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RESEARCH ARTICLE

Investigation of the Emergence of Avian and Swine Influenza among Respiratory Patients in Assiut University Hospital, Egypt

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ABSTRACT

The circulation of Avian Influenza A (H5N1) and Swine Influenza A (H1N1) infection in Egypt increased the burden of a reassortant virus which may increased the human infection rate because it is unknown to the human immune system. This study was done from 2010 through 2016 to detect influenza viruses in Assiut Governorate among respiratory patients admitted to the Assiut University hospital using Real time PCR (rRT-PCR), as well as exhibit the factors associated with infection. Four (5.8%) and 19 (27.5%) out of 69 patients were infected with H5N1 and H1N1 subtypes respectively. Influenza virus activity was increased in cold weather resulted in increased influenza infection rate in both poultry and humans. Also, the clinical outcome toward patient recovery was increased with early detection and treatment of virus infection. In addition, people in contact with poultry, patients with chronic diseases, and those in contact with infected patients are highly significant for A (H5N1) and A (H1N1) infection. Strict implementation of control measures to eliminate the infection in both poultry and human is essential for reducing the risk of zoonotic transmission and human infection with influenza diseases.

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INTRODUCTION

Influenza A viruses are enveloped, single stranded RNA viruses belonging to the family of *Orthomyxoviridae*. Further subtyping into 18 Hemagglutinin (HA) and 11Neuraminidase (NA) is based on the antigenic differences between the two surface glycoproteins (WHO, 2016 a). Co infections of poultry with subtypes H5N1 and H9N2, and subclinical infection of pigs and humans with H1N1 and H5N1 subtypes may raise the potential for reassortment of these viruses. Moreover, adjustment of influenza A virus genomes, particularly the H5N1 subtype to optimize their evolution toward efficient transmission in human is progressing in Egypt (Abdelwhab and Abdel-Moneim, 2015).

Egypt is a hotspot for the evolution of a pandemic potential virus either via antigenic drift of the H5N1 which increased its adaptation to humans, or through reassortment with other influenza A virus subtypes (Fuller +*et al*., 2013). Infection of domestic poultry with A (H5N1) since February 2006 in Egypt caused enormous losses in poultry industry and the poultry slaughter campaign has overwhelmed on the Egyptian resources (Abdelwhab and Hafez, 2011). Poor hygiene, lack of awareness, and random rearing of domestic poultry without control especially in the rural areas of Egypt increased the chance of virus transmission (Bahgat *et al*., 2009). Between 2003 and 2016, 956 human cases of avian influenza A (H5N1) virus infection were reported to World Health Organization (WHO) with 452 deaths from 16 countries. Egypt has the highest number of human cases reported by a country since the emergence of the influenza virus (WHO, 2016b).

The successful zoonotic transfer of influenza A virus containing gene segments from avian, swine and human origin to humans along with consistent human to human transmission on each of the world's continents fulfills each of the current criteria for a pandemic strain (Brockwell-Staats *et al*., 2009). On 11 June 2009, the WHO raised the pandemic alert from Level 5 to Level 6

and declared the start of an influenza H1N1 pandemic (Chen *et al*., 2000). Although on 10 August, 2010, the director general of the WHO has announced that the world is no longer in phase 6 of influenza pandemic alert and we are now moving into the post pandemic period, the virus transmission is still highly active in many countries (WHO, 2009). Therefore, multidisciplinary researches and communication between Health and Agriculture Ministries was required for scientists and policymakers to evaluate the pandemic risk posed by zoonotic viruses.

MATERIALS AND METHODS

The clinical throat swab specimens used in this study were received from the Assiut University Hospital from November 2010 through May 2016; samples were collected from 69 respiratory patients who were suspected to be infected with influenza virus subtypes, such as H5N1 and H1N1. Swabs were immediately kept at 4ºC in transport medium consisting of phosphate buffer saline supplemented with streptomycin, penicillin and amphotricin B, then transported to the Molecular Biology Research Unit laboratories in Assiut University, Assiut, Egypt (ISO/IEC 17025Accredit No.211026) as fast as possible for molecular examination by Real time PCR (rRT-PCR) according to Spackman *et al*. (2003) and Carr *et al*. (2009). Demographic, clinical and epidemiological data were obtained using a standardized case investigation form. Data were collected by Assiut University Hospital staff to identify the risk factors of the disease, also if there were other suspected cases infected with A (H5N1) and A (H1N1) virus. These data comprised age, gender, clinical finding, medical history, the time from the onset of symptoms to hospital admission, and direct exposure to infection as breeding birds, handling sick or dead poultry, visit live poultry markets, presence of infected poultry in the neighborhood and contact with infected persons.

