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ORIGINAL ARTICLE

Detection of causative agents of bacterial pneumonia in hospitalized hajj and umrah cases by multiplex real-time polymerase chain reaction

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ABSTRACT

Background and Objectives: Middle East Respiratory Syndrome Coronavirus (MERS-CoV) is commonly detected in pneumonia patients who travel from the Middle East regions. Besides MERS-CoV, many other pathogenic agents cause pneumonia. Detection of such organisms must be done swiftly, especially in case of the negative MERS-CoV samples. The aim of this study was to identify the pathogenic agents that might account for bacterial pneumonia, from Hajj and Umrah pneumonia cases.

Materials and Methods: We conducted a cross-sectional study, 38 pneumonia clinical samples from suffering of Hajj and Umrah in 2017 with negative MERS-CoV were selected. The laboratory testing was done at National Reference Laboratory in Jakarta and performed by multiplex real-time PCR using a FTD respiratory pathogens.

Results: *Haemophilus influenzae* (26.4%) was the most frequent bacteria detected. Other causative agents of bacterial pneumonia identified were *Moraxella catarrhalis* (20.8%), *Klebsiella pneumoniae* (13.2%), *Streptococcus pneumoniae* (9.4%), and *Staphylococcus aureus* (5.7%). From 38 samples showed that 25 (65.79%) samples were positive with bacteria, including five samples with coinfection. The coinfection were combinations among *S. aureus* and *S. pneumoniae* (1/20), *S. pneumoniae* and *K. pneumoniae* (1/20), *S. pneumoniae* and *M. catarrhalis* (2/20), *S. pneumoniae* and *H. influenzae* (2/20), *K. pneumoniae* and *H. influenzae* (5/20), and *M. catarrhalis* and *H. influenzae* (5/20).

Conclusion: *Haemophilus influenzae* is the most recurrent bacteria to be identified in samples of pneumonia of hajj and umrah cases.

Keywords: Pneumonia; Multiplex real-time polymerase chain reaction; Bacteria; Hajj and umrah

INTRODUCTION

Middle East Respiratory Syndrome Coronavirus (MERS-CoV) is a viral respiratory infection caused by the MERS-coronavirus. Since 2012 MERS-CoV has become endemic in the Arabian Peninsula countries including the Kingdom of Saudi Arabia. MERS-CoV is commonly detected in pneumonia patients who had a history of travel from the Middle East region. Therefore, pneumonia patients who had trav-

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eled from Arabian Peninsula usually called MERS-CoV suspected patients (1). Cases of pneumonia in Hajj pilgrims or Umrah continue to increase after the discovery of MERS-CoV. The results of studies conducted at seven hospitals at the pilgrimage sites (Mina and Arafat) showed a high number of pneumonia cases during the annual Islamic pilgrimage and the numbers had reached 19.7% and 22% in 2003 and 2004 even before the discovery of MERS-COV, while the incidence of pneumonia increased to 63.9% in 2014 due to MERS-CoV infections (2, 3).

This situation raises our concern since each year around 200,000 Indonesian people going to Saudi Arabia for Hajj. The study from Pane et al. (2017) found that respiratory system diseases was one of top two causes of mortality among Hajj pilgrims (4). Pneumonia can occur due to infection with various pathogenic agents such as viruses, bacteria, and fungi, causing swelling of the lung parenchyma. The major cause of mortality and morbidity of pneumonia is bacteria (5). Bacterial detection can be evaluated by staining and culturing, enzyme-linked immunosorbent assay (ELISA), and serology. Unfortunately, these methods are quite time consuming (6). Therefore, this study used the easier method of detection to evaluate the possibility of bacterial caused pneumonia in 38 samples of pneumonia suspected cases from Hajj and Umrah. This technique is more effective and efficient due to its high sensitivity. The aim of this study was to find the pathogenic bacterial caused pneumonia from Hajj and Umrah patients using Multiplex Real-Time RT-PCR analysis. This assessment provides an estimate of the prevalence of each kind of bacterial pneumonia in addition to the efficient laboratory inspection to diagnose the etiology of pneumonia.

MATERIALS AND METHODS

This study is a descriptive cross-sectional study which used 38 archived clinical samples of hajj and umrah pilgrims suffering from pneumonia in 2017. The upper and lower respiratory tract samples were collected from the hospitalized cases. The samples were sent to the Laboratory of Center for Research and Development of Biomedical and Basic Health Technology (CRDBBHT), National Institute of Health Research and Development, Ministry of Health Republic of Indonesia, Jakarta as National Reference Laboratory for further testing. However, due to the PCR examination of the virus, it was found that all the samples were negative for MERS-CoV, therefore we did the detection of the other bacterial pathogens.

