

ORIGINAL RESEARCH

INTENSIVE CARE

Toll-like receptor –2 and 4 as a diagnostic method for ventilator-associated pneumonia

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Abstract

Background & objective: Ventilator-associated pneumonia (VAP) is a nosocomial infection associated with high mortality, especially in the critical care settings. VAP extends the duration and cost of hospitalization and increases the antibiotic usage. Toll-like Receptor (TLR) detects and responds to bacteria, enabling innate immune response. We explored the performance of TLR–2 and TLR–4 assessment as an additional diagnostic tool for VAP.

Methodology: A prospective cohort study was conducted, from November 2019 to April 2020 in three teaching hospitals in Surakarta, Central Java, Indonesia, in intubated and mechanically ventilated patients, aged between 19 and 65 y. Blood samples for TLR–2 and TLR–4 were obtained from the venous blood. Blood sampling was done on the 24th and 48th hour after intubation. TLR–2 and TLR–4 expression was evaluated using RT–PCR (Real-Time Polymerase Chain Reaction). We used IBM SPSS Statistics 25 for data analysis. The Mann–Whitney test was carried out to determine the expression difference of TLR–2 and TLR–4 between VAP and non–VAP patients. The results were considered significant when the $P < 0.05$.

Results: TLR–2 and TLR–4 were expressed higher at the 24th hour after mechanical ventilation, especially in non–VAP patients. TLR–2 and TLR–4 assessment had low sensitivity, specificity, and performance for diagnosing VAP.

Conclusion: Patients with VAP have lower expressions of TLR-2 and TLR-4 compared to non-VAP patients. TLR–2 and TLR–4 assay may not be beneficial as an additional diagnostic method for VAP.

Abbreviations: VAP - Ventilator-associated pneumonia; TLR - Toll-like Receptor; ICU - Intensive Care Unit; CPIS - The Clinical Pulmonary Infection Score; RT-PCR – Real Time - Polymerase Chain Reaction; ROC - Receiver operating characteristic; AUC - Area under curve; TIR - Toll-interleukin-1 receptor; ITTMs - immunoreceptor tyrosine-based inhibitory motifs; IRAK - IL–1 receptor-associated kinase; GM-CSF – Granulocyte Macrophage-Colony Stimulating Factor; PAMP - pathogen-associated molecular pattern; DAMPs - damage-associated molecular patterns

Key words: Ventilator-associated pneumonia; VAP; TLR–2; TLR–4

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1. Introduction

Ventilator-associated pneumonia (VAP) remains one of the most common forms of nosocomial infection, especially in the Intensive Care Unit (ICU), with mechanically-ventilated patients having the highest risk after 48 h.¹ A study in 2013 reported that 10–50% of patients in critical care units acquired nosocomial infections. A meta-analysis by Bonell et al., showed that the VAP was commonly found in 22.9 out of 1,000 days of mechanical ventilation. Mechanically-ventilated VAP patients are prone to higher mortality risk, more extended hospital stay, more significant antibiotic requirement, and higher cost than those without VAP.² Toll-like receptors (TLR) are proteins that identify pathogenic bacteria in VAP patients, promoting innate immune system activation. Tang et al., in 2015 revealed that a lower expression of TLR-2 and TLR-4 in VAP patients correlated with a higher mortality rate. Human lung alveolar stretching, most commonly found in mechanically ventilated patients, may elevate TLR-2 expression up to six-folds. Mechanical ventilation by itself induces TLR-2 and TLR-4 expression and raises TLR-4 ligands expression in bronchoalveolar lavage.³ This study aims to discover the diagnostic value of TLR-2 and TLR-4 expression for VAP diagnosis in patients being ventilated in ICU.

2. Methodology

An observational, prospective cohort study was conducted from November 2019 to April 2020 in three teaching hospitals in Surakarta, Central Java, Indonesia. The study was approved by Dr. Moewardi General Hospital Health Research Ethics Committee No. 1.064/IX/HREC/2019. Informed consent was obtained from the patients or their legal guardians. The samples were taken by consecutive sampling technique. A minimum sample requirement of 11 samples for each group was calculated using *StatCalc* application version 7.2.4.0. We used a confidence interval value of 95% with 80% power. We predicted that an elevated expression of TLR-2 and TLR-4 may be found in 81% of VAP patients and 19% of the control group patients based on a previous study by Li Bassi et al.⁴ Patients aged between 19 to 65 y old with newly inserted ETT connected to mechanical ventilators and without pneumonia were included in our study. We excluded pregnant patients and those with bronchiectasis as well as fibrosis.⁴

VAP was defined as The Clinical Pulmonary Infection Score (CPIS) of > 6 at 48th h post mechanical ventilation. The recorded data for

