Iraqi Journal of Biotechnology, 2016, Vol. 15, No. 2 , 9-20

Molecular Detection of Pandemic Influenza A(H1N1) Virus in SARI Patient in South Iraq Governorates using Real-Time PCR

Nadira S. Mohamed[1], Sabah M. Abd Al Aema[2], Mohammad J. Al-Jassani[3], Faisal G. Nasser [4], Eman M. Aufi[4]

[1] Forensic DNA Centre for Research &Training, University of AL-Nahrain

[2] Respiratory Infectious Disease Unit, Ministry of Health

[3]DNA Research Center, University of Babylon

[4]Central Public Health Laboratory, Baghdad

Received: November 10, 2015 / **Accepted**: May 5, 2016

Abstract: Human influenza virus surveillance has a pronounced seasonal cycle.Rapid and reliable detection of newly emerging influenza viruses is important to enhance our influenza reasserting in Iraq. A total of 869 samples were collected from hospitalized patients with Severe Acute Respiratory Infection (SARI) from six south Iraq governorates (Najaf, Qadisiyah, Maysan, Muthanna, Dhi Qar and Basrah) during the year 2013. Approximately 29.34% of the cases were belong to the Influenza A (H1N1) pdm09 which predominant on the seasonal flue 20.71% of all suspected SARI patients. Male patients showed higher percentage than female patients 153(59.99%) and 102 (39.99% respectively).The highest peak of H1N1 infection was recorded in age group > 40 years old 69 (27.05%) fallowed by age group 14-19 years old which represented 52 (20.39%) in male patients. In female patient the pattern was different the highest peak was observed in age group 19-40 years old 41(16.07%) fallowed by age group > 40 years old 32(12.54%). Influenza A (H1N1) pdm09 activity in Iraq start increasing in winter season, particularly in January, and toward the end of February in some governorates and my extend to March in others.

Key words: Seasonal influenza, Influenza A (H1N1) pdm09, Real Time PCR.

Corresponding author: should be addressed (Email: nadmohamed2000@yahoo.com)

Introduction

Influenza virus is a common human pathogen that causes serious respiratory illness and death over the past century.It always had potential to cause widespread pandemics whenever a new type of influenza strain appeared in the human population and then spread easily from person to person (1). Influenza A viruses as members of the *Orthomyxoviridae* family are made up of eight gene segments and have antigenically related nucleocapsid and matrix proteins (2). They are classified into subtypes based on their hemagglutinin (H) and neuraminidase (N) antigens. At present, 19 H subtypes (H1–H19) and 11 N subtypes (N1–N11) are recognized (3,4). Seasonal influenza is caused by strains of influenza that circulate continuously in the human population, resulting in a portion of the population that has pre-existing immunity due to prior exposure or exposure to similar influenza strains and is, therefore, protected from infection. Currently, there are two influenza A viruses (H1N1 and H3N2) and one influenza B virus which are responsible for annual epidemics (5).

During the spring of 2009, a novel strain of influenza A (H1N1) virus appeared globally. The new swine-origin influenza strain was genetically distinct from seasonal influenza virus and classified by WHO as a novel influenza virus strain and as a pandemic influenza outbreak (5).

The novel influenza A (H1N1) pdm09 virus was identified in Mexico, USA, Canada and Japan (6,7). The first cases, associated with a number of unexpected deaths in younger persons, were registered in Mexico (7). By September 2009, this influenza infection had been discovered in 191 countries (8). This virus was generated by multiple reassortment events, and each of its precursor gene segments has circulated in swine for more than 10 years (8,9). Annual human influenza epidemics occur during the respective winter seasons in the temperate zones of the northern and southern hemispheres between November and March in the northern hemisphere and between April and September in the southern hemisphere (10). Human influenza infections exhibit a strong seasonal cycle in temperate regions there are two types of environmental conditions associated with seasonal influenza epidemics: “cold-dry” and “humid-rainy” influenza activity peaks during the cold-dry season when specific humidity and temperature are at minimal levels (11). Some epidemiological studies indicate that low levels of specific humidity are associated with the onset of pandemic and epidemic influenza in the USA (12,13). Lipid in viral envelope ordering may contribute to viral stability at lower temperatures, which has been found to be critical for airborne transmission. In addition to this, overcrowding during winter acts as a co-contributing factor in facilitating the spread of the virus. It also has been reported that decrease in environmental temperature increases the physiological stress and energy loss due to thermal regulation, which in turn weakens the immune system and thereby increases the susceptibility of the host to infection (14). This explains the high incidence of H1N1 infection during the winter season in Iraq in January, February and December (15).

Materials and Methods

Sample Collection

A total of 869 samples of hospitalized patients with Severe Acute Respiratory Infection (SARI) were collected by local authorized and trained medical personnel and according to the WHO Influenza-like illness case definition. Five hundred twenty one samples were taken from males and 348 were taken from females from different age groups (<1, 1-6, 7-18, 19-40, >40 years old). The patients were from six southern Iraq governorates, with some or all of the following symptoms (fever, chills, cough, sore throat, runny or stuffy nose, bronchial breathing, muscle or body aches, headache, wheeze, fatigue, sometimes associated with vomiting and diarrhea). Nasal swab samples were collected using dacron or rayon tipped swabs. The samples were transferred to the Ministry of Health, Central Public Health Laboratory (CPHL), in a cooled sealed bag. Samples were refrigerated at 2-4 ºC not up 3 days till investigation or freeze at -70 ºC for long preservation (16).

