Animal Health Research Journal Vol. 7, No. 5, December 2019 pp. 49-56

ISSN: 2356-7767

Genotyping of avian influenza viruses (H5N1-H9N2) isolated from Egyptian field strains using Pyrosequencing technique

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Received in 9/10/2019
Accepted in 13/11/2019

Abstract

Avian influenza (AI), caused by the avian influenza A virus (AIV) which is of significant public health concern globally. For the quick identification of avian influenza, a pyrosequencing approach was developed and to Predict the pathogenicity of the virus. In the current study, the ten collected samples from Egyptian farms in 2016 and 2017 were subtyped preliminarily by real-time RT-PCR based on the sequence of the M gene and a subtype-specific sequence of the HA gene, respectively. The RT-PCR results were then double-checked using the pyrosequencing method. That by A pair of degenerative primers to amplify a fragment of the HA gene containing the cleaving site which is crucial for virulence. Following that, about 50bp region enclosing the cleavage site was determined by pyrosequencing. The pyrosequenced samples results were compared and validated using the traditional Sanger sequencing method, which is commonly used for AI pathotyping. Five specimens of avian influenza A H5N1 subtype with high pathogenicity and five specimens of avian influenza A H9N2 subtype with low pathogenicity were identified using the pyrosequencing technique. The study declared that the method is accurate, fast, and can be used efficiently for identifying the pathogenicity of the avian influenza A virus.

Keywords: *Avian influenza A virus, H5N1, H9N2, Subtype, Pathogenicity, Pyrosequencing.*

Introduction

Avian influenza disease is an acute infectious disease caused by the *Orthomyxoviridae* influenza A virus (AIV). Antigenically AIV was subtyped into 16 HA subtypes and 9 NA subtypes based on hemagglutinin (HA) and neuraminidase (NA) proteins **(Krammer *et al.* 2018).** Moreover, according to the virulence in chickens, it is classified into low pathogenic (LPAIVs) and highly pathogenic (HPAIVs) **(Chatziprodromidou *et al.* 2018).** This classification is genetically based on the deduced amino acid sequence at the cleavage site of the HA0 precursor protein; in presence of multiple basic amino acids, it is considered HPAI genetically **(OIE, 2018).** Mainly laboratory diagnosis of avian influenza virus includes the traditional methods as virus isolation, enzyme-linked immunosorbent assay (ELISA), and hemagglutination inhibition test **(Boivin *et al.*, 2001, Cox and Subbarao, 1999 and Kaiser *et al.*, 1999).** Recently, molecular based techniques were developed including the PCR technique**,** real-time PCR and DNA sequencing **(Deng *et al.*, 2015, Magnard *et al.*, 1999 and Payungporn *et al.*, 2006)**. The PCR and real time PCR which are considered the standard method for diagnosis fail to provide the sequence data, while DNA sequencing can not be used as a routine method for detection of large numbers of samples due to the time and the cost. It is very important to develop a fast and accurate method for detecting and genotyping of avian influenza virus. Pyrosequencing technique based on the release of pyrophosphate (PPi) due to incorporation of the nucleotide by the polymerase. The PPi is converted into ATP to be the energy used to activate luciferase enzyme for luciferin oxidation

and generation of light. The fluorescence intensity increased with the nucleotide incorporation and no fluorescence If dNTP is not complementary to the template. So, we can determine the sequence of the template after the primer. For duel usage detection and pathogenicity differentiation of avian influenza, the pyrosequencing approach is extremely useful and fast for short sequences with high throughput to identify large scale samples **(Zhou *et al.,* 2002, Zhang *et al.,* 2006).**

By using pyrosequencing, the current study aims to detect and genetically characterize the pathogenicity of the Egyptian highly pathogenic AI (HPAI H5N1) and low pathogenic AI (LPAI H9N2), which are both endemic in Egypt. **(Aly *et al.,* 2008 and El-Zoghby *et al.,* 2012)**

