

Original Article

Real-Time RT-PCR Detection of *HCN4* and *ADAM8* Genes in Ventilator-Associated Pneumonia Patients who Hospitalized in Intensive Care Unit

Hamidreza Jamaati¹, Naghmeh Bahrami², Masoum Abniki³, Payam Tabarsi⁴, Behrooz Farzanegan⁵, Atosa Doroudinia⁶, Abdolreza Mohamadnia^{7*}

Abstract

Background: Ventilator-associated pneumonia (VAP) is one of the most serious and prevalent nosocomial infections among patients in the intensive care unit (ICU), which is often recognized after detecting the symptoms. To date, there is no proper clinical and diagnostic marker for early detection of this disease. In this study, two genes (*HCN4* and *ADAM8*) were assessed to be used as biomarkers for recognition of the disease in patients with VAP.

Materials and Methods: This study was a case control study, which was done in Masih Daneshvari Hospital affiliated to Shahid Beheshti University of Medical Sciences, Tehran, Iran, since 2015-2016. Current study consisted of 30 patients with VAP and 30 Healthy people. After extraction of RNA, cDNA synthesis was done. Expression of genes was assessed by Real time PCR.

Results: In peripheral blood samples of the patients, 10 out of 30 did not have positive *HCN4* gene expression. Among the healthy individuals, 6 out of 30 cases did not have positive *HCN4* gene expression. Moreover, in *ADAM8* marker patients, 13 out of 30 did not have positive *ADAM8* gene expression and 8 out of 30 healthy individuals did not have positive *ADAM8* gene expression.

Conclusion: Genes *HCN4* and *ADAM8* assessment with Real Time-PCR in this study can be used as promising markers in early detection of VAP disease. More extensive studies on larger sample sizes may yield higher sensitivity for these molecular markers.

Keywords: *HCN4* gene, *ADAM8* gene, Real Time-PCR, ventilator associated pneumonia

Please cite this article as: Jamaati H, Bahrami N, Abniki M, Tabarsi P, Farzanegan B, Doroudinia A, et al. Real-Time RT-PCR Detection of *HCN4* and *ADAM8* Genes in Ventilator-Associated Pneumonia Patients who Hospitalized in Intensive Care Unit. J Cell Mol Anesth. 2016;1(4):163-7.

Introduction

Ventilator-associated pneumonia (VAP) is One of the most serious and prevalent nosocomial infections among patients in the ICU (1).

VAP is diagnosed among patients admitted in the

1. Chronic Respiratory Diseases Research Center, National Research Institute of Tuberculosis and Lung Diseases (NRITLD), Shahid Beheshti University of Medical Sciences, Tehran, Iran

2. Craniomaxillofacial Research center, Tehran University of Medical Sciences, Tehran, Iran. Oral and Maxillofacial Surgery Department, School of Dentistry, Tehran University of Medical Sciences, Tehran, Iran

3. Department of Biotechnology, Faculty of Sciences, Islamic Azad University, Tehran, Iran

4. Clinical Tuberculosis and Epidemiology Research Center, National Research Institute of Tuberculosis and Lung Diseases (NRITLD), Shahid Beheshti University of Medical Sciences, Tehran, Iran

5. Tracheal Diseases Research Center, National Research Institute of Tuberculosis and Lung Diseases (NRITLD), Shahid Beheshti University of Medical Sciences, Tehran, Iran

6. National Research Institute for Tuberculosis and Lung Disease, Department of Pathology Faculty of Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran

7. Virology Research Center, National Research Institute of Tuberculosis and Lung Diseases (NRITLD), Shahid Beheshti University of Medical Sciences, Tehran, Iran. Department of Biotechnology, School of Advanced Technologies in Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran

Corresponding Author: Abdolreza Mohamadnia, Assistant Professor, Virology Research Center, National Research Institute of Tuberculosis and Lung Diseases (NRITLD), Shahid Beheshti University of Medical Sciences, Tehran, Iran. Post code: 1956944413. Tel: (+98) 912-5146410; Email: mohamadnia.ar@gmail.com Received: July 17, 2016 Accepted: September 9, 2016

ICU about 48 hours after intubation (2). The majority of patients in ICU intubated and mechanical ventilated that provides a direct entry of bacteria into the lower respiratory airways and leads to increased risk of VAP (3, 4). The main bacterial agents such as enteric gram-negative bacilli, *Pseudomonas* and *Staphylococcus aureus* are causes of ventilator associated pneumonia (4). Although, VAP is the most common nosocomial infections in ICU department, the mortality rate is higher compared to other pneumonias (5).