RNA Extraction

Viral RNA was extracted from 140 µl of nasal swaps samples using QIAamp Viral RNA Mini Kit (QIAGEN®, Hilden, Germany) followed the manufacture's instruction .

Real-time Qualitative Reveres Transcription PCR (RT-qPCR)

SuperScript™ III Platinum® One–Step Quantitative RT-PCR kit (Invitrogen) was used according to the manufacture's instruction. Table 1 shows the sets of primers and probes used to detect Influenza A virus. RT-qPCR was performed using ABI Prism 7500 (Applied Biosystem) under the following conditions: (1) Reverse transcription at 50 ºCfor 30 minutes, (2) Tag inhibitor activation at 95 ºC for 2 minutes, (3) PCR (denaturation at 95 ºC for 15 seconds, annealing and extension at 55 ºC for 30 seconds) for 45 cycles. Data were collected during the 55 ºC. (17). t-Test (one sample), t-Test (paired samples), multiple comparisons and correlation analysis were done for statistical data analysis. All p values < 0.05 were considered as statistically significant.

Table 1. Primer and probe sets used for pandemic H1N1 (CDC rRT-PCR) detection

Primers & Probes	**Oligonucleotide sequences (5′ - 3′)**
InfA F	GAC CRA TCC TGT CAC CTC TGA C
InfA R	AGG GCA TTY TGG ACA AAK CGT CTA
InfA Probe[1]	TGC AGT CCT CGC TCA CTG GGC ACG
SW InfA F	GCA CGG TCA GCA CTT ATY CTR AG
SW InfA R	GTG RGC TGG GTT TTC ATT TGG TC
SW InfA probe[2]	CYA CTG CA"T" ACA CAC AAG CAG GCA
SW H1 F	GTG CTA TAA ACA CCA GCC TYC CA
SW H1 R	CGG GAT ATT CCT TAA TCC TGT RGC
SW H1 probe[2]	CA GAA TAT ACA "T"CC RGT CAC AAT TGG ARA A
RnaseP F	AGA TTT GGA CCT GCG AGC G
RnaseP R	GAG CGG CTG TCT CCA CAA GT
RnaseP Probe[1]	TTC TGA CCT GAA GGC TCT GCG CG

Note:-[1] Taq Man ® Probes are labeled at the 5′ end with the reporter molecule 6-carboxyflurescein (FAM), Blackhole Quencher 1 (BHQ1).

-[2] Taq Man ® Probes are labeled at the 5′ end with the reporter molecule 6-carboxyflurescein (FAM) and quenched internally at modified "T" residue with (BHQ1),with a modified 3′ end to prevent probe extension by Taq polymerase (Biosearch Technologies , INC., Novato, CA).

Results and Discussion

Influenza surveillance is an important tool to identify emerging/re-emerging strains and define seasonality. Our study focused on 6 Iraqi governorates (Najaf, Qadeseya, AL-Muthana, Maysan, Dhi Qar, and Basra) representing thesouth regions of the country. Nasal swabs were collected from 869 severe acute respiratory infection (SARI) hospitalized patients with a clinical suspicion of having (H1N1) virus infection. Our finding was 255 (29.34%) of the cases were belong to the Influenza A (H1N1) pdm09 which predominant over the seasonal flue 180 (20.71%), and 434 (49.94%) SARI cases were negative for both Influenza A (H1N1) pdm09 and seasonal flue through the study period from January to December 2013 (Figure 1). The age, sex, and gender play important role in incidence and severity of many infectious diseases. Our finding showed that the positive H1N1 male percentage (59.99%) was higher than female (39.99%) in different age groups. These findings are consistent with the finding of Shaman *et al*., (12), Polozov *et al*., (14) and Ashkenazi *et al.*, (18) in developed countries, such as the United States of America and Spain, they reported incidence of infection with seasonal influenza viruses is higher in males (up to 60% in the United States) than females of diverse ages, ranging from infants to elderly adults. The highest peak of H1N1 infection was recorded in age group >40 years (27.05%) in male patients followed by age group 19-40 years old (20.39%) with a significant deference ($p< 0.036$) between all male age groups. H1N1 infection in female patients have recorder highest incidence in age group 19-40 years (16.07%) followed by (12.54%) in the >40 years age group with a significant difference ($p< 0.041$) between all female age groups (Table 2) and this explained by the CDC report (16) who refers to that female in reproductive age when the estrogen level high was display an increased tendency to die of H7N9 influenza than males. Although, there was no significant differences between the male and female H1N1 positive cases ($p < 0.168$). This agrees with what was reported by Nasser *et al*., (15). We proposed that increased morbidity of age over 40 year and middle-aged adults (19-40) during the 2013 season is primarily a result of low vaccination rates within Iraqi populations and may be that recent H1N1 strains possess a mutation that prevents binding of antibodies in people who have been previously exposed to different H1N1 strains but not that recent strain.

