Original Article

Molecular detection of atypical microorganisms in patients with ventilator associated pneumonia

Shahida Akter, Rehana Khatun and S.M Shamsuzzaman

Department of Microbiology, Dhaka Medical College, Dhaka, Bangladesh

Abstract

Ventilator-associated pneumonia (VAP) is one of the major causes of morbidity and mortality among the critically ill patients of intensive care units (ICU). The present cross sectional study was conducted to isolate and identify bacterial causes of VAP among the patients admitted in intensive care unit (ICU) of Dhaka Medical College Hospital. The study was conducted between July, 2013 to June 2014. A total of 65 endotracheal aspirate (ETA) and blood samples were collected from patients with clinically suspected ventilator associated peumonia(VAP). Samples were collected from patients on mechanical ventilation for more than 48 hours. ETA and blood samples were cultured aerobically. Multiplex PCR was performed with ETA to detect *Mycoplasma pneumoniae*, *Legionella pneumophila and Chlamydia pneumoniae*. Among the atypical bacteria, *M. pneumoniae* were detected in 5 (7.69%), *L. pneumophila* in 4 (6.15%) cases by multiplex PCR in ETA from VAP cases. No *C. pneumoniae* was detected. The study revealed that in VAP cases atypical bacteria should be considered as a possible bacterial agents.

Ibrahim Med. Coll. J. 2015; 9(1): 22-25

Introduction

Patients in intensive care unit are at risk of dying not only from their critical illness but also from secondary processes such as nosocomial infection. Pneumonia is the second most common nosocomial infection in critically ill patients, affecting 27% of all critically ill patients and 86% of nosocomial pneumonias are associated with mechanical ventilation.[1,2] The risk for pneumonia increases 3 to 10 fold in patients receiving mechanical ventilation.[3] Ventilator-associated pneumonia (VAP) is a major cause of morbidity and mortality among the patients of intensive care units (ICU).[4] Most cases of VAP are caused by bacterial pathogens that normally colonize upper respiratory tract and gastrointestinal tract of the patient. External sources like transmission from caregivers, environmental surfaces or other patients have been implicated. Common pathogens include *Enterobacteriaceae, Pseudomonas* species, Gram-positive bacteria and *Haemophilus* species.[5] In addition, atypical bacteria like *M. pneumoniae*, *L. pneumophila*, *C. pneumoniae*, viruses and fungi have also been implicated as causes of VAP.[5,6] However, these atypical bacteria cannot be cultured easily and needs special techniques and facilities. Recently, molecular methods like polymerase chain reaction (PCR) has been used to detect these fastidious organisms in clinical samples.

In Bangladesh, no study has yet been done to find out the role of atypical bacteria in VAP. Therefore, the present study was undertaken to determine the presaence of atypical bacteria in the patients with VAP.

Materials and Methods

Study population and sample collection: Patients in ICU having mechanical ventilation for more than 48 hours with suspected VAP were enrolled in the study. Criteria for suspected VAP include a new and persistent (>48-h) or progressive radiographic infiltrate plus two of the following: temperature of >38°C or <36°C, blood leukocyte count of >10,000 cells/ml or <5,000 cells/ml, purulent tracheal secretions, and

Address for Correspondence:

Dr. Shahida Akter, Assistant Professor, Department of Microbiology, Ibrahim Medical College, 122 Kazi Nazrul Islam Avenue, Shahbag, Dhaka-1000, Bangladesh

gas exchange degradation.[2] Endotracheal tube aspirates (ETA) and blood samples were collected from clinically suspected VAP cases. ETAwas collected using a 50 cm and 14Fr suction catheter, which was gently introduced through the endotracheal tube for a distance of approximately 25-26 cm. The ETA was obtained by suction, without instilling saline and the catheter was withdrawn from the endotracheal tube. Two milliliter of phosphate buffered saline (PBS) was injected into the lumen of the catheter with a sterile syringe to flush the exudates.The exudates were collected into a sterile 50 ml Falcon tube and transported immediately to the laboratory for further processing.[7] Only one ETA sample was collected from each patient.[8]

Sample processing for culture and PCR: ETA was mechanically liquefied and homogenized by vortexing for one minute with glass bead (1-2 glass bead). After vortexing sample was centrifuged at 2000 rpm for 10 minutes. Supernatant was discarded using a sterile pipette and the deposit was further mixed by vortexing. The processed specimen was used for culture in recommended media, Gram staining and PCR.

