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TYPING AND SUBTYPING OF SEASONAL INFLUENZA CASES (ILI & SARI) FROM TEN DISTRICTS IN BANGLADESH

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ABSTRACT

Due to antigenic drift and shift, novel Influenza A virus sometimes appears and causes a pandemic. Virological surveillance is essential to prevent the epidemic and pandemic caused by influenza viruses. The samples were collected from suspected patients with Influenza-Like Illness (ILI) and Severe Acute Respiratory Illness (SARI) from the Hospitals located in nine (09) different districts and one (01) sample from a Medical College Hospital in Dhaka. The study was conducted to identify the prevalence, seasonality, and demographic characteristics of influenza infection in Bangladesh. A total of 2315 samples were collected from May 2018 to October 2018, of those 2071 cases (89.5%) were found as negative and 244 cases (10.5%) were identified as positive. Among the positive cases, 94.3% (230/244) were Influenza A and 5.7% (14/244) were Influenza B. Subtyping analysis revealed that seasonal influenza A subtype H^1pdm09 was more dominant carrying the prevalence rate of 75% (183/244). Under this surveillance, we found the circulating influenza viruses in Bangladesh. The high-risk group (5 to 14 years of age) was identified and vaccination in this group can prevent and reduce the mortality and morbidity rate. The seasonal trends also showed that during the peak period, 26.32% of Influenza Illness (ILI) and Severe Acute Respiratory Illness (SARI) cases were observed due to the influenza virus.

Keywords: *Influenza, ILI, SARI, surveillance, H^1pdm09*

Introduction

Influenza is a contagious disease, commonly known as flu caused by influenza viruses (Centers for Disease Control and Prevention, 2018). The word influenza came from the Italian language meaning 'influence' and referred to the cause of the disease (Saunders, 2003). Almost 2400 years ago, the symptoms of human influenza were narrated by Hippocrates and Livy (Nicholson *et al.*,

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1998). Within the previous 400 years, 12 pandemics have been recorded. *Bacillus influenzae* was regarded as the causative agent of influenza before the discovery of the influenza virus in 1933 by Wilson Smith, Christopher Andrews, and Patrick Laidlow of the National Institute for Medical Research, London (Smith *et al.*, 1933). This invention greatly contributed to the development of the influenza vaccine.

Influenza viruses are from the orthomyxoviridae family, enveloped and single-stranded RNA-dependent RNA-negative sense viruses. Influenza virion is mostly non-infectious and RNA-dependent RNA polymerase is required for its replication (Nicholson *et. al.*, 1998). The viral genome usually consists of eight segments (Nicholson *et al.*, 1998). The influenza viral proteins contribute an expanded role in human respiratory infection. Between these two glycoproteins Haemagglutinin (HA) and Neuraminidase (NA), HA infects cells and is responsible for binding the virus to host cell antibodies. NA helps the virion progeny to be released from the cell surface (Fukuyama and Kawaoka, 2011; Kamps *et al.*, 2006).

There are four types of influenza virus: A, B, C, and D, where types A and B are responsible for seasonal influenza (WHO, 2018). Influenza A and B are of a similar structure whereas Influenza C is more divergent (Taubenberger and Morens, 2008). Influenza A virus is grouped into several subtypes based on the combinations of haemagglutinin (HA) and neuraminidase (NA). To date, 18 different haemagglutinin and 11 different neuraminidase subtypes have been identified (Centers for Disease Control and Prevention, 2018). Currently circulating seasonal influenza subtypes are AH^1N^1 and AH^3N^2 (Centers for Disease Control and Prevention, 2018). Because of the year 2009 pandemic caused by AH^1N^1, it is also written as AH^1N^1pdm09. Only influenza-type A viruses are responsible for pandemics (Centers for Disease Control and Prevention, 2018). Influenza type B is divided into 2 subtypes but can be grouped into lineage or strain. Currently, all Influenza B viruses belong to either B/Yamagata or B/Victoria (Centers for Disease Control and Prevention, 2018).

