Assiut Vet. Med. J. Vol. 60 No. 142 July 2014

AVIAN INFLUENZA VIRUS AND NEWCASTLE VIRUS SURVEILLANCE AND CHARACTERIZATION IN BROILER AND LAYER CHICKEN FLOCKS IN EGYPT

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ABSTRACT

Received at: 16/6/2014

Accepted: 6/8/2014

Considering that highly pathogenic avian influenza H5N1 endemically circulates in the Egyptian poultry population, Active surveillance was undertaken in 195 broiler and layers farms from Eighteen Egyptian governorates resulted in, 34,7 and 24 positive farms for AI H9N2, AI H5N1 and Virulent ND respectively, using RT-PCR. The genetic analysis of avian influenza viruses of HA gene of the H5N1 type revealed that these viruses are highly pathogenic strains as they are tracking clade 2.2.1 of classic viruses which became endemic in Egypt since 2006. However, H9N2 viruses revealed that they are low pathogenic as they are tracking G1-lineage of Asian viruses which transmitted to Middle East scince 2000. Also sequence analysis for F gene for twelve NDV isolates revealed that they are of virulent type and genetically related to genotype VII NDV except two isolates are avirulent type and resemble Lasota- like- strains. Pathogenecity studies for NDV isolates using ICPI was evaluated for the strain (NDV-EG-567f-2012) which of virulent with ICPI value 1.96. The analysis of isolated strains by different diagnostic and analytical techniques implies that the H5N1 and H9N2 silent infection exist in Egypt.

Key words: Pathogenisity- Lasota like strains- lineage- RT_PCR- endemic.

INTRODUCTION

The highly pathogenic avian influenza virus subtype H5N1 was first recognized in poultry in Egypt in 2006 (Aly *et al.*, 2008). The subtype H9N2 is widely circulated in the Middle East region and it was associated with serious disease in poultry (Aamir *et al.*, 2007; Alexander, 2007; Perk *et al.*, 2009). In addition, recent detection of low pathogenic avian influenza H9N2 subtype has been recorded in bobwhite quail in Egypt in 2011 with genetic relatedness to the G1-lineage that represented by A/Quail/Hong Kong/G1/97 (Qa/HK/G1/97) that still circulated in the Middle East region. (El-Zoghby *et al.*, 2012) Standard RT-PCR has been applied previously to the detection of AIV and each of the 15 HA subtypes (Starick *et al.*, 2000; Lee *et al.*, 2001; Munch *et al.*, 2001). Sequence analysis of the viral genes and their respective amino acids is a valuable tool for detection of any genic variation among viruses circulating in the field and also provides information about the source of the initial disease outbreak in a certain geographic district (Duvvvuri *et al.*, 2009). The broad variation in virulence and clinical signs necessitates the careful definition of what constitutes ND for the purposes of trade, control measures and policies. The definition of ND currently in use in all member states of the European Union is defined in Directive 92/66/EEC of the Commission for European Communities (OIE 2012). ND outbreaks occur frequently in Egypt and the source of the virulent NDV in these outbreaks are not known. (Mohamed *et al.*, 2009) reported the complete genome sequence of a NDV strain isolated from an outbreak at a poultry farm in Al-Sharkia province, Egypt in 2005 (chicken/Egypt/1/2005).

In this work, the surveillance were carried out to detect the avian influenza and Newcastle viruses and evaluate their virulence attitude as considered most important respiratory viruses for poultry and specially broiler and layer sectors and its zoonotic

picture. Furthermore discuss the genetic characterizations for Newcastle and AI with its subtypes H5N1 and H9N2.

MATERIALS and METHODS

195 layer and broiler farms' from eighteen Egyptian governorates have been survived during late of 2011-2012. 2448 cloacal swabs and 306 organs like trachrea and lung were collected and examined as shown in Table. (1).

Table 1: Collective Numbers of farms, samples in relation to governorate and type of production.

