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Serological study of Bordetella Pertussis, Mycoplasma Pneumonia and Chlamydia Pneumonia in Iranian hajj pilgrims with prolonged cough illnesses: A follow-up study

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ABSTRACT

Introduction: Hajj pilgrimage is the biggest and longest mass gathering in the Muslim world. Annually, about 50% of more than 2.5 million pilgrims participating in this ritual get involved in severe devastating coughs. Most coughs continue, so the pilgrims turn back home and transmit them to family members and other people. Despite the high prevalence of coughs for many years, what causes them remains unknown. Considering the pertussis-like clinical picture of the so-called "hajj coughs", the researchers conducted a study to measure antibodies against three known common atypical bacteria, namely Bordetella Pertussis, Chlamydia Pneumonia and Mycoplasma Pneumonia.

Patients and methods: The study was done on three out of eleven groups of pilgrims from Yazd province, central Iran. The sample was selected randomly and consisted of 202 pilgrims who completed an informed consent. Their blood samples were taken, and the plasma was separated and then stored at -70 °C. After turning back from the journey, the pilgrims had their second blood samples taken. As many as 52 pilgrims failed to come for the second sampling, and two samples were broken during transportation. The final analysis was performed on the remaining 148 pairs of samples.

Results: Antibodies were already elevated in many pilgrims before the journey probably due to their old age (causing more exposure to pathogens) or unplanned pertussis vaccination. After their return, antibody elevation was only mild, again probably due to the old age of the participants (i.e. due to their weaker immune systems). Some antibodies even fell down without any known reason. In this study, previous hajj journey was assumed as a prophylactic factor, due to acquisition of immunity. Coughs with a prolonged pertussis-like picture were also presumed to be more related than other types of coughs to atypical pathogens. Statistical tests showed that the history of previous journeys had no prophylactic effect. Also, no correlation was found between the clinical pictures of coughs and infection with atypical bacteria.

Conclusion: Even though some rises and falls occurred in the antibodies titer, the variations could hardly be attributed to coughs in this study. Indeed, the variation of antibodies had no meaningful relationship with clinical factors. In this regard, further studies are needed to clarify the reason for the so-called "hajj coughs", but epidemiological studies will be difficult to do until easier and more reliable methods become available for accurate diagnosis.

1. Introduction

Based on the statistics of the royal embassy of Saudi Arabia, more than 2.5 million Muslims annually arrive in the Kingdom of Saudi Arabia (KSA) for the hajj ritual. Amongst them are 97,000 Iranians, the maximum allowed for Iran under the Saudi apportioning pilgrim quota. Iran is, indeed, one of the world's biggest Muslim countries whose pilgrims congregate in the holy Saudi cities of Mecca and Medina. Also, hajj is the biggest and most longstanding annual mass gathering (MG) in the world. Like other MGs, hajj increases spread of many infectious diseases, especially respiratory disorders [1].

During a one-month stay in Medina and Mecca, pilgrims face overcrowding everywhere, from crowded chartered flights to hotels or tents where they stay to taxis and shopping centers. Overcrowding culminates during Kaaba pilgrimage, in such a way that there are seven persons clustered in a square meter and cough/sneeze etiquettes are

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very poor. Transmission of pathogens in such a situation is very probable [2].

Cough is so common during hajj pilgrimage. After getting back home, every pilgrim is expected to be afflicted by the so called 'Hajj cough'. In fact, it affects more than 50% of pilgrims [3]. Coughs are transmissible, and many families and friends become involved with them as hajj's souvenir.

Unfortunately, despite the high prevalence of hajj respiratory illnesses for many years, the overall epidemiology is still poorly understood. Some studies have suggested many viral and bacterial etiologies [4–24] or even assumed non-infectious causes for coughs, such as allergens and toxic materials [25].

For more than 25 years, one of the authors (M.R) kept pilgrims returning home under close watch. His observations have revealed that, after a short incubation and prodromal catarrhal phase without a high fever, coughs usually begin with a burning or tingling sensation in the throat. Nonproductive coughs gradually worsen and, most of the time, convert to paroxysms with whoops, which leads to nighttime awakening and sometimes cyanosis, gagging and vomiting. Paroxysms may be precipitated by deep inspirations or laughing. Patients also have mild generalized weakness and perspiration at this time. Paroxysms are almost always more severe in atopic individuals, and, in fact, a non-atopic companion or spouse on the journey usually has milder or sometimes no symptoms at all. Dyspnea and wheezing are also frequent mostly in atopic patients. Complications such as cough syncope, subconjunctival hemorrhage and rib fractures occur but are not common. Many family members and other closely contacted relatives, especially atopic ones, are also involved thereafter.

Respiratory tract infections are the most common causes of persistent coughs [27]. Among them, infections with B. Pertussis [28–32], Chlamydia Pneumonia (C. Pneumonia) [31,33–36] and Mycoplasma Pneumonia (M. Pneumonia) [31] are the best known. Based on the quality and duration of coughs, exacerbating airway hyper-responsiveness, communicability and no presence of fever, a study was conducted to measure atypical bacterial antibodies in pilgrims of Yazd, a central province of Iran. Due to close clinical resemblance of coughs to pertussis, we assumed them as pertussis-like coughs, as described elsewhere [26]. Previous studies showed that Bordetella Pertussis (B. Pertussis) was common in pilgrims turning back home [18]. Based on the long duration of hajj and the severe degree of overcrowding, especially in last days of the ceremony, when the chance of mixed infections may increase, the present study examines the probability such disorders.

2. Materials, participants and methods

This follow-up seroepidemiological study was conducted to search for evidence of solitary and mixed infections with B. Pertussis, C. Pneumonia and M. Pneumonia among adult pilgrims turning back home from Saudi Arabia to Yazd, Iran in October through November 2014. The ethics committee of Shahid Sadoughi University of Medical Sciences approved the study (NO. 29352/Date: June 1st, 2014).

Three out of eleven groups of Yazd city pilgrims were recruited randomly. These pilgrims had been grouped based on a quota system completely distinct from the study objectives. The exclusion criteria were a history of previous or current unexplained dyspnea or cough, cardiopulmonary diseases, symptomatic gastroesophageal reflux, and angiotensin converting inhibitor consumption within the past month. Patients with known or suspected immunodeficiency or immunosuppressive drugs consumption, who could not produce sufficient antibodies, were also excluded.

At first, the researchers explained the purpose of the research to the pilgrims in a mosque where they were gathered, educated and arranged for the journey. All the pilgrims completed questionnaires (the questionnaire was filled by the researchers if a pilgrim could not read and/or write), and if there were no exclusion criteria, informed consent was obtained. Then, first blood samples were taken by a laboratory phlebotomist. To those who could not read and/or write, the details of the study were discussed, and oral consents were obtained. Fortunately, none of the participants refused to give a blood sample probably because of their interest in elucidation of cough origin that they have already known and feared.