It seems that, there should be a genetic predisposition in patients hospitalized in the ICU. Some of these patients are more susceptible to infectious diseases (6). So, investigation of biomarkers in these patients and evaluation the expression of these genes may be helpful in recognition the patients who are more susceptible to these infections (6). Also, it seems that clinically effective treatments can be provided for these patients in earlier stages (7).

In this study, the Real time PCR method is employed for utilization of the genes as biomarkers (7, 8). *HCN4* encodes a protein named Potassium/sodium hyper polarization activated cyclic nucleotide-gated channel which has a role in heart rhythm (6, 9).

ADAM8 encodes a protein which is an enzyme and called A disintegrin and metalloproteinase domain-containing protein 8 (6). Activation of neutrophils by pro-inflammatory cytokines could induce and stimulate fast transition of *ADAM8* to the cell membrane (10, 11).

This study aims to investigate the expression of these two genes using peripheral blood of patients with ventilator-associated pneumonia.

Methods

This study was a case control study, which was done in Masih Daneshvari Hospital affiliated to Shahid Beheshti University of Medical Sciences, Tehran, Iran, since 2015-2016. The ethical code number was No.IR.SBMU.NRITLD.REC.1394.738. 30 patients with VAP and 30 healthy individuals in control group were selected by ICU experts. The control group revealed no VAP symptom after clinical examinations. The control group was matched

to the patients in terms of age and sex. The consent forms were also completed by both groups.

Sampling

In current study, peripheral blood samples were used. The amount of, 1.5ml peripheral blood was taken from both groups. RNA extraction was performed by using RNA Blood Minikit (Qiagen, Germany) according to manufacturer instruction.

The quality and quantity of the extracted RNA were analyzed by Nano Drop. The cDNA synthesis was performed by using (Sinaclon, Iran) Viva 2-sTep RT PCR kit. The purity and quantity of cDNA were measured by Nano Drop. cDNAs were kept at -80°C until they were used for Real-time PCR. The quality of cDNA for real-time RT-PCR was confirmed by observation of 18SrRNA expression. The required primers for each marker were designed using AlleleID7 software and ordered for synthesis. Specifications of the primers are shown in Table 1.

Real time RT-PCR

To evaluate the presence of *HCN4* and *ADAM8* genes, cDNA vials were used for real Real time PCR by using Cinna Green PCR Mix kit (Sinaclon, Iran). The reaction components included the template (2 μl), master mix (4 μl) and (0.5 μl) of each primer (R and F). The final volume was obtained 20 μl by deionized distilled water. Positive and negative controls were also used for quality control.

Statistical Methods

The results of the tests were analyzed by using the SPSS software Version 20. The data mean and standard deviation were calculated in both groups and the t-test was performed. The sample size was calculated using sample size estimation formula considering type one error of 5% and type two errors of 20%. The relationship between gene expressions was analyzed using the Chi-square test.

Results

The participants in this study were included 30 patients with VAP (21 males and 9 females) and 30 healthy individuals (22 males and 8 females). The mean age was 56 ± 5.91 and 50.52 ± 8.84 for case and control groups respectively ($p=0.589$). Also, No statistically significant difference was noted between two groups ($p=0.698$).

Table 1: Specifications and sequences of primers used in Real time PCR.

	Gene		
	HCN4	ADAM 8	18s rRNA
Forward primer	AGAGCGCGTAGGAGTACTGCTT	AAGCAGCCGTGCGTCATC	GTAACCCGTTGAACCCCAT
Primer length	22	18	20
Reverse primer	GACTGCTGGGTGTCCATCAA	AACCTGTCCTGACTATTCCAAATCTC	CCATCCAATCGGTAGTAGCG
Primer length	20	26	20

Table 2: Compared gene expression in patients with VAP.

gene	Ratio of gene expression	Folding change gene	Folding change gene
		HCN4	ADAM8
HCN4	10/30	2.54	—
ADAM8	13/30	—	3.08

Expression of 18SrRNA reference gene

In this study, the 18srRNA gene was selected as the reference gene and the threshold cycle (Ct) value of 18SrRNA reference gene, which was determined by PCR, was reported for each sample. The average CT was statistically analyzed for patients and control group and it was shown that there is no significant difference between the two groups (p=0.680), and indicated that the selection of this biomarker as the reference gene was appropriate.