(Figure 3) showed a positive but not significant ($p < 0.063$) correlation between female age and H1N1 infection, while there is a positive and significant ($p < 0.007$) correlation between male age and H1N1 infection (Figure 4). Male patients found more vulnerable for endemic Influenza H1N1 infection due to the nature of the Iraqi culture that they are mainly responsible for family finance and support making them more exposed to the infectious agents in large crowded governorates as mention by Nasser *et al*., (15).

For the above findings, Influenza vaccination should be covered the highly risk groups among Iraqi population including infants, pregnant women, health care workers, elderlies, and solders.

Influenza A (H1N1) pdm09 activity in Iraq start increasing in winter (mainly in January toward the end of February) and disappeared in the rest of the year as in Najaf, Muthanna, Dhi Qar and Basrah (Figures 5, 8, 9) and 10 respectively and this result was consistent with the Nasser *et al*., (15) a previous study for the central Iraq governorates. The H1N1 incidence was extended to March in Qadisiyah (Figure 5) and Maysan (Figure 6) governorates. The Seasonal influenza began increasing in mid-January and oscillating through the year and rise when temperature dropsin winter.This explain disappearing of influenza infection in summer months in Iraq when the temperatures rise to more than 48 ºC when people spend more time indoors which decrease exposure to viral particles. Low temperature lead to increasein the physiological stress and energy loss due to thermal regulation, which in turn weakens the immune system and thereby increases the susceptibility as what was reported by (16). The cold and dry atmosphere appeared facilitating the H1N1 infection as it appear in Najaf, Qadisiyah and Maysan governorates while in Muthanna Dhi Qar and Basrah where the humidity is high, the infection rate is low (Figure 10). Dry air may accelerate the nasal mucous membrane dehydration which weakens the human respiratory system against viral infection. These finding is agreement with Shaman *et al*.,(12), Shaman *et al*., (13), CDC (16), and Lowen ,(19).

The annual rate of H1N1 infection and seasonal flu in all six southern Iraq governorates has appeared significant (p <0.00) but in Maysan where no seasonal flu cases recorded (Table 3).

From the above results, it obviously clear that atmosphere temperature and humidity play an important role in H1N1 infection and viral spreading despite the infected area in crowded or active in trading with multinational population like Basrah province (portal area).

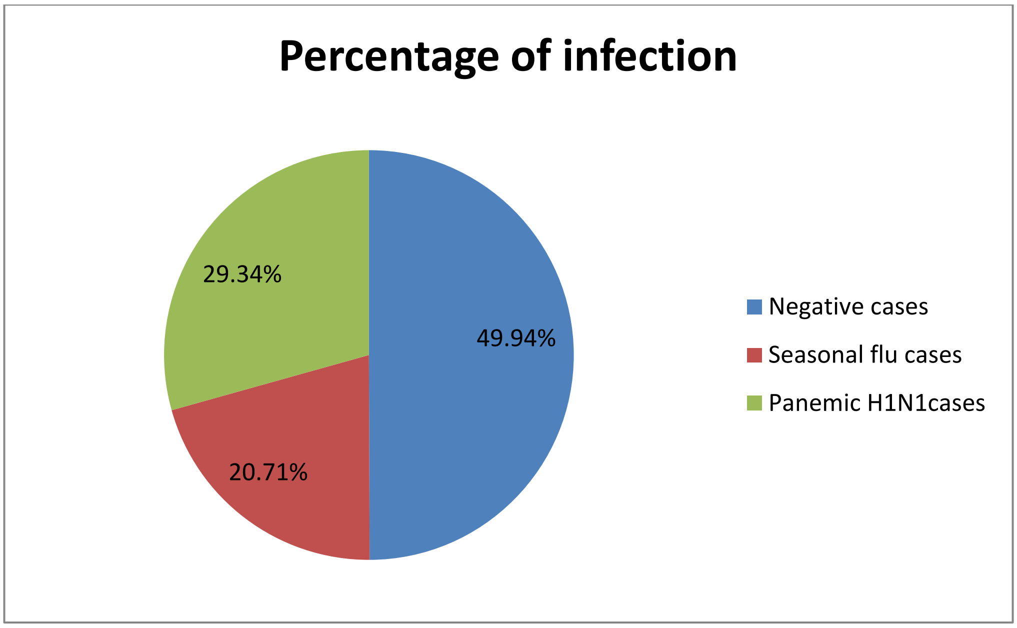

Figure 1: The total percentage of infected cases with pandemic H1N1 and seasonal flue

Table 2: The percentage of male and female H1N1 infected patients in different age groups

Age groups	Male %	Female %
<1 year	8 (3.137)	6 (2.352)
1-6	13 (5.098)	13 (5.098)
7-18	11 (4.313)	10 (3.921)
19-40	52(20.392)	41(16.078)
>40	69 (27.058)	32(12.549)
Total	153 (59.998)	102(39.998)

