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Relationship of Asthma Severity With *Haemophilus influenzae* Type A Infection in Patients With Asthma Compared to Healthy People

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ABSTRACT

Background: Asthma is a chronic respiratory disease characterized by bronchospasm, airway inflammation. The occurrence of microbial infections is related to the severity of asthma symptoms; thus, their development can be prevented by controlling microbial agents. This study aims to determine the relationship of asthma symptoms severity with *Haemophilus influenzae* type A infection in patient with asthma compared to healthy people

Materials and Methods: In this case-control study, sputum samples of 31 patients with asthma (16 men and 15 women; mean age=58.58±14.13) and 31 healthy individuals (16 men and 15 women; mean age=52.03±14.72 years) who referred to a hospital were collected. DNA extraction was done using the kit (Cinnagen Co.). The prevalence of *H. influenzae* type A was investigated using the *bexA* gene primer by real-time PCR method. The collected data were analyzed in Excel and SPSS software, version 20.

Results: 24 sputum samples (77.4%) from patients and 13 samples (41.9%) from healthy people were positive for *H. influenzae* type A. The increase in the severity of asthma symptoms, cough and shortness of breath showed that the severity of the infection decreased with the increase in asthma symptoms. Also, in terms of the asthma control test (ACT) score, spirometry indices, fractional exhaled nitric oxide index, and percentages of lymphocytes, macrophages, neutrophils and eosinophils, there was a significant difference between patients and healthy people (P=0.0001).

Conclusion: Further studies are needed to determine the role of *H. influenzae* type A in the development of lung diseases, including asthma.

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Introduction

Allergic diseases including asthma, are common globally. Asthma has affected approximately 300 million people worldwide. This disease is a chronic inflammation of the airways that leads to an increase in airway responsiveness and causes various symptoms such as wheezing, shortness of breath, chest tightness, and cough during the night or early morning [1]. Extensive research on the mechanism and treatment of asthma has been conducted due to its increasing prevalence. By changing the resting potential of airway smooth muscle cells, inflammatory mediators change their stimulation threshold. Hypertrophy and hyperplasia of these cells have also been reported in patients with asthma [2]. The prevalence of this disease in Iran is 9% in adults and 10% in children and adolescents [3]. The global prevalence of asthma is 1-18%. It is predicted that the population of people with asthma will reach 400 million people by 2025 [4]. Asthma is classified according to the severity of symptoms, the number of attacks and the time of onset of symptoms as following: Mild intermittent (having symptoms fewer than or equal to two days per week and fewer than or equal to two nights per month), mild persistent (having symptoms more than two days per week but less than daily and more than two nights per month), moderate persistent (having symptoms every day and more than one night per week), and severe persistent (having symptoms every day and every night) [5]. Asthma involves the respiratory mucosa of the trachea to the terminal bronchioles, and one of the main goals of treatment is to reduce this inflammation. None of the main inflammatory cells involved in asthma (mast cells, macrophages, dendritic cells, eosinophils and neutrophils) is superior to the others. Mast cells by releasing histamine, leukotrienes, cytokines and growth factors and neurotrophils have a role in the initiation of acute bronchoconstrictor inflammatory response to allergens, smoke and hyperventilation [6].

Haemophilus influenzae or *Pfeiffer's bacillus* is one of the important pathogens in the respiratory system that can cause acute middle ear infections, sinusitis, pneumonia and acute bronchitis attacks [7]. Recently, the occurrence of resistance or reduced sensitivity to antibiotics has been observed in many strains of this organism [8]. This bacterium is classified into two groups based on having a polysaccharide capsule. The first group has a polysaccharide capsule, based on which it is classified into six serotypes from A to F, among which, type B is more clinically important. The second group is the strains without polysaccharide capsule, which are less aggressive [9]. *H. influenzae* is more common in children and infants [10]. It is part of the normal flora of the respiratory system in 60-90% of healthy people [7]. It is often transmitted by the direct contact with respiratory droplets through coughing and sneezing [11]. It is one of the gram-negative bacilli that were first described in an influenza epidemic in 1892 by Richard Pfeifer [12]. It is a member of the Pasteurella family. This bacterium is aerobic, but it can also grow in facultative anaerobes. It was mistakenly considered as the cause of influenza by 1933, until the viral agent of influenza was discovered; however, it can cause various diseases in humans [13]. *H. influenzae* was the first organism whose genome was completely sequenced in 1995 [14]. It is an oxidase-positive and catalase-positive bacterium, and it needs the vessel environment to be supplemented with two growth-stimulating factors found in the blood, including hemin (X factor) and NAD (V factor) [15].