No.	Governorates	No. of farms	No of examined samples		Type of production	
			Cloacal swabs	Organs (Tr& lung)	Layers	Broilers
1	Al Fayoum	51	920	65	5	46
2	Al Qalubia	20	150	14	1	19
3	Al Dakahlia	19	294	34	3	16
4	Al Ismalia	18	85	22	0	18
5	Benisueif	15	204	26	3	12
6	Domiatte	11	95	47	2	9
7	Luxor	12	22	9	0	12
8	Al Giza	9	186	10	4	5
9	Al Sharkia	10	180	8	3	7
10	Al Alexandria	8	90	26	4	4
11	Kafr el sheikh	5	46	10	0	5
12	Al Behira	5	64	11	0	5
13	Al Monefia	3	44	2	0	3
14	Al Gharbia	3	20	11	0	3
15	Al Minia	2	38	2	0	2
16	North sinai	2	4	6	0	2
17	Aswan	1	-	1	0	1
18	Port said	1	6	1	0	1
	Total	**195**	**2448**	**306**	**25**	**170**

Real time PCR:

This test was done for the different samples to detect the AI common gene and haemagglutinin genes of H5 and H9 by multiplex PCR also the method was done to detect the F gene for Newcastle and determine the virulence of the positive strain.

RNA was extracted from samples was performed using the QIAamp viral RNA Mini kit (Qiagen, Germany, GmbH) according to the manufacturer's recommendations. Briefly 140 µl of the sample suspension was incubated with 5.6 µl of carrier RNA and 560 µl of AVL buffer at room temperature for 10 min. After incubation, 560 µl of 100% ethanol was added to the lysate. The mixture was then transferred to the silica column then centrifuged and washed following the manufacturer's recommendations. Nucleic acid was eluted with 60 µl of elution buffer (AE) provided within the kit] NDV, and AI: Primers were utilized in a 25- µl reaction containing 12.5 µl of Quantitect probe rt-PCR master mix (Qiagen, Germany, GmbH), 0.5 µl f each primer of 50 pmol concentration, 0.125 µl of the specific probe, 4.5 µl of water, 0.25 µl of the rt-enzyme and 6 µl of RNA extract.. The reaction was performed in a Stratagen MX3005P real time PCR machine (Stratagene, USA). Oligonucleitide primers are described as in Table (2).

Table 2: RT- PCR primers used in this study were supplied from Metabion (Germany).

RNA Virus	Gene	Primer/ probe sequence 5'-3'	Ref.
AI	Matrix	Sep1 AGATGAGTCTTCTAA CCGAGGTCG	Slomoka *et al.* 2007
		Sep 2 TGCAAAAACATCTTC AAGTCTCTG	
		SEPRO [FAM]TCAGGCCCC CTCAAAGCCGA [TAMRA]	
	H5	H5LH1 ACATATGACTAC CCACARTATTCA G	
		H5RH1 AGACCAGCT AYC ATGATTGC	
		H5PRO [FAM]TCWACA GTGGCGAGT TCCCTAGCA[TAMRA]	
	H9	H9F GGAAGAATTAATTATTATTGGTCGGTAC	Ben Shabat *et al.* 2010
		H9R GCCACCTTTTTCAGTCTGACATT	
		H9 Probe [FAM]AACCAGGCCAGACATTGCGAGTAAGATCC[TAMRA]	
ND	Vir	F+4839 TCCGGAGGATACAAGGGTCT	Wise *et al.* 2004
		F-4939 AGCTGTTGCAACCCCAAG	
		F+4894 [FAM]AAGCGTTTCTGTCTCCTTCCTCCA[TAMRA]	
	Matrix	M_4100 5_-AGTGATGTGCTCGGACCTTC-3_	
		M_4169 5_-[FAM]TTCTCTAGCAGTGGGACAGCCTGC[TAMRA]-3_	
		M_4220 5_-CCTGAGGAGAGGCATTTGCTA-3_	

Virus isolation:
The positive PCR samples were directed to virus isolation according to OIE 2008. Also virulence test evaluation for low pathogenic avian influenza virus (H9N2) and Newcastle virus. Depending on OIE 2012.

