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Molecular detection of selected zoonotic respiratory pathogens and the presence of virulence and antibiotic resistance genes via PCR among Kelantan Hajj pilgrims

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ABSTRACT

Aims: Respiratory tract infections (RTIs) among Malaysian pilgrims are caused by exposure to zoonotic-potential respiratory pathogens, symptomatically and asymptomatically affected by rigorous pilgrimage rituals, overcrowding and other stressors. This study aimed to determine the prevalence, virulence and antibiotic resistance genes of selected zoonotic respiratory pathogens using polymerase chain reaction (PCR) assays among Hajj pilgrims from Kelantan state, Malaysia.
Methodology and results: Throat swab specimens were obtained from 189 Kelantan Hajj pilgrims in 2016 and examined by PCR for the identification of respiratory pathogens. Thirteen samples (6.88%) were positive for *Streptococcus pneumoniae* and four (2.11%) were positive for *Klebsiella pneumoniae*. All the samples were negative for Influenza A virus, MERS-CoV and *Mycobacterium bovis*. One sample was positive for *S. pneumoniae* virulence *lytA* gene. One sample was positive for *K. pneumoniae* virulence *magA* and *K2A* genes respectively, and three samples were positive for *K. pneumoniae rmpA* genes. Ten and seven samples were positive for *S. pneumoniae mefA* and *pbpA* antibiotic resistance genes respectively. Two samples were positive for *K. pneumoniae* bla_{KPC} and bla_{OXA-48} antibiotic resistance genes.
Conclusion, significance and impact of study: This work provided insight into the existence of zoonotic respiratory pathogens inducing Hajj RTIs in Kelantan pilgrims. It showed promising findings for zoonotic studies in Hajj settings. The findings could be relevant in potential control measures for the management of zoonotic infections among Hajj pilgrims.

Keywords: RTI, Hajj, PCR, respiratory pathogens, zoonotic

INTRODUCTION

A mass gathering is a spontaneous or non-spontaneous gathering at certain times and usually involving more than 25000 people (Barasheed *et al.*, 2014). One example of mass gatherings is the Hajj pilgrimage in Mecca and Medina, conducted annually to embrace the Muslim's Islamic identity to God. Hajj is one of the mass gatherings which can increase the spread of many transmissible diseases and the risk of respiratory infections (Gautret *et al.*, 2013). Previous studies have reported evidence of common respiratory pathogens infections among Malaysian Hajj pilgrims (Deris *et al.*, 2010; Hasan *et al.*, 2015). Therefore, screening zoonotic pathogens in the same population is relevant, as is often overlooked due to common human clinical symptoms.

Respiratory diseases in Hajj have increased over the years due to direct contact among pilgrims in extremely overcrowded condition (Al-Asmary *et al.*, 2007). A previous study showed that 40.1% of Malaysian pilgrims had common respiratory symptoms such as cough, subjective fever, sore throat and headache, typically caused by many factors including extended stays in crowded areas during Hajj pilgrimage (Deris *et al.*, 2010). Other factors including mass crowding, limited space and facilities, vigorous rituals and unsuitable weather during Hajj pilgrimage increased the acquisition risk of respiratory pathogens by up to 8 folds (Barasheed *et al.*, 2014; Nor Radhiah *et al.*, 2017). Many communicable diseases, including respiratory tract infections involving bacterial and viral aetiologies, were often reported during Hajj's pilgrimage (Memish, 2010). It is said that the number of zoonotic diseases in the Arabian Peninsula may occasionally affect the flow of Hajj pilgrimage and Umrah visits. Many zoonotic diseases have been reported in the Arabian Peninsula, and this has raised concerns for

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the spread of zoonotic diseases in the region, especially when mass pilgrimages such as Hajj and Umrah occur annually (Almutairi *et al.*, 2018). Zoonotic exposure affecting abattoir employees in Mecca reported that 8.75% of employees had contract zoonotic infections such as Alkhurma haemorrhagic fever (AHF) and Brucella infections due to high exposure to animals and butchering during Hajj (Almasri *et al.*, 2019). During the pilgrimage, there are high interactions among pilgrims, workers, and animals, where the sacrifice of these animals is one of Hajj pilgrimage rituals.

In community-acquired infections, during Hajj pilgrimage, high traffic among pilgrims increased the risk of respiratory tract infections. Some of these infections involved potential zoonotic pathogens, e.g. evidence of MERS-CoV camel-human transmission in the Kingdom of Saudi Arabia, confirming the region's risk of zoonotic disease. Nonetheless, insufficient knowledge of basic disease transmission had acquired several possibilities of transmitting zoonotic diseases in large gatherings, including Hajj pilgrimage (Lessler *et al.*, 2014). Most zoonotic infections still have tremendous information gaps in transmission, treatment, and prevention, making it difficult to control and contain, particularly when occurrences happen in mass gatherings. In addition to the presence of symptomatic and asymptomatic carriers of zoonotic pathogens in humans and animals, there are minimal data on the point of transmission and acquisition, especially on respiratory pathogens in mass gatherings. Respiratory pathogens with zoonotic potential among Malaysian Hajj pilgrims returning with respiratory tract infections (RTI) symptoms has not been investigated. So far, there is no report on zoonotic pathogens among Hajj pilgrims from Kelantan, Malaysia. Therefore, this study aimed to determine the prevalence, virulence and antibiotic resistance genes of selected zoonotic respiratory pathogens using polymerase chain reaction (PCR) assays among the Hajj pilgrims from Kelantan.