Real time PCR (rRT-PCR): Viral RNA was extracted from throat swab samples using QIAamp viral RNA Mini kit (Cat No.: 5290) according to the manufacturer's instructions with the following cycling condition; at 55ºC for 10 min, 95ºC for 2 min, then 50 cycle at 95ºC for 10 sec and 60ºC for 60 sec. Real time PCR (rRT-PCR) was performed using OASIG lyophilised One Step q RT-PCR Mastermix (UK) (Licks *et al*., 2004) Avian Influenza A (H5N1) detection, and Applied Biosystem Real-Time (rRT-PCR) (P/N 4441242C) kit for the detection of Influenza A (H1N1) (CDC, 2009). A (H1N1) primers and probes were InfA [Forward (F): GAC CRA TCC TGT CAC CTC TGA C], InfA [Reverse (R): AGG GCA TTY TGG ACA AAK CGT CTA], InfA [Probe (P): 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 [P: CYA CTG CAA GCC 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 [P: 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] and RnaseP [P: TTC TGA CCT GAA GGC TCT GCG CG]. The cycling condition was; at 50ºC for 30 min, 95ºC for 10 min and 45 cycles at 95ºC for 15 sec and 55ºC for 30 sec as described by Licks *et al*. (2004).

Statistical analysis: Data was analyzed with SPSS version 14 (SPSS, Inc., Chicago, IL, USA) to detect the relation between patients characteristics and their infection with A (H1N1). $P<0.05$ is considered statistically significant.

RESULTS

From 2010 through 2016; among 69 patients admitted to the Assiut University Hospital with influenza like symptoms; infection with A (H5N1) and influenza A (H1N1) was four (5.8%) and 19 (27.5%) respectively. The highest infection rate was in 2011 while 2013 not reported any cases (Table 1). Clinical symptoms at the hospital admission of 69 respiratory patients were reported in Table 2; all patients were suffered from fever and most of them were reported upper respiratory symptoms as coughing 63 (91.3%), sore throat 54 (78.3%), rhinorrhea 45 (65.2%). Some patients showed lower respiratory symptoms as dyspnea 49 (71.01%) and gastrointestinal symptoms as diarrhea 25 (36.2%) and vomiting 15 (21.7%), as well as 15 (21.7%) patients were suffered from myalgia. The four influenza A (H5N1) patients were exposed to poultry (one died and three improved). The dead patient was a pregnant female and suffers from chronic obstructive pulmonary disease (COPD) (Table 3).

Table 1: Distribution of influenza A (H5N1) and A (H1N1) in respiratory patients admitted to the Assiut University Hospital, Assiut, Egypt from 2010 through 2016

Year	No. of examined patients	Patients with A (H5N1)		Patients with A (H1N1)	
		No.	%	No.	%
2010	20	0	0	4	20
2011	29	2	6.9	12	41.4
2012	7	1	14.3	1	14.3
2013	4	0	0	0	0
2014	4	0	0	1	25
2015	3	1	33.3	0	0
2016	2	0	0	1	50
Total	69	4	5.8	19	27.5

Referring to influenza A (H1N1); 12 (63.2%) out of 19 patients were exposed to infection through contact with infected influenza A (H1N1) patients, 11 (57.9%) were smokers and 17 (89.5%) were medically having other chronic diseases than influenza such as diabetes mellitus (DM) (26.3%), renal failure (21.1%), liver cirrhosis, bronchial asthma, rheumatic heart disease (10.5%), heart failure, COPD and hepatitis C (5.3%). In addition; some patients were suffered from more than one disease. Infection with A (H1N1) was increased in patients with chronic diseases, pregnant women, who in contact with infected patients ($P<0.01$) and also in smokers ($P<0.05$).