I: Pathogenicity of H9N2 virulence: protocol using 8 weeks SPF chicken. Virulence was evaluated for randomly selected two isolates:

II: Pathogenicity of NDV virulence: was done by injecting the virus in SPF day old chicks using ICPI.

Genotyping of isolates: sequencing for 20 positive isolates. It was performed by purification of PCR products using QIAquick PCR Product extraction kit. (Qiagen, Valencia). The sequence reaction was done using Bigdye Terminator V3.1 cycle sequencing kit (Perkin-Elmer) and the sequence reaction was purified using Centrisep spin column (ABI, USA). DNA sequences were obtained using Applied Biosystems 3130 genetic analyzer (ABI, USA). A phylogenetic tree was created by the MegAlign module of Lasergene DNAStar.

The sequencing method was done for HA gene of AI virus and for F gene of Newcastle virus.

RESULTS

A total 41 farms out of 195 examined are positive for AIV with a percentage of 21%, It appeared that 34 farm were positive for AI H9 with a percentage 83% and 7 farms were positive for AI H5 with a percentage of 17% Also 41 farms were positive for matrix gene of Newcastle virus with a percentage of 21%, Twenty four farms were positive for the virulent strain of Newcastle with a percentage of 58.5%.

Table 3: The results of PCR test for the examined Avian influenza and Newcastle viruses in relation to each governorate.

Gov.	No of examined farms	AI	The PCR results			
					ND	
		Matrix	AIH9	AI H5	Matrix ND	vNDV
1- Faioum	51	15	14	1	12	7
2- Qalioubia	20	7	6	1	4	2
3- Dakahli	19	2	2	0	3	3
4- Ismalia	18	0	0	0	3	2
5- Benisueif	15	4	2	2	0	0
6- Domiatt	11	0	0	0	1	0
7- Luxor	12	2	1	1	1	0
8- Giza	9	0	0	0	2	2
9- Sharkia	10	3	1	2	3	1
10- Alexandria	8	3	3	0	7	4
11- Kafr el sheikh	5	1	1	0	3	2
12- Behira	5	1	1	0	1	0
13- Monefia	3	1	1	0	0	0
14- Gharbia	3	1	1	0	0	0
15- Minia	2	1	1	0	0	0
16- North Sinai	2	0	0	0	0	0
17- Aswan	1	0	0	0	0	0
18- Port said	1	0	0	0	1	1
Total	195	41	34	7	41	24
Incidence rate		21%	17.4%* 83%**	3.5%* 17%**	21%	12%* 58.5%**

Note: * the percent in relation to No of examined samples
** the percent in relation to the positive farms.

Virulence evaluation for different viruses:
I: evaluation of H9N2 virulence:
Virulence was evaluated for (2) isolates:

1-A/Chicken/Egypt/114940v/NLQP/2011/H9N2.

2-A/Chicken/Egypt/114922v/NLQP/2011/H9N2.
The results showed that the viruses that have been tested were low pathogenic.

II: virulence evaluation of isolated NDVs:
Using ICPI showed that, all evaluated viruses (NDV-EG-567f-2012) were virulent strains (ICPI=1.96).