MATERIALS AND METHODS

Study design

Throat swab specimens were collected from Malaysian Hajj pilgrims arriving in Sultan Ismail Petra Airport, Kelantan, Malaysia after completing their 2016 Hajj. A cross-sectional study was conducted in 2016 at Universiti Sains Malaysia (USM), Universiti Malaysia Kelantan (UMK) and Sultan Ismail Petra Airport, Kota Bharu, Kelantan.

Ethical approval

Ethical approval was granted by Human Research Ethics Committee Universiti Sains Malaysia, USM, Malaysia (Reference number: USMKK/PP/JEPeM[266.3(3)]). All pilgrims were selected based on their consent. Additionally, only pilgrims aged 18 years old and above were selected for this study.

Sample collection

A simple random sampling method was applied. All throat swab specimens were collected from Kelantan pilgrims with RTI symptoms at Sultan Ismail Petra Airport, Kelantan after Hajj pilgrimage in between the period of October to November 2016. Verbal consent was obtained before sampling. Briefly, the pilgrims were explained about the study's purpose and the specimen needed for this study. Their demographic and symptoms details were recorded in a questionnaire. RTI symptoms recorded from the participated subjects include cough, subjective fever, sore throat, dyspnea, abdominal pain, vomiting, runny nose, myalgia or arthralgia. The symptoms were validated by medical personnel during the sampling period. Two throat swab samples were taken from each person and stored accordingly in a viral transport medium and an amies charcoal transport medium (LabChem, Malaysia).

Nucleic acid extraction

The collected throat swabs were inoculated in brain heart infusion (BHI) broth overnight and its turbidity for growth was checked using a turbidometer. The DNA was then extracted from the overnight broth using a commercially available DNA extraction kit from Promega Corporation (USA) according to manufacturer's instructions. Extracted DNA was stored at –20 °C until further use. RNA extraction was also performed for virus identification directly from the viral transport medium. Viral RNA was extracted from the samples using Trizol reagent from Ambion, Inc. (USA) as described by the manufacturer's instructions. The extracted RNA was stored at –80 °C until further processing. Quantity and quality of the extracted DNA and RNA were evaluated using a spectrophotometer (Eppendorf, Germany).

Positive controls used in this study were obtained from different sources. Influenza A virus RNA was obtained from Faculty of Veterinary Science, Universiti Malaysia Kelantan and MERS-CoV RNA was obtained from Hospital Raja Perempuan Zainab 2 (HRPZ 2), Kelantan. On the other hand, *Mycobacterium bovis* DNA, *Streptococcus pneumoniae* and *Klebsiella pneumoniae* were obtained from Microbiology and Parasitology Laboratory, Universiti Sains Malaysia. Nuclease-free water was used for negative PCR control in this study.

The extracted nucleic acid was subjected to the PCR assays. For the pathogen detection, five organisms of interest in this study were Influenza A virus, Middle East Middle East Respiratory Syndrome coronavirus (MERS-CoV), *M. bovis*, *S. pneumoniae* and *K. pneumoniae*.

Detection of respiratory bacteria and viruses

Influenza A virus and MERS-CoV were detected using singleplex reverse-transcriptase PCR (RT-PCR), while *M. bovis*, *S. pneumoniae* and *K. pneumoniae* were detected using singleplex PCR assays. The primers used for the detection of specific target genes were mentioned in

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Table 1: Primers and thermal cycling conditions for respiratory pathogens.

Pathogen	Target gene	Primers	Thermal cycling conditions	Amplicon size (bp)	Reference
Influenza A virus	*Matrix*	Fwd: 5'-ATGAGYCTTYTAACCGAGGTCGAAACG-3' Rev: 5'-TGGACAAANCGTCTACGCTGCAG-3'	55 °C/30 min, 95 °C/15 min, (95 °C/30 sec, 53 °C/60 sec, 72 °C/30 sec)× 30, 72 °C/10 min	244	(WHO, 2012)
MERS-CoV	*upE*	Fwd: 5'-GCAACGCGCGATTCAGTT-3' Rev: 5'-GCCTCTACACGGGACCCATA-3'	55 °C/30 min, 95 °C/15 min, (95 °C/30 sec, 55 °C/60 sec, 72 °C/30 sec)× 30, 72 °C/10 min	92	(Corman *et al.*, 2012)
M. bovis	*IS6110*	Fwd: 5'-AGTTTGGTCATCAGCCGTTC-3' Rev: 5'-CGAACTCAAGGAGCACATCA-3'	95 °C/15 min, (95 °C/30 sec, 60 °C/60 sec, 72 °C/30 sec)× 30, 72 °C/10 min	135	(Thacker *et al.*, 2011)
K. pneumoniae	*mdh*	Fwd: 5'-ACTGCCTTCAGGTTCAGAGC-3' Rev: 5'-TTCAAAGGCGCTGAGTTTGC-3'	95 °C/4 min, (94 °C/30 sec, 50°C/60 sec, 72 °C/30 sec)× 30, 72 °C/10 min	780	(Ismadi, 2017)
S. pneumoniae	*ply*	Fwd: 5'-TTGACCCATCAGGGAGAAAG-3' Rev: 5'-CTTGATGCCACTTAGCCAAC-3'	95 °C/15 min, (94 °C/30 sec, 60 °C/30 sec, 72 °C/60 sec)× 30, 72 °C/10 min	349	(Nik Mohd Noor, 2019)

