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Original article

Diagnostic detection of intended bacteria associated with respiratory tract infections among Kelantanese Malaysian Hajj pilgrims by a ready-to-use, thermostable multiplex PCR assay

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A R T I C L E I N F O

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A B S T R A C T

Bacterial respiratory tract infections (RTIs) are prone to be associated with serious health problems during the annual Hajj pilgrimage and are a public health concern due to the potential of pathogens transmission across continents. This study aimed to perform a diagnostic screening of intended bacteria associated with RTIs among Malaysian Hajj pilgrims by using a newly developed PCR assay. Expectorated sputum specimens ($n = 202$) and sociodemographic characteristics of the returning Hajj pilgrims were collected upon arrival in Kelantan, Malaysia. Diagnostic screening of bacterial respiratory pathogens was performed using a thermostabilized multiplex PCR assay in parallel with the sputum culture. Of the six intended bacteria: *Haemophilus influenzae, Klebsiella pneumoniae, Mycobacterium tuberculosis, Pseudomonas aeruginosa, Staphylococcus aureus* and *Streptococcus pneumoniae*, the sputum specimens were found positive for *H. influenzae* ($n = 139$), *K. pneumoniae* ($n = 20$), and *S. pneumoniae* ($n = 19$) by the multiplex PCR assay. The sensitivity, specificity, positive- and negative predictive values (PPV and NPV) of this assay were 100% (95% confidence interval (CI): 97.85% to 100.00%), 92.23% (95% CI: 85.27% to 96.59%), 95.51% (95% CI: 91.61% to 97.64%) and 100.00%, respectively. The accuracy of this assay was 97.07% (95% CI: 94.31% to 98.73%). Overall, *H. influenzae* was found to be the predominant organism in the pilgrims' sputa by both molecular and microbial culture methods. The multiplex PCR assay would enable a simple, faster and reliable means for the massive screening of intended bacteria compared to the sputum culture, especially during the Hajj pilgrimage.

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1. Introduction

Hajj is the annual Muslim pilgrimage to Mecca and specified holy sites in the Kingdom of Saudi Arabia (KSA). During the years before the COVID-19 pandemic, over two million Muslims from more than 180 countries around the world gathered in Mecca to participate in the rituals of Hajj. This pilgrimage was hence inevitably associated with various communicable and non-communicable health risks due to the massive gathering of pilgrims who were closely surrounded in the confined area, doing the same thing at the same time (Shujaa and Alhamid, 2015). The crowd density of pilgrims during Hajj can reach about eight to nine people per square meter (ppm^2) (Shujaa and Alhamid, 2015) and at a certain time to 12 ppm^2 during tawaf and closing to Kaaba (Rahman et al., 2017).

The massive gathering also could encourage disease transmission, especially of airborne pathogens. Respiratory tract infections (RTIs) have been reported to represent the top communicable diseases and accounted for the highest hospital admissions during Hajj (Alzeer, 2009; Goni et al., 2019). The severity of RTIs may vary from mild respiratory symptoms to severe pneumonia and tuberculosis, which require hospitalization or end in death. Moreover, among the major concerns is the potentially severe consequences

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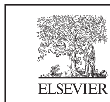

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of RTIs due to the importation or exportation of the pathogens. The spread of pathogens among pilgrims and back to their home countries would contribute to the globalization of respiratory infections (Goni et al., 2020; Shujaa and Alhamid, 2015). The causative pathogens can be easily transmitted in a crowded environment by the air, droplet, or direct hand-to-hand contact with infected secretions. The pathogens subsequently pass through the respiratory tract and produce symptoms corresponding to the area being infected (Mossad, 2013).

Sputum cultures and microscopy have been used for microbiological diagnosis of RTIs in the laboratories. The advantage of this method for broad screening of various microorganisms and antibiotic susceptibility testing is undeniable. However, some of the major drawbacks of this technique due to its lengthy-time (at least 24 h from culture to bacterial positivity) and laborious procedure could delay the appropriate treatment and management of RTIs, especially during Hajj. The crowd density and restricted duration of Hajj rituals in confined areas, plus the conditions of fatigue and elderly pilgrims, are among the challenges that require a prompt and accurate diagnosis to minimize the risks of horizontal spread, mortality, prolonged hospitalization, and multidrug-resistant organisms. Hence, detection by molecular techniques such as PCR-based assay could facilitate a more reliable and faster means to identify the microbial associated with RTIs.