At this stage, blood samples were taken from two hundred and two participants. Plasma was then separated from the blood and saved at -70 °C. The researchers numbered the samples, but the patients' names remained unknown to the laboratory technicians. About one month after the journey, the pilgrims turned back gradually during a ten-day period. During the first round of taking blood samples, the pilgrims were requested to turn back after the pilgrimage for the second sample. Even if they did not turn back voluntarily during one week after return, we would invite them for the second blood sample by phone. During blood sampling, the researchers filled a short questionnaire for the presence or absence of cough and its quality. If the pilgrims refused to come for the second sampling, the first sample was also discarded. Once a second sample was taken, the plasma was separated from it and saved at -70 °C. Upon the completion of sampling, the plasma samples were paired, and defrosted, and laboratory investigation started. Totally, 52 pilgrims did not accept to take part in the second sample despite frequent phone calls; nearly all of them claimed they had no cough. During transport of the frozen samples to the laboratory, two of them were broken, and the analysis was done on 148 remaining paired samples (72 female and 76 male).

2.1. Cough characteristics

For the purpose of statistical analyses, first, the pilgrims were divided into two groups of coughers and non-coughers. One assumption of this study was that, if coughs had pertussis-like characteristics and a long duration, the possibility of their relationship with atypical bacteria would be more likely. To examine the probabilities, we divided the coughing patients into four categories based on the acuteness or prolongation of coughs and pertussis- or non-pertussis-like pictures. Acute coughs were those with a duration of less than 21 days. Coughs were considered as pertussis-like if the coughing pilgrims had two or more of the three symptoms including paroxysmal coughs, night-time coughs and post-tussive emesis. It should also be mentioned that acute coughs may become prolonged if adequate time passes on.

The probability of milder symptoms in pilgrims with a history of a previous hajj journey (within 12 years) was also tested. The test was decided on because of the probability of acquiring immunity during a previous journey.

2.2. Laboratory assessments

Immunoglobulins M and G for B. pertussis, C. Pneumonia and M. Pneumonia were measured respectively by Enzyme-Linked ImmunoSorbent Assays (ELISA) methods (abbreviated as B. IgM and B. IgG, C. IgM and C. IgG, and M. IgM and M. IgG).

To measure B. Pertussis' IgM and IgG, MASTAZYMETM BORDTELLA kits were used (Mast Diagnostica GmbH; FeldstraBe 20, DE-23858 Reinfeld, Germany). Also, measurements of C. Pneumonia, M. Pneumonia's IgM, and IgG were made with Medac Company's kits (Medac; Gesellschaft für klinische, Spezialpräparate mbH, Theaterstrasse 6, D-22880 Wedel, Germany).

All the laboratory procedures were precisely followed as instructed by the manufacturers. To avoid interference with rheumatic factors, for B. IgM, B. IgG, C. IgM and M. IgM, serum pre-absorption with IgG/RF was done (MASTSORBTM. Mast Diagnostica GmbH; FeldstraBe 20, DE-23858 Reinfeld, Germany); however, no pretreatment of the samples, e.g. inactivation, was necessary for C. IgG and M. IgG.

Sero-prevalence (seropositive: IgM + /IgG-, IgM + /IgG + , IgM-/IgG + or seronegative: IgM-/IgG-), sero-conversion (seronegatives

developed antibodies and became seropositives during the study period) and sero-reversion (seropositives became seronegatives during the study period) were detected according to the manufacturers' instructions. Mixed infections were described as infections with two or more pathogens.

Values lower than, within or higher than the gray zone were classified as negative, equivocal and positive respectively. The gray zones (cut-off ± x %) were 8–12 U/mL (10 ± 20%) for B. IgM, 16–24 U/mL (20 ± 20%) for B. IgG as calculated by standard curves, 0.9–1.1 OD (1 ± 10%) for C. IgM and G and M. IgM calculated semi-quantitatively, and 9–11 arbitrary unit (AU) (10 ± 10%) for M. IgG calculated by one-point quantification.

Equivocal samples (borderline results) may indicate commencing or subsiding infections (despite clinical symptoms) and should be retested together with a fresh specimen taken 14 days later. In the present study, follow-up was desirable, but our cases were not accessible after the required 14-day period; therefore, we just determined sero-conversion or sero-reversion after about 40 days. To tackle with this problem, we performed sensitivity analyses, considering the equivocal results as negative or positive.

2.3. Statistical analysis

A statistical analysis was done using SPSS software for Windows (Release 22.0, SPSS Inc., Chicago, U.S.). Two-tailed p-value of < 0.05 was considered significant.

Differences in mean values of pre- and post-hajj changes in immunoglobulins were assessed in each participant by the Student t-test. A paired-samples *t*-test was used to evaluate the differences between pre- and post-hajj immunoglobulins in all the participants. In addition, where the immunoglobulins were considered as a dichotomous variable (negative or positive), a McNemar test was used. This test was also used when examining solitary or mixed infections. This test was also performed in the case of subgroups of types of cough and previous Hajj trips.

To examine the concept of mixed infection more consistently, we performed McNemar and Chi-square tests by considering different combinations of pathogens. They included B. Pertussis associated with C. Pneumonia, B. Pertussis with M. Pneumonia, C. Pneumonia with M. Pneumonia, 'any two pathogens' and 'all three pathogens'.

Three-way Mixed ANOVA was performed to assess the interactions between pre- and post-hajj differences of immunoglobulins with regard to the types of cough in the categories of coughers and non-coughers. This test was also used to understand any three-way interaction between the previous hajj journeys, the types of cough and the current hajj journey. Differences of the mean values of pre- and post-hajj changes in immunoglobulins between coughers and non-coughers were measured by the Student's t-test.

The differences of immunoglobulins between the subgroups of types of cough and the previous hajj journey were determined by using a chi-square test of homogeneity. This test was also used when examining solitary or mixed infections. Pairwise comparisons (with Bonferroni adjustment) were done for statistically significant, but not equal, group proportions. In addition, we conducted a sensitivity analysis, considering equivocal results as negative or positive.

3. Results

The main characteristics of the 148 participants are shown in Table 1. Of these 148, 93 (63%) complained of cough, and their average duration of cough was 22.3 ± 8.6 days. The main characteristics of coughs are also presented in Table 1. For practical and statistical purposes, we divided coughs into four categories including acute, chronic, pertussis-like, and non-pertussis-like coughs.

As shown in Table 2, the Student's t-test did not reveal any differences in the mean values of pre- and post-hajj immunoglobulins between the coughers and the non-coughers, except for B. IgG (which is not practically important).

Table 1
Demographic data and cough characteristics in 148 Iranian hajj pilgrims.

Age	47.6 ± 9.9 Years		
Gender	74 (50%) Males		
Post-tussive emesis	19 (12.8%)		
Paroxysmal cough	56 (37.8%)		
Night cough	71 (48%)		
Types of cough[a]	**Previous hajj journey**		**Total**
	No	**Yes**	
No cough	30	25	55
Acute non-pertussis-like	15	13	28
Acute pertussis-like	9	8	17
Prolonged non-pertussis-like	10	4	14
Prolonged Pertussis-like	19	15	34
Total	83	65	148

[a] "Pertussis-like coughs" mean those coughs of at least two of these three types: posttussive emesis, paroxysmal coughs and night-time coughs. Acute coughs mean coughs going on for less than 21 days. Coughs longer than that are named "prolonged coughs".

