Influenza species and subtypes circulation among hospitalized patients in Laleh hospital during two influenza seasonal (2016-2017 and 2017-2018) using a multiplex Real Time-Polymerase Chain Reaction

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Abstract

The introduction of polymerase chain reaction (PCR) techniques has improved the detection of respiratory viruses, particularly with the use of multiplex real-time technique with the capability of simultaneous detection of various pathogens in a single reaction. The aim of this study was to apply the above technology for the diagnosis of influenza infections and at the same time to differentiate between common flu species between hospitalized patients in Laleh hospital (Iran) between two flu seasons (2016-2017 and 2017-2018). Different respiratory specimens were collected from 540 patients from a period of December 2016 to May 2018 and were sent to the laboratory for molecular diagnosis. RNAs were extracted and subsequently, a multiplex real time PCR identifying flu A, flu B and typing flu A (H1N1) was carried out. The mean age of patients was 47.54±23.96. 216 (40%) and 321 (60%) of subjects were male and female, respectively. 219 out of 540 (40.5%) were positive for influenza infection including flu A (n=97, 44.3%), flu A (H1N1) (n=45, 20.7%) and flu B (n=77, 35%). Flu A was the dominant species on 2016-2017 and flu B was the major species on 2017-2018. Flu A (H1N1) was comparable in both time periods. Flu infections were most frequently diagnosed in age groups 21-40. Flu-positive patients suffered more from body pain and sore throat than flu-negative patients with significant statistical difference (P values <0.001). The mean duration of hospitalization was shorter for flu-positive patients (P value = 0.016). Application of multiplex real time PCR could facilitate the influenza diagnosis in a short period of time, benefiting patients from exclusion of bacterial infections and avoiding unnecessary antibiotic therapy. Influenza diagnosis was not achieved in up to 60% of flu-like respiratory infections, suggesting the potential benefit of adopting the same methodology for assessing the involvement of other viral or/and bacterial pathogens in those patients.

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Introduction

To date, based on the immunological and biological properties, three types of influenza viruses including A, B, and C have been recognized.[1,2] Due to their fatal potential and highly contagious nature, influenza viruses have more predominant effects on communities rather than common respiratory illnesses.[3] Risk factors such as diabetes, respiratory and cardiovascular disorders are particularly making subjects (especially elderly patients) susceptible to hospitalizations and complications.[4] Influenza epidemics of variable extent and severity impose an immense burden in terms of morbidity, mortality and economic and social costs.[5]

Influenza viruses cause respiratory tract infections and may result in complications, which can lead to substantial morbidity and in some cases to death.[4,6] Therefore, rapid and accurate diagnosis is important for clinical patient management and infection control purposes. Due to the presence of effective antivirals against different types of influenza viruses, rapid laboratory diagnosis may result in less antibiotic prescription and more frequent use of those antivirals. The introduction of polymerase chain reaction (PCR) techniques has improved the detection of respiratory viruses, particularly with the use of multiplex real-time techniques with the capability of simultaneous detection of various pathogens in a single reaction. Currently, this new method has been successfully applied to the routine diagnosis and epidemiological detection of respiratory infections including influenza in many centers with reasonable specificity and sensitivity.[7-9] On the other hand, current diagnostic methods for respiratory illnesses have some deficiencies in Iran. Due to inexpensive and low ,cost of antibiotic values across the country, physicians are unwilling about application of new diagnostics tools including molecular-based techniques. This may result in a prolonged stay of patients in hospital as well as in increased morbidity, possibly associated with inappropriate therapy. The aims of this study were to apply the above technology for the diagnosis of influenza infections and at the same time to differentiate between common flu types among hospitalized patients in Laleh hospital.

Materials and Methods

Study subjects

The study population comprised of 540

patients who had been hospitalized in different wards in two flu seasons (2016-2017 and 2017- 2018) between December 2016 and May 2018. Informed consent was obtained from the adults and the legal guardians of the children. The enrolled subjects included patients admitted for moderate to severe onset of respiratory symptoms or for acute onset of fever within the previous week, especially elderly persons and infants, and patients admitted for acute exacerbations of pre-existing chronic medical conditions (e.g. chronic lung disease, asthma, cardiovascular disease, stroke or diabetes). According to guidelines in Laleh hospital, all flu A positive individuals routinely received oseltamivir (Tamiflu) 120 mg for adults and 60 mg for children per day. The study protocol was approved by Laleh hospital ethical committee.