Table 1. For influenza A virus and MERS-CoV, the reaction mixture was made up of 2 µL RNA template, 0.8× AccessQuick™ Master Mix (Promega, USA), 0.4 µM-6 µM forward and reverse primers, 0.1 U AMV Reverse Transriptase (Promega, USA) and nuclease-free water to a final volume of 50 µL in one-step RT-PCR mix. For *M. bovis*, *K. pneumoniae* and *S. pneumoniae*, the reaction mixture was made up of 1× MyTaq™ Red Reaction Buffer (Bioline, USA), 0.4 µM–6 µM forward and reverse primers, 0.05 U–0.1 U MyTaq™ Red DNA polymerase (Bioline, USA), 2 µL DNA template and nuclease-free water to a final volume of 50 µL. Positive controls and negative PCR controls were also included in the PCR assays. For negative PCR control, nuclease-free water was added to replace the DNA template. The PCR and RT-PCR assays were conducted on Mastercycler Nexus (Eppendorf, Germany). Thermal cycling conditions for each assay were shown in Table 1.

Detection of virulence and antibiotic resistance genes

Specific primers used for detection of the target virulence and antibiotic resistance genes were mentioned in Table 2. Positive controls and negative PCR controls were also included in the assays. For *K. pneumoniae* virulence genes, the reaction mixture was made up of 1× MyTaq™ Red Reaction Buffer (Bioline, USA), 0.75 µM of each primer, 0.05 U MyTaq™ Red DNA polymerase (Bioline, USA), 2 µL DNA template and nuclease-free water to a final volume of 50 µL in a multiplex PCR mix. For *S. pneumoniae lytA* and *pspC* virulence genes, the reaction mixture was made up of 1×MyTaq™ Red Reaction Buffer (Bioline, USA), 0.5 µM of each primer, 0.05 U MyTaq™ Red DNA polymerase (Bioline, USA), 2 µL DNA template and nuclease-free water to a final volume of 50 µL in a multiplex PCR mix. For *S. pneumoniae rrgA* virulence gene, the reaction mixture was made up

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Table 2: Primers and thermal cycling conditions for virulence and antibiotic resistance genes.

Target gene	Primer	Thermal cycling conditions	Amplicon size (bp)	Reference
Virulence genes				
K. pneumoniae				
K2A	Fwd: 5'-CAACCATGGTGGTCGATTAG-3' Rev: 5'-TGGTAGCCATATCCCTTTGG-3'	95 °C/15 min, (95 °C/30 sec, 60 °C/90 sec, 72 °C/60 sec)× 30, 72 °C/10 min	531	(Remya *et al.*, 2018)
magA	Fwd: 5'-GGTGCTCTTACATCATTGC-3' Rev: 5'-GCAATGGCCATTTGCGTTAG-3'	95 °C/15 min, (95 °C/30 sec, 60 °C/90 sec, 72 °C/60 sec)× 30, 72 °C/10 min	1283	(Remya *et al.*, 2018)
rmpA	Fwd: 5'-CATTAGAGTATTGGTTGACAG-3' Rev: 5'-CTTGCATGAGCCATCTTTGA-3'	95 °C/15 min, (95 °C/30 sec, 60 °C/90 sec, 72 °C/60 sec)× 30, 72 °C/10 min	461	(Remya *et al.*, 2018)
S. pneumoniae				
lytA	Fwd: 5'-CAACCGTACAGAATGAAGCGG-3' Rev: 5'-TTATTCGTGCAATACTCGTGCG-3'	94 °C/2 min, (94 °C/10 sec, 58 °C/15 sec, 72 °C/60 sec)× 30, 72 °C/10 min	319	(Gholamhosseini-Moghaddam *et al.*, 2015)
pspC	Fwd: 5'-AAGATGAAGATCGCCTACGAACAC-3' Rev: 5'-AATGAGAAACGAATCCTTAGCAATG-3'	94 °C/2 min, (94 °C/10 sec, 58 °C/15 sec, 72 °C/60 sec)× 30, 72 °C/10 min	1000-1200	(Gholamhosseini-Moghaddam *et al.*, 2015)
rrgA	Fwd: 5'-CACTTTTATACGCTTTTGCTA-3' Rev: 5'-TAATACGACTCACTATAGGTGCCATCCGTATTGTTTTTC-3'	95 °C/2 min, (95 °C/30 sec, 51 °C/30 sec, 72 °C/90 sec)× 30, 72 °C/10 min	373	(Gholamhosseini-Moghaddam *et al.*, 2015)
Antibiotic resistance genes				
K. pneumoniae				
bla_{KPC}	Fwd: 5'-CATTCAAGGGCTTTCTTGCTGC-3' Rev:5'-ACGACGGCATAGTCATTTGC-3'	95 °C/5 min, (94 °C/45 sec, 60 °C/45 sec, 72 °C/60 sec)× 35, 72 °C/10 min	538	(Al-Agamy *et al.*, 2018)
bla_{NDM}	Fwd: 5'-GCAGCTTGTCGGCCATGCGGGC-3' Rev:5'-GGTCGCGAAGCTGAGCACCGCAT-3'	95 °C/5 min, (94 °C/45 sec, 60 °C/45 sec, 72 °C/60 sec)× 35, 72 °C/10 min	782	(Doyle *et al.*, 2012)