The next six tables and 12 plots show two-way and three-way interactions, respectively, between the immunoglobulins of the current Hajj pilgrims (IgM and IgG for B. Pertussis, C. Pneumonia and M. Pneumonia), history of previous hajj journey, and types of cough, as in Figs. 1–3 consecutively. There were significant two-way interactions just in two instances (the current hajj journey and the history of previous hajj journey for B. IgM; and the current hajj journey and rhe types of cough for B. IgG).

Table 3 shows the results of paired-sample t-tests for the effect size of the difference between pre- and post-hajj immunoglobulins for B. Pertussis, C. Pneumonia and M. Pneumonia according to previous hajj journey and types of cough in 148 Iranian hajj pilgrims.

We used McNemar's test to show the sero-prevalences after converting the equivocal pre- and post-hajj immunoglobulins to negative or positive assumptions (Table 4). The last column examines the prevalence of mixed infections. This test had no statistically significant results when the equivocal samples were considered as negative. This means there was no difference between the pre- and post-hajj immunoglobulins. In addition, considering the equivocal samples as positive, the sensitivity analysis provided results which were not sufficiently different so as for one to draw different conclusions from them.

A chi-square test of homogeneity showed no statistically significant difference between rate of coughing and the history of previous hajj. For a few instances, due to the insufficient sample size, the test results were not valid. As a valid equivalent of this test, Fisher's exact tests were not statistically significant either. Considering the equivocal samples as both negative and positive, the sensitivity analysis provided results that were not sufficiently different so as for one to draw different conclusions from them (The sensitivity analysis data are not shown). The mixed infection differences between the subgroups of types of cough and the previous hajj journey are presented in Table 5.

The next six tables and 12 plots show two- and three-way interactions, respectively, between the immunoglobulins of the current hajj pilgrimage (IgM and IgG of B. Pertussis, C. Pneumonia and M. Pneumonia), the history of previous hajj journey, and the types of cough (with five categories) as in Figs. 4–6 consecutively.

McNemar and Chi-square tests were performed with a consideration of different combinations of pathogens for mixed infections (B. Pertussis and C. Pneumonia, B. Pertussis and M. Pneumonia, C. Pneumonia and M. Pneumonia, "any two pathogens" and "all three pathogens"). It provided results that differ insufficiently for different conclusions to be drawn, for both (data not shown).

Table 2
Student's t-test for the effect size of differences in the mean values of pre- and post-hajj changes in immunoglobulins of B. Pertussis, C. Pneumonia and M. Pneumonia between the coughers and the non-coughers in 148 Iranian hajj pilgrims.

	Immunoglobulins[a]	Bordetella Pertussis		Chlamydia Pneumonia		Mycoplasma Pneumonia	
		IgM	**IgG**	IgM	IgG	IgM	IgG
Types of cough	No cough (N = 55)	−1.7	**0.23**	0.13	−0.2	0.93	0.45
	Cough (N = 93)	−1.2	**−0.2**	0.19	−0.3	0.86	0.54

Significant at the level of < 0.05 (Bold).
[a] Logarithmically transformed and outliers were removed.

4. Discussion

Pilgrims of hajj are frequently affected by severe prolonged coughs [3]. In this study, antibodies against three bacteria, which are known to cause severe coughs in the general population, were measured in pilgrims before and after the hajj journey. These bacteria were B. Pertussis, M. Pneumonia and C. Pneumonia. The concept of mixed infection was also examined.

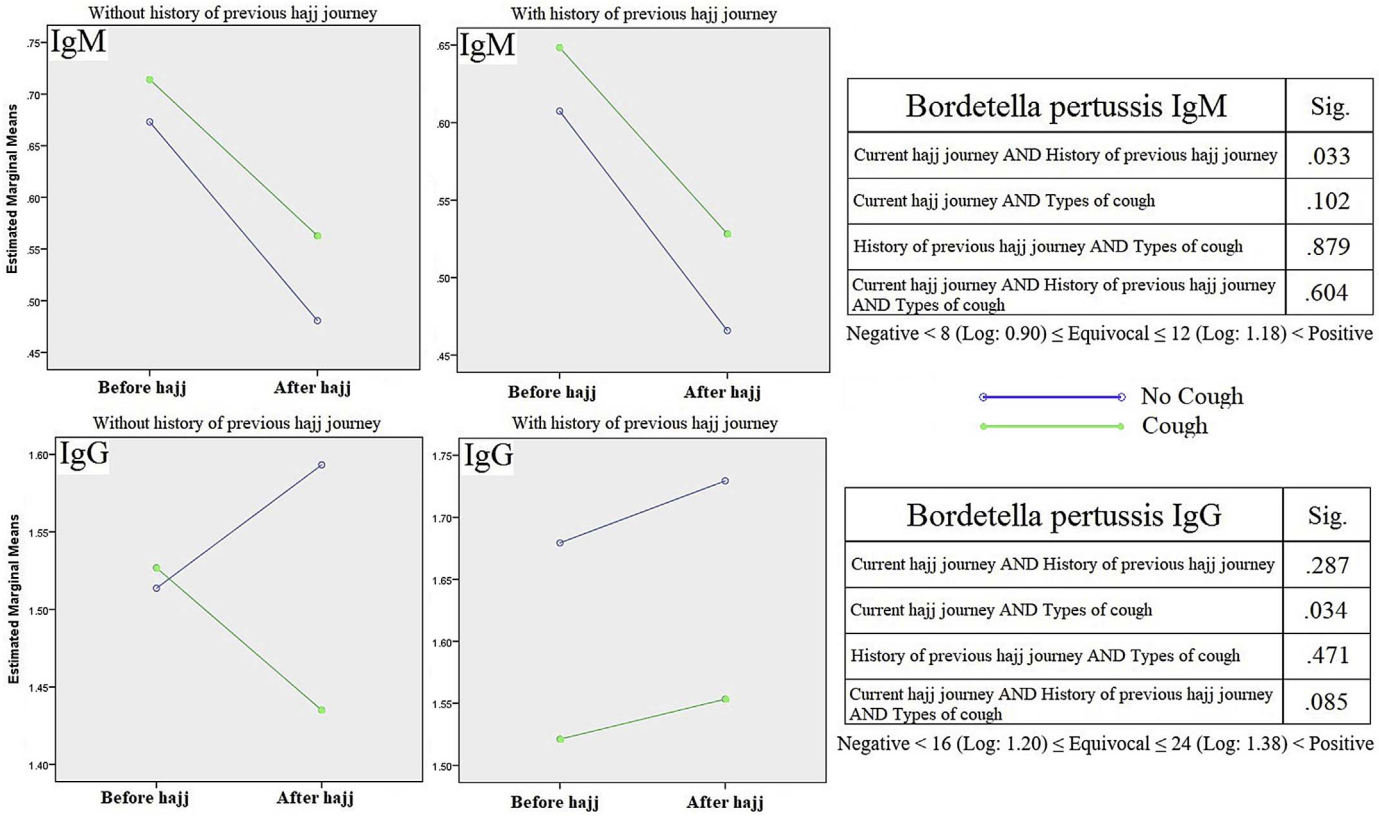
The collateral assumptions of the study were 1) these three bacteria cause severe pertussis-like cough and not a mild cough, and 2) if the pilgrim has a history of recent (within past 12 years) hajj journey, involvement of him or her by these three bacteria must be milder due to acquisition of bacteria in previous journey/s and presence of immunity against them.