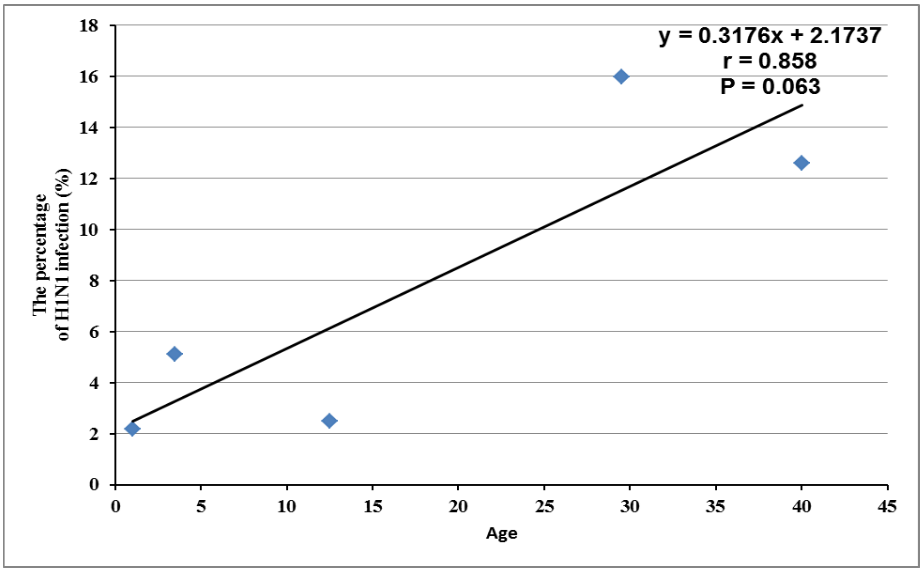

Figure 2: The Correlation between females age and H1N1 infection

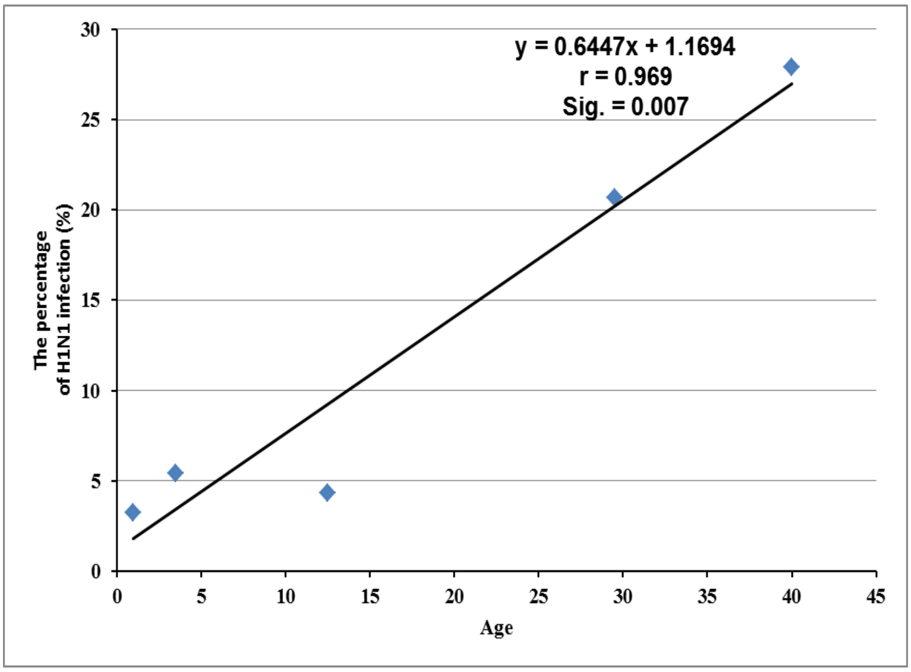

Figure 3: The Correlation between males age and H1N1 infection

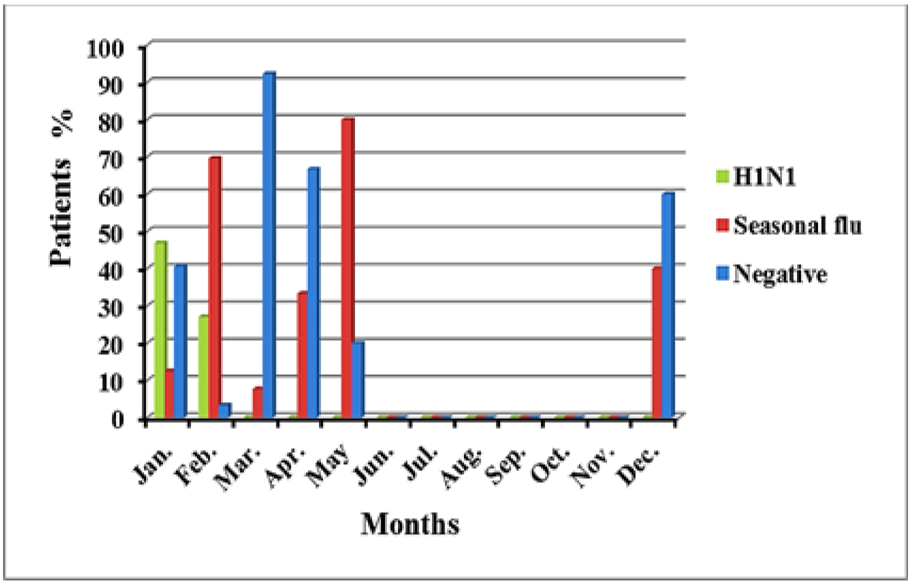

Figure 4: The percentage of pandemic H1N1 compared with seasonal influenza in AL-Najaf governorate