Respiratory specimens received from different wards including sputum, nasopharyngeal and throat swabs were collected and were transported in viral transport medium (VTM) (Copan, Brescia, Italy) to preserve viral nucleic acids during transfer to the laboratory for molecular analysis.

Table 1. Demographical and clinical characteristics of patients.

Variable	All patients (n=540), %	Flu-positive (group I) (n=219), %	Flu-negative (group II) (n=321), %	P-value
Age, year (Mean± SD)	47.54±23.96	47.23±23.98	47.98±23.94	0.992
Gender				0.744
Male	216 (40)	77 (35.2)	139 (43.3)	
Female	321 (60)	142 (64.8)	182 (56.7)	
Pulmonary Disorders				0.232
Yes	36 (6.7)	18 (8.2)	18 (5.6)	
No	504 (93.3)	201 (91.8)	303 (94.4)	
Heart Disorders				0.652
Yes	86 (15.9)	33 (15.1)	53 (16.5)	
No	454 (84.1)	186 (84.9)	268 (83.5)	
Hypertension				0.717
Yes	87 (16.2)	37 (16.9)	50 (15.6)	
No	453 (83.8)	182 (83.1)	271 (84.4)	
Diabetes Mellitus				0.200
Yes	58 (27.4)	19 (8.7)	39 (12.1)	
No	392 (72.6)	200 (91.3)	282 (87.9)	
Smoking				0.749
Yes	30 (5.6)	13 (5.9)	17 (5.3)	
No	510 (94.4)	206 (94.1)	304 (94.7)	
Headache				0.059
Yes	194 (35.9)	89 (40.6)	105 (32.7)	
No	346 (64.1)	130 (59.4)	216 (67.3)	
Body pain				<0.001
Yes	203 (10.2)	107 (48.9)	96 (29.9)	
No	337 (89.8)	112 (51.1)	225 (70.1)	
Chest pain				0.225
Yes	30 (5.6)	9 (4.1)	21 (6.5)	
No	510 (94.4)	210 (95.9)	300 (93.5)	
Sore throat				<0.001
Yes	38 (7)	27 (12.3)	11 (3.4)	
No	502 (93)	192 (87.7)	310 (96.6)	
Dyspnea				0.333
Yes	89 (16.5)	32 (14.6)	57 (17.8)	
No	451 (83.5)	187 (85.4)	264 (82.2)	
Sputum				0.460
Yes	122 (22.6)	53 (24.2)	69 (21.5)	
No	418 (77.4)	166 (75.8)	252 (78.5)	
Cough				0.154
Yes	176 (32.6)	79 (36.1)	97 (30.2)	
No	364 (67.4)	140 (63.9)	224 (69.8)	
Duration of Hospitalization (days)	4.71±4.42	4.39±3.86	5.06±4.93	0.016
Specimen				0.408
Nasopharyngeal swab	328	186	142	
Throat swab	115	25	90	
Sputum	10	5	5	
Throat swab + swab sputum	6	2	4	
Throat + Nasopharyngeal swab	2	1	1	
Others	79	0	79	

DNA extraction and polymerase chain reaction

Viral nucleic acids from different specimens were extracted using a viral RNA/DNA nucleic acid extraction kit (ROCHE, Mannheim, Germany) according to manufacturer's recommendation. Internal control was added to each specimen before loading the trays. The use of an internal control of each reaction tube excluded false negatives due to nonspecific inhibitors of the PCR enzymes. DNA was eluted using 50 µL of elution buffer. Qualitative Multiplex Real Time PCR was carried out on 5µL of extracted materials using Flu kit, for the detection of flu A, flu A (H1N1) and flu B (Fast-track diagnostics/SIEMENS, Luxembourg), according to manufacturer's instructions. This kit could differentiate between the pandemic influenza A (H1N1)pdm strain and other (untyped) influenza A strains; the results were classified as and flu A (H1N1) if a sample was positive for to both influenza A and H1, and flu A if a sample was positive to influenza A and negative to H1. If a positive laboratory test result was not compatible with the clinical picture of the patients, the assay was repeated by another technician on the same sample. The time period from extraction to the end of result was between 24 and 36 hours (excluding public holidays).