In general, the results of this study showed that, even though some rises and falls occurred in the antibodies against these three bacteria, there were no correlations between these changes in the antibodies and the clinical features of coughs (or history of previous hajj journey).

As the plots in Figs. 1–6 show, there are increases as well as decreases in the titer of some immunoglobulins G and/or M for all the three pathogens. As Table 3 suggests in details, the effect size was negative for B. Pertussis IgM (decrease in immunoglobulin) after the pilgrims came back home. In this regard, the level of significance was < 0.01 in all the five types of cough and in both groups with and without a history of previous hajj journey. The reason for this fall in the antibodies remained obscured. On the other hand, the effect size was positive for both IgM and IgG for M. Pneumonia (increase in immunoglobulins). The level of significance was < 0.01 in all the four types of cough subgroups after returning home.

Examining the interactions by three-way mixed ANOVA showed that there were no two- or three-way interactions among the history of previous hajj journey, the current hajj journey and the subtypes of cough (except in two instances with no important clinical significance). The paired-samples *t*-test (Table 3), McNemar test (Table 4) and Chi-square test (Table 5) were used to retest this finding; all of these tests confirmed the ANOVA results, which means that there is no relationship between antibody titers and previous history of hajj journey or types of cough. This finding rejects the hypothesis that a previous hajj journey may protect pilgrims with the presence of antibodies in their blood. The findings also showed that pertussis-like type of cough could not be clinically attributed to atypical bacterial infections.

These results should be interpreted as confirmatory of exposure to



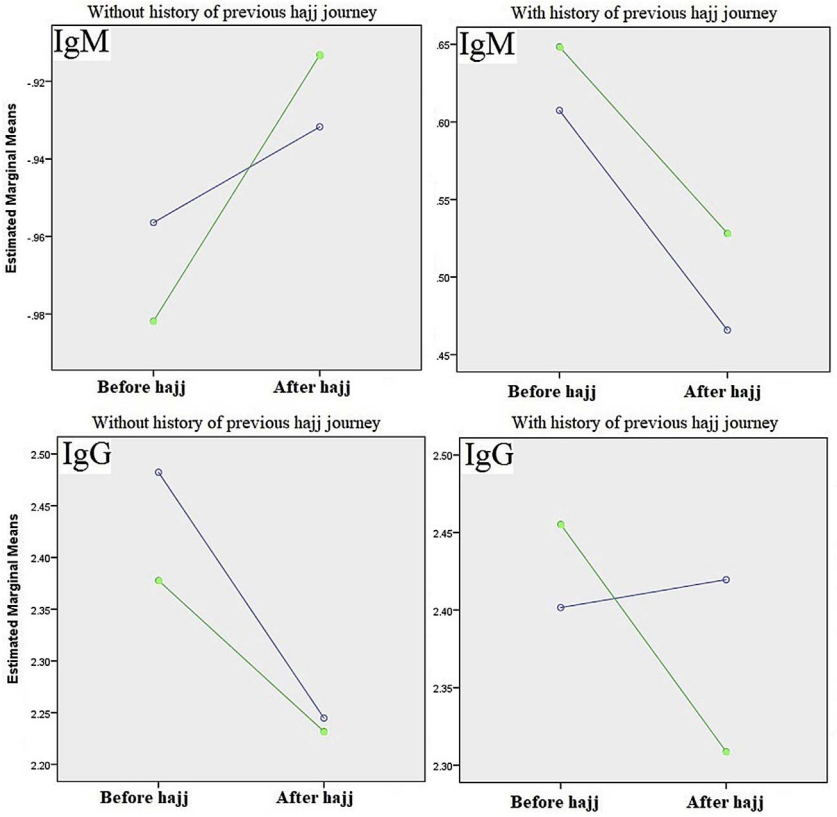
Bordetella pertussis IgM	Sig.
Current hajj journey AND History of previous hajj journey	.033
Current hajj journey AND Types of cough	.102
History of previous hajj journey AND Types of cough	.879
Current hajj journey AND History of previous hajj journey AND Types of cough	.604

Negative < 8 (Log: 0.90) ≤ Equivocal ≤ 12 (Log: 1.18) < Positive

Bordetella pertussis IgG	Sig.
Current hajj journey AND History of previous hajj journey	.287
Current hajj journey AND Types of cough	.034
History of previous hajj journey AND Types of cough	.471
Current hajj journey AND History of previous hajj journey AND Types of cough	.085

Negative < 16 (Log: 1.20) ≤ Equivocal ≤ 24 (Log: 1.38) < Positive

Fig. 1. Two-way mixed ANOVA (table) and three-way mix ANOVA (tables and plots) between the current Hajj journey, the previous Hajj journey and the types of cough (with two categories) for pre- and post-Hajj differences in Bordetella pertussis immunoglobulins M and G of 148 Iranian Hajj pilgrims.



Chlamydia pneumonia IgM	Sig.
Current hajj journey AND History of previous hajj journey	.320
Current hajj journey AND Types of cough	.586
History of previous hajj journey AND Types of cough	.509
Current hajj journey AND History of previous hajj journey AND Types of cough	.398

