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Original article

Characteristics of the hemagglutinins antibody responses in influenza A patients in Suez Governorate, Egypt

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ABSTRACT

Background: Influenza is an acute respiratory illness caused by the influenza virus. There are 3 types of influenza virus A, B and C. Infection with the influenza virus exhibits broad immune responses and protection from re-infection by the same type of virus. The protective humoral immune response depends mainly on antibodies to the virus surface glycoprotein, hemagglutinin, and neuraminidase. Our study aims to reveal the characteristics of hemagglutinin reactive serum antibody responses in influenza patients in Suez Governorate. **Methods**: 160 cases were enrolled according to their clinical picture and symptoms onset not more than 10 days from the beginning of illness. Nasopharyngeal swabs were obtained from each case and tested for the influenza virus by rapid influenza antigen detection test. The proven influenza A samples were typed by real-time PCR. Each sample was mapped for their hemagglutinin (HA) antibodies rising titer by ELISA: group 1 HAs (H1, H5, H9) and group 2 HAs (H3 and H7). **Results:** 15 cases were diagnosed as influenza A with subtype H1N1. There was a rise in group 1HAs more than group 2 HAs when comparing the antibody titer of the 1st and 2nd serum samples. Group 1: H1 showed a 20-fold rise, H5 showed a 5-fold rise and H9 showed a 2-fold rise. Group 2: H3 and H9 showed a 5-fold rise. **Conclusion:** Following natural infection; influenza A(H1N1) pdm09 virus induces a strong anti-HA immune response.

Introduction

Influenza is an acute respiratory disease caused by the influenza virus. It often occurs in outbreaks and epidemics worldwide. Droplets can spread illness because a significant quantity of influenza virus particles found in the respiratory secretions of infected individuals. In immunocompetent adult patients, influenza virus shedding typically lasts 5 days, but in children, older adults, patients with chronic illnesses, and immunocompromised hosts, it can last up to 10 days or longer. The classic course of influenza is a sudden onset of high fever, myalgia, headache, and malaise, accompanied by respiratory tract symptoms such sore throat, nonproductive cough, and nasal discharge. Hospitalization may be necessary if other organs are impacted [1].

Influenza viruses are members of the family Orthomyxoviridae. Their ribonucleoprotein (NP) and matrix (M) protein antigens differ in antigenicity, which divides them into three main types: A, B, and C. The majority of influenza virus hosts are humans. Many mammalian and avian species are infected with influenza A virus, which are closely linked to the viruses common in humans [2].

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A lipid bilayer membrane made from the plasma membrane of the host cell envelops a nucleocapsid that contains eight segments of negative-sense single-stranded RNA, which is the component of both influenza A and B viruses. There is a layer of matrix protein (M1) specific to the virus on the inside of the envelope. Hemagglutinin (HA) and neuraminidase (NA), two glycoproteins specific to viruses, are embedded in the lipid bilayer envelope's outer layer and protrude as spikes on the surface of the virion [3,4].

To initiate the infectious cycle, hemagglutinin binds to the terminal sialic acid residues on glycoproteins and glycolipids, attaching virions to cells. NA completes the infectious cycle by cleaving terminal sialic acids, which release virions [5].

Since HA is the virus's surface protein that the humoral immune response can readily recognize, antibodies to it are thought to be the most protective since they neutralize the virus upon re-exposure [3]. Neutralizing antibodies are reactive against the HA stalk and frequently cross-react between different HA subtypes because of the conserved structure of the HA stalk.

Eighteen hemagglutinin subtypes of the influenza A virus exist. These subtypes are divided into two phylogenetic groups according to their antigenic characteristics: group 1 is made up of H1, H2, H5, H6, H8, H9, H11, H12, H13, H16, H17, and H18; group 2 is made up of H3, H4, H7, H10, H14, and H15. The majority of stalk-reactive antibodies can only bind to group 1 or 2 HAs [6].

Individuals' immunological responses vary quantitatively from one another. Eventually, the virus can also impair the antibody response over repeated exposures due to antigenic changes, commonly known as drifts and shifts. The antigenic variations provide the viruses the ability to elude important antibodies produced by vaccines, prior infections, or both [7].