Influenza virus is changing frequently in two ways: antigenic drift and antigenic shift. Due to antigenic shift and variability of subtypes, Influenza A is infectious and easily evades the vaccine mechanism (How the Flu Virus Can Change: “Drift” and “Shift, 2018). The most famous and lethal outbreak was the Spanish influenza pandemic in 1918 caused by type influenza A, subtype H^1N^1 (Beveridge, 1991). It lasted from 1918 to 1920 and killed about 20 to 100 million people which were 2.5-5.0% of the world’s population. This pandemic is described as "the greatest medical holocaust in history" (Waring, 1971). A new strain of Influenza A virus with quadruple segment translocation in its RNA caused an outbreak of human infection in April 2009 in the USA and Mexico. It was classified as Influenza AH^1N^1 2009 (Al-Muharrmi, 2010). In the temperate zone of the northern hemisphere, influenza activity was observed at inter-seasonal levels. Increased influenza detections were reported in some countries of Southern and South-East Asia. In the temperate zones of the southern hemisphere, influenza returned to nearly inter-seasonal levels. Worldwide, the seasonal influenza subtype A virus is accounted for the majority of influenza (WHO, 2018).

The objective of the study is to identify the circulating influenza types and subtypes, and explore the prevalence and seasonality of influenza virus infection among Influenza-like Illness (ILI) and Severe Acute Respiratory Illness (SARI) cases in Bangladesh. This study was part of an ongoing project of National Influenza Surveillance, Bangladesh (NISB) organized by the Institute of Epidemiology, Disease Control & Research (IEDCR, Dhaka).

Materials and Methods

Nasal swabs, throat swabs of Influenza-like Illness (ILI), and nasal swabs, throat swabs, and sputum swabs samples of Severe Acute Respiratory Illness (SARI) were collected from patients from ten districts throughout the country. Then the specimens were collected into viral transport media (1-3 ml sterile Copan UTM RT viral transport medium, Copan Diagnostics Inc., USA) and were kept in a dry shipper at around -17^{o}C for a week until transported to the laboratory. After aliquoting the samples, viral RNA was extracted from the aliquot by QIAamp Viral RNA Mini Kit (Qiagen, Germany), a fast spin-column kit to extract RNA from the study samples according to the manufacturer's instructions. The purified viral RNA sample was eluted from the spin column with RNase-free water.

Influenza typing was performed by one-step Real-Time PCR kit QuantiFast Pathogen RT-PCR+IC Kit (Qiagen, Germany), and the following oligonucleotides (0.1µM) and TaqMan probes were used (Table 1) for influenza typing as per the manufacturer's protocol.

Table 1. Oligonucleotides and probes for the typing of Influenza

Virus (Target)	Name	Sequence (5'→ 3')	Concentration (µM)
Influenza A Virus (M)	FLUAM-7-F	CTTCTAACCGAGGTCGAAACGTA	0.1
	FLUAM-161-R	GGTGACAGGATTGGTCTTGTCTTTA	
	FLUAM-49-P6	CFO560-TCAGGCCCCCTCAAAGCCGAG-BHQ-1	
Influenza B Virus (HA)	FLUBHA-940-F	AAATACGGTGGATTAAACAAAAGCAA	0.1
	FLUBHA-1109-R	CCAGCAATAGCTCCGAAGAAA	
	FLUBHA-994-P4	FAM-CACCCATATTGGGCAATTTCCTATGGC-BHQ1	

PCR Amplification: After master-mix preparation, the qualitative thermal cycling was performed in a Real-Time qRT-PCR Thermal cycler (Applied Biosystems 7500 Fast Dx Real-Time PCR, ThermoFisher Scientific, USA) under the following conditions. The reverse transcription was carried out at 50^{o}C for 20 min, and after initial PCR activation at 95^{o}C for 5 min, the thermal cycling was performed for 45 cycles with denaturation at 95^{o}C for 15 sec followed by annealing/extension at 60^{o}C for 45 sec.

The positive Influenza A samples were further subjected to subtyping with Invitrogen SuperScript® III Platinum® one-step kit (ThermoFisher Scientific, USA), and the following oligonucleotides (0.1µM) and TaqMan probes were used (Table 2). The reverse transcription was carried out at 50°C for 20 min, and after initial PCR activation for 5 min at 95°C, the thermal cycling was performed for 45 cycles with denaturation at 95°C for 15 sec with annealing/extension at 60°C for 45 sec.