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bla$_{OXA-48}$	Fwd: 5'-GCGTGGTTAAGGATGAACAC-3' Rev:5'-CATCAAGTTCAACCCAACCG-3'	95 °C/5 min, (94 °C/45 sec, 60 °C/45 sec, 72 °C/60 sec)× 35, 72 °C/10 min	438	(Doyle *et al.*, 2012)
S. pneumoniae				
ermB	Fwd: 5'-CAAAACGTACTCAACCAAATA-3' Rev:5'-ACTAACCGTACAATTCTTATTTAC-3'	95 °C/5 min, (93 °C/35 sec, 55 °C/30 sec, 72 °C/45 sec)× 35, 72 °C/10 min	642	(Wang *et al.*, 2017)
mefA	Fwd: 5'-AGTATCACTAATTAATCCTGC-3' Rev:5'-TTCTTCCTACTGTAAAAGTCC-3'	95 °C/5 min, (93 °C/35 s, 55 °C/30 s, 72 °C/45 s)× 35, 72 °C/10 min	353	(Wang *et al.*, 2017)
pbpA	Fwd: 5'-AAACAAGGTCGGACTCAACC-3' Rev:5'-ATATACATTGGTTTATAGTAAGTT-3'	95 °C/5 min, (93 °C/35 sec, 55 °C/30 sec, 72 °C/45 sec)× 35, 72 °C/10 min	171	(Nagai, 2001)

of 1× MyTaq™ Red Reaction Buffer (Bioline, USA), 0.25 µM of each primer, 0.05 U MyTaq™ Red DNA polymerase (Bioline, USA), 2 µL DNA template and nuclease-free water to a final volume of 50 µL in a singleplex PCR mix.

For *S. pneumoniae* and *K. pneumoniae* antibiotic resistance genes, the reaction mixture was made up of 1× MyTaq™ Red Reaction Buffer (Bioline, USA), 0.75 µM of each primer, 0.05 U MyTaq™ Red DNA polymerase (Bioline, USA), 2 µL DNA template and nuclease-free water to a final volume of 50 µL in a multiplex PCR mix. For negative PCR control, nuclease-free water was used to replace the DNA template. These assays were conducted in Mastercycler Nexus (Eppendorf, Germany).

Agarose gel electrophoresis

Following amplification, PCR products were electrophoresed on 1.5% (w/v) agarose gel in 1× TBE buffer for 45 min at 100 V. Briefly, 1.5 g of agarose was added to 100 mL of 1× TBE buffer, before being heated in a microwave for 3 min until it dissolved completely. A total of 1.5 µL GelRed® fluorosafe (1st BASE, Singapore) was added to the agarose solution upon cooling and mixed well. Molten agarose was poured into a gel casting chamber and left to solidify for 45 min at room temperature. In the gel electrophoresis, molecular weight markers were added into the first lane, followed by positive and negative controls in the next lanes. PCR products were loaded into the remaining lanes.

RESULTS

Detection of zoonotic respiratory pathogens

Out of 189 pilgrims recruited in this study, 17 pilgrims (9%) were tested positive for *K. pneumoniae* and *S. pneumoniae*. None of the samples was positive for *M. bovis*, influenza A virus and MERS-CoV (data not shown). Four (2.12%) samples were positive for *K. pneumoniae mdh* gene (Figure 1); and 13 samples (6.88%) were positive for *S. pneumoniae ply* gene (Figure 2). There was no mixed infection of both pathogens detected.

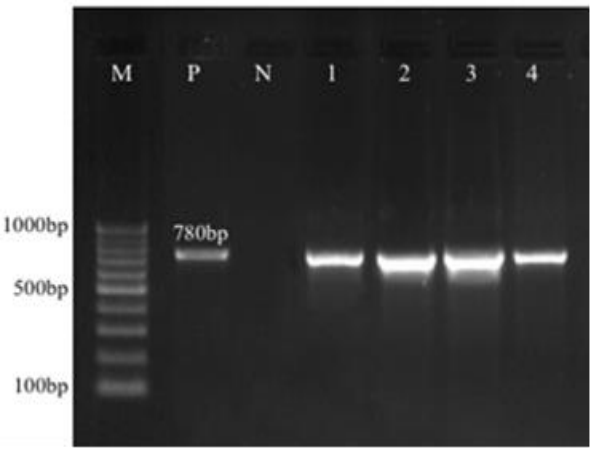

Figure 1: PCR products of *K. pneumoniae mdh* gene. Lane M: 100 bp DNA ladder, Lane P: *K. pneumoniae mdh* gene (positive control), Lane N: Negative control, Lane 1-4: Samples positive for *mdh* gene.