Considering that the prevalence of asthma is increasingly in the world and since the general vaccination against *H. influenzae* bacteria is one of the future goals of the country, there is need to investigate the prevalence of *H. influenzae* in patients with asthma for find the possible relationship between them and development of anti-*H. influenzae* vaccines. Therefore, this study aims to investigate the prevalence of *H. influenzae* type A and its relationship with inflammatory cells in the sputum samples of patients with asthma compared to healthy individuals.

Methods

This is a case-control study that was conducted in 2021-2022 in Imam Reza Hospital, Mashhad, Iran. The study population consists of patients with asthma (definitively diagnosed with asthma by a specialist and based on having a history of at least six months of asthma symptoms such as cough, sputum production, alternative and/or variable shortness of breath, wheezing, chest pressure, and meeting spirometry criteria) and healthy people (no history of asthma in the individuals and their first- and second-degree relatives). The random sampling method was used in this study. The sample size was determined 31 per group based on the goals of the study. Exclusion criteria were defects in files, history of non-respiratory diseases such as chronic heart failure, history of respiratory diseases other than asthma, lobar pneumonia, bronchiectasis, tumor, cystic fibrosis, and history of smoking in the family.

After obtaining ethical approval from the Research Ethics Committee of Islamic Azad University, Mashhad

Branch. The asthma control test (ACT) was performed and an informed consent form was signed by each participant. The test was conducted by an clinical immunology/allergy specialist. Information related to age, gender, clinical symptoms (such as cough and shortness of breath) and spirometry examination of the patients were taken by a specialist. Airway inflammation was assessed by measuring fractional exhaled nitric oxide (FeNO) using NObreath device (Bedfont Co.). Based on spirometry indicators, asthma is divided into four categories: Mild intermittent (PEF or FEV ≥80%, PEF or FEV variability >20%), mild persistent (PEF or FEV ≥80%, PEF or FEV variability >20-30%), moderate persistent (PEF or FEV ≥60%, PEF or FEV variability >30%), and severe persistent (PEF or FEV ≤60%, PEF or FEV variability >30%) [16].

Sputum sampling

The morning sputum samples were immediately collected in sterile screw-capped tubes and transferred to the laboratory in a flask containing ice. The saliva contamination was removed by sampler. Homogenization of samples was done by adding 4 cc of 1% DTT to the samples. Then, some of the sputum sample was placed on a microscope slide using a swab and examined for the presence of epithelial cells and the ratio of epithelial cells to white blood cells (neutrophils, eosinophils, macrophages and lymphocytes).

DNA extraction was done using a kit (Roche, GmbH, Germany) according to the protocol included in the kit. Then, the extracted DNAs were concentrated. To ensure the correctness of the sample extraction, a quantitative evaluation was done by the spectrophotometry method. Then, to confirm the quality of the examined samples, the ratio of 260 OD to 280 OD was measured with a nanodrop device. The obtained samples were kept at -20 ˚C until the next stages of the research.

Polymerase chain reaction

BexA primer was used to detect *H. influenzae* type A. For polymerase chain reaction (PCR) test, 2 µL of template DNA, 5.5 µL of Master Mix (Cinnagen Co.), 16.5 µL of sterile distilled water, 0.5 µL of forward primer (ATCTTACAACTTAGCGAATAC) and 0.5 µL of reverse primer (GAATATGACCTGATCTTCTG) were mixed together [17]. The schedule of PCR for the detection of *H. influenzae* type A was as following: Initial denaturation for 5 minutes at 95 ˚C, 25 cycles of denaturation at 95 ˚C for 60 seconds, annealing at 60 ˚C for 30 seconds, extension at 72 ˚C for 90 seconds, and final extension at 72 ˚C for 4 minutes. In the end, the PCR products were transferred on a 1% agarose gel containing ethidium bromide, and electrophoresed [18]. In all stages of this research, *H. influenzae* type A strain ATCC9006, which was purchased from the microbial bank of Pasteur Institute of Iran, was used as a positive control and distilled water was used as a negative control.