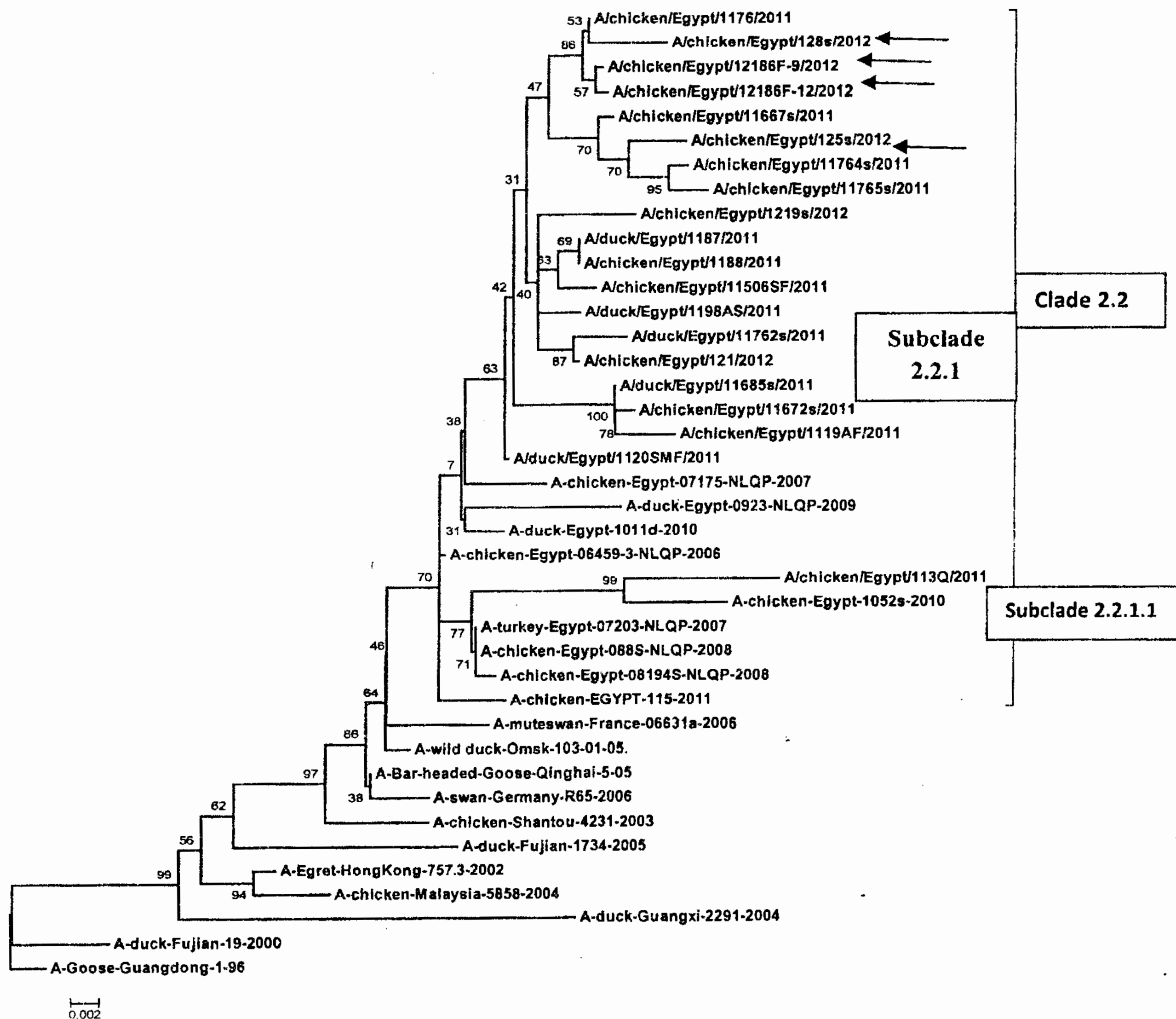
Sequence analysis:
The genetic analysis for 4 strains of avian influenza viruses HA gene of the H5N1 type [A/chicken/Egypt/128s/2012, A/chicken/Egypt/12186F-9/2012, A/chicken/Egypt/12186F-12/2012 and A/chicken/Egypt/125s/2012] revealed that these viruses are highly pathogenic as well as tracking clade 2.2.1 virus which is endemic and widespread in Egypt since 2006 and no cases of Egyptian H5N1 variant strain which follows clade 2.2.1.1 and described in Table (4).

The genetic analysis of HA gene of 4 isolates of H9N2 revealed that they are of low pathogenic in the phylogenic tree they are tracking G1-lineage of Asian viruses which spread at Middle East and they are nearly similar to other Egyptian viruses from 2011 (Fig.2).