Statistical analysis

Statistical analysis was performed by using the Statistical Package for Social Science (SPSS 21, SPSS Inc., Chicago, Illinois, USA). Data were expressed as percentages for categorical variables and means ± standard deviations (SDs) for continuous variables. The intergroup differences of numerical values were performed by using the Student's t-test. Categorical variables were expressed as percentages, and differences between groups were judged for significance using the chi-squared test. For all comparisons, P-value <0.05 was considered as statistically significant.

Results

This cross-sectional study was carried out on 540 of hospitalized patients from Laleh hospital between two flu seasons (2016-2017 and 2017-2018). Demographical and clinical characteristics of patients are shown in Table 1. The age of patients ranged between 3 and 80 years, with a mean of 47.54±23.96. Two hundred and sixteen (40%) and 321 (60%) of subjects were male and female, respectively (Table 1). Two hundred and nineteen out of 540 (40.5%) were positive for influenza infection including flu A (n= 97, 44.3%), flu A (H1N1) (n=45, 20.7%) and flu B (n=77, 35%) (Table 1 and Figure 1A).

In a two years period analysis, Flu A was the dominant species in the 2016-2017 season; on the other hand, flu B was the major species in the 2017-2018 season (Figure 1B). Flu A (H1N1) was comparable with both time periods (Figure 1B). In the two year period, the peaks of seasonal influenza occurred in January, December, February and March in the order of magnitude, respectively (Figure 1B). Flu A predominated from December 2016 to February 2017 (Figure 1C). On the other hand, flu B predominated from January 2017 to March 2018, and was the unique influenza type detected in March 2018 (Figure 1D).

The 540 patients were grouped into two groups: flu positive (group I) and flu negative (group II). The differences of mean ages between groups I and II were not statistically significant (47.23±23.98 and 47.98±23.94, respectively, P=0.992) (Table 1). Despite we observed several peaks of flu cases between age groups, flu infections were most prevalent in age groups 30-40 followed by 70-100 years old (Figure 2A). Overall the period of study, flu A was the dominant type for <60 years old; however, for age groups >60 years old, flu B was the dominant type (Figure 2A). Similarly, in period between 2016 and 2018, flu A was circulated among different age groups in 2016-2017 somehow different from flu B which was distributed mostly among older

Table 2. Rate of Influenza diagnosis and duration of hospitalizations according to different age groups.

Age groups	2016-2017	2017-2018	FluA	FluB	FLU A (H1N1)	Duration of Hospitalization (Mean, days)	P Value
1-10	All: 17	All: 9	All:11	All:7	All:8	3.07	0.485
M:	6	3	5	2	2		
F:	11	6	6	5	6		
11-20	2	4	2	2	2	3.33	0.020
M:	0	4	1	2	1		
F:	2	0	1	0	1		
21-30	8	9	10	1	6	2.5	0.816
M:	2	2	2	0	2		
F:	6	7	8	1	4		
31-40	23	18	21	11	9	4.5	0.151
M:	10	5	9	3	3		
F:	13	13	12	8	6		
41-50	8	16	5	13	6	5	0.195
M:	1	9	0	8	2		
F:	7	7	5	5	4		
51-60	15	10	17	4	4	5.2	0.442
M:	4	0	3	0	1		
F:	11	10	14	4	3		
61-70	12	22	15	17	2	5.9	0.160
M:	1	9	5	3	2		
F:	11	13	10	14	0		
71-100	19	27	13	26	7	5.6	0.057
M:	10	11	9	10	2		
F:	9	16	4	16	5		

ages in 2017-2018 (Figure 2B). Also, gender difference between the two groups was not statistically significant (P=0.744) (Table 1). Histories of heart and pulmonary disorders, hypertension, diabetes mellitus and smoking were found in different proportions of patients, however, no significant differences were found between the two groups (Table 1, P=0.013).