Annual seasonal influenza A and B outbreaks in temperate zones are primarily caused by antigenic drifts involving different dominant antigenic types and subtypes. On the other hand, pandemic influenza A is caused by an antigenic shift, wherein a significant alteration in HA/NA gives rise to a new virus and subtype. Because influenza can mutate numerous times and spread across species, the remarkable capacity to experience more developing pandemics is enhanced by the possibility for avian, porcine, and human strains to recombine [8].

Except in certain situations where the diagnosis of influenza requires laboratory confirmation using readily available tests like nucleic acid tests (e.g., polymerase chain reaction (PCR) or rapid diagnosis kits), the majority of influenza cases are diagnosed based only on their clinical manifestations, negating the need for laboratory testing [1].

The current study was conducted to characterize the HA reactive serum antibody response among influenza patients in Suez governorate.

Patients and methods

This cross-sectional study was carried out in the Suez Governorate's General Suez Hospital and the Zagazig University Faculty of Medicine's Medical Microbiology and Immunology Department. On August 25, 2019, the Faculty of Medicine Ethics Committee granted ethical permission with approval number ZU-IRB #5467/25-8-2019.

Sample size

Assuming that rate of admission of patients with influenza at Suez General Hospital is 300 cases per month and prevalence of type A influenza is 13 %, So sample size is calculated by Openepi program to be 159 cases with confidence level 95 %.

Study patients

The patients with influenza-like symptoms shown in the outpatient clinic in Suez General Hospital during the study period were included, only patients who refused to participate were excluded. Inclusion criteria were: body temperature of 38°C and more with two or more of the following: (a)respiratory symptoms; cough, dyspnea, sore throat, nasal congestion or rhinorrhea, headache, myalgia or arthralgia (b) Contact with sick or dead bird or poultry prior 14 days of symptom onset [1]. Written informed consents were signed by the participants. They received guarantees that their data would be handled anonymously and confidentially.

Specimen collection

On the first day of enrolment in the study, two samples were collected from each participant applying the infection prevention standard precautions and standard sampling protocol [9]; two nasopharyngeal swabs in viral transport media tubes

(UTM® Universal Transport Medium, Copan Diagnostics, Italy) and first whole blood sample (5 ml). The second whole Blood sample (5ml) was collected 21-28 days later. Serum separation from both blood samples was done. Nasopharyngeal swabs,1st and 2nd serum samples were stored at temperature (-80°C). One nasopharyngeal swab was used for rapid antigen detection test for detection of influenza A and Influenza B while the other was used for real-time polymerase chain reaction (RT-PCR) for subtyping of influenza A. 1st and 2nd serum samples were used for measurement of serum antibody titer by Enzyme-Linked Immunosorbent Assay (ELISA).

Rapid antigen detection test

It was carried out by Quick Navi-Flu2 which offers 94.8% sensitivity and 98.4% specificity for influenza A virus and 97.8% sensitivity and 96.8% specificity for influenza B virus (Otsuka Pharmaceutical Co., Japan). Following the manufacturer's instructions; a suspension buffer was used to soak swab samples, and three drops of the buffered sample were added to each test strip. It takes two to five minutes to get the readings. Control line is checked for the validity of the test. Positive reading then observed as colored line in A or B lane of the test strip.

Qualitative detection of influenza viruses in patients' respiratory samples by Real-time Polymerase Chain Reaction (RT-PCR)

The second nasopharyngeal swab was used for subtyping of influenza A. Automated RNA extraction was carried out by The QIAsymphony DSP Virus/Pathogen Mini Kit (Qiagen, Germany) using Qia symphony automated extraction machine (Qiagen, Germany) following the manufacturer instructions. The subtyping was performed according to CDC Real-Time RT-PCR (rRT-PCR) protocol for detection and characterization of influenza virus by Ambion AgPath-ID™ One-Step RT-PCR Kit (Thermo Fisher Scientific, USA) using real-time PCR detection system by with a 96-well format thermocycler reaction block (ABI 7500, OR Strategen, USA). For each sample RNA extract was tested by separate primer/probe sets: InfA, InfB, and RNaseP (RP) as screening. The RNaseP primer and probe set targeted the human RNase P gene and thus served as an internal positive control for human nucleic acid.