Materials and Methods

Samples collection: -

Different samples including Tracheal, cloacal swabs and organs such as Brain, trachea, lungs, small intestine and pancreas from ten chicken farms were collected from different Egyptian governorates. These farms showed suspected clinical signs as respiratory, nervous signs and diarrhea with or without high mortality. The viral RNAs of the ten samples were extracted from the samples using QiAmp Viral RNA Mini kit (manufacturer's instructions. RT-PCR was carried out using quantiTect qRT-PCR kit (Qiagen). The primers used are listed in (Qiagen, Hilden, Germany) according to the Table 1) and the RT-PCR was conducted in the Stratagene 3005P MXpro Real-Time PCR System (Stratagene,USA).

RT-PCR and the partial sequence of Hemaglutinin using Sanger method: -

Qiagen One Step Enzyme Mix kit was used for PCR amplification according to the manufacturer's instructions, using a set of primers as shown in Table 2. The DNA band of the correct size was excised and purified using the QIAquick Gel Extraction Kit (Qiagen). The purified RT-PCR amplicons were sequenced using BigDye TerminatorV3.1 cycle sequencing kit (Perkin Elmer, Foster City, CA) and Applied Biosystems 3500xl genetic analyzer (ABI, USA). By using Bioedit 7.2 software, a comparative analysis of deduced amino acids of the sequenced cleavage site was created**. (Hall, 1999).**

Pyrosequencing: -

The primer sets used for this investigation covered the cleavage site of the HA gene in the H5N1 and H9N2 subtypes. The forward Primer was biotinylated, for the pyrosequencing assay. The reverse PCR primer was used as a sequencing primer (Table 2). According to the manufactuer's instructions, the PyroMark One Step RT-PCR Kit (Qiagen, Hilden, Germany) was used to amplify the specific fragment.

RNA was Extracted added to 45 of reaction mix containing the specific primers. A 30 min of reverse transcription step at 50ºC was followed by 15 min initial denaturation at 95ºC and then the cycling starts with denaturation at 94ºC for 30s, then annealing at 64ºC for 30s and the extention at 72ºC for 40s and the final extention H9N2 subtypes. The forward primer was biotinylated, for the pyrosequencing assay. The reverse PCR primer was used as a sequencing primer (Table 2). According to the manufacture's instructions, the PyroMark is 70ºC for 10 min. Finally, the reactions were loaded into the PyroMark Q96 ID instrument (Qiagen).

Table (1). The sequences of primers used for real time RT-PCR for AI H5N1 and AI H9N2 viruses.

Primer Name	Primer Sequence	References
H5LH1	ACATATGACTACCCACARTATTCAG	**(Slomka *et al,* 2007)**
H5RH1	AGACCAGCTAYCATGATTGC	
H5 PROBE	[FAM]TCWACAGTGGCGAGTTCCCTAGCA[TAMRA]	
H9 FOR	GGAAGAATTAATTATTATTGGTCGGTAC	**(Ben Shabat *et al.,* 2010)**
H9 REV	GCCACCTTTTTCAGTCTGACATT	
H9 PROBE	[FAM]AACCAGGCCAGACATTGCGAGTAAGATCC[TAMRA]	

Table (2). The sequences of primers used for Sanger Sequencing and Pyrosequencing

Gene	Sequencing type	Primer Name	Primer Sequence	References
H5N1- HA	**Sanger Sequencing**	H5-HGGT	CTC TTC GAG CAA AAG CAG GGG T	**Veterinary legal agency**
		H5- KH3	TAC CAA CCG TCT ACC ATK CCYTG	
H9N2-HA		H9-F800	TGG GAA TCT AAT TGC TCC ATG GTTTGG ACA	**(Ben Shabat *et al.*, 2010)**
		H9-R1300	TCATCAATC TTG TTA TTGATC ATA	
H5N1-HA	**Pyrosequencing**	F H5	Biotin-TATGCATACAAAATTGTCAAG	**Wang *et al.*, 2016**
		*R/S	ACCTGCTATAGCTCCAAATAG	
H9N2-HA		F H9	BiotinTTCAGGAGAGAGCCAC-GGAAG	
		R/S	ACCTGCTATAGCTCCAAATAG	

* R/S this primer is a reverse primer for amplification and at the same time it is used as a sequencing primer for pyrosequencing

Results

Detection and amplification of H5N1 and H9N2 by real time RT-PCR: -

All collected samples (10) were positive by real time RT-PCR using specific primers and probes for both H5N1 and H9N2.