Regardless of fever, which was seen in all patients, in general common symptoms included body pain, myalgia, headache, chest pain, sore throat, cough and sputum. However, patients in group I suffered more from body pain and sore throat than patients in group II (P <0.001, Table 1).

Period of hospitalization ranged between 1 and 34 days with a mean duration of 4.71±4.42 days for all patients. However, this period was shorter for patients in group I (4.39±3.86 and 5.06±4.93 days for groups I and II, respectively, P, 0.016; Tables 1 and 2). In age group comparisons, on average, patients under 40 years old tended to be hospitalized for less than 5 days, whereas, for those who were more than 40 years old this duration increased to more than 5 days (Table 2). Most flu positive cases that were diagnosed according to the type of clinical specimen included: nasopharyngeal (65%), throat swab (30%) and sputum (5%) samples, suggesting higher sensitivity of the test when applied to nasopharyngeal swab as compared to other respiratory specimens (Table 1).

Discussion and Conclusions

Early and rapid detection of influenza viruses is crucial in controlling the severity and spread of the infection, due to the existence of neuraminidase inhibitors as therapeutic option. Nevertheless, differentiation between influenza subtypes is of clinical importance because flu A infection may be associated with higher morbidity and mortality especially among older adults and immunocompromised individuals.[3] In the present study, we clearly showed that 40.5% of hospitalized patients admitted to Laleh hospital due to acute respiratory symptoms were infected by different types of influenza virus. Similar to our finding, others reported influenza prevalence between 34.5% and 52.2% circulating in Iran.[10-13] Contrary to our results that showed subtype fluA H1N1 has least common prevalence, previous studies performed in Iran including prevalence of 10.6% to 17.5%.[14-17]

The pattern of flu subtypes was different in two years period. Flu A was the dominant species in the 2016-2017 season,