DISCUSSION

Most of influenza A (H5N1) and A (H1N1) cases was detected in winter months, followed by autumn and summer, while in spring months, only H1N1 subtype was detected (Fig. 1). Low temperature and increased humidity in winter months will promote the spread of influenza viruses; those conditions favor the aerosol borne influenza virus, which survives longer on surfaces under

colder temperature (Necibe and Maia, 2013). The most common influenza symptoms of four (5.8%) A (H5N1) and 19 (27.5%) A (H1N1) cases were fever in 23 (100%) patients, coughing in 19 (82.6%) and dyspnea in 18 (78.3%) patients, followed by diarrhea in 13 (56.5%) patients, sore throat 12 (52.5%), vomiting 10 (43.5%), myalgia 9 (39.1) and rhinorrhea in 7 (30.4%) patients. This result goes parallel with that of Marty *et al.* (2014). From this results it is clear that infection with influenza viruses have no specific symptoms and it is difficult to be diagnosed based only on clinical signs because influenza symptoms may be similar to several diseases caused by other infectious agents (CDC, 2016). So, a further sensitive and rapid method for identification of influenza viruses' is important for early detection as Real time PCR (Fig. 3 & 4), effective treatment and also, detection of the suspected cases (Boivin *et al*., 2000). As the results explained in figure 2; Survival of influenza A (H5N1) and A (H1N1) was higher in patients admitted early to hospital after the onset of symptoms than patients who received treatment with advanced stage of illness; one (25%) out of four patients with A (H5N1) and nine (47.4%) out of 19 patients with A(H1N1) were died as they receive the treatment within six days after the onset of symptoms which longer than the recommended 48 hours (De Jong and Hien, 2006). These results were consistent with Kandun *et al*. (2008) who illustrated that patients who received treatment earlier showed significant association with its survival. From this study we found that most of patients not attend the hospital as soon as possible for treatment after the onset of symptoms because they suppose that the infection is not serious and they will cure without treatment. So, increased the public awareness about the disease is important to reduce the risk of disease complications. Table 3 showed that all patients with A (H5N1) were infected by contact with poultry either through backyard birds, handled sick or dead poultry, visit live poultry markets or presence of infected poultry in the neighborhood. This indicated that contact with infected poultry is the primary route of bird to human transmission and also considered the main risk factor of avian influenza infection. In addition, a history of close contact with poultry may be helpful in identifying the infected patients with A (H5N1) virus. This result goes parallel with the results reported by Ferial *et al.* (2012). In Egypt, backyard poultry is considered a primary economic income for many populations in villages. Live birds with different species and ages were illegally sold in live bird markets under poor hygienic measures. Therefore, increasing the biosecurity measures encourage the public to notify about the infected region, as well as compensations in case of depopulation of infected birds is the primary line of defense. So, more information from the veterinary authority about A (H5N1) virus outbreak regions through continuous veterinary and public health surveillance is helpful for early identification of the infected foci.

Infection with chronic diseases (chronic obstructive pulmonary disease (COPD), diabetes mellitus (DM) and Rheumatic arthritis), pregnancy and smoking may enhance influenza infection rate. Our result was similar to the results reported by Yaogang *et al*. (2015) but due to the small number of cases, the study was unfit to compare the differences between the died and improved cases.

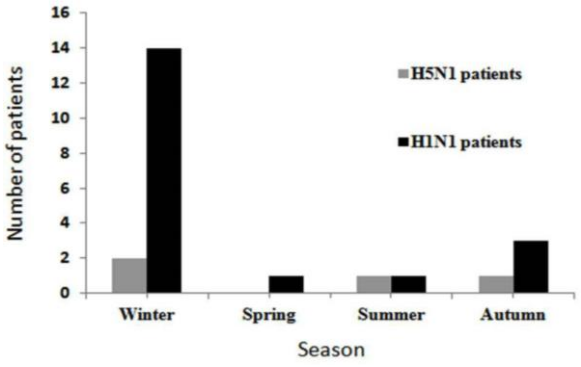

Fig. 1: Seasonal variation of influenza A (H5N1) and A (H1N1) infection in respiratory patients admitted to Assiut University Hospital, Assiut, Egypt from 2010 through 2016.