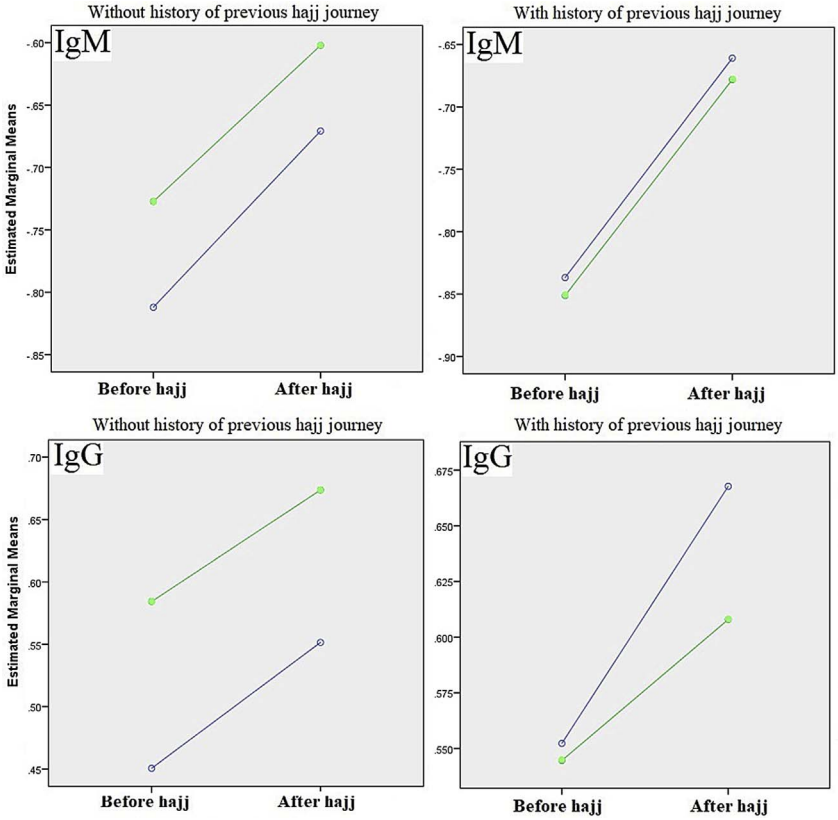
Negative < 0.9 (Log: -0.05) ≤ Equivocal ≤ 1.1 (Log: 0.04) < Positive

No Cough
Cough

Chlamydia pneumonia IgG	Sig.
Current hajj journey AND History of previous hajj journey	.231
Current hajj journey AND Types of cough	.732
History of previous hajj journey AND Types of cough	.932
Current hajj journey AND History of previous hajj journey AND Types of cough	.229

Negative < 0.9 ≤ Equivocal ≤ 1.1 < Positive

Fig. 2. Two-way mixed ANOVA (table) and three-way mixed ANOVA (tables and plots) between the current hajj journey, the previous hajj journey and the types of cough (with two categories) for pre- and post-hajj differences in Chlamydia Pneumonia immunoglobulins M and G of 148 Iranian hajj pilgrims.



Mycoplasma pneumonia IgM	Sig.
Current hajj journey AND History of previous hajj journey	.155
Current hajj journey AND Types of cough	.744
History of previous hajj journey AND Types of cough	.388
Current hajj journey AND History of previous hajj journey AND Types of cough	.820

Negative < 0.9 (Log: -0.05) ≤ Equivocal ≤ 1.1 (Log: 0.04) < Positive

No Cough
Cough

Mycoplasma pneumonia IgG	Sig.
Current hajj journey AND History of previous hajj journey	.857
Current hajj journey AND Types of cough	.321
History of previous hajj journey AND Types of cough	.318
Current hajj journey AND History of previous hajj journey AND Types of cough	.528

Negative < 9 (Log: 0.95) ≤ Equivocal ≤ 11 (Log: 1.04) < Positive

Fig. 3. Two-way mixed ANOVA (table) and three-way mixed ANOVA (tables and plots) between the current hajj journey, the previous hajj journey and the types of cough (with two categories) for pre- and post-hajj differences in Mycoplasma Pneumonia immunoglobulins M and G of 148 Iranian hajj pilgrims.

Table 3
Paired-samples *t*-test for the effect size of the difference between pre- and post-hajj immunoglobulins of B. Pertussis, C. Pneumonia and M. Pneumonia according to previous hajj journey and types of cough in 148 Iranian hajj pilgrims.

	Immunoglobulins[a]	Bordetella Pertussis		Chlamydia Pneumonia		Mycoplasma Pneumonia	
		IgM	IgG	IgM	IgG	IgM	IgG
	Total	**−1.9**	−0.1	0.1	**−0.3**	**1.0**	**0.6**
	No cough (N = 55)	**−1.7**	0.1	0.0	**−0.3**	**1.0**	**0.4**
	Acute non-pertussis-like (N = 28)	**−3.0**	0.1	0.1	−0.4	**1.0**	**0.6**
	Acute pertussis-like (N = 17)	**−1.6**	−0.2	0.4	−0.2	**0.9**	**1.1**
	Prolonged non-pertussis-like (N = 14)	**−2.2**	−0.3	−0.2	−0.1	**1.3**	0.6
	Prolonged pertussis-like (N = 34)	**−1.8**	−0.2	0.0	**−0.4**	**0.9**	**0.6**
Previous hajj journey	No	**−1.9**	−0.1	−0.1	**−0.4**	**0.9**	**0.7**
	Yes	**−1.8**	0.0	0.2	−0.2	**1.1**	**0.5**

Significant at the level of < 0.05 (Bold).
Significant at the level of < 0.01 (Bold and underlined).
[a] Logarithmically transformed values and outliers were removed.

these three atypical bacteria at least at the laboratory level. Even in some instances, infections, especially mixed types, seemed to have occurred. However, this process could not explain the typical clinical presentation of severe prolonged coughs ('hajj cough') in Iranian pilgrims.

Jackson et al. showed that Bordetella infections were common among 319 young adult university students (at the incidence of 9%), and, surprisingly, a large number of those infections were mixed with other respiratory pathogens (e.g. influenza A and B, adenovirus, respiratory syncytial virus, mycoplasma, and chlamydia). Like in the present study, there were no major differences in clinical findings between the infected and non-infected participants [42]. In two studies on adult patients with persistent coughs [30,36], Wright SW et al. found B. Pertussis and C. Pneumonia infections in a similar proportion (20%) of patients, but infections concurrent with B. Pertussis or M. Pneumonia were not found in these studies. These studies also confirmed that no clinical symptom or sign could differentiate the infected from the non-infected, except for fever.

Incidences of coughs due to these pathogens in hajj pilgrims are differently reported in available studies. The difference may be due to the demographic features studied (e.g. age of the pilgrims), country of origins of the pilgrims, methods of measuring, criteria for diagnosing infections, etc. It also could be related to fluctuations in different season when hajj takes place; indeed, rather than being held at a fixed time, the ceremony is held in different times in different years. (It is to be noted that the Islamic calendar is a lunar one in which the months are shorter than the Gregorian months for eleven days) [2,24,37,38]. In addition, B. Pertussis and C. Pneumonia epidemics follow a cyclic pattern, with peaks coming every 2–5 years [39,40].

Annelies Wilder-Smith et al. [18] showed that, before hajj, pertussis IgG was positive for 81.8% of pilgrims. However, they reported that only 2.4% of prolonged coughs in their pilgrims were due to pertussis (Surprisingly, this is far lower than that reported in adolescent and adult populations with prolonged coughs in industrialized countries, i.e. 19–26%) [32].

Judy M. Vincent et al. [41] studied antibodies against three pathogens (the same as us) in soldiers on duty in South Korea. They did a case-control study and selected a control group of soldiers in Hawaii. Having measured more antibodies and done PCR, they proved that 76% of the cases were lately involved by one of the three pathogens. In addition, they found that mixed infections were very common. In contrast with old persons in our study, their cases were young soldiers with intact immune systems.