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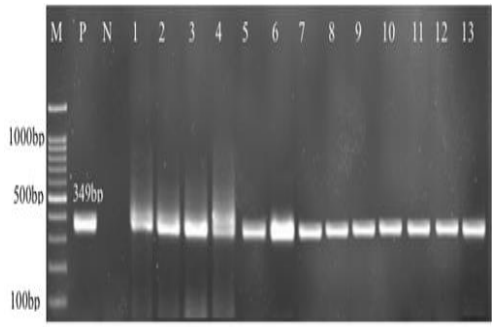

Figure 2: PCR products of *S. pneumoniae ply* gene. Lane M: 100 bp plus DNA ladder, Lane P: *S. pneumoniae ply* gene (positive control), Lane N: Negative control, Lanes 1-13: Positive samples for *ply* gene.

Detection of virulence and antibiotic resistance genes

For the detection of *K. pneumoniae K2A*, *magA* and *rmpA* virulence genes, four samples positive with *K. pneumoniae* were subjected to another PCR assay. One sample was positive for *magA* (1283 bp) and *rmpA* (461 bp) genes, one sample was positive with *K2A* (531 bp) and *rmpA* (461 bp) genes, and one sample was positive with *rmpA* gene (461 bp) only (Figure 3). From the 13 samples positive for *S. pneumoniae ply* gene, multiplex PCR assay was conducted to determine the presence of *lytA*, *pspC* and *rrgA* virulence genes. All samples were negative for *pspC* and *rrgA* genes and only one sample was positive for *lytA* gene (319 bp) (Figure 4).

For *K. pneumoniae* antibiotic resistance genes (*bla*$_{KPC}$, *bla*$_{NDM}$ and *bla*$_{OXA-48}$), the result showed that none of the samples was positive for *bla*$_{NDM}$ gene (Figure 5). However, two out of four samples were positive for both *bla*$_{KPC}$ and *bla*$_{OXA-48}$ genes. For *S. pneumoniae ermB*, *mefA* and *pbpA* genes, all 13 samples were negative for *ermB* gene. Five samples were positive with *mefA* gene (353 bp); five samples were positive with *mefA* (353 bp) and *pbpA* (171 bp) genes, and two samples were positive with *pbpA* (171 bp) gene (Figure 6). The presence of virulence and antibiotic resistance genes of *K. pneumoniae* and *S. pneumoniae* were summarized in Table 3.

RTI symptoms

A total of 109 (57.67%) female and 80 (42.33%) male of Hajj pilgrims participated in this study. The male: female ratio was 1:1.36 with a mean age of 58.23±9.28 years old for males and 55.28±8.61 years old for females. Table 4 showed the distribution of RTI symptoms experienced by Hajj pilgrims from 2016 pilgrimage. The most common symptom experienced by Kelantan Hajj pilgrims was cough which was in 93.65% (n=177) of the pilgrims. This is followed by sore throat in 63.49% (n=120), fever in 49.74% (n= 94) and runny nose in 46.03% (n= 87) of the

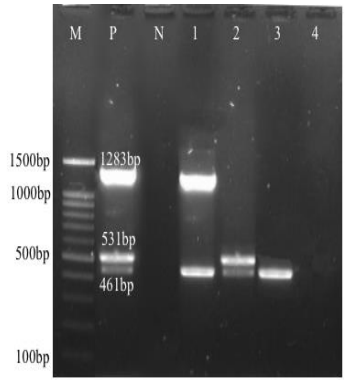

Figure 3: PCR products of *K. pneumoniae magA*, *K2A* and *rmpA* genes. Keys; Lane M: 100bp plus DNA ladder, Lane P: Positive control (*magA*: 1283 bp, *K2A*: 531 bp, *rmpA*: 461 bp), Lane N: Negative control, Lane 1: Sample positive for *magA* and *rmpA* genes, Lane 2: Sample positive for *K2A* and *rmpA* genes, Lane 3: Sample positive for *rmpA* gene, Lane 4: Sample negative for all genes.

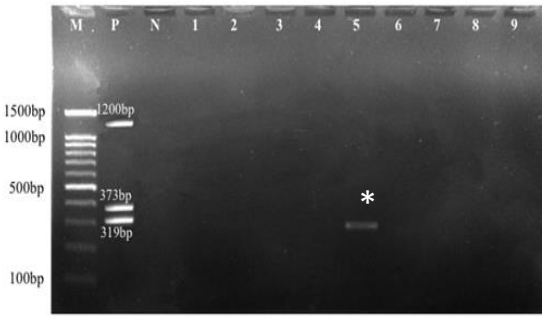

Figure 4: Representative PCR products of *S. pneumoniae lytA*, *pspC* and *rrgA* genes. Lane M: 100 bp plus DNA ladder, Lane P: Positive control (*pspC*: 1200 bp, *rrgA*: 373 bp, *lytA*: 319 bp), Lane N: Negative control, Lanes 1-4 and 6-9: Samples negative to all three genes, Lane 5: Sample positive for *lytA* gene (*).

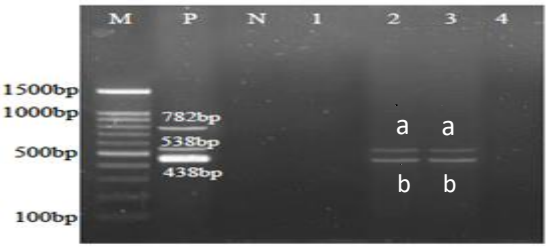

Figure 5: PCR products of *K pneumoniae bla*$_{KPC}$, *bla*$_{NDM}$ and *bla*$_{OXA-48}$ genes. Lane M: 100 bp plus DNA ladder, Lane P: Positive control (*bla*$_{NDM}$: 782 bp, *bla*$_{KPC}$: 538 bp, *bla*$_{OXA-48}$: 438 bp), Lane N: Negative control, Lanes 1 and 4: Samples negative for blaNDM, *bla*$_{KPC}$ and *bla*$_{OXA-48}$, Lanes2 and 3: Samples positive for *bla*$_{KPC}$ (a) and *bla*$_{OXA48}$ (b).