Data analysis

The collected data were analyzed in Excel and SPSS software, version 20 applications. The statistical methods were: Shapiro-Wilk test, Mann-Whitney U test, t-test and Fisher's exact test. Also, Mean±SD were used to describe the data. The statistical significance level was set at 0.05.

Results

Mean age of 31 patients with asthma was 58.58±14.13 years and the mean age of 31 healthy people was 52.03±14.72 years. Table 1 shows the mean levels of study variables at baseline in patients and healthy people. As can be seen, there was a significant difference between the two groups in the ACT score, spirometry indices, FeNO level and percentages of lymphocytes, macrophages, neutrophils and eosinophils (P=0.0001). Based on the results of PCR test, 24 patients (77.4%) had *H. influenza* type A infection and 7(22.6%) had no *H. influenza* type A infection in their sputum samples. In the control group, 13 people (41.9%) had *H. influenzae* type A infection and 18(58.1%) did had no infection. Table 2 shows the results of *H. influenzae* type A infection based on demographic variables and severity of symptoms in patients with asthma. There was a significant difference in the infection with *H. influenzae* type A between different severity levels of asthma (P=0.0001), where the increase in *H. influenzae* type A infection caused a decrease in asthma severity. No significant difference was observed between other variables in *H. influenza* type A infection.

Discussion

The results of this study showed a significant difference between patients with asthma and healthy people in the ACT score, spirometry indices, FeNO level, and percentages of lymphocytes, macrophages, neutrophils and eosinophils. Also, there was a significant relationship between *H. influenzae* type A infection and the severity of asthma, such that with the increase of infection, the asthma severity decreased. *H. influenzae* type A infection was more common in patients with less severe asthma

Table 1. Mean levels of study variables at baseline in patients and healthy people

Variables	Mean±SD		Test Results	
	Controls	Patients	Statistic	P
ACT score	22.29±1.40	11.03±2.12	t=24.69	0.0001**
FEV1	86.03±9.15	51.35±13.98	t=11.59	0.0001**
FeNO	7.71±4.12	43.10±23.48	t=07.95	0.0001**
LYM%	13.32±3.92	21.26±8.54	t=-4.69	0.0001**
MQ%	81.77±5.00	22.45±5.55	t=45.94	0.0001**
NEU%	3.74±1.69	36.45±9.92	t=-25.82	0.0001**
EO%	1.03±0.18	18.71±5.95	t=-17.04	0.0001**

**Significant difference at the error level of <1%.

symptoms. Therefore, infection with *H. influenzae* type A bacteria can make the disease worse by obstructing the airways and neutrophilic inflammation. Controlling *H. influenzae* type A infection in people with less severe asthma symptoms can prevent the worsening of asthma.

A study was conducted in San Francisco by Huang et al. for investigating the airway microbiome in 30 patients with severe asthma using 16S rRNA-based methods. They showed that certain types of bacteria such as *proteobacteria* and *actinobacteria* are associated with certain clinical characteristics, some of which may cause asthma by activating certain pathways [19]. Carroll et al. assessed the relationship of infant bronchiolitis with the risk of early childhood asthma. In their study, 90 infants with bronchiolitis were compared to 90 healthy children. Their results showed that children hospitalized with bronchiolitis during infancy had increased early childhood asthma morbidity compared to children with no bronchiolitis visit [20]. This confirms that infectious lung diseases play an important role in having asthma. The results the two studies can be consistent with the results of the current study. Based on our results, there is a significant relationship between the simultaneous de-

Table 2. The *H. influenzae* type A infection based on demographic variables and severity of symptoms in patients with asthma

Variables		Min	Max	Mean±SD	Test Results
Age (y)	<50	16.39	48.54	31.77±12.80	t=0.98 P=0.337
	≥50	9.57	46.99	27.32±11.40	
Sex	Female	9.57	43.86	24.61±11.19	t=1.58 P=0.124
	Male	11.90	48.54	31.37±11.77	
Severity of asthma	Mild intermittent	44.38	48.54	46.14±1.74	F=66.94 P=0.0001**
	Mild persistent	27.55	44.19	34.96±5.51	
	Moderate persistent	17.94	25.71	22.50±3.29	
	Severe persistent	9.57	22.24	15.68±4.01	
Cough	1 and 2	15.83	48.54	33.93±11.42	t=2.03 P=0.051
	3 and 4	9.57	46.99	25.48±11.17	
Shortness of breath	1 and 2	9.57	48.54	26.21±11.67	t=-1.79 P=0.083
	3 and 4	11.90	46.99	34.10±10.86	

**Significant difference at the error level of <1%.

velopment of asthma and *H. influenzae* type A infection. This infection can lead to airway obstruction and neutrophilic inflammation, making the disease worse.