In another study on university students by Mink CM. et al. [43], patterns of antibodies against B. Pertussis antigens were different in the case of patients and control subjects, and 3 of 4 young adults with M. Pneumonia infections had significant titer increases or high values of antibodies against B. Pertussis antigens.

Table 4
Sero-prevalence in the pre- and post-hajj immunoglobulins of B. Pertussis, C. Pneumonia and M. Pneumonia and the mixed infections in 148 Iranian hajj pilgrims.

		Bordetella pertussis			Chlamydia pneumonia			Mycoplasma pneumonia			Mixed Infection
		IgM	IgG	Infection	IgM	IgG	Infection	IgM	IgG	Infection	
Considering equivocal samples as **negative**	Both negative	143(96.6)	46(31.1)	45(30.4)	145(98)	21(14.2)	21(14.2)	145(98)	125(84.5)	125(84.5)	52(35.1)
	Sero-conversion	0	14(9.5)	15(10.1)	1(0.7)	2(1.4)	2(1.4)	2(1.4)	3(2)	3(2)	12(8.1)
	Sero-reversion	3(2)	17(11.5)	17(11.5)	2(1.4)	5(3.4)	5(3.4)	0	0	0	13(8.8)
	Both positive	2(1.4)	71(48)	71(48)	0	120(81.1)	120(81.1)	1(0.7)	20(13.5)	20(13.5)	71(48)
Considering equivocal samples as **positive**	Both negative	**135(91.5)**	28(18.9)	23(15.5)	145(98)	17(11.5)	17(11.5)	142(95.9)	**116(78.4)**	**113(76.4)**	29(19.6)
	Sero-conversion	**0**	8(5.4)	8(5.4)	1(0.7)	2(1.4)	2(1.4)	3(2)	**7(4.7)**	**8(5.4)**	7(4.7)
	Sero-reversion	**9(6.1)**	10(6.8)	13(8.8)	1(0.7)	0	0	1(0.7)	**0**	**1(0.7)**	11(7.4)
	Both positive	**4(2.7)**	102(68.9)	104(70.3)	1(0.7)	129(87.2)	129(87.2)	2(1.4)	**25(16.9)**	**26(17.6)**	101(68.2)

The data are presented as percentages.
Infection with a pathogen was considered to be present if IgM and/or IgG against it was/were positive.
Mixed infection was considered to be present if antibodies against two or more pathogens were positive.
Sero-conversion means negative test results were converted to positive during the study period.
Sero-reversion means positive test results were converted to negative during the study period.
Significant at the level of < 0.05 (Bold).
Significant at the level of < 0.01 (Bold and underlined).

Table 5
Chi-square test of homogeneity for mixed infections; differences between the subgroups of types of cough and the history of previous hajj journey (considering equivocal samples as negative).

			Types of cough					Sig.	History of previous hajj journey		Sig.	Total
			No cough	Acute		Prolonged						
				Non-pertussis like	Pertussis like	Non-pertussis like	Pertussis like		No	Yes		
Mixed Infection	Pre-hajj	Negative	23 (41.8)	12 (42.9)	9 (52.9)	5 (35.7)	15 (44.1)	0.91	37 (44.6)	27 (41.5)	0.71	64 (43.2)
		Positive	32 (58.2)	16 (57.1)	8 (47.1)	9 (64.3)	19 (55.9)		46 (55.4)	38 (58.5)		84 (56.8)
	Post-hajj	Negative	21 (38.2)	10 (35.7)	10 (58.8)	5 (35.7)	19 (55.9)	0.25	40 (48.2)	25 (38.5)	0.25	65 (43.9)
		Positive	34 (61.8)	18 (64.3)	7 (41.2)	9 (64.3)	15 (44.1)		43 (51.8)	40 (61.5)		83 (56.1)
Total			55 (100)	28 (100)	17 (100)	14 (100)	34 (100)		83 (100)	65 (100)		148 (100)

Data are presented as percentages for the variables of types of cough or history of previous hajj journey.

In another study by Miyashita N et al. on adults with persistent coughs [35], C. Pneumonia infection was found to be the main cause of coughs, but B. Pertussis (18.5%) and M. Pneumonia (1.1%) were prevalent at a much lower rate. Dual infections with C. Pneumonia and B. Pertussis were found in three patients. In general, few studies on patients with prolonged cough have failed to show mixed infections.

Skowronski DM et al. studied an epidemy of a pertussis-like illness in a junior high school [44] and determined C. Pneumonia as its cause. The serology and/or the cultures for B. Pertussis, B. Parapertussis, M. Pneumonia, Chlamydia Trachomatis, Chlamydia Psittaci or viruses were all negative. Again, the clinical data could not differentiate the infected patients from the non-infected ones.

Due to the probability of high prevalence of antibodies against these atypical bacteria in the general population, studies which measure the antibodies just after a hajj journey may estimate the prevalence wrongly. However, the present study has the advantage of measuring antibodies before and after exposure to pathogens during such a journey.

In diagnosing pertussis-like coughs, most definitions advocate at least one of the three symptoms, namely paroxysms, inspiratory whoop, and posttussive vomiting [26]. However, this study focused on ≥2 symptom to increase the specificity of the diagnosis. Inevitably, some patients with milder pertussis-like coughs were missed, but this could not distort the results.

The time frame of coughs lasting more than two weeks has been used by some authors as the expected time frame for diagnosing prolonged coughs for B. Pertussis [45,46] and C. Pneumonia [36]. In the present study, three weeks was taken into consideration because the median cough duration in this study was 23 days. Thus, this led to more equal groups for statistical analyses (though the analyses brought about the same results with a two-week time frame).

