCT, and positive blood cultures did show statistically important differences with positive sepsis, but had lower predictive values. CRP and other cultures were not statistically significant (Table 3). New cut-off values that could more accurately predict sepsis were also calculated. For CD64 index, CRP and PCT were slightly higher (1.58, 163.5 mg/L, 1.73 µg/L, respectively). Blood cultures, urine cultures, or cultures of endotracheal aspirate alone could not predict VAP, outcome, or survival. The pathogens isolated from taken sample are listed in Table 4.

For further predictions of outcome and survival, logistic regression analysis using new calculated cut-off values for biomarkers was performed, which showed that no parameter could predict the outcome of disease. The survival could be predicted only by CD64 index (p = 0.046) or the combination of positive blood culture and culture of endotracheal aspirate though (p = 0.047). For further evaluation, the Kaplan-Meier survival plot showed that subjects with CD64 index lower than 1.58

Table 1. Basic characteristics of the study population

Characteristics	Number of patients (n = 32)
Age (years)	61.8 ±17.8
Gender (male/female)	22/10
VAP	
VAP with no sepsis	7 (21.9%)
VAP with sepsis	25 (78.1%)
Positive BC	12 (37.5%)
Positive EA	19 (59.4%)
Positive BC or EA	24 (75.0%)
Positive Sanford	7 (21.9%)
Died	15 (46.9%)

VAP: ventilator-associated pneumonia; BC: blood culture; EA: endotracheal aspirate

Table 2. Difference in average values of biomarkers with standard deviations in groups of patients with or without VAP-induced sepsis

	Clinically confirmed sepsis		
	VAP with no sepsis (n = 7)	VAP and sepsis (n = 25)	P value
CD64 index	1.83 ± 1.61	6.60 ± 4.82	0.016
CRP [mg/L]	108.7 ± 55.5	243.1 ± 100.2	0.002
PCT [µg/L]	0.82 ± 0.48	53.02 ± 46.83	0.415
Leucocytes [x10^9 /L]	9.0 ± 5.2	17.7 ± 14.6	0.139
Neutrophils [%]	105.7 ± 58.6	70.6 ± 1.7	0.014

VAP: ventilator-associated pneumonia; CRP: C-reactive protein; PCT: procalcitonin

Table 3. Values of ROC analysis to detect possible bacterial infection of blood after VAP (AUC, sensitivity and specificity, cut-offs, PPV, NPV) and logistic regression prediction p-values for tested biomarkers and culturing.

	AUC (95% CI)	Cut-off	Sensitivity [%]	Specificity [%]	PPV [%]	NPV [%]	P value to predict clinical sepsis*	P value to predict VAP*	P value to predict outcome*	P value to predict survival*†
CD64 index	0.929 (0.793–1.000)	1.58	100.0	85.7	83.3	100.0	0.006	0.098	0.100	0.046
CRP [mg/L]	0.869 (0.743–0.996)	163.5	83.3	85.7	79.3	50.0	0.338	0.106	0.131	0.734
PCT [µg/l]	0.909 (0.749–1.000)	1.73	81.8	100.0	95.5	50.0	0.026	0.001	0.902	0.664
Leucocytes [x109 /L]	0.657 (0.467–0.847)	12.6	60.0	85.7	93.7	37.5	0.033	1.000	0.723	0.383
Neutrophils [%]	0.179 (0.012–0.345)	80.5	37.5	28.6	60.0	6.3	0.025	0.254	0.104	0.468
BC	0.750 (0.578–0.922)	-	50.0	100.0	100.0	36.8	0.017	0.546	0.756	0.912
EA	0.524 (0.255–0.793)	-	71.4	33.3	78.9	25.0	0.822	0.116	0.706	0.065
BC+EA	0.614 (0.364–0.865)	-	80.0	42.9	83.3	37.5	0.217	0.217	0.306	0.047
Sanford	0.576 (0.296–0.856)	-	35.3	80.0	85.7	26.7	0.519	0.563	0.290	0.605

*P values obtained by binary logistic regression; †P values were obtained by analysis of calculated new cut-off values for biomarkers; AUC: area under curve; PPV: positive predictive value; NPV: negative predictive value; CRP: C-reactive protein; PCT: procalcitonin; BC: blood culture; EA: endotracheal aspirate.

Figure 2. Kaplan-Meier survival plots for patients with VAP-induced sepsis using a CD64 index cut-off of 1.58, which shows that patients with CD64 index under this value survived longer.