However, most of the PCR reagents conversely require cold chain transportation and cold storage, due to their heat sensitivity. Thus, there will be additional expenses for packaging and storage, plus inconvenience in the reaction setting up, down to the freeze-thawing procedure. Out of these, degradation of the reagents' stability will be the major problem because this will lead to inconsistent and irreproducible results. To overcome the limitations, a thermostabilized multiplex PCR assay was developed by our research group (Nik Zuraina et al., 2021). The PCR assay had 100% accuracy performance when initially tested on bacterial isolates (n = 145) and had the lowest limit of detection (LOD) of 200 bacterial cells/reaction (or 1×10^5 cells/ml) (Nik Zuraina, 2020). This assay was found stable at ambient temperature, which requires no conventional cold transport/storage and is ready for immediate use. The ready-to-use, dried reagent-based PCR might be helpful in the management of communicable RTIs, especially under mass gatherings and other challenging conditions. This study was therefore aimed to perform a diagnostic screening of respiratory bacteria associated with RTIs using the newly developed PCR assay on sputum specimens collected from Malaysian Hajj pilgrims.

2. Materials and methods

2.1. Collection of sputum specimens

This cross-sectional study was conducted during the year 2016 Hajj pilgrimage. Sample collection was done at the arrival hall of Sultan Ismail Petra Airport, Kelantan, Malaysia upon the return of the pilgrims from Hajj. Participation in this study was completely on a voluntary basis. The returning pilgrims who had the symptoms of RTIs during the Hajj were eligible to join this study. Following the written consent, data for demographic of the pilgrims and the presence of respiratory symptoms such as fever, shortness of breath, cough, runny nose and sore throat were recorded. Pilgrims under the age of 18, absence with any respiratory symptoms or incapable of providing deep cough sputum were not eligible to participate in this study. For the collection of expectorated sputum specimens, the enrolled pilgrims were requested to cough up sputum into a sterile container. All the specimens were labelled accordingly and stored temporarily in cool boxes containing ice packs. The sputum specimens were transported to the laboratory within one hour after the specimen collection and immediately processed for microbial identification by using sputum culture, as described in the following section. Subsequent to microbial sputum culture, an aliquot of 0.5–1 ml sputum was collected into 1.5 ml tube for further DNA extraction.

2.2. Microbial identification by sputum culture

Microbial identification by sputum culture, gram staining and standard biochemical tests was performed by medical laboratory technologists in accordance with the Clinical and Laboratory Standard Institute standards. Observation of haemolytic reaction, growth of gram negative and *Haemophilus* species bacteria were identified by culturing the good quality sputa (>25 leukocytes and <10 squamous epithelial cells/low power field) onto trypticase soy agar with 5% sheep blood, McConkey agar and chocolate agar with bacitracin (Thermo Scientific, Malaysia), respectively. The cultured media were incubated at 37 °C overnight in the presence of 5% carbon dioxide. Further confirmation tests include biochemical tests for gram negative bacteria, optochin discs (Oxoid, United Kingdom) for *Streptococcus pneumoniae*, X (hemin), V (nicotinamide adenine dinucleotide) and X + V factor discs (Oxoid, United Kingdom) for *Haemophilus* species differentiation, latex agglutination test for *Staphylococcus aureus*, and Vitek II system (BioMérieux, France) to further validate the organism. Simultaneously, the presence of *Mycobacterium tuberculosis* in the sputum specimens was also checked by using Ziehl–Neelsen staining, followed by cultivation on Ogawa medium for at least two weeks at 37 °C.

2.3. Sputum processing and DNA template preparation for PCR assay

Leftover sputum specimen from microbial sputum culture was initially treated in the ratio of 1:1 vol with liquefying solution containing 4% sodium hydroxide (NaOH) and 1 % N-acetyl-L-cysteine (NaLC) to reduce the sputum viscosity. After 15 min of the liquefying procedure, the sputum matrix was collected by centrifugation at 13,000 × g for 5 min. Supernatant was decanted and 1 ml of normal saline (0.9% sodium chloride) was added to wash the pellet. The solution was re-centrifuged, and the sputum pellet was collected for DNA extraction method. Following the sputum processing, DNA template for PCR was prepared using the QIAamp DNA Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instruction. The extracted DNA was analyzed using the developed multiplex PCR assay for the detection of six targeted bacterial pathogens from previous study (Nik Zuraina et al., 2021).