Specimens preparation. The specimens used in this research were upper (throat swabs) and lower respiratory tract specimens (tracheal aspirate and sputum), which were stored frozen at -80ºC prior to nucleic acid extraction. The sputum was retrieved and diluted with 1:1 dithiothreitol (DTT) and phosphate buffer saline (PBS), and then incubated at room temperature for 30 min according to manufacture instruction for extraction preparation. Tracheal aspirate is the other option if the patient is no longer in a position to yield sputum. The upper respiratory tract specimens were taken by Dacron swabs and swabbed with the epithelium, and then the swabs immediately inserted into the tube containing virus transport medium (VTM) (7).

Nucleic acid extraction and negative control extraction. Nucleic acid samples and negative control were extracted using QIAamp® (Qiagen, Hilden, Germany) viral RNA/DNA mini kit according to manufacturer's instructions. Negative control is required for the validation test conducted in the diagnosis of nucleic acids in the laboratory. One hundred forty microliters of each respiratory specimen were eluted to a final volume of 60 µL of elution buffer.

Multiplex one step real-time PCR method. The FTD kit (respiratory pathogens 33) can detect the presence of pathogen specific sequences in samples with high sensitivity. This kit consists of eight panels to detect 33 pathogens causing respiratory tract infections in a sample in a single PCR reaction according to manufacturer's instructions. Three panels (RespBac, KLepSa and MoBoCH) were used to detect bacterial pathogens in the present study. The RT-PCR reaction mix for 38 samples was prepared using the reagent reaction mix kit FTD (respiratory pathogens 33). The reaction mix consists of 12.5 µL 2× RT-PCR buffer (Fast-track master mix), 1.5 µL primer and probe, and 1 µL 25× RT-PCR enzyme mix.

Multiplex one step real-time RT-PCR analysis. The extracted nucleic acids were amplified using Bio-Rad Real-Time thermocycler in multiplex RT-PCR reaction. Initially, RNA genome samples were

reverse transcribed into cDNA using designated primers and reverse transcriptase. Next, the DNA and cDNA from different pathogens were amplified simultaneously in the same tube. The presence of specific pathogen sequences in each sample of the reaction will be detected by the increase in the fluorescence intensity of the probe, and interpreted as Cycle Threshold (Ct) value by Real-Time Thermocycler according to manufacturer's instructions.

Interpretation of results and validation of test. The interpretation of the results and the validation of the test were adjusted according to the manual of FTD Respiratory pathogens 33. The cut-off point value Ct>33 indicates negative sample, according to manufacturer's instructions.

Data analysis. Descriptive data analysis of bacterial that were found from the patients was described by multiplex real-time RT-PCR results.

Ethical consideration. The study was part of surveillance activity to elaborate the cause of the pneumonia. There is no individually identifiable and all specimens were fully anonymized and could not be tracked back.

RESULTS

Detection of bacterial pneumonia in suspected MERS-CoVcases using a Multiplex real time RT-PCR analysis successfully amplified the DNA and cDNA fragments from the bacterial genomes of the samples. This investigation indicates that the detection results meet all validation requirements of the FTD kit and the method is considered reliable and accuracy.

The results showed that out of 38 samples of pneumonia patients, there were 25 (65.79%) samples positive for bacterial infections. The percentages of each of the bacterial species detected in the positive samples have been presented in Table 1. This study found that *Haemophilus influenzae* was the most frequent bacteria detected in samples of the pneumonia cases; which was 26.4% (14/53) of the samples. The other bacterial pneumonia agents detected were *Moraxella catarrhalis* (20.8%; 11/53), *Klebsiella pneumoniae* (13.2%; 7/53), *Streptococcus pneumoniae* (9.4%; 5/53), and *Staphylococcus aureus* (5.7%; 3/53).

The results of bacterial detection were not only detected bacteria in a mono infection, but could also detect co-infected cases in these samples. Our assessment outcome shows that the frequency of co-infection was 80% (20/25) of the population of positive samples. The co-infection determined in samples were combinations between *S. aureus* and *S. pneumoniae* (1/20; 5%), *S. pneumoniae* and *K. pneumoniae* (1/20; 5%), *S. pneumoniae* and *M. catarrhalis* (2/20; 10%), *S. pneumoniae* and *H. influenzae* (2/20; 10%), *K. pneumoniae* and *H. influenzae* (5/20; 25%), and *M. catarrhalis* and *H. influenzae* (5/20; 25%).

