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Trends of Influenza A Virus Infection in Paediatric Patients

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ABSTRACT

Objectives: Influenza A virus infection causes significant morbidity and mortality worldwide. India continues to have Influenza A (H1N1) pdm09 positivity in patients, especially in Rajasthan. We studied the epidemiological profile of influenza A infection in paediatric patients presenting with Influenza like Illness (ILI) / Acute Respiratory Infections(ARI), during the period 2014-2016.
Methods: Throat or nasopharyngeal swab samples were collected in viral transport medium (VTM) from 1315 patients with ILI in 0-5 years age group, attending paediatric Hospital and transported to the laboratory. Samples were tested for influenza A, its subtypes, and for H275Y mutation as per recommended real-time RT-PCR protocols.
Result: Among 1315 patients 227 (17.2%) were positive for Influenza A, out of which 7.1% were Influenza A H3N2 and 10.1% were (H1N1) pdm09 positive. Among the positive patients 129(9.7 %) were males of which 55(4.2 %) were positive for Influenza A H3N2, and 74(5.6 %) for Influenza A (H1N1) pdm09. A totalof 98(7.5%) femaleswere positive for Influenza A,of which39(3%) were positive for H3N2 and 59(4.5%) for H1N1 pdm09. The positivity was higher in winter months. All 60 Influenza A (H1N1) pdm09 positive samples were negative forH275Y mutation.
Conclusions: Both, H3N2 and (H1N1) pdm09 subtypes of Influenza A are prevalent in our area; prevalence is higher during the winter season, especially in males. All 60 Influenza A (H1N1) pdm09 positive samples were negative for H275Y mutation. This epidemiological information can be helpful in planning vaccine strategies and for prevention and control of infection and for proper treatment of patients.

Keywords: H275Y mutation, Influenza A (H1N1) pdm09, Real time PCR.

Introduction

Respiratory illnesses due to Influenza virus infection are a major cause of mortality and morbidity worldwide [1]. Influenza viruses are of 3 types: A, B, and C; types B and C are only found in humans. Type A influenza viruses are responsible for most influenza pandemics, epidemics, and sporadic cases with significant mortality and morbidity [2]. Influenza type A viruses can infect humans, pigs, birds, seals, horses, and some other animals. Influenza A virus possesses eight segmented genomes encoding 11 proteins. Based on the variation of surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA), Influenza A viruses are categorized into 18 HA (H1–H18) and 11 NA (N1–N11) subtypes [3].

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Influenza A viruses evolve rapidly due to both antigenic drift and reassortment (antigenic shift), and in humans, new antigenic variants emerge constantly to give rise to yearly epidemics and pandemics due to lack of immunity in the population to new subtype. The pandemic Influenza A H1N1 2009 virus emerged in humans in April 2009 in Mexico, and then spread worldwide, it evolved due to genetic reassortment between human, swine, and avian strain [4]. India also reported many H1N1 cases and deaths during the H1N1 pandemic in 2009-10 and continues to have outbreaks with significant mortality and morbidity [5]. In 2015 outbreak a large number of cases and deaths were reported in India and some reports indicated that the 2014 Indian H1N1 strain had undergone mutations leading to its increased virulence. Sequencing of influenza isolates from 2015 outbreak by National Institute of Virology, Pune, revealed that the virus hadnot undergone any genetic changes that could have affected H1N1 virulence or leading to resistance to antiviral drug, oseltamivir [6].Oseltamivir and zanamivir are the two primary antiviral agents recommended for the prevention and treatment of influenza. Three unrelated cases of resistance to oseltamivir were observed in Denmark, Japan, and Hong Kong [7]. Resistance to oseltamivir can occur due to a point mutation in any of several regions of the neuraminidase protein of the virus. A single nucleotide mutation in the NA gene from C to T resultingin an amino acid change from histidine (H) to tyrosine (Y) at N1 position 275 i .e.H275 to Y275 by N1 nomenclature is known to be associated with oseltamivir resistance in H1N1 Influenza virus. Monitoring mutations conferring the antiviral resistance in influenza is critical to public health epidemiology and pandemic preparedness activities [8]. The present study was aimed to study the epidemiological profile of Influenza A infection and presence of H275Y mutation in paediatric patients attending JK lone hospital presenting with ILI /ARI.