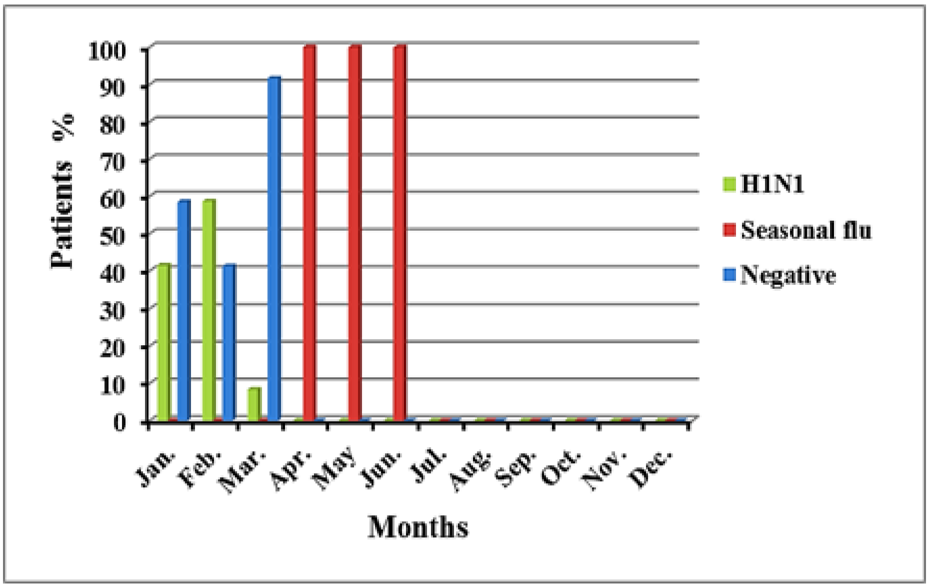

Figure 5: The percentage of pandemic H1N1 compared with seasonal influenza in AL-Qadisiyah governorate

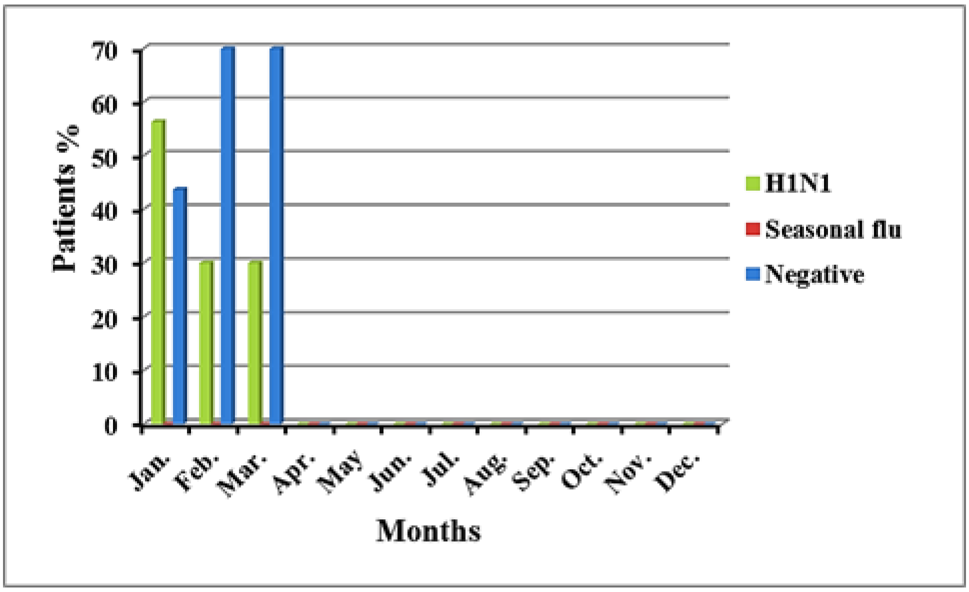

Figure 6: The percentage of pandemic H1N1 compared with seasonal influenza in Maysan governorate