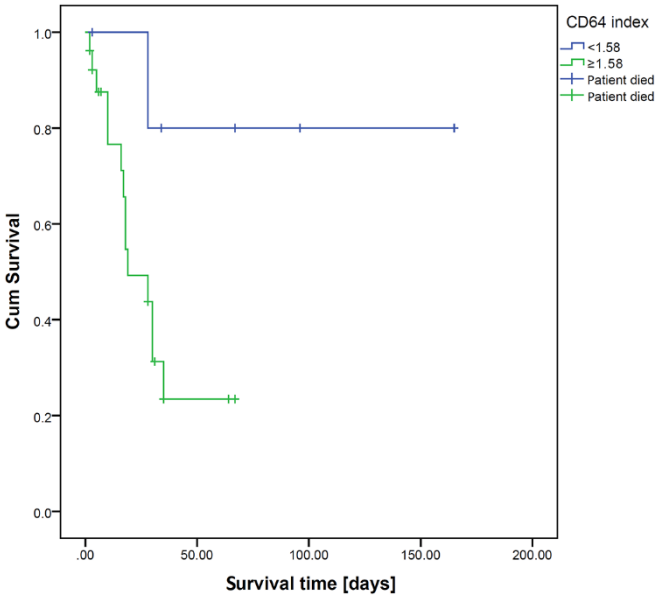
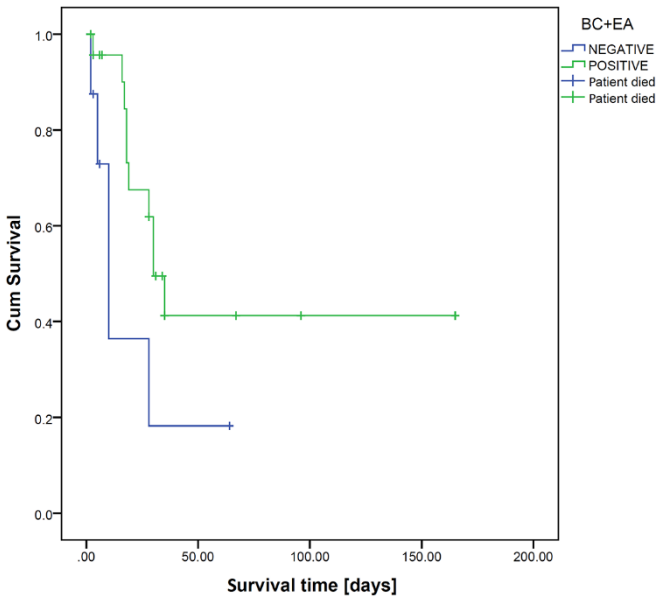


Figure 3. Kaplan-Meier survival plots for patients with VAP-induced sepsis using results of positive blood culture or culture of endotracheal aspirate. Positive cultures detect the right pathogen, which leads to more targeted antibiotic therapy that allows longer survival of such patients.



were more likely to survive longer (Figure 2). Furthermore, subjects with positive blood culture or endotracheal aspirate culture were also likely to survive longer (Figure 3). Survival time in days for each culture and CD64 index is displayed as box-plots (Figure 4).

Discussion

In our pilot observational study, we included 32 subjects: those with proven VAP and no concomitant sepsis and those with VAP-induced sepsis. Despite the low sample size, we tried to evaluate CD64 index predictions for sepsis and mortality in subjects who already had pneumonia, compared to the other most commonly used biomarkers in the diagnosis of VAP. The tested biomarker has to have high diagnostic accuracy for an early recognition of potential infection [9,22]. Levels of all tested biomarkers were elevated in both examination groups; however, in the sepsis group, the levels were even higher compared to the VAP-only diagnosed subjects. Pneumonia alone elevated the levels of biomarkers, which is generally expected. CD64 index and CRP were statistically significant between the groups ($p = 0.016$ and 0.002, respectively). Bacterial infection activates the neutrophils and the expression of CD64 receptor for IgG on neutrophils.