CPIS were; body temperature, leukocyte count, tracheal secretion characteristic, oxygenation, the bacterial culture results from the tracheal aspirate, and chest X-ray. Venous blood was obtained at the 24th and 48th h after mechanical ventilation for TLR-2 and TLR-4 examination. TLR-2 and TLR-4 expression was evaluated using RT-PCR (Real-Time Polymerase Chain Reaction) with Exicycler3 version Exicycler 96 Run 3.55.6P. Relative expression for TLR-2 and TLR-4 was calculated using Livak's relative expression formula.⁵

We used IBM SPSS Statistics 25 for data analysis. The Mann-Whitney test was carried out to determine the expression difference of TLR-2 and TLR-4 between VAP and non-VAP patients. The results were considered significant when the $P < 0.05$. We used the ROC (Receiver operating characteristic) curve to calculate each parameter's sensitivity, specificity, and cut-off points. TLR-2 and TLR-4 performance in distinguishing VAP from non-VAP patients were determined by AUC (area under curve) value, in which an AUC below 0.50 is deemed as "badly-performed" or unable to distinguish between the two groups, whereas an AUC of 0.5 was interpreted as "well-performed".

3. Results

We obtained 28 mechanically ventilated patients during the study period, and 14 of them had a confirmed VAP

Table 1: Clinical Pulmonary Infection Score⁶

Parameter	Result	Score
Temperature (°C)	36.5–38.4	0
	38.5–38.9	1
	≤ 36 or ≥ 39	2
Leukocytes (cells/mm ³)	4.000–11.000	0
	< 4.000 or > 11.000	1
	≥ 500 band cells	2
Tracheal secretions (subjective)	None	0
	Mild/non-purulent	1
	Purulent	2
Radiographic findings on chest X-ray	No infiltrates	0
	Diffuse/patchy infiltrates	1
	Localized infiltrate	2
Culture result (respiratory sampling)	No/mild growth	0
	Moderate/florid growth	1
	Moderate/florid growth and pathogen consistent with gram stain	2
Oxygenation status (PaO ₂ /FiO ₂ ratio)	> 240 or ARDS	0
	≤ 240 and absence of ARDS	2

diagnosis. The VAP patients were older (43.36 ± 16.66 y) than those in the control group (40.57 ± 19.50 y). Patients in the control group had a longer length of hospitalization (11.29 ± 5.97 days) than VAP patients (8.07 ± 4.38 days). Most subjects received mechanical ventilation due to post-operative indication (Table 2).

TLR-2 and TLR-4 relative expression using Livak's formula is shown in Table 3. TLR-2 expression at the 48th hour in both VAP patients and the control group was 1.32 folds lower (0.76 ± 1.21) than at the 24th. We also found that TLR-4 expression at the 48th-hour post mechanical ventilation was 1.61 folds lower (0.62 ± 0.99) than those at the 24th. We conducted a comparative analysis using the Mann-Whitney test to find the significance between these different results.

We found that TLR-2 was slightly higher in the control group compared to VAP patients ($P = 0.982$). The same result was found for TLR-4, in which patients without VAP had higher expression of TLR-4 compared to VAP patients ($P = 0.854$). However, the difference between these two groups was not statistically significant.

TLR-2 assessment for VAP yielded sensitivity and specificity of 42.9% and 64.33%, respectively. TLR-2 assay performed poorly in differentiating VAP from non-VAP patients, but this result was statistically insignificant ($AUC = 0.497$; $P = 0.982$). TLR-4 assay had a higher sensitivity of 71.4% and specificity of 42.99%. We discovered that TLR-4 assay might be able to distinguish VAP patients from non-VAP better than TLR-2 assessment ($AUC = 52.0\%$; $P = 0.854$).

Both TLR-2 and TLR-4 were found to have relatively low sensitivity and specificity for diagnosing VAP. Still, TLR-4 assessment performed better than TLR-2 in diagnosing VAP.

4. Discussion

In this study, we reported the diagnostic value of TLR-2 and TLR-4 based on their relative expression. The mean TLR-2 and TLR-4 expression at 48th-hour post mechanical ventilation was lower than the first 24th hour and was especially higher in the control group. We discovered that both TLR-2 and TLR-4 had no adequate sensitivity and specificity for diagnosing VAP and showed low ability in differentiating VAP from non-VAP patients.