Materials and Methods

This study was conducted at the Indian Council of Medical Research (ICMR)/DHR Grade-I Virology reference Laboratory-Advanced Research Laboratory, Department of Microbiology, S. M. S. Medical College, Jaipur over a period of three years from January 2014 to December -2016.

Sampling

All the pediatricpatients under 5 years of age, presenting withInfluenza Like Illness (ILI) / Acute Respiratory Infections (ARI)attending J.K. Lone paediatricHospital, a hospital attached to Sawai Man Singh (SMS) Medical College, Jaipur.Samples were collected from patients of medical units having OPD and admissions on three fixed days each week, during the study period with the prior consent of the parent/guardian.

Inclusion Criteria

Patients with ILI /ARI were enrolled as per WHO case definition. ILI defined as acute onset within the last 10 days following respiratory symptoms, measured fever of $\geq 38^{\circ}$ C and cough. ARI defined as, sudden onset of respiratory infection symptoms;cough, sore throat shortness of breath, coryza [9]. All clinical signsand symptoms, co- morbidities, Complete Blood Count (CBC) and X-ray findings were noted down in the prescribed performa.

Sample Size

Considering the prevalence of ARIs in the age group under five in children in Rajasthan to be 15.9 per cent as reported by Annual Health Survey (AHS 2012-13), the sample size was

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calculated as 1285 using the formula $n=z^2*p*(1-p)/e^2$[10].Where: z = 1.96 for a confidence level (α) of 95%, p = known prevalence (expressed as a decimal), e = margin of error, set as 2 %.(z = 1.96, p = 0.159, e = 0.02,n = 1.96^2 * 0.159 * (1 - 0.159) / 0.02^2, n = 0.5137 / 0.0004 = 1284.237, n ≈ 1285) rounded off to 1315 for samples collected during 3 years.

Sample Processing and Storage

Throat swab/ Nasopharyngeal Swab samples were collected in Viral Transport Medium (VTM) under good illumination. For throat swab collection, the patient was asked to open his/her mouth. A sterile swab, partially dipped in a vial containing normal saline was taken, and both the tonsils and the posterior pharynx were swabbed vigorously, till the patient gagged. For Nasopharyngeal Swab collection, the patient's head was tilted back 70 degrees and then the swab was inserted into the nostril and was allowed to leave in place for several seconds to absorb secretions. It was then slowly removed while rotating it. Both the swabs were placed in the same tube containing VTM and transported to the laboratory maintaining cold-chain. On reaching the laboratory, unique identification number was allotted.RNA extraction was done from 200 µL of the specimen by using the QIAGEN Viral RNA Mini Kit,60µL RNA was eluted from each sample. The real-time RT-PCR master mix was prepared using the AgPath One-Step RT-PCR Master Mix Reagent (Applied Biosystems, Foster City, CA, USA) and Taqman assays, separate master mix was made for each of the four target genes viz InfA, SwA, SwH1 and RNaseP (Applied Biosystems) in a 20 µl volume as per CDC protocol[11].For each gene:12.5 µl 2X PCR buffer, 1 µlEnzyme Mix, 0.5 µl of Assay mix and 6 µl ofNuclease free water was taken, 5 µL of RNA was added to 20µL amplification mixture to make the final reaction volume to 25µl. Thermo cycling conditions were: 50°C for 30 min, 95°C for 2 min, PCR amplification (45 cycles) 95°C for 15 sec, 55°C for 30 sec. A specimen was considered positive for Influenza A virus if the InfAamplification curves crossed the threshold line within 40 cycles and was considered positive for SWInf A/H1 if both the Inf A and the respective subtype (swInfA or swH1) amplification curves crossed the threshold line within 40 cycle[11].Influenza A positive samples were subtyped by real-time RT-PCR one-step duplex for the detection of H1N1 pdm09 and subtype H3, usingAgPath One-Step RT-PCR Master Mix Reagent kit (Applied Biosystems, Foster City, CA, USA) andprimers and probes as perWHO protocol[12].Randomly 60 Influenza A (H1N1) pdm09 positive samples were selected and screened for the presence of H275Y mutation using AgPath One-Step RT-PCR Master Mix Reagent kit (Applied Biosystems, Foster City, CA, USA)and primers and probes as per WHO allelic discrimination Real-Time PCR protocol [13]. This is a multiplex one-step RT-PCR that uses a pair of primers with two TaqMan MGB probes each targeting a different allele i.e. wild type and mutant alleles that differ by a single base substitution or single nucleotide polymorphism (SNP) Using this method, a single nucleotide mutation in the NA gene from C to T which is known to be associated with Oseltamivir resistance in H1N1 Influenza virus was detected.