Table 2. Oligonucleotides and probes for the subtyping of Influenza A

Subtype	Primer and probe	Sequences (5'→ 3')
Influenza A subtype H1N1pdm09 (HA gene)	GRswH1-349Fw	GAGCTAAGAGAGCAATTGA
	GRswH1-601Rv	GTAGATGGATGGTGAATG
	GRswH1-538Probe(-)	FAM-TTGCTGAGCTTTGGGTATGA-BHQ1
Influenza A subtype H3 (HA gene)	H3-266-F	ACCCTCAGTGTGATGGCTTTCAAA
	H3-373-R	TAAGGGAGGCATAATCCGGCACAT
	H3-315-P	FAM-ACGAAGCAAAGCCTACAGCAACTGTT-BHQ1

Influenza B subtyping was accomplished using Qiagen One-Step RT-PCR Kit (Qiagen, Germany) with the following (Table 3) oligonucleotides (0.1µM) and TaqMan probes. The reverse transcription was carried out at 50°C for 30 min and after initial PCR activation for 15 min at 95°C, the thermal cycling was performed for 45 cycles with denaturation at 95°C for 10 sec with annealing/extension at 54°C for 50 sec.

Table 3. Oligonucleotides and probes for the subtyping of Influenza B

Type/subtype	Gene	Primer	Sequence (5'→ 3')
B	HA	BHA-188F	AGACCAGAGGGAAACTATGCCC
B	HA	BHA-270R	TCCGGATGTAACAGGTCTGACTT
B (Victoria lineage)	HA	Probe-VIC2	Yakima Yellow-5'-CAGACCAAAATGCACGGGGAAHATACC-3'-BHQ
B (Yamagata lineage)	HA	Probe-YAM2	FAM-5'-CAG**R**CCAATGTGTGTGGGGA**Y**CACACC-3'-BHQ

Data Interpretation: Amplification of viral RNA target and Internal Control (IC) had confirmed successful amplification. Influenza typing was a qualitative TaqMan RT-PCR assay. In the case

of influenza type A and B amplification, the early CT value was considered the dominant type and the CT value <40 was considered a positive result. The signal of reporter dyes FAM indicated the successful amplification of the influenza target sequence and the VIC signal indicated the successful amplification of the specific sequence of internal control (IC).

Results and Discussion

Results

During the study period from May 2018 to October 2018, total 2315 samples with suspected ILI (1239) and SARI (1076) respiratory complications were collected. The Influenza typing of 2315 samples revealed that 2071 (89.5%) cases were identified as influenza negative, 230 (9.9%) cases as Influenza type A which was predominant in circulation, and 14 (0.6%) cases as Influenza type B positive (Fig. 1), i.e. a total of 244 (10.5%) influenza-positive cases were found out of 2315 samples. No positive cases of Influenza AH^{3v}, AH^{5} and AH^{7} were observed.

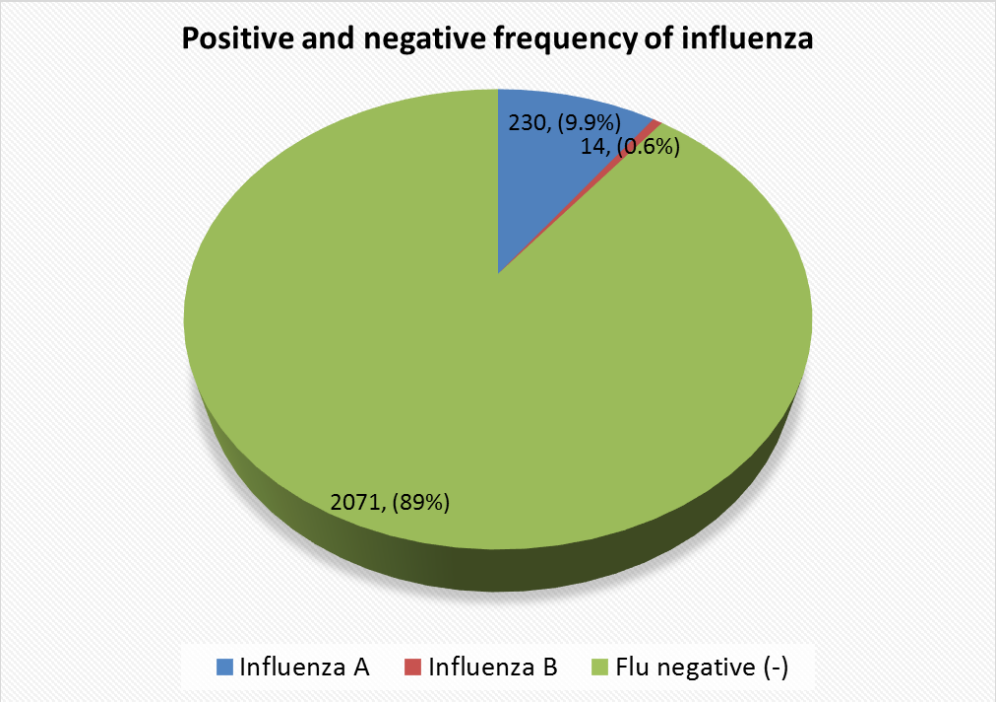

Fig. 1. Prevalence of influenza virus throughout the year 2018.