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Table 3 Percentage of positivity for virulence and antibiotic resistance genes of *K. pneumoniae* and *S. pneumoniae* in this study.

Pathogen	*K. pneumoniae*		*S. pneumoniae*	
PCR Screening (n=189, %)	4 (2.12%)		13 (6.88%)	
	(n=4)		(n=13)	
Virulence genes	*K2A*	1/4	*lytA*	1/13
	magA	1/4	*pspC*	0/13
	rmpA	3/4	*rrgA*	0/13
	magA + *rmpA*	1/4		
	K2A + *rmpA*	1/4		
Antibiotic resistance genes	bla_{KPC}	2/4	*ermB*	0/13
	bla_{NDM}	0/4	*mefA*	10/13
	bla_{OXA-48}	2/4	*pbpA*	7/13
	bla_{KPC} + bla_{OXA-48}	2/4	*mefA* only	5/13
			pbpA only	2/13
			mefA + *pbpA*	5/13

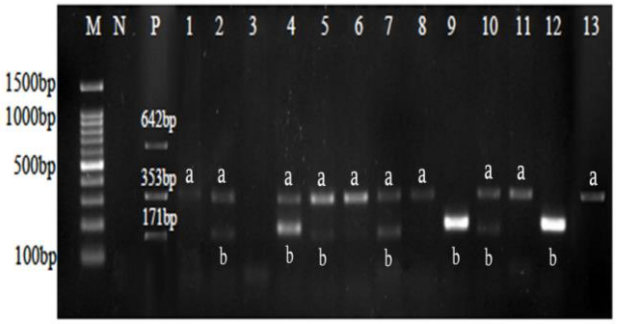

Figure 6: PCR products of *S. pneumoniae ermB*, *mefA* and *pbpA* genes. Lane M: 100 bp plus DNA ladder, Lane N: Negative control, Lane P: Positive control (*ermB*: 642 bp, *mefA*: 353 bp, *pbpA*: 171 bp), Lanes 1, 6, 8, 11 and 13 : Samples positive for *mefA* (a), Lanes 2, 4, 5, 7 and 10: Samples positive for *mefA* (a) and *pbpA* (b) genes, Lane 3: Sample negative for *mefA* (a) and *pbpA* (b) genes, Lanes 9 and 12: Samples positive for *pbpA* (b) gene.

pilgrims. About 32.28% (n=61) of the pilgrims produced sputum after their pilgrimage. Other symptoms such as dyspnea, abdominal pain, vomiting, myalgia and arthralgia were recorded in less than 7% of hajj pilgrims.

Table 4: Distribution of RTI symptoms for Kelantan pilgrims from 2016 Hajj.

Symptoms of RTIs	With symptoms (n=189, %)[a]	Male (n=80, %)[b]	Female (n=109, %)[b]
Cough	177 (93.65)	74 (92.50)	103(94.49)
Sore throat	120 (63.49)	45 (56.25)	75 (68.80)
Fever	94 (49.74)	35 (43.75)	59 (54.12)
Runny nose	87 (46.03)	30 (37.50)	57 (52.29)
Sputum	61 (32.28)	25 (31.25)	36 (33.03)
Arthralgia	12 (6.35)	2 (2.50)	10 (9.17)
Dyspnea	10 (5.29)	7 (8.75)	3 (2.75)
Vomiting	6 (3.17)	2 (2.50)	4 (3.70)
Myalgia	5 (2.65)	3 (3.75)	2 (1.83)
Abdominal pain	3 (1.59)	1 (1.25)	2 (1.83)

[a]Percentage within total hajj pilgrims (n=189).
[b]Percentage within total number of pilgrims according to gender.

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DISCUSSION

Kelantan Hajj pilgrims participating in this study suffered from various respiratory symptoms such as cough, fever, sore throat, runny nose and sputum production. Dzaraly *et al.* (2016) reported that that Malaysian pilgrims suffered from coughing, while Deris *et al.* (2010) and Hashim *et al.* (2016) found that Malaysian hajj pilgrims are often experienced influenza-like illness after pilgrimages. Although influenza A virus and MERS-CoV is not detected in this study, some symptoms such as arthralgia and myalgia among pilgrims may indicate a viral infection but this possibility could be further investigated. In this study, 147 (77.78%) Hajj pilgrims aged more than 50 years old. It was reported that most of the Islamic countries prioritized Hajj pilgrimage for people above 40 years of age (Shirah *et al.*, 2017). However, older pilgrims are susceptible to infections during pilgrimage including fatigue, inadequate rest and nutritional stress. Besides, they had a higher risk of infection due to overcrowding, vigorous rituals and underlying diseases during the pilgrimage (Nor Radhiah *et al.*, 2017).