Extraction of DNA: One hundred μl lytic buffer (composition-tris–HCL, proteinase-K and Tween 20 solution) was added to the pellet and vortexed thoroughly. The mixture was incubated at 60°C for 2 hours. After incubation the tube was placed in a block heater (DAIHA Scientific, Seoul, Korea) at 100°C for 10 minutes. Then it was-immediately transferred to the ice and kept for 5 minutes. The solution was then centrifuged at 13000 rpm at 4°C for 10 minutes. The supernatant was used as template DNA.

Primers and PCR assay: The primers as shown in the Table-1 were used for the detection of *Legionella pneumophila, Mycoplasma pneumoniae* and *Chlamydia pneumoniae*.

PCR was performed in a final reaction volume of 25μl in a PCR tube, containing 10 μl of master mix (mixture of dNTP, taq polymerase, $MgCl_2$ and PCR buffer), 2 μl forward primer and 2 μl reverse primer (Promega corporation, USA) 3 μl extracted DNA and 8 μl of nuclease free water. After a brief vortex, the PCR tubes were centrifuged in a micro centrifuge for few seconds.

PCR assays were performed in a thermal cycler (Eppendorf AG). After initial denaturation at 94°C for 10 minutes, the reaction was subjected to 36 cycles. Each cycle consisted of denaturation at 94°C for one minute, annealing at 60°C for one minute and elongation at 72°C for 90 seconds followed by final extension at 72°C for 10 minutes. Then the product was held at 4°C. After amplification products were processed for gel documentation or kept at -20°C till tested. Amplified PCR product was analyzed by electrophoresis in 1.5% agarose gel containing ethidium bromide (0.5 μg/ml) in TBE buffer (0.04 M Tris acetate, 0.001 M EDTA, (pH 8.6) and photographed under UV illumination. In all assays, DNA from known *M. pneumoniae, L. pneumoniae and C. pneumoniae* were included as positive control. A tube without DNA served as no template DNA control.

Table-1: *The primers used for the PCR detection of atypical bacteria.*[9-11]

Organism	Primer Sequence	Product size
C.pneumoniae	F5'-GTTGTTCATGAAGGCCTACT-3'	437
C.pneumoniae	R5'-TGCATAACCTACGGTGTGTT-3'	
L.pneumophila	F5'-AGG GTT GAT AGG TTA AGA GC-3'	386
L.pneumophila	R5'-CCA ACA GCT AGT TGA CATCG-3'	
M.pneumoniae	F5'-TCAATCTGGCGTGGATCTCT-3'	180
M.pneumoniae	R5'-GTCACTGGTTAAACGGACTAO-3'	

Result

A total of 65 suspected VAP cases were enrolled. Out of 65 VAP cases, *M. pneumoniae* and *L. pneumophila* were detected in 5 (7.69%) and 4 (6.15%) cases respectively by multiplex PCR (Table -2 and Fig -1). No *C. pneumoniae* was detected. Out of 9 positve cases which showed presence of *M. pneumoniae* and *L.*

Table-2: *Distribution of atypical bacteria identified by PCR from ETA of VAP patients (n=65)*

Name of the organism	Positive by PCR	
	N	(%)
M. pneumoniae	5 (4* +1)	7.7
L. pneumophila	4 (3*+1)	6.1
C. pneumoniae	0	0
Total	9 (7*+2)	13.8

Note: **Indicate cases where other bacteria were isolated.*

Table-3: *Distribution of other organisms isolated from VAP cases positive for atypical bacteria*

Atypical bacteria	No. of case	Other organisms isolated
M. pneumoniae	4	Case 1: *Staphylococcus aureus* Case 2: *Streptococcus pneumoniae* Case 3: *Acinetobacter baumannii* Case 4: *Pseudomonas aeruginosa*
L. pneumophila	3	Case 1: *Klebsiella pneumoniae* Case 2: *Acinetobacter baumannii* Case 3: *Pseudomonas aeruginosa*

pneumophila, only 2 cases did not have any other pathogen by culture. Seven cases had mixed infection (Table-3) along with the presence of atypical bacteria.