2.4. PCR detection of respiratory bacteria

DNA samples from pilgrims' sputa were proceeded with PCR amplification using the thermostabilized, multiplex PCR assay as described previously (Nik Zuraina et al., 2021). The lyophilized PCR assay vials contained an optimized concentration of primers, enzyme stabilizer and other PCR components (Nik Zuraina et al., 2021). The assay vials were initially rehydrated with 18 µl of water and allowed to completely dissolve for about 10 min at room temperature. The vials for samples, positive amplification control (PAC) and negative amplification control (NAC) were respectively added with 2 µl of extracted DNA from sputa, 2 µl of PAC template mix and 2 µl of water and were labelled accordingly. A standard PCR cycling condition was set up with an initial denaturation step at 95 °C for 5 min plus 30 repeating cycles for denaturation (95 °C for 30 s), annealing (60 °C for 30 s) and elongation (72 °C for 30 s). Once the cycles completed, a final elongation step at 72 °C for 5 min was added to complete the polymerization process. Five microliter of PCR amplification product was analysed on 1.5% agar-

ose gel electrophoresis, which was run at 90 v for 60 min. The distribution of bacteria isolated from Malaysian Hajj pilgrims was analysed. Sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of the developed PCR assay were determined based on the clinical evaluation and compared with sputum culture as the reference method.

3. Results

3.1. Characteristics of the study participants: Kelantanese Malaysian Hajj pilgrims

Altogether, 202 sputa were used for PCR screening of intended bacteria and subsequently for the validation of PCR assay accuracy. Of these 202 qualified sputum specimens, only 192 pilgrims had completed demographic data and symptomatic RTIs records (Table 1). All of the pilgrims were local Malaysians, and the ratio of male to female was 90 (46.9%) to 102 (53.1%). Their age ranged from 26 to 80 years old with the mean (standard deviation) of 56.7 (9.5).

3.2. Detection of bacteria from Hajj pilgrims using the thermostabilized multiplex PCR assay

An in-house multiplex PCR assay was used to detect six common bacteria associated with RTIs from the Hajj pilgrims' sputa. The representative agarose gel image showed the presence and absence of PCR amplicons from the sputum specimens (Fig. 1). Of the 202 total specimens, the multiplex PCR assay detected positive amplicons from 144 sputa (71.3%), comprising both single- and multi-amplicon bands. Overall, the sputum specimens were found positive for *H. influenzae* (69%, n = 139), *K. pneumoniae* (10%, n = 20), and *S. pneumoniae* (9%, n = 19). In detail, most of the *H. influenzae* positive were observed as single amplicons (n = 111), in contrast to *K. pneumoniae* (n = 2) and *S. pneumoniae* (n = 3) single amplicons. The results indicated that *H. influenzae* appeared predominantly as single organism, whilst, *K. pneumoniae* and *S. pneumoniae* were mostly present in multiple targets. None of the specimens were found positive for *S. aureus, P. aeruginosa* or *M. tuberculosis* by this multiplex PCR assay. The results were compared with sputum culture.

Table 1
Characteristics of the study participants, Malaysian Hajj pilgrims (n = 192).

Characteristics	Frequency, n	Percentage (%)
Nationalities		
Malaysian	192	100
Gender		
Male	90	46.9
Female	102	53.1
Age distributions (years)		
21–40	9	4.7
41–60	113	58.9
61–80	70	36.4
minimum age	26	
maximum age	80	
mean (SD)	56.72 (9.53)	
Symptoms of RTIs		
Fever	61	31.8
Cough	192	100.0
Sore throat	89	46.4
Nasal discharge	52	27.1
Difficulty in breathing	5	2.6
Prescriptions of antibiotics		
Yes	103	53.6
No	47	24.5
Unknown	42	21.9
Total, *n*	**192**	**100.0%**