In this study, the prevalence of *K. pneumoniae* and *S. pneumoniae* was 2.12% and 6.88% respectively. The findings were positively related to pathogens where acquisition rates of *K. pneumoniae* and *S. pneumoniae* were 3.9% and 12% respectively among the pilgrims in 2013 Hajj (Memish *et al.*, 2015). Similarly, 28.6% from hospitalized Malaysian pilgrims in the Kingdom of Saudi Arabia (KSA) were positive with *K. pneumoniae* and *S. pneumoniae* (Dzaraly *et al.*, 2016). Similarly, 9.2% of pilgrims suspected for pneumonia were positive for *K. pneumoniae* during the 2005 Hajj season in Makkah, Saudi Arabia (Asghar *et al.*, 2011) and *K. pneumoniae* was also isolated from 29% of Hajj pilgrims who visited the emergency department in Madinah, Saudi Arabia from 2004-2013 (Shirah *et al.*, 2017). Besides, it was also reported that 18% of the Hajj pilgrims from Saudi Arabia with suspected community-acquired pneumonia (CAP) were positive with *S. pneumoniae* from 2016 Hajj pilgrimage (AlBarrak *et al.*, 2018). In this study, the percentage of *S. pneumoniae* detected using PCR assays was lower (6.88%), as compared to other Hajj pilgrims from Indonesia, Pakistan and Egypt. It was reported that the prevalence rate of *S. pneumoniae* reached up to 30% in those countries after 2013 Hajj pilgrimage (Memish *et al.*, 2015).

Mycobacterium bovis, influenza A and MERS-CoV were not detected using PCR testing in this study. To date, there have been no cases of *M. bovis* infection among Hajj pilgrims and low *M. tuberculosis* infections among Hajj pilgrims. It was found that pilgrims had strong tuberculosis immune responses 90 days after Hajj, which were associated with a two to twelve week incubation period. It is advisable to get another sample at least a month after pilgrimage to validate the accuracy of tuberculosis diagnosis among pilgrims (Wilder-Smith *et al.*, 2005; Ryu, 2015). During the pilgrimage season, there is a high risk of respiratory disease transmission among pilgrims, including influenza A virus spread. Influenza A virus was detected in China and India pilgrims (Ma *et al.*, 2017; Koul *et al.*, 2017) but it was not present in this study. This is almost in agreement with a report by Memish *et al.* (2012) in where only 0.2% of the departing Hajj pilgrims from Jeddah had acquired influenza A virus H1N1. MERS-CoV was also not present in the throat swab specimens of in this study among Malaysian Hajj pilgrims. Similarly, in other studies, there is no MERS-CoV infection reported among other Hajj pilgrims from other countries such as China, France, North India, Egypt, Ghana, KSA and the United Kingdom (Rashid *et al.*, 2008; Gautret *et al.*, 2014; Amer *et al*., 2016; Koul *et al.*, 2017; Ma *et al.*, 2017; Refaey *et al.*, 2017). However, there were evidence of limited human-to-human transmissions of MERS-CoV in KSA region, which need further investigations about the circulation of the virus among pilgrims during Hajj pilgrimage season (WHO, 2019).

Within this research, *K. pneumoniae* virulence genes such as *magA*, *rmpA*, and *K2A* were investigated. These genes are involved in the hypermucoviscosity of hypervirulent *K. pneumoniae*. The mucoid phenotype (*rmpA*) gene regulator or the plasmid-based extracapsular polysaccharide synthesis regulator can positively control the mucoid phenotype. This strain can overproduce extracellular polysaccharides, therefore can be recognized stringy on the plates (Yu *et al.*, 2006). Coexistence of *rmpA* and *magA* substantially increased the frequency of hypermucoviscosity phenotype expression (Lee *et al.*, 2010). In addition, the severity of *K. pneumoniae* infection often depends on its serotypes. However, there are other determinants such as *rmpB* gene and *Lon* protease regulator (Yu *et al.*, 2006), showing that a complex regulator mechanism could play a role in *K. pneumoniae* hypermucoviscosity. *lytA*, *pspC* and *rrgA* genes encode certain *S. pneumoniae* virulence determinants including autolysin, surface protein and pili, frequently associated with fatal serotypes and severe infections. These three virulence genes are encoded for proteins and enzymes that play roles in pneumococcal infection colonization as well as multiple effects against host immune responses (Iannelli *et al.*, 2004). Ganaie *et al.* (2018) also reported that 2016 Indian Hajj pilgrims acquired multiple serotypes of *S. pneumoniae* (21.8%).

In this study, a pilgrim with *S. pneumoniae* virulence gene was a 63-year-old male who had symptoms such as cough, sore throat, runny nose and sputum. On the other hand, pilgrims with *K. pneumoniae* virulence genes were 54-year-old male, 62-year-old-female and 78-year-old male. All of them had cough and sore throat, while the 78-year-old male had runny nose, fever, and sputum. This shows that the pilgrims experienced common RTI symptoms such as fever, which could be overlooked in clinical settings during Hajj. Generally, the presence of virulence genes in bacteria will cause more severe symptoms in patients (Peterson, 1996). For the detected samples, most of them did not harbor selected *S. pneumoniae* virulence factors such as *lytA*, *pspC*, and *rrgA* genes. There is no differences in the symptoms caused by bacteria with different virulence factors.