The genetic analysis of F gene of 12 selected isolates of NDV revealed that ten strains are virulent genotype VII) and also the recent isolated strains from Middle East like Jordan (apmv1/chicken/Jordan/Jo 11/2011) and Israel (buzzard/Israel/714/2011) with a percentage of 99.98% while not similar to vaccinal strains as lasota with a percentage of 72% based on partial F gene. As well as 2 isolates of NDV were classified as avirulent that like vaccinal strain (Lasota – like NDV (lentogenic strain), (Fig.3).

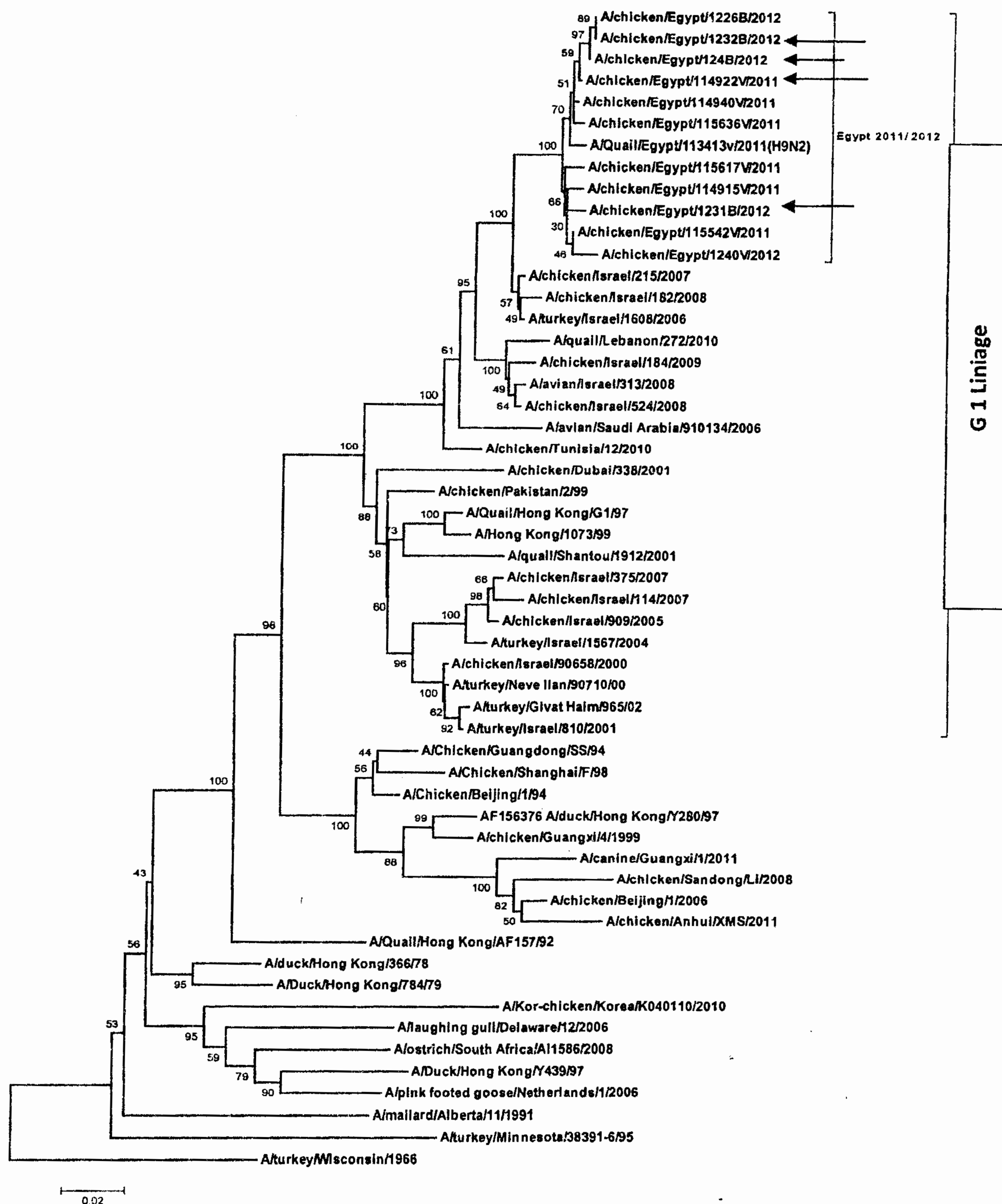
Table 4: No. and type of isolates of each virus.