Positive cases detected in the Influenza typing were further subjected to subtyping. 75% (183/244) cases were identified as Influenza AH^{1}pdm09, 17.2% (42/244) as Influenza AH^{3}, and 2% (5/244) cases as co-infection of Influenza AH^{1}pdm09 and AH^{3}. Among Influenza B, 1.2% (3/244) was detected type B/Victoria and 4.5% (11/244) of type B/Yamagata was observed (Fig. 2).

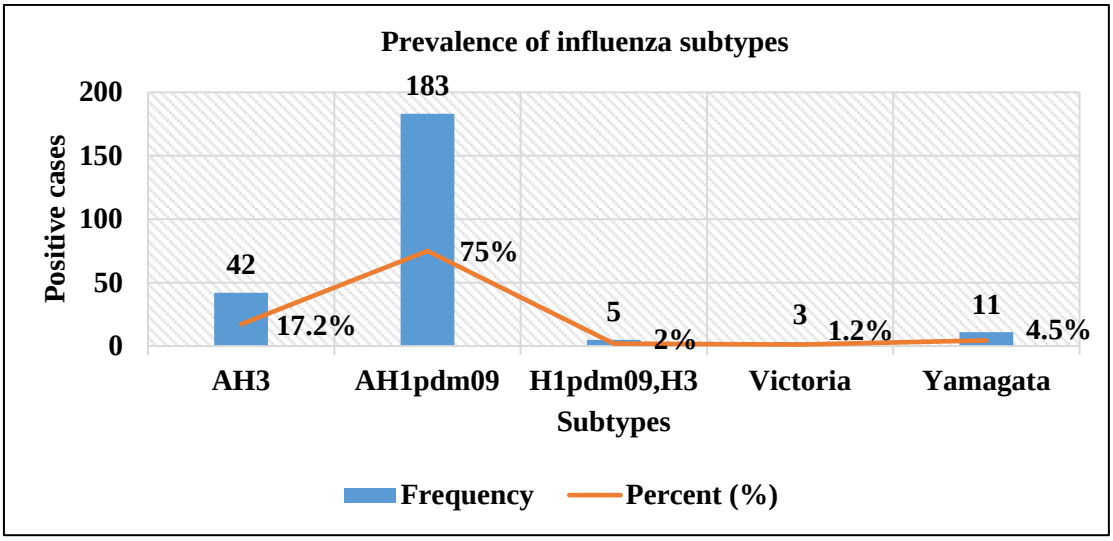

Fig. 2. Prevalence trend and positive number of influenza subtypes during the study period of 2018.

In the study period of 2018, Influenza AH^1pdm09 was the most prevailed and the next common strain was Influenza AH^3 grabbing the positivity of 75% and 17.2%respectively.

Discussion

The study focused on the data-based analysis of hospital samples collected from ten different study areas of Bangladesh, to evaluate a current aspect of influenza throughout the country and create awareness for the control of emerging outbreaks. This surveillance of influenza was also important to assess the risk and prevalence of influenza in specific age groups, regions and the necessity of effective vaccination.

Prevalence of influenza: From this study, it was lucid that the most prevalent type was Influenza A (Figure 1) holding a prevalence rate of 9.9% (230/2315). NISB Influenza study from January 2017 to December 2017 reported 336 (12.22%) positive cases out of 2748 cases (NISB News Letter, 2018). Hence, it was evident that the prevalence of influenza in 2018 decreased slightly and similar results were also observed in the previous year. This study manifested the steady presence of influenza, and the serotype AH^1pdm09 was observed more prevalent than other serotypes, and its recent trend was found to surpass the serotype AH^1N^1. People especially children were observed to have no or lesser herd immunity against this strain than other serotypes (Pielak and Chou, 2011) which might aid the prevalence of circulating Influenza.