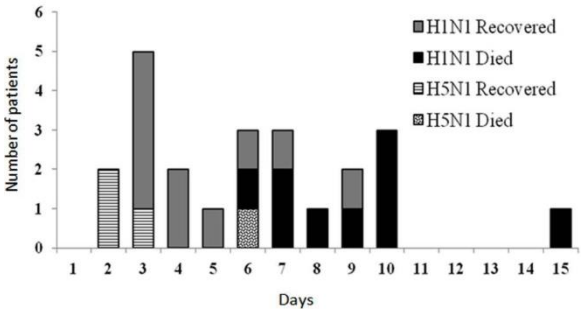

Fig. 2: Clinical outcome of patients with A (H5N1) and A (H1N1) from the onset of symptoms and hospital admission for treatment.

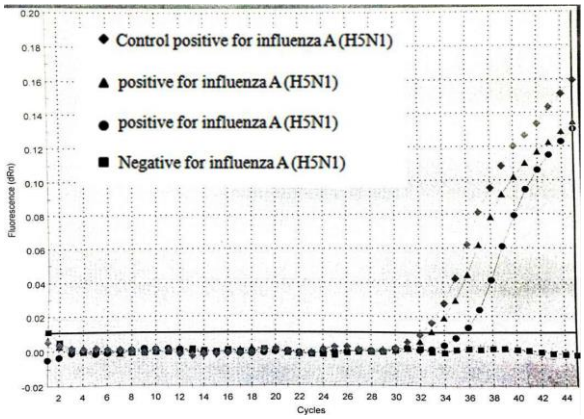

Fig. 3: Amplification of real-time RT-PCR for detection of influenza A (H5N1).

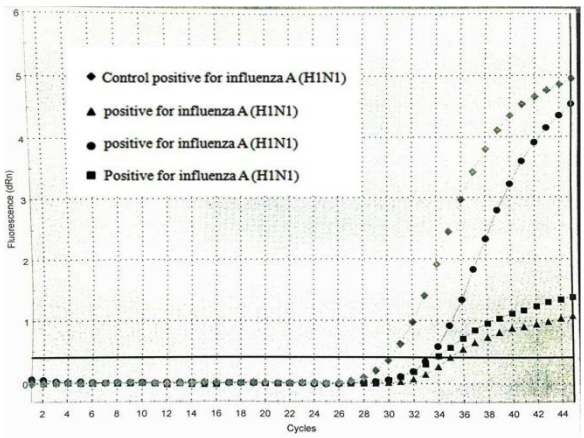

Fig. 4: Amplification of real-time RT-PCR for detection of influenza A (H1N1).

Table 2: Clinical symptoms of patients with A (H5N1) and A (H1N1) upon the hospital admission

Clinical symptoms	All patients n=69		patients with A (H5N1) n=4		patients with A (H1N1) n=19		Total n=23	
	No	%	No	%	No	%	No	%
Fever	69	100	4	100	19	100	23	100
Coughing	63	91.3	4	100	15	78.9	19	82.6
Dyspnea	49	71.01	3	75	15	78.9	18	78.3
Sore throat	54	78.3	1	25	11	57.9	12	52.2
Vomiting	15	21.7	2	50	8	42.1	10	43.5
Diarrhea	25	36.2	2	50	11	57.9	13	56.5
Myalgia	15	21.7	2	50	7	36.8	9	39.1
Rhinorrhea	45	65.2	2	50	5	26.3	7	30.4

*Some patients had more than one symptom.

Table 3: Characteristics of four patients infected with A (H5N1) virus admitted to Assiut University Hospital, Assiut, Egypt.