3.3. Detection of bacteria from Hajj pilgrims by conventional microbial culture method

Identified bacteria from sputum specimens by the microbial culture methods were listed in Table 2. The results showed that the predominant bacterium was *H. influenzae* (n = 139), which appeared as either the sole isolated organism (n = 88), or in parallel with other bacteria as dual (n = 42), triple (n = 7), or quadruple (n = 2) isolates. *K. pneumoniae* (n = 20) and *S. pneumoniae* (n = 11) were among the isolated bacteria from the specimens. Whilst, none of the other target bacteria of this study; *S. aureus, P. aeruginosa* and *M. tuberculosis*, were identified via the reference methods. Despite these target bacteria, the sputum culture concurrently isolated other bacteria such as *M. catarrhalis* (n = 17), *H. parainfluenzae* (n = 13), *Klebsiella* spp. (n = 10), and a few more, as listed in Table 2. In summary, of 202 sputum specimens, 163 sputa were positive with a total of 226 identified bacterial isolates.

3.4. Comparison of the thermostabilized multiplex PCR assay with the reference method

The results from sputum culture and the developed multiplex PCR were compared and analyzed as presented in Supplementary Table 1. True positive (n = 170), true negative (n = 95), false positive (n = 8), and false negative (n = 0), were recorded. The results showed that all targeted bacteria that were positive by sputum culture, were found positive by the multiplex PCR assay. Similarly, the sputum culture negative and other non-target bacteria were also negative by this developed assay. However, the multiplex PCR assay detected the presence of additional *S. pneumoniae* DNA from a few specimens, which resulted in false positive discordancy. None of the PCR detection on these sputum specimens was recorded as false negative or invalid.

The summary of bacterial identification by sputum culture and multiplex PCR assay was presented in Table 3. The multiplex PCR assay successfully detected all the 170 target bacteria that were positive by sputum culture, in addition to the extra positive amplicons (n = 8) corresponding to *S. pneumoniae*'s target DNA. These false-positive amplicons were detected from the sputum specimens that were simultaneously positive for other pathogens. In further details, the false positive outcomes of *S. pneumoniae* were either amplified as single amplicon (n = 1) or simultaneously amplified with *H. influenzae* as duplex amplicons (n = 5), and as triplex amplicons in the presence of *H. influenzae* and *K. pneumoniae* (n = 2). When the results were compared with sputum culture, the single amplicon of false positive *S. pneumoniae* was found to have originated from the sputum specimen that was positive for *H. parainfluenzae* (n = 1). Meanwhile, one of the false positive *S. pneumoniae* of the duplex amplicons was positive for *Streptococcus* Group G.

3.5. Analyzing the performance of multiplex PCR assay on the sputum specimens from Kelantanese Malaysian Hajj pilgrims

The diagnostic performance of multiplex PCR assay on the sputum specimens collected from Kelantanese Malaysian Hajj pilgrims was analyzed by using the Medcalc Software. The overall scorings for true positive, false positive, true negative and false negative were computed in a 2 $\times$ 2 table as shown in Table 4a. The result in Table 4b showed that the sensitivity and specificity of this multiplex PCR assay were 100.00% (95% CI: 97.85% to 100.00%) and 92.23% (95% CI: 85.27% to 96.59%), respectively. The PPV and NPV were 95.51% (95% CI: 91.61% to 97.64%) and 100.00%, respec-