Seasonality of Influenza: The study was conducted between May 2018 and October 2018; and the data demonstrated that the prevalence of influenza infection was highest in the months of July (22.80%) and August (26.32%) than in other months. These data were found consistent with the surveillance result of a study carried out on influenza infection by IEDCR from 2011 to 2017 that

described the occurrences of influenza infection as high between the periods of May to August being the peak in August (NISB News Letter, 2018). This period of the year was considered a rainy season in Bangladesh, and it is possible that, during the monsoons, people usually spend a large span of time inside homes with poor ventilation systems which might have contributed to the increased rate of transmission of the Influenza infection. An analysis of Influenza surveillance data collected between 2006 and 2011 in Bangladesh, Cambodia, India, Indonesia, Laos, Malaysia, the Philippines, Singapore, Thailand, and Vietnam revealed that influenza activity showed a distinct peak between July and October (Cox, 2018). This study is compatible with that analysis possessing the discrete peak seasonality of July and August.

Table 4. Seasonal variation of occurrence of Influenza infections

Month	Number of samples	Influenza positive cases	Percentage of positive cases (%)
May	360	2	0.56
June	314	23	7.32
July	364	83	22.80
August	395	104	26.32
September	395	25	6.32
October	487	7	1.43
Total	2315	244	10.54

The Table 4 asserted distinct peak in Influenza occurrence was in the month of August, and the seasonality observed were the months of July and August, which were the prominent monsoon period in Bangladesh, and beyond this period, the reduced infection rate was observed.

The prevalence of influenza among the study areas: The samples of this study were collected from ten sentinel sites specifically the hospitals of nine districts and one medical college hospital in Dhaka, and it was observed that influenza prevalent sites (Figure 3) were Thakurgaon (16.30%), Habiganj (14.56%), Gazipur (12.60%) and Potuakhali (12.32%); and Satkhira had the lowest rate of prevalence (2.16%). Fluctuation of this prevalence rate might depend on weather conditions, temperature, rainfall, humidity and wind speed of the specific zones, and also greatly depended on the patient's socio-economic conditions who did seek treatments in hospitals.

Seasonal influenza cases were depicted in the graph (Fig. 3) and it is apparent that Thakurgaon and Habiganj were the most prevalent Influenza occurrence sites.

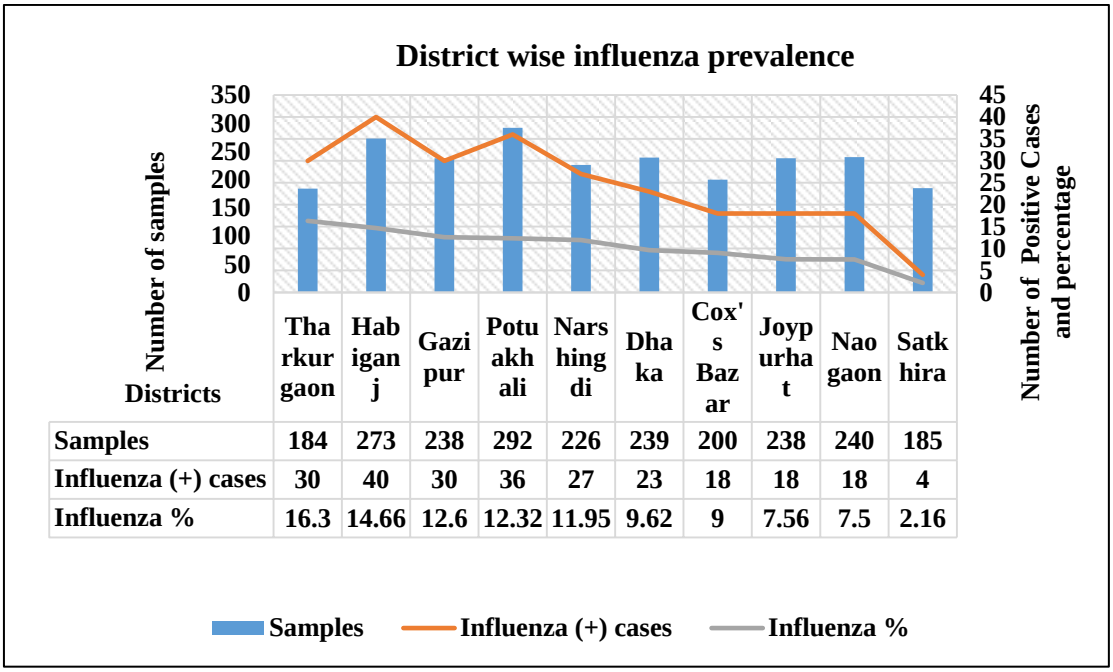

Fig. 3. Prevalence of influenza infection among study-conducted districts.