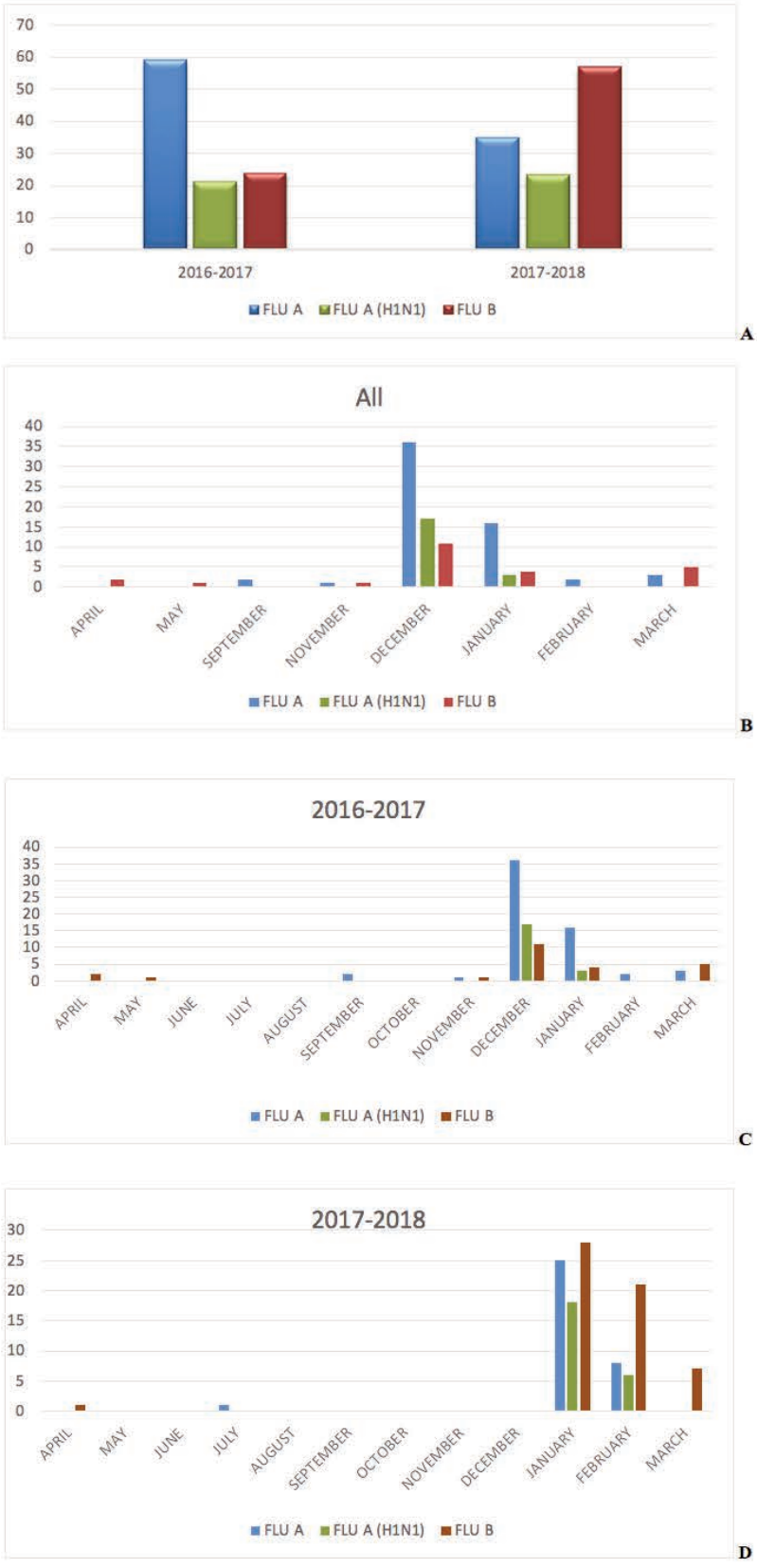

Figure 1. Frequency of samples positive to flu A, flu B and flu A (H1N1) during the influenza seasons 2016-2017 and 2017-2018. A) Overall frequency of Flu types in the two influenza seasons. B) Montly distribution of fluA, fluB and flu A (H1N1) cases in the whole study period; C) Montly distribution of fluA, fluB and flu A (H1N1) cases in the 2016-2017 season. D) Montly distribution of fluA, fluB and flu A (H1N1) cases in the 2017-2018 season.

which correlates with Yavarian *et al.* and Alavi *et al.* who found similar result.[4,5] On the other hand, flu B was the major type in the 2017-1018 seasons. Flu B was predominated from January 2017 to March 2018 which was the unique influenza type detected in March 2018. Yet, no data reported in Iran that consists of incidence of flu B in 2018. Moreover, flu A was predominantly seen in early winter, while flu B was found in mid-winter and tended to be the exclusive type during spring. Circulation of different influenza types seemed to be seasonal which might be correlated with outbreaks in different living circumstances,[18,19] although this relationship was not investigated in this study.

Our data also indicated that the incidence of flu B in hospitalized patients was relatively high, in contrast with previous Iranian reports of 2.5% to 2.8% flu B prevalence among hospitalized patients.(4, 6) Other studies have evaluated distribution of types and subtypes of influenza viruses in patients. Horthongkham *et al.* reported 3.1% for flu B and they concluded that Victoria lineage was significantly associated with the duration of hospitalization.[7] However, in here, we did not analyze the lineage of flu B viruses in hospitalized patients. Cohen et al reported prevalence of 8% for influenza, A (H3N2) 37% and flu B (34%).[8] Garg *et al.* showed that fluA (H3N2) 62.8% and flu B (28.5%) had the highest rates for 2017-2018 influenza seasonality.[9]

Among general and specific respiratory illness symptoms, we found that body pain and sore throat were the most significant symptoms among those who were flu positive compared to those who were flu negative. Other studies found similar findings, however with different frequencies.[10] Similar to other studies, cough was frequent in flu-positive patients, consistent with the fact that this is a common symptom in these patients.