If the sample is influenza A positive, the sample was tested for A/H3, A/H5, A/pdm (post-pandemic), and A/pdm H1. No template controls and positive template controls for all primer/probe sets were included in each run [10].

The thermocycler program was: reverse transcription at 50°C for 30 min, Taq inhibitor activation at 95°C for 10 min, and PCR amplification (45 cycles) at 95°C for 15 sec then at 55°C for 30 sec by then the fluorescence data (FAM) was collected. Two detectors for influenza A post pandemic were used; the first one is for detection of matrix gene and the second one is for the detection of hemagglutinins. The circular threshold (Ct) is inversely proportional with the viral load. Ct more than 30 indicates low viral load detected in the specimen.

Quantitative assay of anti-hemagglutinin in the serum samples by ELISA

The assay of rising titer of anti-hemagglutinin was done using Enzyme-Linked Immunosorbent Assay (HA ELISA Kit; Sino Biological, China) with 2.31 pg/mL sensitivity. Following the protocol described by Nachbagauer et al. [11]; (H1 pre- pandemic, H1 post-pandemic, H3, H5, H7, and H9) were used for detection of HA antibodies, B- yama (Yamagata lineage influenza type B) and B –vic (Victoria lineage influenza type B) were used as a negative control and anti-human IgG as a positive control. The ELISA plates were read and measured at 450nm wave length.

Statistical analysis

Data analysis was performed by SPSS software, version 25 (SPSS Inc., PASW Statistics for Windows version 25. Chicago: SPSS Inc.). Qualitative data were described using numbers and percentages. Quantitative data were described using mean± Standard deviation for normally distributed data after testing normality using the Kolmogrov-Smirnov test. The significance of the obtained results was judged at the (0.05) level.

Results

One hundred sixty cases of influenza-like illness were included in our study. Seven cases (4.3%), and fifteen cases (9.4%) were positive for influenza A and B, respectively. Conversely, 138 patients (86.3%) were negative for both the influenza A and influenza B viruses. Fourteen cases were gathered in season 2019-2020 and 8 cases in season 2020-2021.

In the first season, there were seven instances of influenza A and B combined, but in the second, there was only one diagnosis of influenza A.

The mean age of the studied influenza A cases was 33.47±12.42 ranging from 19 to 60 years old. More than half of the cases (53.3%) were females and the other (46.7%) were males. Only one case (6.7%) was diabetic and two cases (13.3%) were asthmatic patients. In all cases, no influenza vaccine was received. About (20%) of them were in contact with birds. Two cases (13.3%) were taking anti-influenza drugs. In comparison, the mean age of the studied influenza B cases was 25.14±9.34 ranging from 16 to 43 years old. Most cases (71.4%) were females and the other (28.6%) were males. In all cases, the patient didn't receive any vaccine or had a history of contact with birds **(Table 1)**.

Regarding subtypes of influenza A strains detected by real-time PCR; all were subtyped in H1N1 strain pdm09 only.

Influenza A patients showed a rise in H1 post-pandemic antibody response with a mean value (22.6) folds of rise followed by H1 pre-pandemic antibody response with a mean value (17.47) folds. There was a rise in H5, H3, and H7 with a mean value (5) folds for each. There was a rise in H9 with a mean value (2) folds. The rise in group 1 hemagglutinins antibodies responses among influenza A patients was statistically significant **(Table 2)**. There was no statistically significant difference between group 1 & group 2 influenza A HA antibodies responses regarding history of bird contact. There was a statistically significant positive correlation between HA Pre and post-pandemic H1 Ab folds of rise (r= 1, two tailed P value 0.567).

Table 1. Basic characteristics of positive influenza A virus and influenza B virus cases.