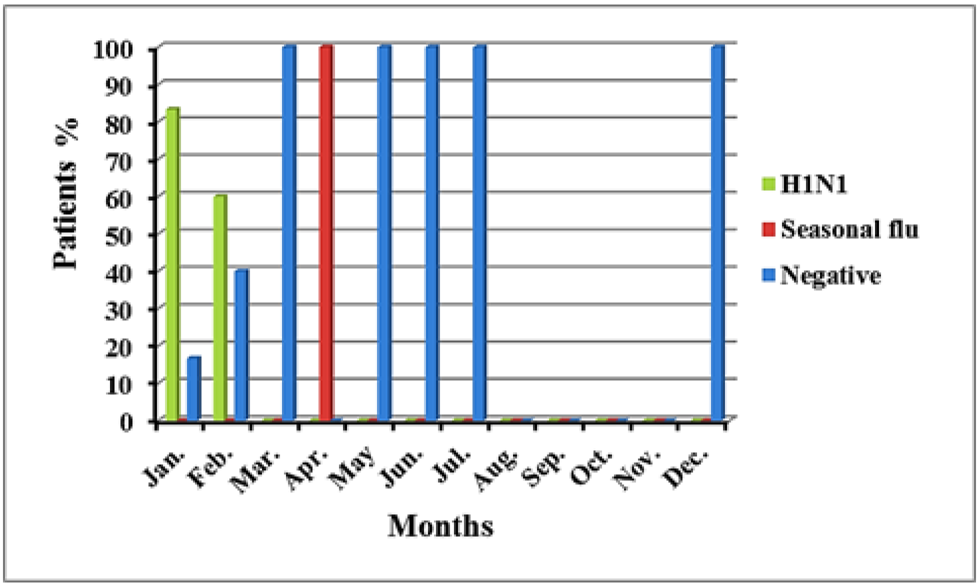

Figure 7: The percentage of pandemic H1N1 compared with seasonal influenza in AL-Muthana governorate

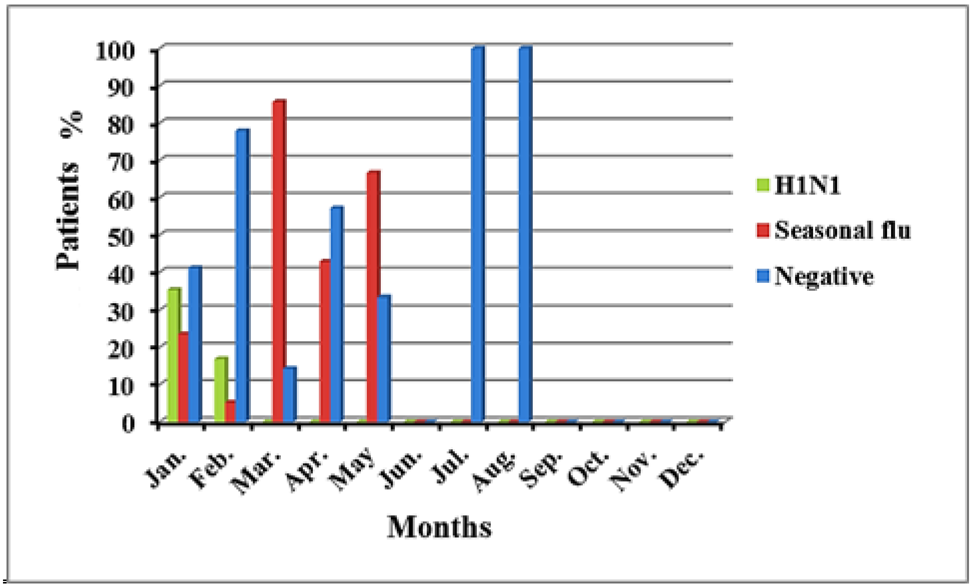

Figure 8: The percentage of pandemic H1N1 compared with seasonal influenza in Dhi Qar governorate

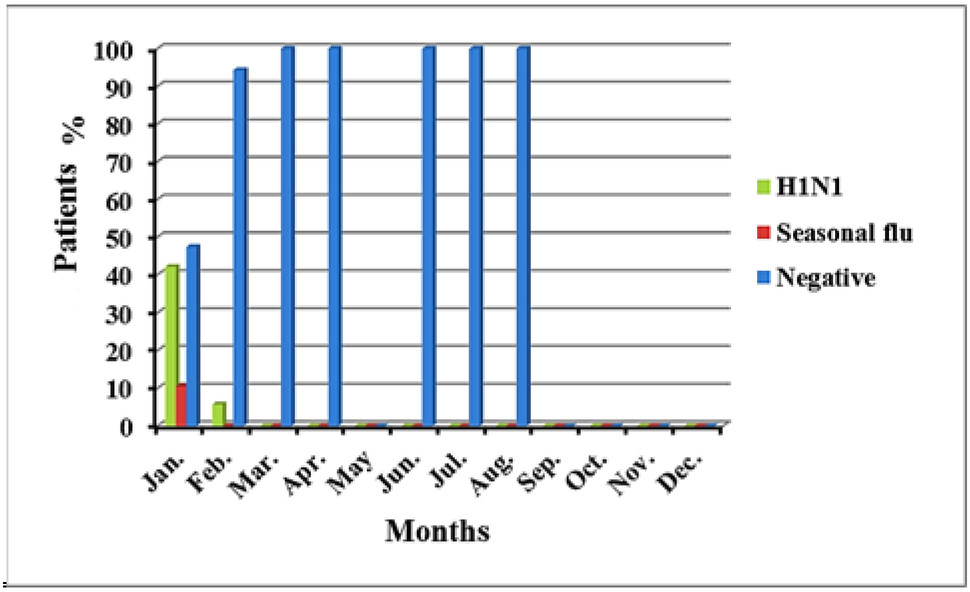

Figure 9: The percentage of pandemic H1N1 compared with seasonal influenza in AL-Basrah governorate