Simpson et al. assess the prevalence of *H. influenzae* in 46 people with asthma using the PCR method. They found that *Haemophilus* occurred most often in younger atopic males with higher neutrophils. Also, 76% of participants with asthma had *H. influenzae* [21]. A study by Green et al. was conducted in Australia for investigating the bacterial species in the sputum of 28 patients with asthma and its relationship with clinical findings and inflammatory markers. The dominant species was *Moraxella catarrhalis* or a member of the *Haemophilus* or *Streptococcus* genera. They stated that these microbial agents can worsen the disease with more severe airway obstruction and neutrophilic airway inflammation [22]. Essilfie et al. conducted a study on the relationship between *H. influenzae* infection and neutrophilic asthma in an animal model (mice). Infection, allergic airways disease, steroid sensitivity and immune responses were assessed in mice infected with *H. influenzae*. They concluded that the combination of infection and allergic airways disease promotes bacterial persistence, leading to the development of a phenotype similar to steroid-resistant neutrophilic asthma [23]. The results these studies can be consistent with the results of the current study. According to this study, there is a significant relationship between asthma and *H. influenzae* type A infection which is more common in patients with less severe symptoms. Therefore, infection with *H. influenzae* type A bacteria can make the disease worse by obstructing the airways and neutrophilic inflammation.

In a study, Tan et al investigated the prevalence of common respiratory viruses among patients with near-fatal asthma and chronic obstructive pulmonary disease (COPD) and the relationship of these findings with acute respiratory symptoms. They sampled 17 hospitalized patients with asthma and 14 patients with COPD. They concluded that respiratory viruses are associated with hospitalizations for near-fatal asthma and COPD. Therefore, respiratory viruses are a target for the prevention and perhaps the treatment of these conditions [24]. Bizzintino et al. assessed the prevalence of human rhinovirus C and severity of acute asthma in 128 children with asthma aged 2-16 years. Their results showed that rhinovirus C infection increases the severity of symptoms in children with asthma [25]. Gerke et al. investigated the relationship between seasonal influenza and the severity of asthma symptoms. Based on time series regression models, a strong, significant association was found between concurrent influenza activity and incidence of asthma hospitalizations. Their results confirm that influenza activity is associated with increased severity of asthma symptoms. They suggested that improvements in influenza surveillance, prevention and treatment may decrease hospitalizations of asthma patients [26]. In 2020, Trinh et al. also investigated the relationship between influenza and the severity of asthma symptoms. Their results showed that having influenza increases the severity of asthma symptoms. Therefore, influenza prevention strategies targeting at-risk populations can reduce the severity of symptoms in patients with asthma [27]. The results these studies can be consistent with the results of the current study. According to this research, it is possible to prevent the worsening of asthma by controlling *H. influenzae* type A infection in people with less severe symptoms.

Based on the obtained results, asthma classification cannot be only evaluated by the severity of symptoms; a set of immunological and inflammatory mechanisms are induced in the body regarding the relationship between infections and lung diseases, including asthma. Therefore, a more comprehensive assessment of the relationship between asthma and immune system responses is needed. If more dimensions of this relationship are investigated, it can be possible to prevent the worsening of the disease by preventing *H. influenzae* type A infection in people with less severe symptoms of asthma.

Ethical Considerations

Compliance with ethical guidelines

The study was approved by the Research Ethics Committee of Islamic Azad University, Mashhad Branch (Code: IR.IAU.MSHD.REC.1400.023). An informed consent form was signed by each participant.

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Authors contribution's

Conceptualization: Mohammadreza Khakzad and Jina Khayatzadeh; Study design and data analysis: Mohammad Reza Pourmohammad; Data collection: Hasan Alammar; Consultation: Mohammadreza Khakzad; Witing: Jina Khayatzadeh and Mohammadreza Khakzad.

Conflict of interest

The authors declared no conflict of interest.

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