Characteristic		Influenza A(n=15)		Influenza B(n=7)	
		No.	**%**	**No.**	**%**
Sex	**Female**	8	53.3	5	71.4
	Male	7	46.7	2	28.6
History of receiving vaccine	**No**	15	100	7	100
Bird contact	**No**	12	80	7	100
	Yes	3	20	None	-
Comorbidities	**Asthma**	2	13.3	1	14.3
	Diabetes	1	6.7	None	-
	Hypertension	None	-	1	14.3
	None	12	80.0	5	71.4
Receiving anti influenza drug		2	13.3	None	-

Table 2. Folds of rise of group 1 hemagglutinins and group 2 hemagglutinins antibodies among influenza A patients

Antibodies to influenza A virus	**NO.(%) influenza cases**	**Fold rise Mean ± SD**	**F (P-Value)**
Group 1 Hemagglutinins	H1 Pre-Pandemic	17.47 ± 1.75	959.732 0.0001*
	H1 Post-Pandemic	22.60 ± 2.12	
	H5	5 ± 0	
	H9	2 ± 0	
Group 2 Hemagglutinins	H3	5 ± 0	
	H7	5 ± 0	

*** Statistically significant differences**

Discussion

Influenza is an acute infectious viral respiratory disease that creates yearly epidemics and, on occasion, pandemics. In temperate countries, transmission mostly takes place in the winter, but in tropical climates, especially in

populated areas, it happens all year round. Influenza can cause serious complications, hospitalization, and even death, particularly in elderly people, young children, expectant mothers, obese people, and people with chronic diseases [12].

There are four different types of influenza viruses: A, B, C, and D. Only influenza type A has the ability to start a pandemic [13]. Certain antibodies against HA are a correlate of protection against influenza virus infection [6].

This study included one hundred and sixty patients with influenza-like illness. Fifteen of them were diagnosed as influenza type A by rapid influenza antigen detection test and were further subtyped (H1N1) by real-time PCR. Seven of them were diagnosed as influenza type B. The influenza type A patients were mapped for their antibodies immune responses against type 1 and type 2 hemagglutinins.

In our study, overall influenza patients were (13.7%). When compared to previous studies done in Egypt, it was consistent with earlier reports [14,15]. Different influenza incidences 17% [16] and 9.7% [17] were reported in Egypt and Eastern Mediterranean region respectively.

The total number of influenza positive patients in 2019-2020 season was 17.5%, which approximately met another study in Egypt [18].

In patients with influenza-like illnesses, the overall positive rate of influenza virus decreased from 17.5% in the 2019–2020 season to 10% in the 2020–2021 season. Similar to what is shown by [19,20] and [21] in Bangladesh, Canada, and China, respectively. During the COVID-19 pandemic, significant drops in influenza transmission were also noted in Singapore, Thailand, Taiwan, Australia, South Africa, Chile, and Iran [21,22]. eventually, this was a worldwide decline as reported by CDC [23]. The following explanations could be given for this decline: First, as influenza and COVID-19 are respiratory infections spread by droplets, the COVID-19 preventive standard may reduce the incidence of influenza infections. Second, people's behavior in seeking medical attention changed during the COVID-19 pandemic recalling that influenza is a self-limiting illness. Thirdly, physical distancing reduced the number of large gatherings, which may have contained influenza outbreaks. Lastly, one of the possible causes of the reduced influenza virus circulation could be the viral interference between SARS-CoV-2 and influenza virus [19].

The same concept was underscored by Kandeel et al. who charted a sharp decline in the transmission of common respiratory viruses, such as influenza and RSV during COVID-19 pandemic, across various geographic regions and climate zones. Varying incidences, hospitalization rates, and circulation patterns due to variations in community preventive measures and immunity levels during the winter season (2022–2023) were then featured the post pandemic rise of influenza and RSV infections [24].

On the other hand, in Romania 2019-2020; reports of high influenza positivity rate (49.5%) was noticed. Different diagnostic approaches might explain this discrepancy (cases were diagnosed and subtyped by real-time PCR followed by next-generation sequencing) [25].

According to the current study, there are more influenza A cases overall than influenza B cases. This finding was also supported by earlier research [26,27]. This suggests that during the study period, there was a higher incidence of influenza A viruses circulating in the population. In contrary to Avni et al. the percentage of laboratory-confirmed cases of influenza B (62%) was higher than that of influenza A (38%) during 2017–2018 influenza season [28].

Detection rates of influenza B virus infections vary between countries and over time. The current study rate was 8.75% in 2018-2019 season and zero in 2019-2021 season. Same reported by studies done in Egypt [29] and Sweden [30].