The length of stay was shorter for flu-positive than for flu-negative subjects, implying the usefulness of rapid diagnosis for physicians to make decisions such as discontinuation of antibiotics and prompt patient discharge.[10-13]

A majority of flu-positive patients had either pulmonary or heart disease backgrounds, in line with their ages. Most often older adults or those with chronic pulmonary, cardiac, and metabolic or other disease have a more complicated influenza illness with subsequent secondary bacterial infections.[2,14-16] Different specimen types have been proposed by different for influenza virus detection. Flocked nasal and/or throat swabs or gargling are the best choices in this regard.[17,18] However, the frequency of positive results decreased from nasopharyngeal (65%) to throat swab (30%) to sputum (5%) samples. This finding suggests that the molecular influenza tests show different sensitivity depending on the specimen types, being the difference in favor of nasopharyngeal swabs.

This study suffers from some limitations. First, the multiplex panel only allowed to discriminate flu A from flu B, and, among flu A, only flu A (H1N1) subtyped was identified. Identification of flu A subtypes different from H1 was not possible with this panel. Finding of different flu species, subtypes and variants would be useful for epidemiological investigations and for tracking outbreaks and also predict flu vaccine efficiency. Moreover, other flu-like illnesses such as respiratory syncytial virus and human metapeumovirus infections were missed from differential diagnosis in patients who were flu-negative. Moreover, coinfections between these viruses were also ignored. These latter issues are due to the absence of more comprehensive diagnostic tools for respiratory viral infection evaluation at least in our center. In conclusion, despite no other respiratory pathogen were investigated, these results showed that almost 40% of patients admitted to Laleh hospital were infected by influenza virus. Influenza diagnosis was not achieved in up to 60% of flu-like respiratory infections, suggesting the potential benefit of adopting the same methodology for assessing the involvement of other viral or/and bacterial pathogens in those patients. This study clearly revealed that the rapid diagnosis due to the application of a multiplex molecular test would result in a shorter period of hospitalization and would benefit patients avoiding unnecessary antibiotic therapy due to the exclusion of bacterial infections.

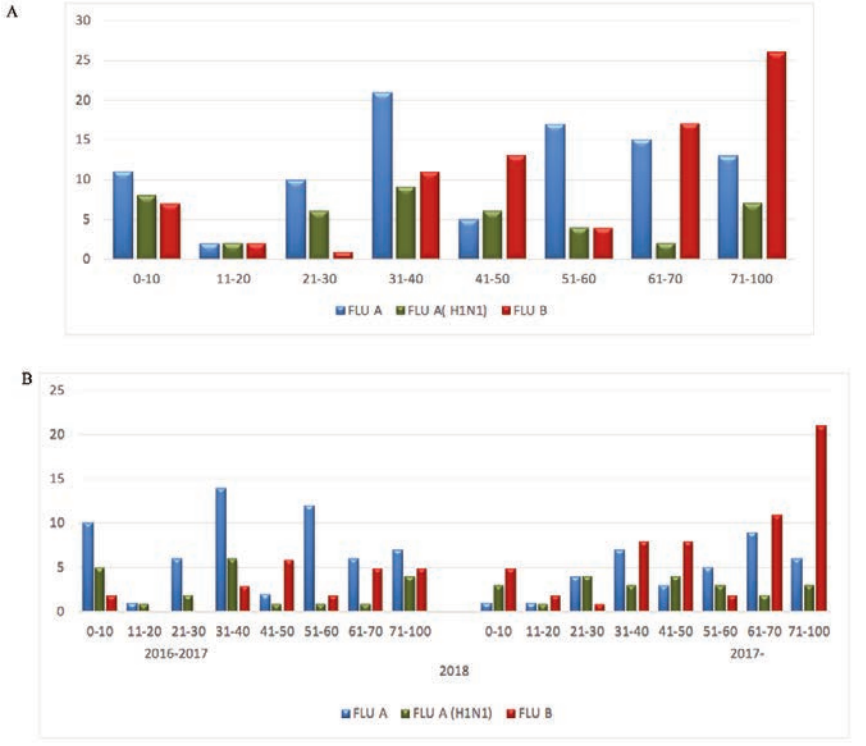

Figure 2. Frequency of patients with Flu A, Flu A (H1N1) and Flu B according to different age groups. A: results referring to the whole study duration (2016-2018); B: break down of the results according to the 2016-2017 and 2017-2018 seasons.

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