One of the problems with the present study is that there were no

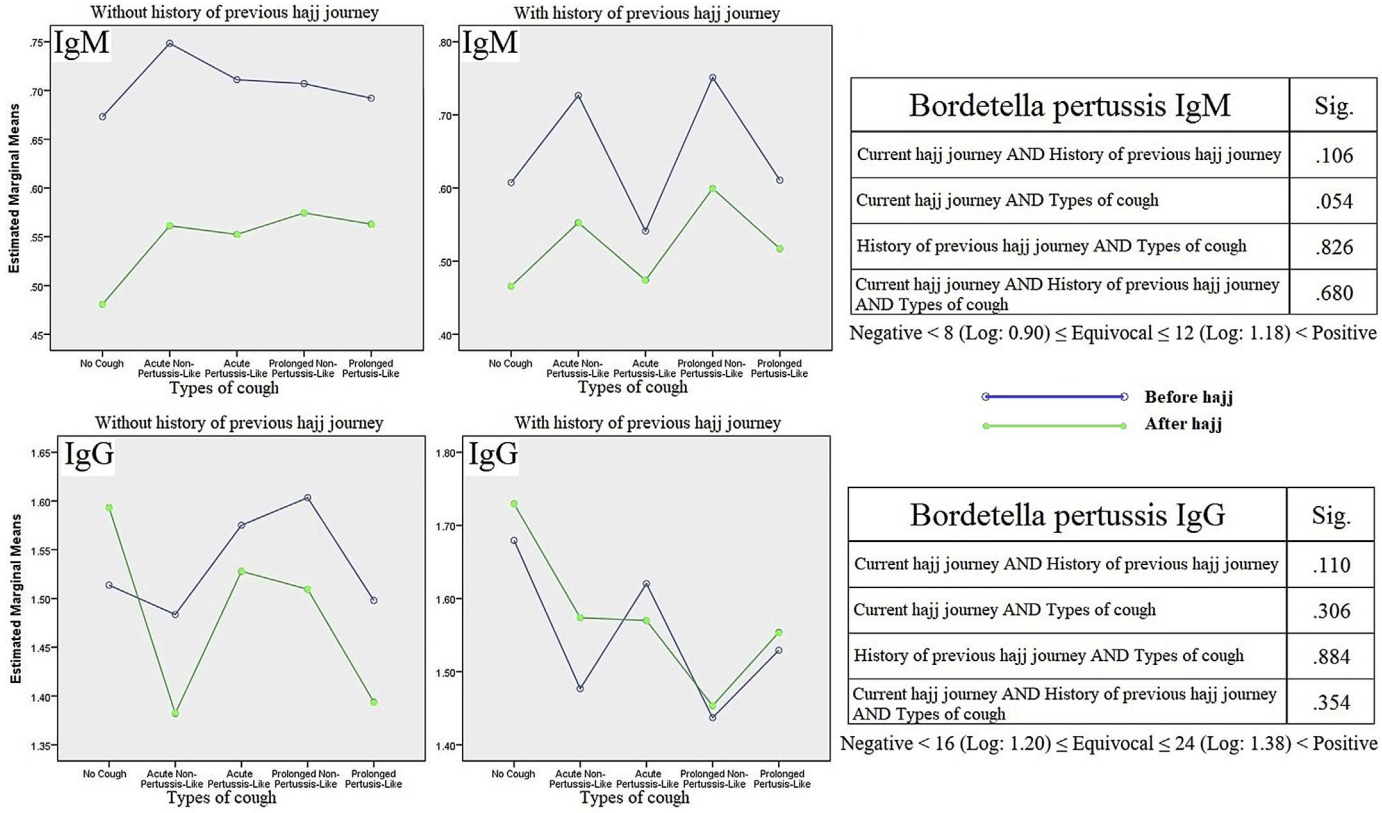
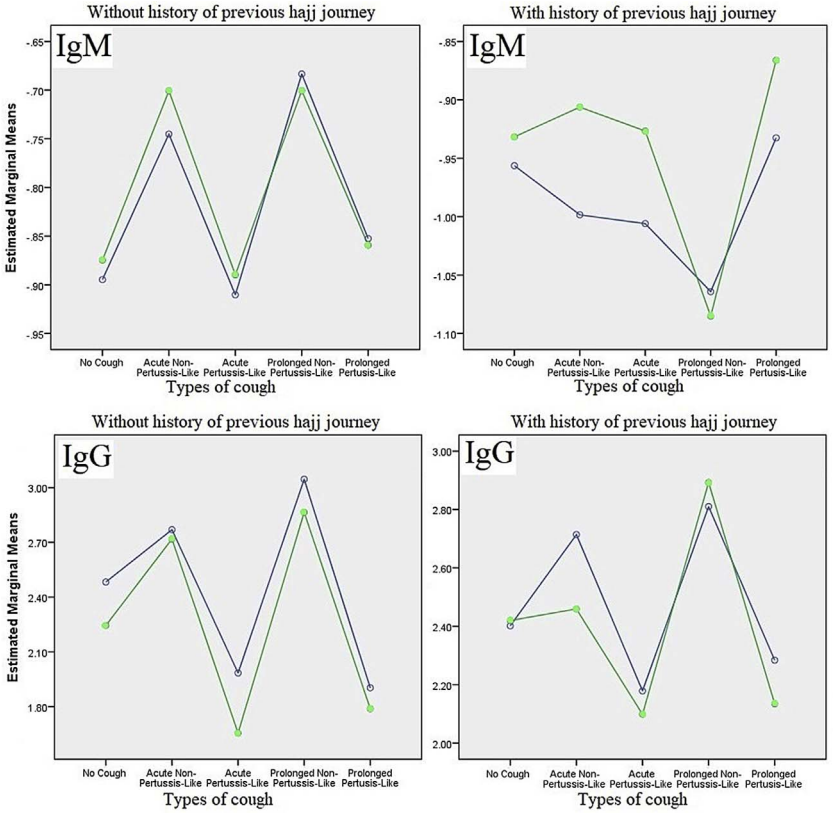


Fig. 4. Two-way mixed ANOVA (table) and three-way mixed ANOVA (tables and plots) between the current hajj journey, the previous hajj journey and the types of cough (five categories) for pre- and post-hajj differences of immunoglobulins M and G for Bordetella Pertussis in 148 Iranian hajj pilgrims.



Chlamydia pneumonia IgM	Sig.
Current hajj journey AND History of previous hajj journey	.317
Current hajj journey AND Types of cough	.697
History of previous hajj journey AND Types of cough	.689
Current hajj journey AND History of previous hajj journey AND Types of cough	.919

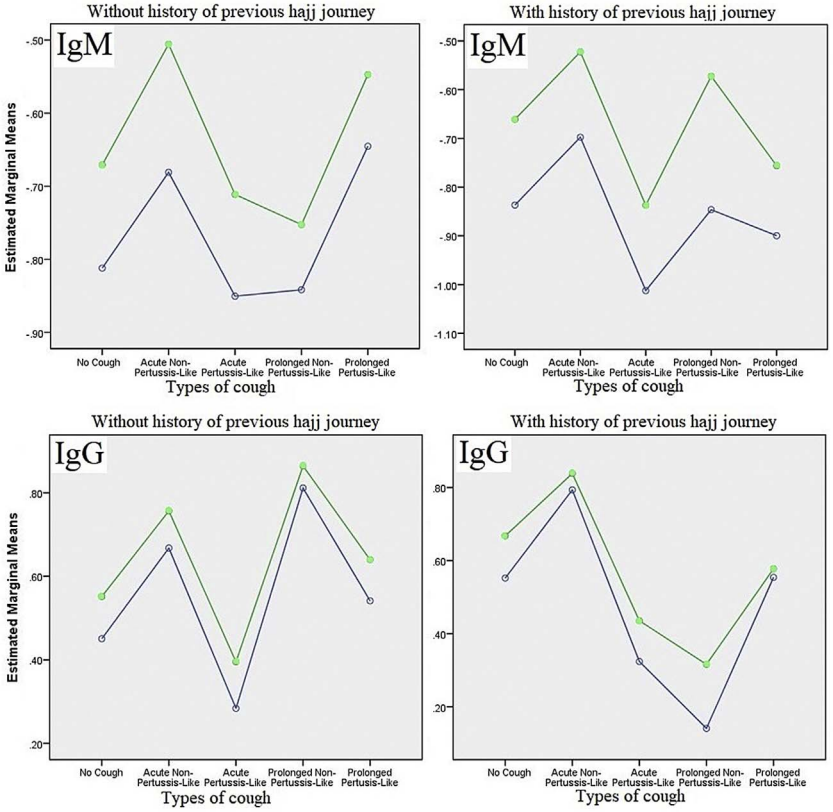
Ncgativc < 0.9 (Log: -0.05) ≤ Equivocal ≤ 1.1 (Log: 0.04) < Positivc