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Likewise, *K. pneumoniae*'s virulence genes based on the pathogen's hypermucosviscosity showed no variations in symptoms encountered by pilgrims in this sample. This contradicts previous reports of *K. pneumoniae* hypermucoviscosity, as it is frequently associated with severe illness and death. Hypervirulent *K. pneumoniae* typically reported in cases of secondary bacteraemia and purulent disease (Yu *et al.*, 2006). K2 serotypes are frequently isolated from urinary tract infections, pneumonia, or bacteraemia (Podschun and Ullmann, 1998). According to Yu *et al.* (2007), the mucoid strain was closely related to death than capsular strains.

Throughout the years, antibiotic use has been common in respiratory disease treatment. Nearly 47.6% of Hajj pilgrims in France used antibiotics in respiratory tract infection and increased antibiotic use was associated with resistant bacteria and recurrent post-Hajj pilgrim symptoms (Hoang *et al.*, 2019). Extended-spectrum beta-lactamase (ESBL) producers in the Saudi Arabia region were previously reported (Al-Agamy *et al.*, 2018). Saudi Arabia is at risk of spreading and transmitting ESBL bacteria and carbapenem-resistant *Enterobacteriaceae* (CRE) with increased trips to the area. International travel, especially from risky areas such as India, Africa and Pakistan, contributed 23.3% of CRE patients in Saudi Arabia (Alotaibi, 2019). Moreover, France pilgrims were detected with *Escherichia coli* and *K. pneumoniae* bla_{CTX-M} genes in 2013 and 2014 Hajj pilgrimages were 31% and 34.83% respectively (Leangapichart *et al.*, 2017).

Several *K. pneumoniae* confer bla_{KPC} and bla_{OXA-48} antibiotic resistance genes responsible for carbapenemase resistance. In this study, the percentage of positive *K. pneumoniae* was half for bla_{KPC} and bla_{OXA-48} genes. These genes also resist beta-lactam antibiotics, including penicillin and ampicillin. *Klebsiella pneumoniae* carbapenemase (KPC)-producing strains can hydrolyze most β-lactam antibiotics worldwide (Kitchel *et al.*, 2010). It was also confirmed that some French Hajj pilgrims acquired *Acinetobacter baumannii* with bla_{OXA-72} and *Escherichia coli* with bla_{NDM-5} with carbapenemase resistance after 2014 Hajj pilgrimage (Leangapichart *et al.*, 2016). Furthermore, *Klebsiella* spp. with bla_{KPC-2} was found in the pilgrimage area (Hala *et al.*, 2019). *OXA-48* and *NDM* have been common genes of carbapenemase resistance in the Middle East region since 2014 (Al-Zahrani and Alasiri, 2018). In Iran, a report on *NDM-1* gene-positive isolates recovered from different hospital wards showed spread through public travel (Shoja *et al.*, 2018). This condition is particularly critical in Hajj settings, as pilgrims travel from place to place to complete the ritual. In this study, some *S. pneumoniae* samples carried *mefA* and *pbpA* genes conferring macrolide and penicillin resistance. Among the 2016 Indian Hajj pilgrims, the prevalence of multidrug-resistant *S. pneumoniae* was detected (32%) (Ganaie *et al.*, 2018). There are few reports on antibiotic resistance transmission and emergence in Hajj. In a 2009-2010 study, *Streptococcus* spp. with resistance to multiple antibiotics caused admission of Hajj pilgrims from different countries for pneumonia to Makkah and Medina health centres ICU (Mandourah *et al.*, 2012). It was also recorded that the acquisition rate of multiple resistant *S. pneumoniae* increased from 0.6% to 2.2% post-Hajj and approximately one-third of pilgrims acquired them during Hajj (Benkouiten *et al.*, 2014; Memish *et al.*, 2015). The prevalence of pneumococcal colonization is increased by mass gatherings such as Hajj. Multiple pathogen carriage and antibiotic resistant isolates increased from pre-Hajj to post-Hajj cohort by 40% in 2013 Hajj (Memish *et al.*, 2015). The crowding factor, excessive human-human and human-animal interactions, increased antibiotic use and stress factor could affect this spread of infections (Alzeer, 2009).

CONCLUSION

Our findings on selected zoonotic pathogens among Kelantan Hajj pilgrims in year 2016 showed that only *S. pneumoniae* and *K. pneumoniae* were present in pilgrims' throat swab specimens. Detection of their related genes showed that *S. pneumoniae* was found to carry *lytA* gene for autolysin, *mefA*, and *pbpA* genes for macrolide and penicillin resistance. *K. pneumoniae* carried *magA*, *K2A* and *rmpA* genes representing both K1 and K2 serotypes and mucoid phenotype as well as bla_{KPC} and bla_{OXA-48} genes associated for carbapenemase resistance. There are some limitations in this research, including insufficient clinical data of the participants, limited pathogen testing and no additional conventional testing done to further confirm the results, including the use of standard antibiotic sensitivity test for antibiotic resistance and culture method for phenotypic expressions of the virulence genes. Future studies should therefore address these limitations. This work provided insight into the existence of zoonotic respiratory pathogens inducing Hajj RTIs in Kelantan pilgrims. Clinically significant respiratory pathogens could be easier identified using molecular methods, such as PCR assays. This research showed promising findings regarding pathogens of concern and could be used for zoonotic studies in Hajj settings. The findings of the current research will result in potential control measures for the management of zoonotic infections among Hajj pilgrims.

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