Results

Typing and Subtyping

Out of 227 Influenza A positive cases, 94 (7.1 %) cases were Influenza A H3N2, and 133(10.1 %) cases were Influenza A (H1N1) pdm09.

Gender-Wise and Age Wise Distribution

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Gender and age wise distribution of Influenza A (H1N1) pdm09 and H3N2 subtypes are given in Table 1 and Table 2 respectively. Positivity was higher in males than females (Table 1) and in 1-12 months age group,94 (41.4%) casesas compared to other age groups(Table 2).

Table 1. Gender wise distribution of Influenza A (H1N1) pdm09 and H3N2 subtypes

Sex	Total Tested N= 1315	Total Influenza A positive	Influenza A H3N2 positive	Influenza A H1N1 pdm09 positive
Male	863 (65.6 %)	129 (9.7 %)	55 (4.2 %)	74 (5.6 %)
Female	452 (34.4 %)	98 (7.5 %)	39 (3 %)	59 (4.5 %)
Total	1315	227 (17.2%)	94 (7.1%)	133 (10.1%)

Table 2. Age wise distribution of Influenza A (H1N1) pdm09 and H3N2 subtypes (n=227)

Virus	1-12 months	13-24 months	25-36 months	37-48 months	49-60 months	P value
Influenza A H3N2	33 (14.5 %)	16 (7%)	21 (9.3%)	13 (5.7%)	11 (4.8%)	0.0148
Influenza A (H1N1) pdm09	61 (26.9%)	19 (8.4%)	26 (11.5%)	15 (6.6 %)	12 (5.3%)	0.0001
Total	94	35	47	28	23	-

Distribution of Out Patient Departments(OPD) and In Patient Department (IPD) Cases

Out of 94(7.1%) influenza A H3N2 positive cases, 5(0.4 %) were IPD cases and 89(6.8 %) were from OPD. Out of 133(10.1 %) influenza A (H1N1) pdm09 positive cases, 70 (5.3 %) were from IPD and 63(4.8 %) from OPD.

Clinical Feature of Influenza a Virus with Different Age Group

Profile of signs and symptoms in Influenza A H3N2 and Influenza A (H1N1) pdm09 positive cases is given in Table-3.

Table 3. Sign and symptoms in Influenza A (H1N1) pdm09 and H3N2 positive cases(n=227)

Signs and symptoms	Influenza A H3N2	Influenza A (H1N1) pdm09
Fever	89 (39.2 %)	133 (58.6 %)
Cough	83 (36.6%)	123 (54.2 %)
Sore throat	81 (35.7 %)	120 (52.9 %)
Rhinorrhoea	51 (22.5 %)	73 (32.2 %)
Breathlessness	38 (16.7 %)	58 (25.6 %)
Nasal catarrh	24 (10.6 %)	56 (24.7 %)