Before hajj
After hajj

Chlamydia pneumonia IgG	Sig.
Current hajj journey AND History of previous hajj journey	.379
Current hajj journey AND Types of cough	.971
History of previous hajj journey AND Types of cough	.841
Current hajj journey AND History of previous hajj journey AND Types of cough	.513

Negative < 0.9 ≤ Equivocal ≤ 1.1 < Positive

Fig. 5. Two-way mixed ANOVA (table) and three-way mixed ANOVA (tables and plots) between the current hajj journey, the previous hajj journey and the types of cough (with five categories) for pre- and post-hajj differences of immunoglobulins M and G for Chlamydia Pneumonia in 148 Iranian hajj pilgrims.



Mycoplasma pneumonia IgM	Sig.
Current hajj journey AND History of previous hajj journey	.067
Current hajj journey AND Types of cough	.724
History of previous hajj journey AND Types of cough	.395
Current hajj journey AND History of previous hajj journey AND Types of cough	.648

Negative < 0.9 (Log: -0.05) ≤ Equivocal ≤ 1.1 (Log: 0.04) < Positive

Before hajj
After hajj

Mycoplasma pneumonia IgG	Sig.
Current hajj journey AND History of previous hajj journey	.920
Current hajj journey AND Types of cough	.714
History of previous hajj journey AND Types of cough	.192
Current hajj journey AND History of previous hajj journey AND Types of cough	.582

Negative < 9 (Log: 0.95) ≤ Equivocal ≤ 11 (Log: 1.04) < Positive

Fig. 6. Two-way mixed ANOVA (table) and three-way mixed ANOVA (tables and plots) between the current hajj journey, the previous hajj journey and the types of cough (with five categories) for pre- and post-Hajj differences of immunoglobulins M and G for Mycoplasma Pneumonia in 148 Iranian hajj pilgrims.

facilities to measure IgA and PCR. However, ELISA for detecting IgM and IgG had high specificity and sensitivity in the appraisal of the infection status. It should be mentioned that cross-reaction with antigens against other bacteria can make false positive results [41,47–51]. As an example, M. Pneumonia has many glycolipid and glycoprotein antigens, which have cross-reactions with many infectious and non-infectious agents, one of which is B. Pertussis [43]. Høiby et al. [51] showed that two B. Pertussis antigens have cross-reactions with many bacteria especially gram-negative ones.

Diagnosing B. Pertussis by serology depends on the vaccination history. However, IgG anti-PT (i.e. pertussis toxin determined in our ELISA kits) can indicate acute/recent infection with B. Pertussis if more than a year has gone from pertussis vaccination. Therefore, one big confounder can be pertussis vaccination prior to a hajj journey. Even though immunity lasts for 4–12 years after adult-type acellular pertussis vaccination [52–54] and a booster vaccination is not obligatory for Iranian pilgrims, some pilgrims had a spontaneous vaccination which, unfortunately, was not taken into account in this study. This may be one of the causes for high pre-hajj B. Pertussis IgM titer in our study.

It should also be mentioned that many of the cases in this study were of old age (with the mean age of 47.6 ± 9.9 years), which could cause two problems in relation to antibody production. Firstly, the chance of exposure to pertussis and, therefore, antibody production against it was higher, especially Bordetella IgG [55,56]. This may have caused a high titer of antibodies at the start of the study and, therefore, a non-significant rise of it. Secondly, weak immune systems may have caused suppressed antibody production against microbes and, thus, lower antibodies' titer rise in spite of heavy exposure to microbes. Unfortunately, there was no possibility of making adjustments for the effects of age on the immunoglobulin production because there was not sufficient variability in the age of our pilgrims (i.e. most of them had high ages). These can be explanations for a decrease in some immunoglobulins.

As mentioned, there are many unresolved issues with no agreement on them about hajj's respiratory diseases (e.g. 'hajj cough'). To overcome such a confusion, the authors of this study suggest that respiratory diseases that occur during hajj inside the KSA be separated from those that occur after pilgrims' returning home. Inside the KSA, the biggest issues are severe acute respiratory problems mainly occurring in old-age people who already have certain underlying diseases. Their respiratory diseases are often caused by influenza [57], as the most common vaccine-preventable respiratory virus infection identified among hajj pilgrims [58]. Due to patients' weakness, this infection frequently leads to hospitalization even in intensive care units [38]. It is also marked with a high mortality rate, as reported in some studies [20,23,24,59,60]. As opposed to these respiratory troubles, there is 'hajj cough' which mostly occurs in younger healthier pilgrims upon their returning home. It is not grave, and the patients are usually treated as outpatients [3]. In accordance with this suggestion is the fact that those researchers who work outside the KSA (even Saudis) do not recommend influenza vaccination based on their experiences and studies performed on pilgrims turning back to their own countries, when the influenza problems are finished [13,37,61–65]. Rashid et al. conducted a study on pilgrims who returned to UK, emphasizing that epidemiology of influenza and the effect of its vaccination on mass gatherings are not well understood [68]. However, inside KSA, researchers have found influenza vaccination crucial because influenza causes severe respiratory diseases in pilgrims and may even lead to hospitalization and mortality [57,66,67,69].

Hajj cough is not only a matter of concern in pilgrims but also a public health problem especially in infants who usually acquire pertussis from an infected adult [70]. Also, many pilgrims return to countries where pertussis vaccination may not be a part of childhood immunization programs.

5. Conclusion

Exposure to some pathogens exists, at least, at a laboratory level, and, even in some cases, there occur infections especially mixed-type ones. However, these pathogens cannot account for the so-called 'hajj cough'. Lack of a definite explanation in this regard might be due to the weak points of the present study, some bacterial infections, viral infections, or presence of toxic or allergic materials. It should be kept in mind that epidemiologic and even treatment studies will be difficult until easier and more reliable methods of diagnosis become available. Finally, the authors hypothesize that 'hajj cough' is not just an atypical infection; there is an atopy case involved too.

Ethics committee approval

This study was approved by the local ethics committee of Shahid Sadoughi University of Medical Sciences, Yazd, Iran. Informed consent was taken from all the participants.

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Authors' contributions

Dr. Masoud Rahimian and Mahdi Hosseini B had full access to all the data. They contributed to the study design, data interpretation and writing of the manuscript. They take the responsibility for data acquisition and confirm that the tests followed guidelines and the results are valid. They also guarantee the integrity and accuracy of data analysis.

Conflict of interests statement

There is no conflict of interests with any other authors, nor is there any financial/non-financial disclosure.

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