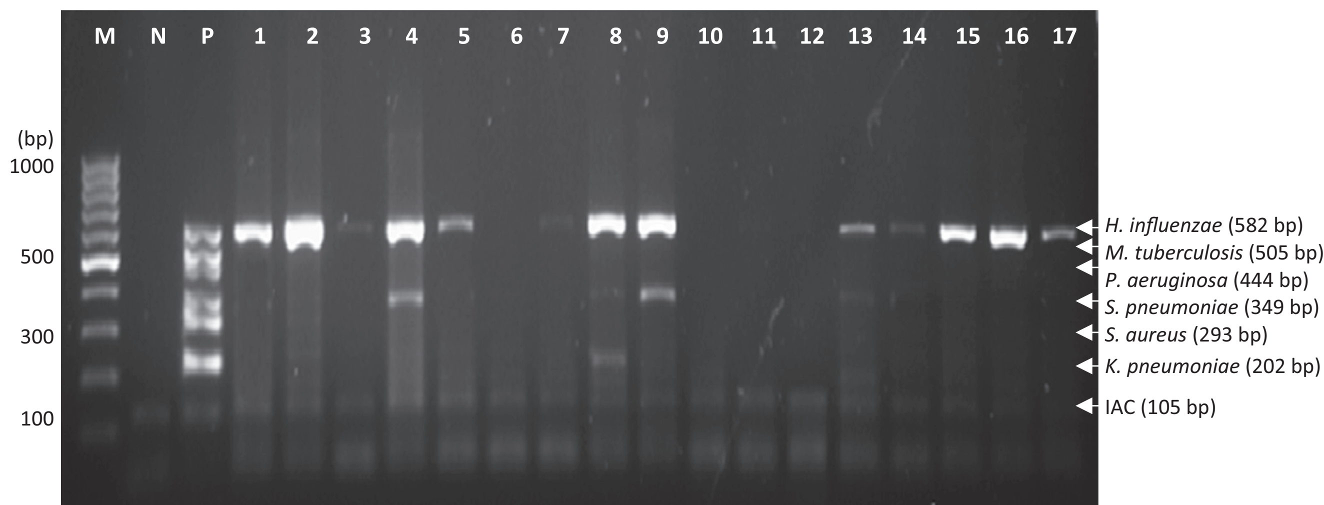

Fig. 1. Representative agarose gel image for the diagnostic evaluation of the developed multiplex PCR assay on the sputum specimens from Malaysian Hajj pilgrims (n = 17). This image shows the presence of target amplicons as single and multiple bands [in double (Lanes 4, 9 and 13) or triple bands (Lane 8)] on positive sputa, as well as the absence of amplicon on negative sputa. Lane M, 100 bp DNA ladder; lane N, no template control; lane P, positive amplification control; lane 1–17, presence or absence of PCR amplicons from individual Hajj pilgrims' sputa.

Table 2
Distribution of bacteria isolated from Malaysian Hajj pilgrims.

Bacterial pathogens	Single isolates (n = 111)	Multiple isolates (n = 52)			Total sputa (n = 202)
		two pathogens (n = 43)	three pathogens (n = 7)	four pathogens (n = 2)	
H. influenzae	88	42	7	2	**139**
K. pneumoniae	1	13	4	2	**20**
M. catarrhalis	2	11	3	1	**17**
H. parainfluenzae	12	1	0	0	**13**
Streptococcus group G	2	8	1	1	**12**
S. pneumoniae	2	4	4	1	**11**
Klebsiella spp.	3	6	1	0	**10**
Streptococcus group A	1	1	0	0	**2**
E. coli	0	0	1	1	**2**
Total bacterial isolates	**111**	**86**	**21**	**8**	**226**

Table 3
The summary of bacterial identification from Hajj pilgrims by the reference methods and multiplex PCR assay.

Identification by the gold standards	Frequency	PCR positive	PCR negative	PCR inhibited
Culture positive				
Panel of bacteria included in the multiplex PCR:				
H. influenzae	139	139	–	–
K. pneumoniae	20	20	–	–
S. pneumoniae	11	19[a]	–	–
P. aeruginosa	–	–	–	–
S. aureus	–	–	–	–
M. tuberculosis	–	–	–	–
Total	170	178	0	0
Panel of bacteria excluded from the multiplex PCR:				
M. catarrhalis	17	–	17	–
H. parainfluenzae	13	–	13	–
Streptococcus group G	12	–	12	–
Klebsiella spp.	10	–	10	–
Streptococcus group A	2	–	2	–
Escherichia coli	2	–	2	–
Total	56	0	56	0
Culture negative				
Normal flora	35	–	35	–
Unsuitable for culture (rejected)	3	–	3	–
Total	38	0	38	0

– indicates zero interpretation for the respective subjects.
[a] presence of additional amplicon corresponding to *S. pneumoniae* in positive sputum culture.

Table 4a
Computation of the overall positive- and negative- detections from pilgrims' sputum specimens by multiplex PCR assay.

Test	Disease				Total
	Present	*n*	Absent	*n*	
Positive	True Positive	a = 170	False Positive	c = 8	a + c = 178
Negative	False Negative	b = 0	True Negative	d = 95	b + d = 95
Total	a + b	170	c + d	103	

Table 4b
The performance of multiplex PCR assay on pilgrims' sputum specimens.