Table: 4 Clinical features of Influenza A virus positive patients among different age groups (n=227)

Clinical	1-12	13-24	25-36	37-48	49-60	P value

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Features	months	months	months	months	months	
Fever	92 (40.5 %)	34 (15 %)	46 (20.3 %)	27 (11.9 %)	23 (10.1 %)	0.0001
Cough	86 (37.9 %)	31 (13.7 %)	44 (19.4 %)	25 (11 %)	20 (8.8 %)	0.0181
Sore throat	71 (31.3 %)	19 (8.4 %)	32 (14.1 %)	21 (9.3 %)	19 (8.4 %)	0.0737
Rhinorrhoea	36 (15.9 %)	12 (5.3 %)	21 (9.3 %)	13 (5.7 %)	11 (4.8 %)	0.0004
Breathlessness	23 (10.1 %)	17 (7.5 %)	21 (9.3 %)	10 (4.4 %)	16 (7 %)	0.3126
Nasal catarrh	19 (8.4 %)	10 (4.4 %)	12 (5.3 %)	9 (4 %)	8 (3.5 %)	0.1234

Table 5. Complete blood count and radiological profile in positive patients

Hb levels	No. of patientsN=227	Percentage
Hb levels<11gms/dl	145	64%
Hb levels>11gms/dl	82	36%
Total Leukocyte count		
<4500 (Leukopenia)	35	15.4%
4501-9999 (Normal)	145	63.9%
>10000 (Leukocytosis)	47	20.7%
Platelet count		
<1.50 lacs	48	21.2%
>1.50 lacs	179	78.9%
Radiological features		
Bilateral chest infiltration	54	23.8%
Patchy pneumonia	14	6.2%
Hyper infiltration	7	3.1%
Normal Chest X ray	152	67%

Fever and cough were the commonest clinical manifestations followed by sore throat, rhinorrhea, breathlessness, and nasal catarrh. Clinical features in different age groups are given in Table-4, children in 1-12 months age group had higher rate of signs and symptoms than other age groups.Five deaths were reported due to Influenza A (H1N1) pdm09 in the year 2015 and one in 2016, 27(11.9%) patients (18 H1N1 positive and 9 H3N2 positive patients had co morbid conditions, 24/227(10.5%) had bronchial asthma, 2 (0.8%) had acute bronchiolitis, 1(0.4%) had cystic fibrosis. The correlation of Complete Blood Count (CBC), X-ray and Influenza positivity is given in Table-5.

Seasonal Trends

Trends of Influenza A virus infection over a period of 36 months from January 2014 till December 2016 are shown in Figure 1.

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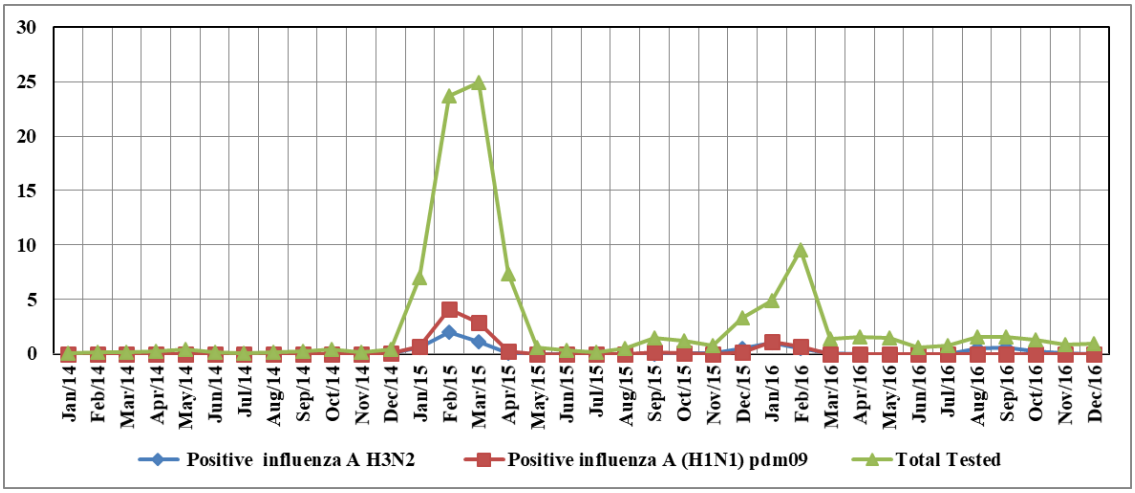