Discussion

The present study has revealed that atypical bacteria are important causes of VAP, besides typical bacteria which are routinely detected by culture of ETA or bronchoalveolar lavage. In the present study, 13.84% VAP cases had infection with atypical bacteria like *M. pneumoniae* and *L. pneumophila*. But it is to be noted that except 2 cases, majority of the cases had mixed infection with other bacteria. Studies in other countries also reported the presence of such atypical bacteria in VAP cases. The reported rate of infection ranged from 6.6% to 15%.[12,13]

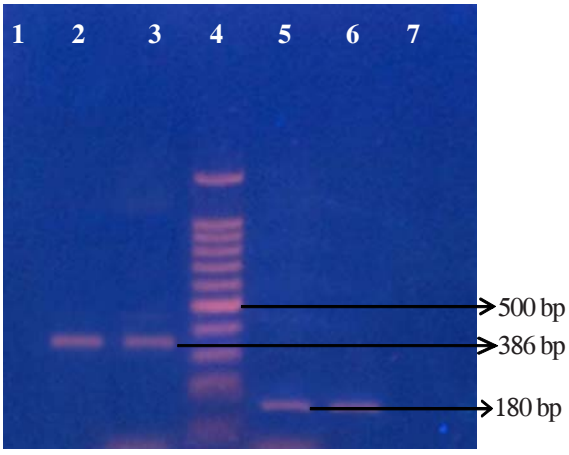

Fig-1. Multiplex PCR showing amplified DNA of *L. pneumophila* and *M. pneumoniae*. Lane 1: negative control (DNA of *Ps. aeruginosa*). Lane 2: positive control of *Legionella pneumophila*. Lane 3: ETA test sample. Lane 4: 100bp DNA ladder. Lane 5: ETA test sample. Lane 6: Positive control of *M. pneumoniae* Lane 7: Negative control (DNA of *K. pneumoniae*)

The findings imply that atypical bacteria should be looked for in VAP cases for specific or targeted antibiotic treatment. Additional serological investigations may be done in these cases to determine the active infection by organism like *M. pneumonia*. Serological investigation may be useful to diagnose infection by the atypical bacteria in absence of molecular diagnostic facilities. The identification of atypical bacteria causing VAP is important as it would reduce unnecessary antibiotic use.

References

1. Richards MJ, Edwards JR, Culver DH, Gaynes RP, *et al*. Nosocomial infections in Medical intensive care units in the United States. *Crit Care Med* 1999; **27**: 887-892.
2. Koenig SM, Truwit JD. Ventilator–Associated Pneumonia: Diagnosis, treatment and prevention. *Clin Microbiol Rev* 2006; **19**(4): 637-657.
3. Chastre J, Fagon JY. Ventilator-associated pneumonia. *Am J Respir Crit Care Med* 2002; **165**(7): 867–903.
4. Craven DE, Chroneou A, Zias N, Hjalmarson KI. Ventilator-Associated Tracheobronchitis. The impact of targeted antibiotic therapy on patient outcomes. *Chest* 2009; **135**: 521-528.
5. Park DR. The Microbiology of Ventilator-Associated Pneumonia. *Respiratory Care* 2005; **50**(6): 742-765.
6. Dey A, Bairy I. Incidence of multidrug resistant organisms causing ventilator-associated pneumonia in a tertiary care hospital: a nine month prospective study. *Ann Thorac Med* 2007; **2**(2): 52-57.
7. Park DR. Antimicrobial treatment ofVentilator-associated pneumonia. *Respiratory Care* 2005; **50**(7): 932-955.
8. Goel V, Hogade SA, Karadesai SG. Ventilator associated pneumonia in a medical intensive care unit: Microbial aetiology, susceptibility patterns of isolated organisms and outcome. *Indian Journal of Anaesthesia* 2012; **56**(6): 558-562.
9. Verkooyen R, P, Willemse D, Casteren S.C.A.M, Joulandan S.A.M, *et al*. Evaluation of PCR, Culture and Serology for Diagnosis of *Chlamydia Pneumoniae* Respiratory infections. *J Clin Microbiol* 1998; 2301-2307.
10. Wellinghausen N, Frost C, Marre R, Detection of *Legionellae* in Hospital Water Samples by Quantitative

Real – Time Light-Cycler PCR. Appl Environ Microbiol 2001; 3985-3993.

11. Afrin S. Bacterial causes of ventilator associated respiratory tract infection. (M.Phil Thesis) Dhaka, Bangladesh: Department of Microbiology, DMC 2013.
12. Mokhless NAS, Mofty MFE, Hanafi NF, Fayed AM, Asser SF. Atypical Bacteria in Ventilator Associated Pneumonia; an Egyptian University Hospital Esperience. *Journal of American Science* 2010; **6**(12): 1074-1079.
13. Abukhabar H, Atta S, Bedewy K, Youssef A, Moustafa A. Ventilator associated pneumonia: Therole of typical, atypical bacteria and fungi; comparison between endotracheal aspirate and bronchoalveolar lavage. *Egyptian Journal of Bronchology* 2007; **1**: 53-61.