Patients characteristics	P1	P2	P 3	P4
Age (year)	33	18	28	27
Gender	Female	Female	Male	Male
Exposure to the infection				
Bird breeder	Yes	0	0	0
Handled sick poultry	yes	0	0	0
Visit live poultry market	0	0	yes	yes
Presence of infected poultry in the neighborhood	0	Yes	0	0
Medical history chronic obstructive pulmonary disease (COPD)	yes	0	0	0
Diabetes mellitus (DM)	0	0	yes	0
Hepatitis C	0	0	0	0
Bronchial asthma	0	0	0	0
Rheumatic heart disease	0	0	0	0
Rheumatic arthritis	0	0	0	Yes
Pregnancy	yes	0	0	0
smoking	0	0	1	1
Clinical outcome	Died	Improved	Improved	Improved

***P**=patient

Table 4: Characteristics of 19 patients infected with A (H1N1) virus admitted to Assiut University Hospital, Assiut, Egypt

Risk factors	No. of patients n=69		patients with H1N1 n=19		P-value
	No.	%	No.	%	
Age [b]					0.775
16-25	14	20.3	3	15.8	
26-35	9	13.04	3	15.8	
36-45	11	15.9	4	21.1	
46-55	17	24.6	3	15.8	
>55	18	26.1	6	31.6	
Gender [b]					0.708
Female	23	33.3	7	36.8	
Male	46	66.7	12	63.2	
Exposure to infection [a]					
Contact with infected persons	13	18.8	12	63.2	0.01
Medical history * [a]	23	33.3	17	89.5	0.01
Renal failure	5	7.2	4	21.1	
Liver cirrhosis	4	5.8	2	10.5	
Heart failure	3	4.3	1	5.3	
Chronic obstructive pulmonary disease (COPD)	3	4.3	1	5.3	
Diabetes mellitus (DM)	6	8.7	5	83.3	
Hepatitis C	2	2.9	1	5.3	
Bronchial asthma	3	4.3	2	10.5	
Rheumatic heart disease	3	4.3	2	10.5	
Pregnancy [a]	3/23	13.04	2	10.5	0.01
Smoking [a]	24	34.8	11	57.9	0.05

*Some patients suffer from more than one disease; [a]Significant factors; [b]Non-significant factors

Regarding to A (H1N1) infection; characteristics of 19 patients with H1N1 subtype in Table 4 explained that age and gender of patients were not correlated with influenza infection. Also, patients suffered from other diseases than influenza (renal failure, liver cirrhosis, heart failure, COPD, DM, Hepatitis C, Bronchial asthma, Rheumatic heart disease and Rheumatic arthritis) were highly significant for A (H1N1) infection ($P<0.01$). This result in agreement with the results reported by Izurieta *et al*. (2000) who stated that patients with chronic medical conditions are at high risk for influenza complications. Furthermore, Influenza infection is significantly higher in pregnant patients ($P<0.01$) and smokers ($P<0.05$). Also, persons in contact with infected patients with influenza exhibited high significant for A (H1N1) infection ($P<0.01$). The circulation of A (H1N1) virus in A (H5N1) endemic areas raises the fears of emergence of highly pathogenic virus which has the efficient at human to human transmission (Kayali *et al*., 2010). Therefore, controlling of influenza among birds, human and several animal species is important to stop the virus evolution and adaptation in different host species which may causing a reassortment of these viruses toward adaptation and increased the virulence to human.

Conclusions: To control influenza risk factors, the level of public knowledge should be improved through obligated health education programs which conducted by the national government including health precautions for both A (H5N1) and (H1N1) such as wearing protective gloves and masks during handling sick or dead poultry, notification about the infected foci. Also, early hospital attendance just after the onset of symptoms especially for people with chronic medical condition and avoid contact with infected patients. Television is the excellent media for dissemination of these health messages through short messages, films or cartoon.

Efforts should be done by the authorities to ensure the application of biosecurity measures through vaccination of live poultry and be sure that the vaccine matches the circulating strain of the virus, as well as prevent live bird markets, strict isolation of the infected foci and traffic control especially during outbreaks. Also, training and education programs should be conducted to poultry farm employers and workers as well as, it is important to compensate those affected by the execution of infected birds.

Sharing databases among the affected countries and further researches are needed to develop better diagnostic methods for early detection and treatment of influenza cases. The collaboration between the veterinary, medical

and agricultural profession is critical to set a strategy in the eventual control and eradication of influenza viruses.

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Authors contribution: AAH conceived the idea, AAH and AAH designed the study, SS cases investigation, collected the data and samples, RE and MA performed lab work under the supervision of AAH, AAH analyzed the data, wrote and prepared the manuscript for submission. All authors critically revised the manuscript for intellectual content and approved the final version.

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