Gram-positive and negative bacteria remain the leading cause of VAP in critical care settings. TLR-mediated lung inflammation occurs when TLR recognizes the bacterial lipopolysaccharide (LPS) layer on the outer membrane vesicle. This process ignites pro-inflammatory cytokine and neutrophil recruitment. TLR-4 plays a significant role in identifying Gram-negative LPS, while TLR-2 mainly recognizes Gram-positive bacteria. TLR-2 may also work in hand with TLR-4 to induce the production of pro-inflammatory interleukin from lung epithelial cells. While TLR-4 majors in identifying bacterial LPS, TLR-2 plays a supportive role in generating systemic inflammation.⁷

TLR-4 is mainly found in alveolar macrophages. A decrease in TLR-4 indicates lower macrophage, leukocyte, and neutrophil levels in Gram-negative-induced sepsis in rats.⁷ Lipopeptide on Gram-positive bacteria cell wall such as *Staphylococcus aureus* (*S. aureus*) is a recognizable component of TLR-2. A study revealed that mechanically ventilated mice had higher expression of TLR-2 in lung epithelial cells at the 24th hour after induction of sepsis by *S. aureus*.⁸ Gram-positive bacteria such as *S. aureus* promote expressions of TLR-2 and TLR-4 from the interaction between diacyl lipopeptide ligands. Elevated TLR-2 expression might play a protective role in disease progression, activating the innate immune response. *S. aureus* may suppress TLR-2 activity by forming TLR-2/6 heterodimers, which mimic the toll-interleukin-1 receptor (TIR) domain and activates immune system

Table 2: Demographic data of the patients

Parameter	VAP	Non-VAP
Age (years)	43.36 ± 16.66	40.57 ± 19.50
Gender (Male/Female)	7/7	8/6
Duration of stay (days)	8.07 ± 4.38	11.29 ± 5.97
Indication for ventilation		
Post-operative	7	9
Respiratory	6	1
Neurologic	0	4
Cardiologic	1	0

Table 3: Relative expression of TLR-2 and TLR-4

Parameter	Relative Expression (Folds)
TLR-2	0.76 ± 1.21
TLR-4	0.62 ± 0.99

Table 4: Comparative analysis of TLR-2 and TLR-4

Parameter	VAP	Non-VAP	p-value
TLR-2	0.15 (0.01–2.50)	0.32 (0.01–5.17)	0.982
TLR-4	0.30 (0.01–4.86)	0.33 (0.002–1.72)	0.854

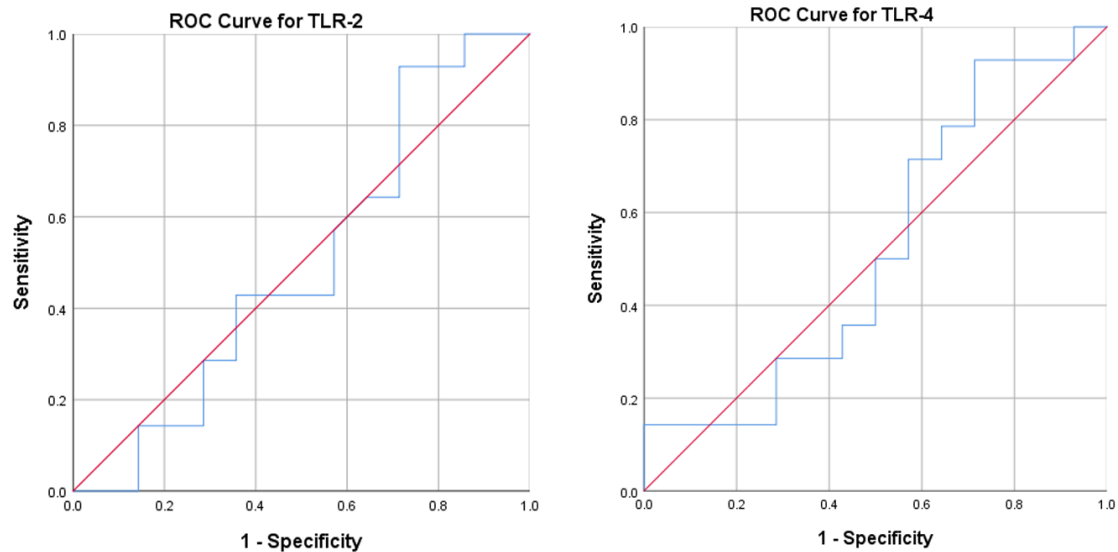


Figure 1 ROC curve for TLR-2 and TLR-4

receptor inhibitors such as immunoreceptor tyrosine-based inhibitory motifs (ITIMs).⁹

A study by Barbar et al. found that mechanical ventilation may excessively induce TLR-2 expression, which is related to impaired lung function.⁷ Other authors provided the same result in which mechanically ventilated patients were found to have higher expression of TLR-4, MD-2, dan CD14.¹⁰ Mechanical ventilation also produces other TLR ligands which are not dependent on TLR-4 but on MyD88 (Myeloid Differentiation primary response gene 88). Lung hyperinflation due to mechanical ventilation with high tidal volume induces TLR-4 expression and IRAK-M (IL-1 receptor-associated kinase-M), suppressing TLR-mediated signaling.⁸ An in vivo study showed that high concentration oxygen exposure might lead to *Klebsiella pneumoniae* infestation due to a decrease of GM-CSF (Granulocyte-Macrophage Colony Stimulating Factor) in bronchoalveolar lavage and more elevated cellular surface TLR-4 expression.¹¹