Figure 1. Month wise distribution of Influenza A (H1N1) pdm09 and H3N2 subtypes

Very few Influenza A positive cases were reported till December 2014, then sudden rise in cases from January 2015till May 2015 was noted, with a peak in February 2015; followed by again decline in number of positive cases. An increase in number of positive cases was observedagain from November 2015 tillMarch 2016 with a peak in January 2016.

Detection of H275Y mutation by allelic discrimination real-time PCR

All the 60 Influenza A (H1N1) pdm09 positive samples selected for the detection of H275Ymutation were found to be negative, 51 samples were from 2015 and 9 samples were from 2016.

Discussions

The present study was performed to study the epidemiological profile of Influenza A virus infection and drug resistance to Oseltamivir in paediatric patients attending tertiary care hospital at Jaipur.Positivity for Influenza A was found to be 17.2%, among these 7.1% samples were Influenza A H3N2 and 10.1% were Influenza A (H1N1) pdm09 positive.This is similar to that earlier reported in a study from Kashmir where 19.4% of pediatric cases were found to be positive for H1N1pdm09 [14].

A study from Chennai reported a positivity of 8.2% for Influenza viruses in ARI cases in children [15]. While a study from New Delhi reported a positivity of 32.6% in Indian children [16]. A study conducted in Nepal from April 2014 to March 2015 reported 10.3% cases to be positive for H1N1 pdm09 and 29.1 % cases to be positive for H3N2 [17].Authorsfrom AndhraPradesh reported 27.6 % positivity for Influenza A (H1N1) pdm09 and only 2.9 % for Influenza A (H3N2) during the 2017- 18 outbreak[18].The pattern of influenza positivity around the world varies from time to time depending upon the climate, humidity, vaccination statusand the strain prevalent etc. In the present study, we observed highest Influenza A positivity in winters with peak positivity in February 2015. Similar to other respiratory viruses, influenza has also been reported to exhibitdistinct seasonal variations [19]. In temperate climates, the disease is thought to exist at a low level throughout the year but exhibits a marked seasonal increase, typically begins to increase in late autumn and peaks during the winter months [20]. In India climate varies substantially from the north region to the south. In south year-round circulation of Influenza A has been reported, though