Virus	Genotype		No. of cases
H5N1	Classic A	Clade 2.2.1	4
NDV	Virulent	Vir VII	10
	Avirulent		2
H9N2	Low pathogenic	Asian G/ lineage	4



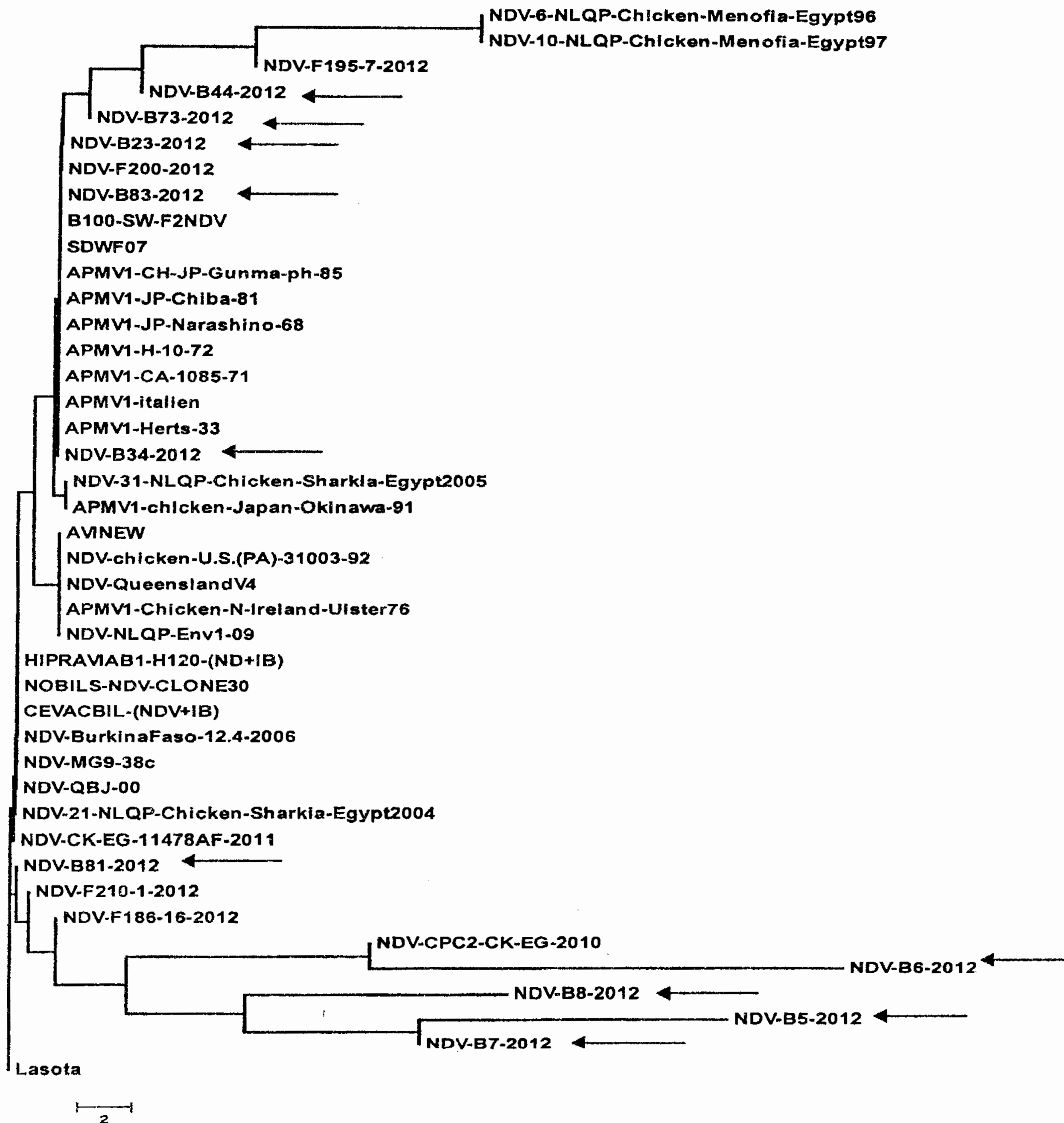
(Fig. 1): Phylogenetic analysis of HA gene of the Egyptian H5N1 isolates in comparison to other strains from Middle East region, Asian and other strains.