Statistic	Value	95% CI
Sensitivity	100.00%	97.85% to 100.00%
Specificity	92.23%	85.27% to 96.59%
Positive Likelihood Ratio	12.88	6.62 to 25.05
Negative Likelihood Ratio	0.00	
Disease prevalence	62.27%	56.23% to 68.04%
Positive Predictive Value	95.51%	91.61% to 97.64%
Negative Predictive Value	100.00 %	94.37% to 99.62%
Accuracy	97.07%	94.31% to 98.73%

tively. The accuracy performance of this assay was 97.07% (95% CI: 94.31% to 98.73%).

4. Discussion

In this study, the multiplex PCR assay was implemented on sputum specimens collected from Kelantanese Malaysian Hajj pilgrims. The purpose of this implementation on Hajj pilgrims was to identify as many as possible significant bacteria that most likely had caused RTIs among the symptomatic Hajj pilgrims, as well as to validate the accuracy performance of this in-house multiplex PCR assay on Hajj sputa specimens. This is important for the input of epidemiological data, as the pathogens associated with RTIs among Hajj pilgrims have been reported to differ from season to season. Moreover, available data on bacterial RTIs among Malaysian Hajj pilgrims is very limited.

All participants involved in this study claimed to experience symptomatic RTIs, which include cough (100%), sore throat (46.4%) and fever (31.8%). Based on the data for prescriptions of antibiotics, more than half of the participants (53.6%, n = 103) had visited healthcare facilities and received treatment or medication. The PCR assay was shown to be sensitive in detecting the intended bacteria (n = 170) in parallel with the reference method (100.00%). It was also specific against all the non-intended target bacteria. Discordance of the PCR assay with the reference method was observed from additional *S. pneumoniae* amplicons (n = 8). Although the discordancy was listed as false positive detection and has reduced the assay specificity to 92.23%, it might show that the PCR assay is more sensitive than microbial culture for detecting *S. pneumoniae*. The overall performance of the assay in this study was good, in which the values for PPV, NPV and accuracy were above 95.00%. The turnaround time from DNA preparation to PCR interpretation is around 4–6 h, which is at least four times faster compared to that of reference methods (sputum cultures and microscopy). This shows that the PCR assay could be helpful for the rapid diagnosis of RTIs, mainly during the massive Hajj pilgrimage.

Based on the spectrum of bacteria detected from the pilgrims, *H. influenzae* was found to be the predominant organism that was isolated from 68.0% (n = 139) of the enrolled pilgrims. According to the previous reports, *H. influenzae* was one of the commonest bacteria responsible for RTIs during Hajj (Alzeer, 2009; Asghar et al., 2011). The contributing cause to the high rate of this organism is unclear. One possibility could be the high uptake of antibiotics among the pilgrims. Previous study reported that this Gram-negative bacillus was more frequently observed among subjects who had received antibiotic treatments (Miyashita et al., 2008). Most importantly, *H. influenzae* has been reported as one of the cosmopolitan pathogens, which is responsible for the globalization of RTIs to the entire pilgrims after the Hajj (Memish et al., 2015). The nasal carriage rate of this organism among Malaysian pilgrims was reported to increase in around 25.0% after Hajj (Memish et al., 2015). However, data for the pre-Hajj carriage rate was not conducted in this study for further comparison.

Meanwhile, *K. pneumoniae* (10.0%, n = 20) was remarked as the second most isolated organism from the pilgrims. The occurrence of *K. pneumoniae*-RTIs was reported around 3.9% to 28.6% cases in the previous Hajj pilgrimages, and it was commonly associated with severe cases of pneumonia (Al-Tawfiq et al., 2016; Asghar et al., 2011; Dzaraly et al., 2016). In this study, all except one specimen which were positive for *K. pneumoniae,* were observed to contain multiple bacteria, mostly in the presence of *H. influenzae* (90.0%, n = 18) and/or *S. pneumoniae* (20.0%, n = 4). Respiratory symptoms for fever (40.9%, n = 9) and dyspnea (9.0%, n = 2) were noted higher within the pilgrims who had positive for these multiple bacteria.