Table 3: The annual rate of infection in the southern governorates during 2013

governorate	The annual rate of H1N1 positive infection (%)	The annual rate of seasonal flu (%)	The annual rate of negative samples (%)
AL-Najaf	5.33 ± 14.41 p = 0.00	12.00 ± 35.94 p = 0.00	6.58 ± 13.69 p = 0.00
AL Qadisiyah	6.41 ± 16.25 p = 0.00	0.91 ± 1.88 p = 0.00	6.66 ± 13.45 p = 0.00
Maysan	5.83 ± 12.21 p = 0.00	0 p = 0.00	8.41 ± 15.25 p = 0.147
AL Muthanna	0.91 ± 2.15 p = 0.00	0.50 ± 1.73 p = 0.00	3.41 ± 5.63 p = 0.00
Dhi Qar	1.58 ± 3.98 p = 0.00	1.75 ± 2.26 p = 0.00	6.83 ± 16.89 p = 0.00
AL Basrah	0.83 ± 2.32 p = 0.00	0.16 ± 0.57 p = 0.00	4.33 ± 9.39 p = 0.00

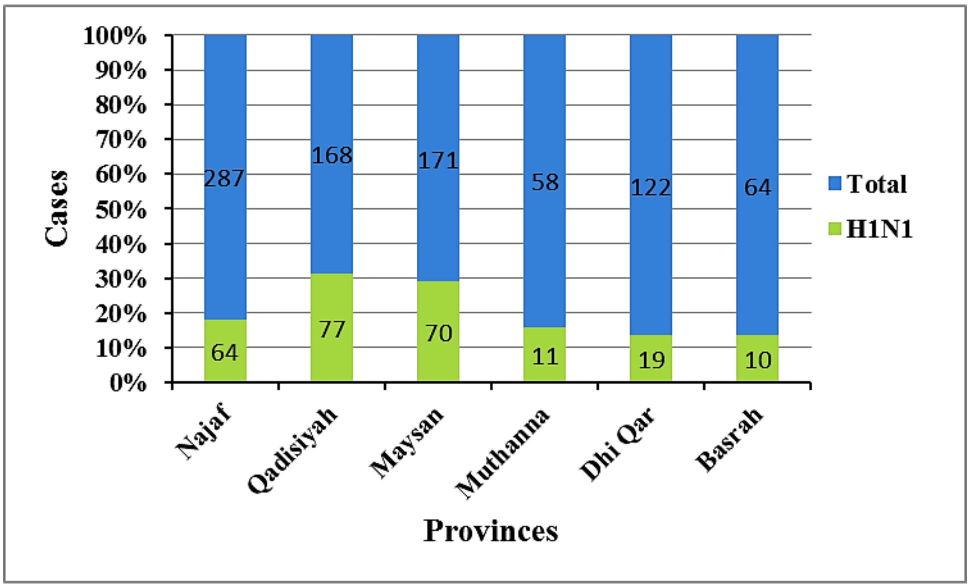

Figure 10: The percentage frequency of pandemic H1N1 positive samples in six southern Iraqi governorates

Conclusion

Our conclusion that during the year 2013 the Influenza A (H1N1) pdm09 which predominant on the seasonal flue of all suspected SARI patients and start increasing in winter season, particularly in January, and toward the end of February in some governorates and my extend to March in others.

References

1- Khanna, M.; Kumar, P.; Choudhary, K. and Kumar, B. (2008). Emerging influenza virus: A serious global threat. J Biosci, 33:475-482.

2- Alvarez, A.C.; Brunck, M.E.G.; Boyd, V.; Lai, R.; Virtue, E.; Chen, L.M.; Bletchly, W.C.; Heine, H.G. and Ross ,B.; (2008). A broad spectrum, one-step reverse-transcription PCR amplification of the neuraminidase gene from multiple subtypes of influenza A virus.Virology Journal, 5:77.

3- Swayne, D.E.; Suarez, D.L. and Sims, L.D. (2013). Influenza. In: Swayne, D.E.; Glisson, J.R.; McDougald, L.R.; Nair, V.; Nolan, L.K. and Suarez, D.L. eds. Diseases of Poultry, Thirteenth Edition., Wiley-Blackwell, Ames, Iowa, USA, 181–218.

4- Tong, S.; Li, Y.; Rivailler, P.; Conrardy, C.; Castillo, D.A.; Chen, L.M.; Recuenco, S.; Ellison, J.A.; Davis, C.T.; York ,I.A.; Turmelle, A.S.; Moran, D.; Rogers, S.; Shi, M.; Tao, Y.; Weil ,M.R.; Tang, K.; Rowe, L.A.; Sammons, S.; Xu, X.; Frace, M.; Lindblade, K.A.; Cox, N.J.; Anderson, L.J.; Rupprecht, C.E. and Donis, R.O.(2012). A distinct lineage ofinfluenza A virus from bats. Proc. Natl. Acad. Sci., 9:4269-4274.