*Note: Black arrows refer to the sequenced strains in this study.



(Fig. 2): Phylogenetic analysis of HA gene of the Egyptian H9N2 isolates in comparison to other strains from Middle East region, Asian and other strains.

*Note : Black arrows refer to the sequenced strains in this study.



(Fig. 3): Phylogenetic analysis of F gene of the Egyptian NDV isolates in the present study 2012 in comparison to other strains.

*Note : Black arrows refer to the sequenced strains in this study.

DISCUSSION

Conventional microbiological methods are sensitive and specific, it takes considerable time (4 to 7 days) to reach a diagnosis; therefore, PCR-based detection and pathotyping assays have been developed to support/substitute for these traditional diagnostic tools and to replace animal experiments (Kant *et al.*, 1997; Aldous *et al.*, 2001; Creelan *et al.*, 2002; Pham *et al.*, 2005 and Wise *et al.*, 2004). Real-time RT-PCR can offer several advantages over standard RT-PCR, such as speed and lower risk of cross contamination of clinical samples with previously amplified products In addition, the detection of the amplicon with a sequence-specific probe further confirms the nature of the target, reducing false positivity (Schweiger *et al.*, 2000; Spackman *et al.*, 2002; Lee and Suarez, 2004; Stone *et al.*, 2004; Di Trani *et al.*, 2006 and Payungporn *et al.*, 2006). Standardized RT-PCR protocol can also be applied easily to other respiratory viruses. our results for AI virus detection by RT- PCR confirm the endemic picture of AI H5 with a percentage of 17% from total examined farms and those strains belongs to classic