Severe infections in elderly pneumonia patients usually present with high TLR-2 and TLR-4 expression.³ A study in 2014 found that CD14 monocyte induced expression of TLR-2 and TLR-4 in pneumonia patients, which in turn also increased IL-1, IL-6, and TNF- α . Expression of TLR-2 was not correlated with TLR-4 expression. High expression of TLR-2 was linked with a severe degree of pneumonia. However, a decrease in TLR-2 and TLR-4 was a mortality risk for pneumonia as it signifies an impaired bacterial clearance. A similar result was found in an earlier study which revealed that TLR-2 is essential in the TLR-2/MAPK pathway, which facilitates bacterial phagocytosis through the production of nucleus pulposus cells (NPC).¹²

Mechanical ventilation with a tidal volume of ≥ 40 ml/kg may induce lung inflammation due to alveolar stretching, marked by an increase of TLR-2, TLR-4, and TLR-9. A latest study from McLeod et al., (2020)¹³ found that detection of TLR-2/6 heterodimer may become evidence of the presence of Gram-positive bacteria. Heterodimer TLR2/6 has a high affinity for Pam2CSK4 as a specific pathogen-associated molecular patterns (PAMPs) in Gram-positive bacteria. Chatzi et al., (2018)¹⁴ stated that TLR-2 polymorphism, especially in TLR-2Arg753Gln, was not correlated to an increase in nosocomial infection, especially in critical care settings. This form of mutation is sporadic, and a correlation between TLR-2 polymorphism and the incidence of nosocomial disease has not been established. It has been stated that *S. aureus* may have undergone an evolution, resulting in lower activation of TLR-2 through inhibition of damage-associated molecular patterns (DAMPs).¹⁵ To function appropriately, TLR-2 requires a heterodimer binding with TLR-1 or TLR-6 and DAMP to develop an immune response. This binding process explains why TLR-2 may not be an accurate assay for VAP diagnosis compared to TLR-4. 96.3% of VAP cases with a CPIS score of ≥ 7 were most likely caused by Gram-negative bacteria, which is not a specific target for TLR-2. Albeit significant to Gram-negative bacteria, and TLR-4 demonstrated a stronger reaction to lipoteichoic acid, a component commonly found in Gram-positive bacteria, rather than TLR-2.^{16, 17}

Cai et al. found that a polymorphism in the TLR-4 A299G gene played a role in the pathogenesis of VAP. TLR-mediated signaling activates nuclear factor-kappa B (NF κ B) transcription factor, a mediator of inflammatory cytokine expression control. Activation of NF κ B requires phosphorylation and degradation of

Inhibitory $\kappa\beta$ ($I\kappa\beta$) into $I\kappa\beta$ kinase α ($IKK\alpha$) and $IKK\beta$. Several TLRs work their way through alternative pathways involving enzymes and their interaction with other IKKs, such as TBK1 (TRAF family member-associated NF- $\kappa\beta$ activator (TANK) binding kinase-1) and inducible $I\kappa\beta$ kinase (IKKi), both of which also stimulate innate immune system response. Activation of these enzymes yields a specific reaction in targeting and fending off viral infection.^{18–20}

We previously suspected low expressions of TLR-2 and TLR-4 in VAP patients. Contrary to our hypothesis. Our study results only showed an increased expression of TLR-2 in VAP patients at 48 hours post mechanical ventilation. VAP can also be caused by fungi or a combination of fungi and bacteria, which might interact with another signaling pathway than TLR-2 and TLR-4. An increase in TLR-2 was also not proved to be related to the VAP incidence.²¹ For example, TLR-1 is also a PAMP with a role in identifying bacteria, but it was not explored in this study.²² Other possible pathways include *C-type lectin receptor*, *nucleotide-binding oligomerization domain-like receptor* (NLR), and *retinoic acid-inducible gene-1-like receptor*, and *absent in melanoma 2 (AIM2)-like receptor*. Activation of PRRs trigger an inflammatory cascade that initiates an immune response to microbial or non-microbial invasion. Our study was only able to incorporate a small number of samples which may affect the significance of the results. Further analysis, which may include more subjects and evaluate other PRR pathways, may reveal another potential biomarker for diagnosis of VAP.^{23,24}

5. Conclusion

Patients with VAP have lower expressions of TLR-2 and TLR-4 compared to non-VAP patients. TLR-2 and TLR-4 assay may not be beneficial as an additional diagnostic method for VAP.

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No internal or external funding was used.

7. Conflict of interest

No conflicts of interest/competing interests were declared by the authors.

8. Authors' contribution

BP: supervised the research

BW: conceived and designed the analysis

P: assessment of research report process

FHD: literature search, collected data, analyzed data, and wrote the article.

9. References

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