S. pneumoniae has been reported as another common pathogen associated with RTIs during Hajj with the prevalence ranged between 4.8% and 62.0% (Benkouiten et al., 2014; Memish et al., 2015; Parker et al., 2018). The frequent rate of *S. pneumoniae* isolated from symptomatic pilgrims was reported around 10.0% (Ridda et al., 2014). Hence, the isolation rate of *S. pneumoniae* by reference methods in this study can be considered as low (5.4%, n = 11). Method of detection by microbial culture was believed to cause the low prevalence of *S. pneumoniae* in previous reports (El-Sheikh et al., 1998; Parker et al., 2018). Taking into account the positive detection by multiplex PCR assay developed in this study, the total detection rate would be 9.4% (n = 19), which is comparable to the reported frequent rate (10.0%). *S. pneumoniae* is among the organisms that has been found difficult to isolate from clinical specimens and has often led to false negative culture (Mandell et al., 2007; Xu et al., 2011). One reason is due to the properties of being a fastidious, facultative anaerobic organism that requires specific nutrients and growth conditions (Bhattacharya, 2006). In addition, the low recovery of this organism could be due to prior antibiotic uptake and the tendency to undergo autolysis at their stationary growth phase (Blaschke, 2011).

On the other hand, the previously reported common organisms for the RTIs during Hajj: *S. aureus*, *P. aeruginosa*, and *M. tuberculosis*, were not isolated in the current study by the microbial sputum culture, nor detected by the developed multiplex PCR assay. These organisms are usually associated with more severe cases of pneumonia, which require further hospitalization (Mandourah et al., 2012). The high rate of antibiotic administration among these pilgrims could be one of the reasons for zero detection of these pathogens. However, records for the types of antibiotic medications were not available in this study.

One of the study limitations is this study was conducted only in Kelantan, an eastern state in Peninsular Malaysia. Thus, the find-

ings should not be generalized to the entire Malaysian Hajj population. Additionally, there was insufficient data on the pilgrims' medication and vaccination records, which could be useful for further investigation on the efficacy of the antibiotic treatments and vaccination. For example, types of the administered antibiotic can be correlated with the identified or unidentified targeted organisms. Meanwhile, the optional pneumococcal vaccination status might be useful for further comparison between *S. pneumoniae*-positive and -negative pilgrims. It has been reported that pneumococcal vaccination may lead to a reduced rate of *S. pneumoniae* acquisition (Rashid et al., 2013). The records were unavailable for this study, due to restriction of space and time during the field sampling.

Overall, the developed single-tube, thermostabilized multiplex PCR assay of this study has the accuracy of above 90.0% during the diagnostic evaluation. Based on the properties and performance (rapidity, simplicity, detection limit and accuracy) of this assay, it could provide an easier, faster, and reliable tool for bacterial detection in the diagnosis of RTIs. As a new developed assay, it can be applied as either a triage and/or an add-on test to the existing diagnostic tools (Bossuyt et al., 2006). However, it is known that qualitative-based PCR assays are restricted by their inability to distinguish between normal flora and pathogenic-bacteria. Hence, in addition to microbiological diagnosis, clinical presentations and radiographic findings are useful to facilitate the diagnosis and therapeutic decision by the clinicians. Despite its limitations, this assay has the potential use as a triage test to facilitate in the initial laboratory detection of the target bacteria from sputum specimens. It can also be used as an add-on test, especially when the sputum culture is negative or when the confirmation of closely related target species is needed. In future studies, the primers used for this assay may be applied in the development of quantitative-based PCR assays, which have been widely used for microbial diagnosis in clinical areas.

Institutional Review Board Statement

This study was approved by the Institutional Review Board of the Human Research Ethics Committee, Universiti Sains Malaysia (Registration Number: 00004494).

CRediT authorship contribution statement

Nik Mohd Noor Nik Zuraina: Methodology, Validation, Formal analysis, Writing – original draft, Writing – review & editing. **Habsah Hasan:** Conceptualization, Writing – review & editing, Supervision. **Suharni Mohamad:** Conceptualization, Validation, Writing – review & editing, Supervision. **Siti Suraiya:** Conceptualization, Validation, Formal analysis, Writing – review & editing, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.sjbs.2022.103349.

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