Influenza prevalence among different age groups: Samples were collected from the different age-group of patients ranging from less than 1 year of age (<1 year) and more than 60 years (>60 years). The study showed (Figure 4) that children aged 5 to 14 were infected (16.9%) comparatively more than other age groups, and children aged 01 to 04 years were the second most infected group (12.43%). CDC also reported that the highest morbidity rate was found among children aged 5-14 years (Centers for Disease Control and Prevention, 2018). In 2009, the AH^1N^1pdm09 strain was the main causative agent of high morbidity in adults while mortality was higher among children and elderly aged (>60) people with this same strain (Centers for Disease Control and Prevention, 2018). The reasons behind this morbidity were due to immature and infirm immunity, repeated exposure to public places such as schools, playgrounds, and carelessness about hand washing along with person-to-person contact. Infants usually were more prone to infection due to weak immune systems and person-to-person contact. Influenza infected children or person could easily spread infection through sneezing and coughing, whereas the 40 to 59 years of age group people were observed to be less infected by influenza, and the prevalence rate (6.47%) was found to be lower because it was probable that they had comparatively stronger immunity and sometimes might have herd immunity (Harper, *et al.*, 2009).

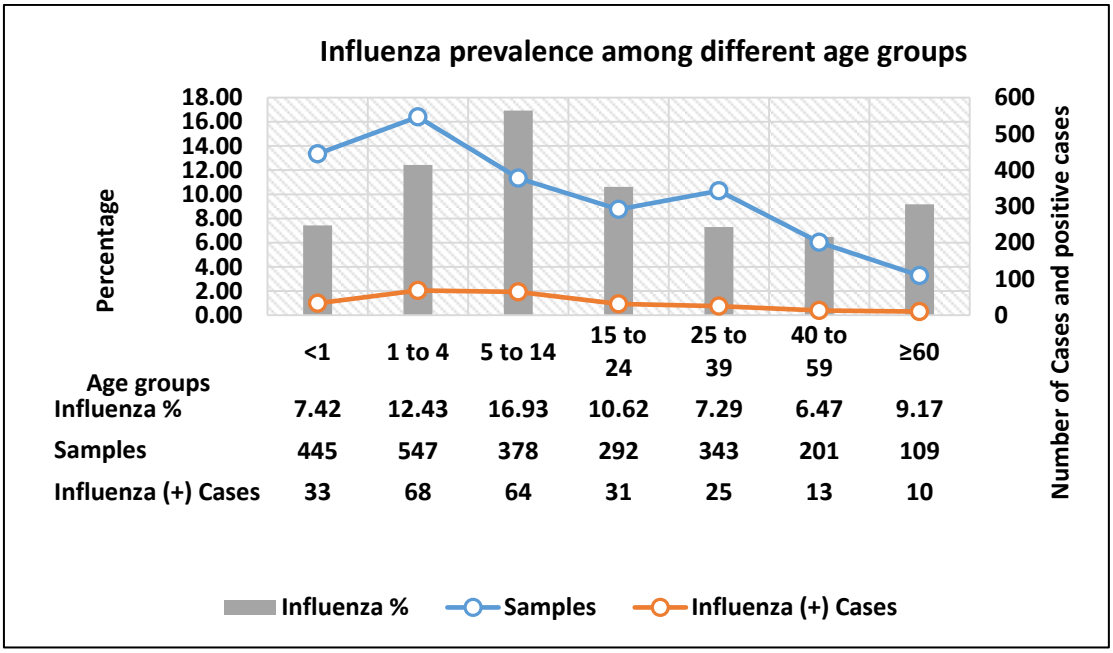

Fig. 4. Influenza prevalence among patients of different age groups.

In the graph (Fig. 4), it's conspicuous that infants and juveniles were observed as more vulnerable to Influenza.

Conclusion

The study revealed the most prevalent serotype, seasons, zones, and age groups of influenza infection. Although we had limitations such as 'duration of the surveillance', the findings of the study showed that influenza was a common infectious agent among all age groups, and showed prevalence during the rainy season. To minimize the prevalence, along with proper vaccination focusing on local variants of influenza, public awareness and personal hygiene are also required to combat influenza infection.

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