5- Centers for Disease Control and Prevention.Outbreak of swine-origininfluenza A (H1N1) virus infection - Mexico, March-April 2009.MMWRMorb Mortal Wkly Rep, 58:467-470.

6- Dawood, F.S.; Jain, S.; Finelli, L .; Shaw, M.W., Lindstrom, S.; Garten, R.J.; Gubareva, L.V.; Xu, X.; Bridges, C.B. and Uyeki, T.M. (2009). Emergence of a novel swine-origin Influenza A (H1N1) virus in humans. N. Engl. J. Med., 360:2605-2615.

7- Chowell, G.; Bertozzi, S.M.; Colchero, M.A.; Lopez-Gatell H.; Alpuche-Aranda C.; Hernandez, M. and Miller, M.A. (2009). Severe respiratory disease concurrentwith the circulation of H1N1 influenza. N. Engl. J. Med., 361:674-679.

8- World Health Organization. Pandemic (H1N1) 2009- update 67. Geneva: Accessed October 6, 2009.

9- Smith, G.J.; Vijaykrishna, D.; Bahl, J.; Lycett, S.J.; Worobey ,M.; Pybus, O.G.; Ma, S.K.; Cheung, C.L.; Raghwani , J.; Bhatt, S.; Peiris, J.S; Guan ,Y. and Rambaut, A. (2009) .Origins and evolutionary genomics of the 2009 swine-origin H1N1 influenza A epidemic. Nature, 459(7250):1122-5.

10- Azziz, B. E.; Dao, C.N.; Nasreen, S.; Bhuiyan, M. U.; Mah-E-Muneer, S.; Al Mamun ,A.; Sharker ,M.A.; Zaman, R.U.; Cheng, P.Y.; Klimov, A.I.; Widdowson, M.A.; Uyeki ,T.M.; Luby ,S.P.; Mounts ,A. and Bresee ,J. (2012). Seasonality, timing, and climate drivers of influenza activity worldwide. J Infect Dis, 206(6): 838-46.

11- Tamerius, J. D.; Shaman, J.; Alonso ,W.J ; Kimberly, B.; Christopher, K.; Uejio, A. Comrie, C. (2013).Environmental Predictors of Seasonal Influenza Epidemics across Temperate and Tropical Climates. PLoSPathog,9:e1003194.

12- Shaman, J.; Pitzer, V. E.; Viboud, C.; Grenfell, B.T. and Lipsitch, M. (2010). Absolute humidity and the seasonal onset of influenza in the continental United States. PLoS Biol., 8: e1000316.

13- Shaman, J.; Goldstein, E. and Lipsitch, M. (2011). Absolute humidity and pandemic versus epidemic influenza. Am J Epidemiol., 173:127–135.

14- Polozov, I.V.; Bezrukov, L.; Gawrisch, K. and Zimmerberg, J. (2008). Progressive ordering with decreasing temperature of the phospholipids of influenza virus. Nat. Chem. Biol., 4: 248–255.

15- Nasser, F.G.; Mohamed, N.S.; Al-Jassani M.J., Aufi1I, M .; Ahmed, M.A.; Ali, K.; Dafi , I.H.; Najim, A.T. and Mohamed, N. and Ghlaim,I. (2015). Detection of pandemic influenza A H1N1 virus in central Iraq using Real-time PCR. CurrRes. Microbiol. Biotechnol., 3:694-700.

16- Centers for Disease Control and Prevention. H1N1 flu (swine flu): Resources for laboratories [Internet]. Centers for Disease Control and Prevention. [Cited 2009 June 2]. Available from: http: // www. cdc.gov/ h1n1flu / lab /.

17- Shu, B.; Wu, K.; Emery, E.; Villanueva, J.; Johnson, R.; Guthrie E., Berman, l., Warnes, C.; Barnes, N.; Klimov, A. and Stephen Lindstrom , L. (2011). Design and Performance of the CDC Real-Time Reverse Transcriptase PCR Swine Flu Panel for Detection of 2009 A (H1N1) Pandemic Influenza. Virus J. Clin. Microbiol., 49 (7): 2614–2619.

18- Ashkenazi, S.1.; Vertruyen A.; Arístegui, J.; Esposito, S.; McKeith, D.D; Klemola., T.; Biolek, J.; Kühr, J.; Bujnowski, T.; Desgrandchamps, D.; Cheng, S.M.; Skinner, J.; Gruber ,W.C. and Forrest, B. D.(2008). CAIV-T Study Group. Influenza vaccine effectiveness among children 6 to 59 months of age during 2 influenza seasons: a case-cohort study. Archives of Pediatrics and adolescent Medicine, 162:943–951.

19- Lowen, A. C. and Steel, J. (2014). Roles of humidity and temperature in shaping influenza seasonality. Journal of Virology., 88 (14) 7692–7695.