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the infection peaks during the rainy season (June-September) [21-23]. However in northern India with very cold winter season (December-February), two peaks of infection are observed, one in the rainy season and one during winters [23]In a multicentric study from India it was reported that in Delhi, Pune, and Kolkata influenza activity was from June to August and in Chennai October to December [24].A study from Andhra Pradesh reported two peaksone in summer and another in winter [18].Authors from Chennai reported peakactivity in September and October followed by a decline in January [25].In the present study, the prevalence of influenza infection was higher in males,9.7% as compared to females,7.5% and positivity was higher for Influenza A (H1N1) pdm09;10.1% as compared to Influenza A H3N2 virus,7.1%. In our study, 4.2% males and 3% females were Influenza A H3N2 positive and 5.6% males and 4.5 % females were Influenza A (H1N1) pdm09 positive. However studies from China [26] and Andhra Pradesh [18] reported no difference in the distribution of influenza virus infection based on sex.In our study, majority of patients had fever, cough, sore throat, rhinorrhea, breathlessness, and nasal catarrh, in Influenza A H3N2 and Influenza A (H1N1) pdm09 positive cases, commonest symptoms werefever and cough. A similar study from Greecereported fever in 94% patients, cough in 92%, and sore throat in 66% and severe illness and deathwas reported in H1N1 pdm09 positive patientstoo [27].In our study, we observed 2.6% (6/ 227) CaseFatality Rate (CFR) due to Influenza A (H1N1) pdm09 which is higher than that reported in an earlier study from stateof 1.8% (14/774) CFR among paediatric patients of all age groupsfrom all overRajasthan [28].In our study co morbidities were found in 11.9% Influenza A positive patients, commonest of all being bronchial asthma(10.5%).In a study from Kashmir, co-morbidities were reported in 28% of the children who tested positive for H1N1 influenza A. Pulmonary disease has been reported to be most common co-morbid condition with bronchial asthma as the leading cause for hospitalization [14].In our study anemia (haemoglobin <11gms/dl) was present in 64 % cases. Similarly a study from Gujarat reported anemia (haemoglobin <11gms/dl) in 74.5% cases [29]. Most of the patients (64%) in our study had normal Total Leukocyte count, while 20.7 % had leucocytosis possibly due to secondary bacterial infections. Similarly, a study from Pune also found that the TLC (Total Leukocyte count)<5000/cu mm in 25% and >14000/cu mm in 16.7% with lymphocytic predominance in 66.7% of them [30]. Similarly study from, Gujarat reported TLC <4000/cu mm in 15.1 % cases and >10000/cu mm in 34.9% cases.

Thrombocytopenia was present in 21.2 % of cases in our study; similarly thrombocytopenia was reported from Gujarat in 25 % cases [29] and from Southern Rajasthan in 27.5 % cases [31].In our study 33. % positive patients had abnormalities in X-ray; 23.8% had bilateral chest infiltration, 6.1% had patchy pneumonia and 3.1% had hyper infiltration. Similarly authors from Bangalore, reported chest X-Ray abnormalities in form of bilateral involvement and extensive pneumonia in 18% cases [32] Studies done in Egypt and Michigan also reported X-ray abnormalities in 14.7% and 50% cases respectively. [33, 34]. In our study, all the 60 (100%) Influenza A (H1N1) pdm09 virus-positive samples tested for H275Y mutation were found to be negative that is they were sensitive to Oseltamivir. Three unrelated cases of resistance to oseltamivir have been reported from Denmark, Japan, and Hong Kong [8]. While in a study from South Korea, 11(16%) patients were found to have drug-resistant Influenza A (H1N1) pdm09 and all isolates had the H275Y mutation in the neuraminidase gene except one isolate which had the I117M mutation [35].In a study conducted by National Institute of Virology Pune, 493Influenza A (H1N1) pdm09 isolates obtained from all over India from 2004 to 2011werefound to be negative for H275Y mutation [36]. The first case of Oseltamivir drug resistance reported in 2015 from India by real-time PCRhad an uneventful recovery [37]. Though mutation conferring resistance has been reported but clinical significance needs to be evaluated in terms of outcome of patient.

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Conclusions

Positivity for Influenza A was 17.2% in pediatric patients, both, H3N2 and (H1N1) pdm09 subtypes are prevalent in our area; prevalence is higher in males as compared to females withpeak positivity in winters.CFR was 2.8%. All Influenza A (H1N1) pdm09 positive samples were sensitive to Oseltamivir as they were negative for H275Y mutation. The epidemiological information will be helpful in planning vaccine strategies and for prevention and control of infection and for proper treatment of patients.

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Conflicts of Interest

None

Ethical approval

The study was approved by the ethics committee of SMS Hospital Jaipur Rajasthan No. MC/EC/2011/227.dated March 3, 2011.

Authors Contribution

Khushbu Trivedi-did experiments, wrote manuscript, Bharti Malhotra- conceived the research, edited manuscript, Widhi Dubey- edited manuscript, Pratibha Sharma- helped in experiments and manuscript, Farah Deeba- helped in experiments, Jitendra Tiwari- helped in experiments, Aradhana Chauhan - helped in experiments.

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