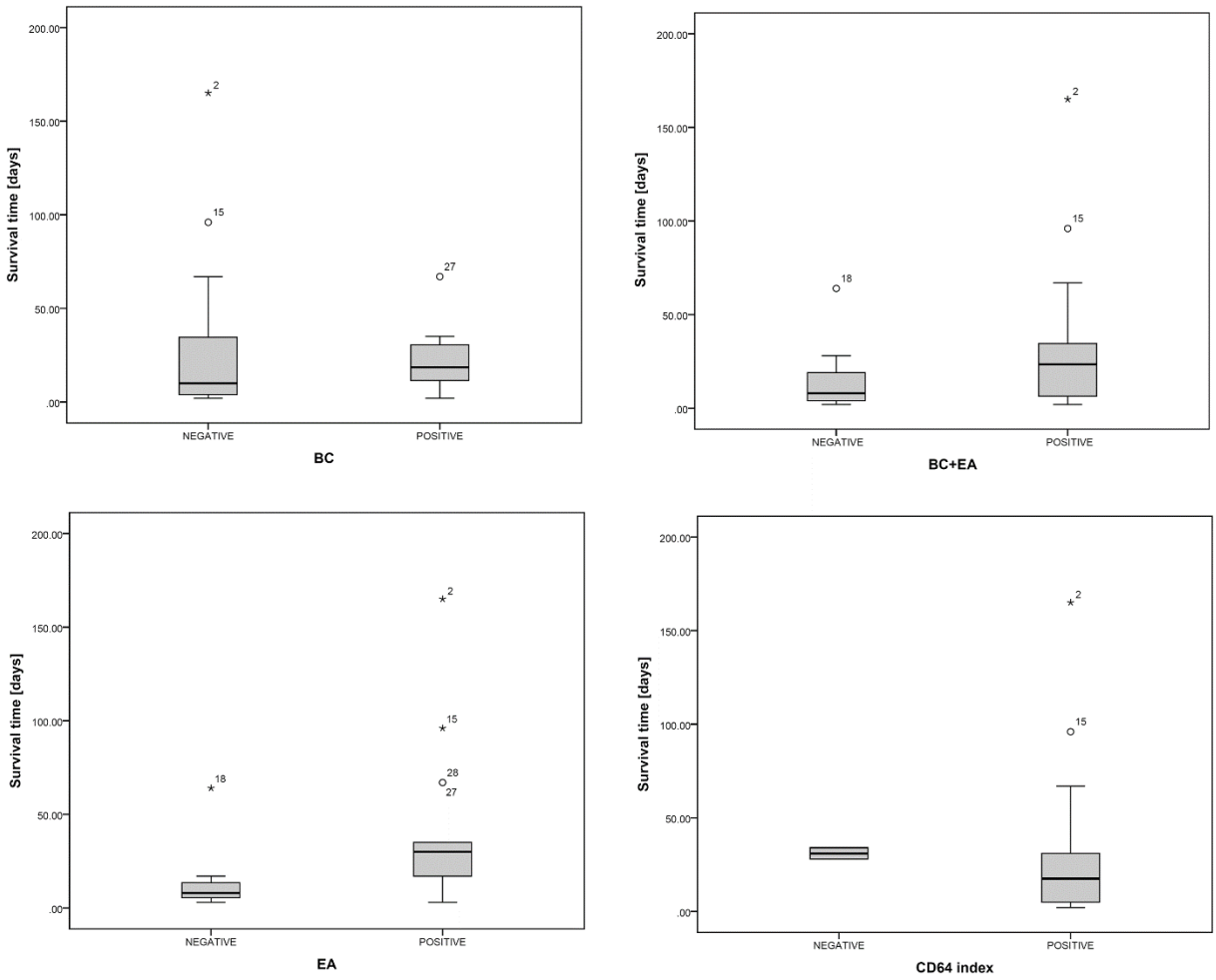
Table 4. List of all identified microorganism taken from the three different samples (blood, trachea, urine).

	Isolated microorganisms from	
Blood cultures (n = 12)	**Endotracheal aspirate (n = 19)**	**Sanford (n = 7)**
Escherichia coli	*Escherichia coli*	*Escherichia coli*
Streptococcus pneumoniae	*Streptococcus pneumoniae*	*Staphylococcus aureus*
Staphylococcus aureus	*Staphylococcus aureus*	*Klebsiella pneumoniae*
Neisseria meningitidis	*Pseudomonas aeruginosa*	*Enterobacter cloacae*
Enterobacter cloacae	*Citrobacter kosei*	*Enterococcus faecalis*
Staphylococcus hominis	*Legionella pneumoniae*	*Candida albicans*
Streptococcus pyogenes	*Klebsiella pneumoniae*	
Enterobacter aerogenes	*Stenotrophomonas maltophila*	
	Enterococcus faecium	
	Enterococcus rophinosus	
	Staphylococcus hemolyticus	
	Enterobacter cloacae	
	Actinomyces spp.	
	Candida albicans	
	Mixed culture	

IgG bind to these sites very specifically, which at the end represents a very sensitive biomarker for sepsis detection [23]. Other biomarkers could not statistically distinguish between the two groups. Leucocyte counts and CRP are widely used in the diagnosis of possible sepsis. Moreover, CRP is a very unspecific acute-phase marker and can be raised in autoimmune diseases, in tumors, in ischemic heart attacks, or in severe virus infections [24], which is why it was not statistically relevant. PCT is meanwhile considered as the best marker compared to commonly used biomarkers and has been, in several studies, demonstrated to have superior diagnostic accuracy for a variety of bacterial infections, including bloodstream infection [9,25]. Our findings showed that the diagnostic accuracy of PCT was higher than that of CRP among the subjects with VAP-induced bacterial sepsis with AUC = 0.909 compared to CRP 0.869, and coincided with the results of Simon *et al*. [12] and Hirakata *et al*. [26]. PCT was also used in many clinical trials as guidance for the duration of antibiotic therapy [27-29]. However, it is also increased in cases of SIRS due to non-infectious disease conditions such as severe congestive heart failure or acute pancreatitis, and viral and parasitic infections [30,31].