Table 1. Frequency of different bacterial infection

Pneumonia Bacteria Species	Total	
	N	%
Staphylococcus aureus	3	5.7
Chlamydia pneumoniae	0	0
Streptococcus pneumoniae	5	9.4
Pneumocystis jiroveci	0	0
Legionella pneumophila	0	0
Legionella long beachae	0	0
Klebsiella pneumoniae	7	13.2
Salmonella sp.	0	0
Moraxella catarrhalis	11	20.8
Bordetella pertussis	0	0
Haemophilus influenzae	14	26.4
Negative	13	24.5
Total Number	53	100

DISCUSSION

The results of our study enabled us to show that more than half of the samples had a positive result. The positive samples were detected with mono-infection and co-infections. The bacteria detected were commonly found in people with pneumonia. As many as 84.6% of bacterial pneumonia agents were detected in patients with pneumonia in Saudi Arabia during the Hajj. The most frequent types of bacteria detected were *Haemophilus influenzae*, *Streptococcus pneumoniae*, and *Moraxella catarrhalis* (2). The bacterial types identified in sputum samples of patients with pneumonia during the Hajj in 2013 were *Haemophilus influenzae, Klebsiella pneumoniae, Streptococcus pneumoniae,* and *Staphylococcus aureus* (2, 8).

H. influenzae is known to be the leading cause of pneumonia in adults (9). In the United State the in-

cidence of *H. influenzae* infection was estimated increase to 6.50 cases per 100,000 population of people aged over 65 years. The cases of *H. influenzae* infection are more commonly caused by *H. influenzae* non type-B and non-typeable *H. influenzae* (10). This is in coherence with the results obtained from the samples used in this study. Samples of pneumonia patients in this study came from Umrah congregation with an average age of more than 60 years. Meanwhile, 8.8% of the bacteria *K. pneumoniae* and 5.4% *S. pneumoniae* were found in sputum samples of patients with pneumonia during the Hajj in 2005 (11). *S. aureus* infection increases the number of pneumonia patient cases up to 32.6% (12).

Co-infection cases generally occur in patients with pneumonia. The most common combination of bacteria that cause coinfection is *H. influenzae* and *S. pneumoniae* (13). *H. influenzae* and *S. pneumoniae* are commensal bacteria in the nasopharynx with the ability to migrate to other organs, causing a myriad of respiratory diseases such as pneumonia. The interaction between *H. influenzae* and *S. pneumoniae* is competitive and cooperative in order to avoid the host immune response. However, the factors that affect the interaction between *H. influenzae* and *S. pneumoniae* are still unknown (14).

The high frequency of co-infection in this study may possibly be attributed to the use of upper respiratory tract samples for detection of pneumonia pathogens. The upper respiratory tract has the same normal flora as that of the mouth. Some pathogenic bacteria such as *S. aureus, S. pneumoniae* and *H. influenzae* can be found in the pharynx or upper respiratory tract in healthy individuals. The presence of these organisms in the upper respiratory tract (throat) infections does not imply that it is the primary etiological agent of the disease. The organism generally colonizes in the upper respiratory tract as a commensal bacterium, while it is rarely found in the lower respiratory tract because the lower respiratory tract is protected by the ciliary epithelial movement (15).

The high co-infection frequency in this study may also be due to the origin of the samples. The samples used in this study were procured from hospitals which might consequently affect the research outcomes. This is because pneumonia can occur due to nosocomial infections. *K. pneumoniae* is the main cause of nosocomial pneumonia or pneumonia in patients who acquire respiratory infections in the hospitals (16). It is reported that as much as 32% of patients with acute lower respiratory tract infections in the hospitals exhibited the presence of *M. catarrhalis* colonization (17).

The limitation of this study is the use of upper respiratory tract samples (throat swabs) for detection of pneumonia bacteria. It possibly causes the commensal bacteria in the upper respiratory tract to be detected, which does not necessarily cause pneumonia. Besides, the use of a test panel that detects only some of the specific pneumonia causing bacteria means missing out on other pathogenic pneumonia causing bacteria not included in the test panel, during the entire detection process. Some pathogenic agents such as *Pseudomonas aeruginosa* and *Candida albicans* have been reported as the causative agents of pneumonia during the Hajj (11).

CONCLUSION

Besides MERS-CoV, many other viral and bacterial agents cause pneumonia. Therefore, detection of such organisms must be done swiftly, especially in case of the negative MERS-CoV samples. Our investigation using a multiplex real time RT-PCR analysis found that *Haemophilus influenzae* is the most common bacteria to be identified in samples of Hajj and Umrah pneumonia cases.

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