Analysis of HCN4 and ADAM8 gene expression

In patient samples 10 out of 30 cases (10/30) did not have positive HCN4 gene expression. Among

the healthy individuals, 6 out of 30 cases did not have positive HCN4 gene expression. In patient samples 13 out of 30 cases (13/30) did not have positive ADAM8 gene expression and 8 out of 30 healthy individuals did not have positive ADAM8 gene expression. To increase the sensitivity, the tests were performed in triplicate.

The difference between gene expression in peripheral blood of patients and healthy individuals

To compare between two groups, $\Delta\Delta Ct$ method was applied. Finally, folding change was calculated by the equation $2^{-\Delta\Delta Ct}$. Comparison of gene expression in patients with VAP was shown in table 2.

Comparing the ΔCt obtained for the HCN4 gene showed statistically significant differences between the two groups of patients and healthy ones (p=0.038).

Comparing the ΔCt obtained for the ADAM8 gene showed statistically significant differences between the two groups of patients and healthy subjects (p=0.040).

Discussion

Nosocomial infections are an important factor resulted in increased mortality rate in patients admitted to ICU (12). VAP is one of the most serious

and common infections in the ICU and are included about 15-20 percent of nosocomial infections (13). According to the reports, this disease can increase mortality up to 30% (14). VAP has two types, early and late. Early onset occurs 48 to 96 hours after intubation and late onset after 96 hours (3, 15).

According to some studies, use of silver-coated endotracheal tubes in ventilation devices, compared with conventional tubes, can delay early-onset VAP (16).

In addition, there are also some strategies which are effective in the prevention of VAP such as hands and mouth hygiene, staff training, putting the open side of the tube at a proper position and so on (16).

Regarding the statistics, the patients admitted to ICU, especially hospitalized patients with underlying conditions who take invasive procedure in their treatment, form a majority of individuals who are mostly at risk of nosocomial infections (17).

Therefore, evaluating and examining genes or biomarkers by using peripheral blood can be one of the preventive methods of infectious diseases in the ICU at different stages. This is similar to examining biomarkers used in cancers (7, 8).

In this study, two *HCN4* and *ADAM8* genes have been evaluated in patients with VAP. There are different methods for the study of biomarkers, but Real time PCR method is a suitable and ideal method for evaluating genes and is of relatively high sensitivity (7, 18).

The use of biomarkers to evaluate a disease by biological samples, such as peripheral blood, is more used for diseases such as cancers. However, with advances in molecular and genomic sciences, usage of these examinations in other diseases is growing (7).

A number of previous studies suggest that there are genetic differences which can affect the risk of nosocomial infections (6).

Furthermore, changes in the pattern of gene expression can also be used in early diagnoses of nosocomial infections such as VAP (19, 20). McDunn et al. evaluated the expression of 85 genes in their study, which can be used in detection and diagnosis of VAP patients (6).

Conclusion

The current study assessed the expression of *HCN4* and *ADAM8* as biomarkers for detection and diagnosis of VAP in primary stages using a peripheral blood sample. It should be noted that use of molecular markers for detection of infections such as VAP is still at a preliminary phase and further studies on other molecular markers are required to obtain more reliable results in this respect.

Acknowledgment

The authors would like to acknowledge the kind efforts of authorities and health care personnel, National Research Institute of Tuberculosis and Lung Diseases (NRITLD), Shahid Beheshti University of Medical Sciences, Tehran, Iran

Conflicts of Interest

The authors declare that there are no conflicts of interest.