The accuracy of CD64 index to detect positive sepsis in subjects with VAP was superior compared to other parameters, which was actually in accordance with other studies [32,33]. We also found that in subjects with severe disease, such as VAP-induced sepsis, slightly higher cut-off values for positivity in CD64 index (1.58), CRP (163.5 mg/L), and PCT (1.73 μg/L) levels are required to obtain the best sensitivity, specificity, and predictive values. Moreover, even low PCT levels can be misleading since septic subjects with PCT levels < 0.5 μg/L were demonstrated to have positive blood cultures in up to 25% of cases [34,35], which was not in concordance with our suggestion of the necessary higher cut-off. In the end, only CD64 index, PCT, and count of leucocytes and neutrophils

Figure 4. Comparison of patients' survival time in days for blood cultures, cultures of endotracheal aspirate, the two cultures together, and CD64 index on neutrophils.



could predict sepsis. To predict VAP with no sepsis, only high PCT levels were accurate enough (p = 0.001). Positive blood cultures logically also showed good prediction of VAP-induced sepsis (p = 0.017), but endotracheal aspirate could not (p = 0.822). Solh *et al*. [36] were able to demonstrate good sensitivity (90%), specificity (77%), and NPV (80%) for endotracheal aspirate compared to bronchoalveolar lavage. They proposed a lower threshold for bacterial concentration because one-third of their subjects had received antibiotics before sampling, which could be regarded as a potential bias because it could have led to a high rate of false-negative results. In our study, we objectively excluded the subjects who underwent antibiotic therapy, so we showed poorer NPV of 25.0% for endotracheal aspirate.

In sepsis, the improvement of the subject's health is considered a good outcome. The worst-case scenario is that the subject shows no improvement despite broad-spectrum antibiotic therapy. We proved that biomarkers or culturing methods could not predict the subject's outcome. The results coincided with the findings of Velasquez *et al*. [37], who concluded that there was also no correlation. They also showed that there was no relationship between survival and CD64 levels. However, we found that a CD64 index cut-off of 1.58 may be optimal for prediction of survival length in subjects with VAP-induced sepsis. The levels were significant (p = 0.046), and survival curve analysis showed much longer time of survival in subjects with lower CD64 index, similar to the findings of Song *et al*. [33] and Muller *et al*. [38]. In addition, the culturing methods are also of great importance because they identify the pathogen, and more focused and targeted antibiotic therapy could be applied, which could prolong the survival of the subject or even cure the disease. Samples of endotracheal aspirate could not predict survival alone (p = 0.065), but in combination with blood cultures, the prediction was significant (p = 0.047). There is a much higher probability of detecting a pathogen when more than just one sample is taken. The findings were confirmed with the list of isolated microorganisms. With blood cultures alone, only 12 samples were proven to be positive. Endotracheal aspirate taken from subjects showed positivity in 19 samples. Meanwhile, when we took into account the positive results of both samples, we detected 24 positive samples. The survival curve demonstrated that positive culture methods and identified microorganisms prolong the time of survival of the subjects due to more focused antibiotic therapy [6]. The problem occurs when the identified pathogen is not the cause of the disease but a contaminant, which could appear at the point of taking the sample or during cultivation. False antibiotic therapy cannot cure the infection, so further samples of blood cultures or endotracheal aspirate for bacterial identification must be taken. Therefore, we suggest taking both cultures at the same time, so in the best case we get same results from both tests. Simultaneously, we improve sensitivity and specificity of blood and endotracheal aspirate culture. However, such testing still takes time, so biomarkers might be necessary to recognize potential infection. Moreover, when testing multiple biomarkers, a better prediction for infection could be obtained.

Our study had one limitation: a small number of subjects examined. The reasons were very strict SIRS criteria, which were fully fulfilled for all subjects, and in the end, only 32 subjects could be included in the analysis. We used an objectively designed common standard of clinical evaluation, radiographic examination of lungs, and microbiologically proven infection. We proved that CD64 index may be a useful marker for prediction of survival time, but less reliable in predicting VAP and outcome, though in some studies it was used as predictor of successful antibiotic therapy. The prognostic value of CD64 index obtained by AUC showed cut-off of 1.58. The predictive potential of CD64 index was considered good and survival length was longer in subjects with a lower index.

Conclusions

Our findings confirmed that CD64 index measured due to VAP may be valuable for prediction of VAP-induced sepsis and survival time, and may be used to determine which VAP subjects require more urgent monitoring in terms of taking samples for cultures, and consequently more focused therapy.

CD64 index could serve as a good prognostic marker in subjects with VAP-induced sepsis and may be an independent predictor of subjects' survival time.

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