strain in Egypt and these observation agree with results of Aly *et al.* (2008) which recorded that the HPAI virus of subtype H5N1 was first recorded in Egypt in 2006 and since then, the disease has become endemic and still causes a significant threat to the poultry industry and humans in Egypt. Detection of two subtypes of AI, HPAI H5N1 and LPAI-H9N2 is also detected in the present study with a percentage of 3.5%, 17.4% from total examined respectively, and this conclusion discussed by Shortridge *et al.*, 1998; Spackman *et al.*, 2002; Salzberg *et al.*; 2007 and Xu *et al.*, 2007 detected the presence of LPAI-H9N2 and the HPAI-H5N1 viruses have continuously co-circulated in domestic poultry in different areas. The high percent of AI H9 in this study is 83% from total positive AI cases it also reflects the low pathogenic nature of the virus which allow the silent spread of such viruses in commercial chickens and this results agree with that of (Nili and Asasi, 2002; Kim *et al.*, 2006; Aamir *et al.*, 2007; Naeem *et al.*, 2007 and Park *et al.*, 2011). Which discussed and recorded that phenomenon of silent spread of LP H9N2 in the Middle East region for several years indicated additional risk factor to the poultry industry. Although H9N2 viruses are characterized as low pathogenic avian influenza (LPAI) viruses, they may cause high morbidity and mortality Since avian H9N2 viruses are currently perceived to represent a significant threat to human health it is important to determine whether or not viruses of this subtype circulating in poultry in various parts of the world have the potential to infect people (Cameron *et al.*, 2000). To determine the genetic relationship of H5N1, H9N2 avian influenza viruses and ND virus currently prevalent in poultry in Egypt using Phylogenicity relationship analysis of the 4 selected strains of H5N1 are highly pathogenic as well as tracking subclade 2.2.1 classical strain and these results confirmed that recorded by Arafa *et al.* (2012a) that recorded the presence of HPAI H5N1, and that group is prevailing mainly in village poultry and had fewer mutations compared to the originally introduced virus in 2006 Since 2009, this group has started to be transmitted back to commercial sectors. On the other hand the Phylogenetic analysis of the HA gene showed that the 4 Egyptian isolates of H9N2 in our study were resemble that isolated from Egypt in 2011 the Gl-ike lineage similar to the circulating viruses in the Middle East with very close phylogeny to the Israeli viruses and these results also resemble the results obtained by Arafa *et al.* (2012b) when they recorded different strains of low pathogenic H9N2 in poultry sector. Currently the isolation of the causative virus using embryonated chicken eggs and its characterization by hemagglutination activity (HA)/ hemagglutination inhibition/in vivo pathogenicity assays is the standard protocol to confirm ND cases (Alexander 2004). The discrimination of a virulent (e.g., vaccine viruses) and virulent NDV strains is very important in regions practicing vaccination as a control measure against ND, especially in the context of the differential diagnosis of avian influenza (Alexander 2004). In this study we use primers specific to amplify the F gene for Newcastle indeed similar primers were used by Kant *et al.* (1997). To amplify the F gene sequence specifically from virulent and non-virulent strains of NDV. In this surveillance 58.5% positive cases of Virulent strains of Newcastle disease virus were detected from total positive (NDV examined farms) this high percent is agree to that mentioned by Kim *et al.* (2013), that the Virulent NDV can cause destructive signs in chickens all over the world. Although the current vaccines are substantially effective, they do not completely prevent infection, virus shedding and disease. All NDV isolates belong to a single serotype. Consistent with this, currently used vaccines, such as strains B1 and LaSota, are known to protect against morbidity and mortality caused by NDV isolates in different parts of the world (Cho *et al.*, 2008). However, recent studies have suggested that NDV strains currently in circulation represent genotypes that differ from that of the vaccine strains (Miller *et al.*, 2010).

We conclude that the active surveillance is very important to detect the co-circulated viruses within the farms as well as evaluation of viruses and genotypic characterization help in the selection of the type of vaccine used.

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استبيان وتوصيف فيروس انفلوانزا الطيور وفيروس النيوكاسل فى قطعان التسمين والبياض فى مصر

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نظرا لانتشار فيروس الانفلوانزا H5N1 في قطاع الدواجن فى مصر منذ مدة فقد اوضح الاستبان النشط فى ١٩٥ مزرعة تسمين وبياض لثمانية عشر محافظة مصرية ٣٤، ٧ ، ٢٤ مزرعة ايجابية ل H9N2 و H5N1 والنيوكاسل الضاري على الترتيب واوضح التحليل الجينى لجين الهيماجلوتينين لفيروس انفلونزا الطيور للعترة H5N1 أظهر أنها عترات عالية الضراوة كلاسيكية من الفصيلة ٢.٢.١ والتي استوطنت وانتشرت في مصر منذ ٢٠٠٦. لكن عترات ال H9N2 كانت منخفضة الضراوة وتنتمي ل G1-lineage من الفيروسات الأسيوية والتي تنتشر في الشرق الأوسط. أيضا تحليل التتابع النيوكليتيدى لجين F لإثنى عشر معزولة نيوكاسل أظهر أنهم شديدى الضراوة ماعدا معزولتين كانتا منخفضى الضراوة وتشابه عترة التحصين لاسوتا. تم تقييم الضراوة لبعض معزولات النيوكاسل من خلال معامل الضراوة بالحقن المخى كما حدث مع العترة (NDV-EG-567f-2012) والتي ظهرت أنها عالية الضراوة وكانت قيمة معامل ضراوة الحقن المخى ١.٩٦. ومن هذه الدراسة تم اثبات تواجد فيروس الانفلوانزا من العترةH5N1 والعترة H9N2 منخفضة الضراوة فى الطيور فى نفس الوقت فى مصر.