References

1. Nassaji M, Mosavi S, Ghorbani R. Incidences of nosocomial pneumonia in patients above 15 years in intensive care units of university hospital in Semnan. *Koomesh*. 2004;5(1):89-94.
2. Charles MP, Easow JM, Joseph NM, Ravishankar M, Kumar S, Umadevi S. Incidence and risk factors of ventilator associated pneumonia in a tertiary care hospital. *The Australasian medical journal*. 2013;6(4):178.
3. Gastmeier P, Geffers C. Prevention of ventilator-associated pneumonia: analysis of studies published since 2004. *Journal of Hospital Infection*. 2007;67(1):1-8.
4. Silva Júnior JMd, Rezende E, Guimarães T, Campos EVd, Magno LA, Consorti L, et al. Epidemiological and microbiological analysis of ventilator-associated pneumonia patients in a public teaching hospital. *Brazilian journal of infectious diseases*. 2007;11(5):482-8.
5. Tsai H-H, Chang S-C, Lin F-C. Intermittent suction of oral secretions before each positional change may reduce ventilator-associated pneumonia: a pilot study. *The American journal of the medical sciences*. 2008;336(5):397-401.
6. Swanson JM, Wood GC, Xu L, Tang LE, Meibohm B, Homayouni R, et al. Developing a gene expression model for predicting ventilator-associated pneumonia in trauma patients: a pilot study. *PloS one*. 2012;7(8):e42065.
7. Karimi S, Mohamadnia A, Nadji SA, Yadegarazari R, Khosravi A, Bahrami N, et al. Expression of two basic mRNA biomarkers in peripheral blood of patients with non-small cell lung cancer detected by real-time rt-PCR, individually and simultaneously. *Iranian*

- biomedical journal. 2015;19(1):17.
8. Mohamadnia A, Karimi S, Yadegar Azari R, Naji SA, Khosravi A, Bahrami N, et al. Expression Of CK19 Gene In Patients With Lung Cancer And Its Comparison With Carcinoembryonic Antigen In Peripheral Blood. *Journal of Payavard Salamat*. 2016;9(5):459-68.
 9. Baruscotti M, Bottelli G, Milanesi R, DiFrancesco JC, DiFrancesco D. HCN-related channelopathies. *Pflügers Archiv-European Journal of Physiology*. 2010;460(2):405-15.
 10. Gómez-Gavero M, Domínguez-Luis M, Canchado J, Calafat J, Janssen H, Lara-Pezzi E, et al. Expression and regulation of the metalloproteinase ADAM-8 during human neutrophil pathophysiological activation and its catalytic activity on L-selectin shedding. *The Journal of Immunology*. 2007;178(12):8053-63.
 11. Smalley D, Ley K. L-selectin: mechanisms and physiological significance of ectodomain cleavage. *Journal of cellular and molecular medicine*. 2005;9(2):255-66.
 12. Chawla R. Epidemiology, etiology, and diagnosis of hospital-acquired pneumonia and ventilator-associated pneumonia in Asian countries. *American journal of infection control*. 2008;36(4):S93-S100.
 13. Rocha LdAd, Vilela CAP, Cezário RC, Almeida AB, Gontijo Filho P. Ventilator-associated pneumonia in an adult clinical-surgical intensive care unit of a Brazilian university hospital: incidence, risk factors, etiology, and antibiotic resistance. *Brazilian Journal of Infectious Diseases*. 2008;12(1):80-5.
 14. Kalanuria AA, Zai W, Mirski M. Ventilator-associated pneumonia in the ICU. *Critical Care*. 2014;18(2):1.
 15. Sabery M, Shiri H, Taghadosi M, Gilasi HR, Khamechian M. The frequency and risk factors for early-onset ventilator-associated pneumonia in intensive care units of Kashan Shahid-Beheshti hospital during 2009-2010. *Feyz Journals of Kashan University of Medical Sciences*. 2013;16(6).
 16. Sole ML, Byers JF, Ludy JE, Zhang Y, Banta CM, Brummel K. A multisite survey of suctioning techniques and airway management practices. *American Journal of Critical Care*. 2003;12(3):220-30.
 17. Craven DE. Epidemiology of ventilator-associated pneumonia. *CHEST Journal*. 2000;117(4_suppl_2):186S-7S.
 18. Yadegarazari R, Hassanzadeh T, Majlesi A, Keshvari A, Esfahani AM, Tootoonchi A, et al. Improved real-time rt-PCR assays of two colorectal cancer peripheral blood mRNA biomarkers: a pilot study. *Iranian biomedical journal*. 2013;17(1):15.
 19. Cobb JP, Moore EE, Hayden DL, Minei JP, Cuschieri J, Yang J, et al. Validation of the riboleukogram to detect ventilator-associated pneumonia after severe injury. *Annals of surgery*. 2009;250(4):531.
 20. McDunn JE, Husain KD, Polpitiya AD, Burykin A, Ruan J, Li Q, et al. Plasticity of the systemic inflammatory response to acute infection during critical illness: development of the riboleukogram. *PloS one*. 2008;3(2):e1564.