Numerous factors influence the risk of influenza infection in various age groups. These include epidemiological attributes such as the number of effective contacts among susceptible individuals, the population density, the family structure (the average number of occupants in a home), social habits, and the pattern of contacts between individuals in different age groups [31]. Another important factor is vaccination, but our study showed that all cases had no vaccination history.

The demographic data in our study showed influenza A patients with mean age (33.47) and influenza B patients with mean age (25.14). Influenza affects all age groups, but influenza B affects younger adults more while influenza A affects young and old adults. These findings met with previous studies [31,32].

Regarding gender; females are more affected than males in both types of influenza, same reported by [29,33]. Another study found an equal distribution of both genders [25], Kandeel et al. reported males being more affected than females [24].

Our study showed that all patients of influenza A were subtyped in H1N1 post-2009 pandemic strain. This finding was met with previous studies on [34-40]. It also met a study done in Egypt in 2020 coinciding with the COVID-19 pandemic [41]. Other studies reported both H1N1 and H3N2 subtypes of influenza A isolated strain [14, 42-45]. While H3N2 was the only subtype of influenza A isolated in studies [46-48]. Some previous studies in Egypt reported pandemic and post-pandemic 2009 H1N1 [15,17], while others reported pre-pandemic and post-pandemic 2009 H1N1 [16].

Most studies showed that the pre-pandemic 2009 has greatly diminished and been replaced by the pandemic and post-pandemic strain till now.

All cited studies were performed at different Eastern Mediterranean and North African regions at various times over 10 years. They showed different epidemiological prevalence of influenza A subtypes. These differences are considered multi-factorial related to the most contagious strains in certain regions, special habits and infection prevention considerations related to a certain region population, vaccination scheduled programs annually for influenza and naturally acquired immunity in a certain region according to previous exposure to different subtypes.

In our study, the complete natural infection occurred after 3-4 weeks with influenza A (H1N1) post-pandemic virus evidenced by HA post-pandemic seroconversion (more than 20 folds of rise) which is comparable to [49-51].

In our study there was induction in group 1 hemagglutinin antibodies response with a mean: of 11.78 folds of rise more than group 2 hemagglutinin antibodies response with a mean of 5 folds of rise. Influenza B was not induced. Nachbagauer et al. got comparable results but the boost against group 2 HAs was almost as strong as that of HA group [11].

Our data showed a cross-reactivity of pre-pandemic strain antibodies with the post-pandemic strain antibodies. However, the hemagglutinin of post-pandemic is extensively different from the previous seasonal strain. The theory that cross-reactive immune response was triggered by secondary exposure to HA of the same group may support this [13].

As part of cross-reactivity, more than four fold increases in hemagglutinin antibody levels were found in other antibodies (H5, H3, and H7). Cross-reactive anti-HA was generated against distinct viral subtypes, according to some investigations [50,52]. In contrast, investigations reported not to have seen cross-reactive antibody responses to the influenza A(H3N2) and influenza B viruses [49].

Another reason for the cross-reactivity of HA antibodies is that antibodies specific to the HA stalk can attach to a variety of viral isolates and subtypes. These antibodies attach to influenza B, influenza A group 1 or 2 HA proteins in some circumstances, this type of antibodies likely makes up the bulk of the cross-reactive antibodies elicited by natural infection, together with HA stalk-specific monoclonal antibodies that bind across viral groups [13].

Conclusion and Recommendation

After contracting influenza A(H1N1) pdm09 naturally, a robust anti-HA immune response is elicited. In the current study the antibody cross-reaction directed against the influenza virus's glycoproteins was presented. A negative association between the COVID-19 pandemic and the influenza season in two consecutive seasons (2019–2020 and 2020–2021) among a specific demographic was identified. The results of this study provide a solid foundation for large-scale sero-surveillance studies to be carried out in the future in order to investigate the features of influenza antibody response.

Since none of the enrolled patients in our study had received the influenza virus vaccination, we advise starting efforts to educate the public about the critical need to become vaccinated.

Limitation of the study

The coinciding COVID 19 pandemic was one of our obstacles for reaching influenza patients. Only one strain has been detected among influenza A patients; which affects our ability for complete characterization of antibodies response to different strains. It was difficult to study the impact of bird contact on influenza infection as the population not completely depend on breeding birds at their home.

Disclosure of potential conflicts of interest

None to declare

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