RT- PCR, Sequence and pyrosequencing: -

Particular bands at 1100bp and 500bp on agarose gel for both H5N1 and H9N2, respectively were detected by using the RT-PCR. These specific bands of partial amplification of the HA gene were sequenced by the Sanger method (Fig 3). While specific bands at 259 bp and 235 bp for both H5N1 and H9N2 respectively were amplified by using the pyrosequencing primers. These amplicons were sequenced by pyrosequencing method. The consensus sequence that covers the cleavage site of the HA gene for five samples has been determined as 5′TCCTCTCTTTTTTCTTCTCTTCTCTCCT TGAGGGCTATTCCTG-AGCCCAGT -3′ and they were tested by NCBI BLAST program. The result indicated that these samples were avian influenza A virus subtype H5. The detected sequence were translated into the amino acid sequence of TGLRNS**PQGEKRRKKRG** by using BioEdit software. According to OIE criteria **(OIE, 2018),** the five samples include multiple basic amino acids and had a high pathogenicity trait genetically (Table 3). The consensus sequences which covers the cleavage site of the other five samples were 5′-CCCTCTACTTGACCTGGCAGGCACA-TTTCTCAGACCGATTGCCAGTTTGAG 3′. They were tested by NCBI BLAST program. The results revealed that these samples were avian influenza A virus subtype H9. The detected sequences were translated into the amino acid sequence of LKLAIGLR**NVPA-RSSRG** by BioEdit software. According to OIE criteria **(OIE, 2018)** the five samples did not include multiple basic amino acids and had a low pathogenicity trait genetically (Table 3). These ten samples' results were compared to those obtained using the Sanger approach on an ABI PRISM 3500xl Genetic Analyzer (Applied Biosystem, USA). Both pyrosequencing and the Sanger method showed similar results (Table 3 and Fig 3&4).

Table (3). Amino Acid Sequence of the cleavage site of HA protein using Pyrosequencing:

Isolate name	Pyrosequencing results	Genotyping	Accessionnumber
A/chicken/Egypt/173CAL/2017	TGLRNS**PQGEKRRKKRG**	H5N1 HPAI	MG192004
A/chicken/Egypt/S52/2016	TGLRNS**PQGEKRRKKRG**	H5N1 HPAI	MF417623
A/chicken/Egypt/CAL9/2016	TGLRNS**PQGEKRRKKRG**	H5N1 HPAI	MF417622
A/chicken/Egypt/CAG139/2016	TGLRNS**PQGEKRRKKRG**	H5N1 HPAI	MF417624
A/chicken/Gharbia/5/2016	TGLRNS**PQGEKRRKKRG**	H5N1 HPAI	KY951990
A/chicken/Egypt/16194V/2016	LKLAIGLR**NVPARSSRG**	H9N2 LPAI	MH762046
A/chicken/Egypt/1685SL/2016	LKLAIGLR**NVPARSSRG**	H9N2 LPAI	MF289430
A/chicken/Egypt/171123V/2017	LKLAIGLR**NVPARSSRG**	H9N2 LPAI	MH762067
A/chicken/Egypt/171595V/2017	LKLAIGLR**NVPARSSRG**	H9N2 LPAI	MH762069
A/chicken/Egypt/1774CAD/2017	LKLAIGLR**NVPARSSRG**	H9N2 LPAI	MH762068

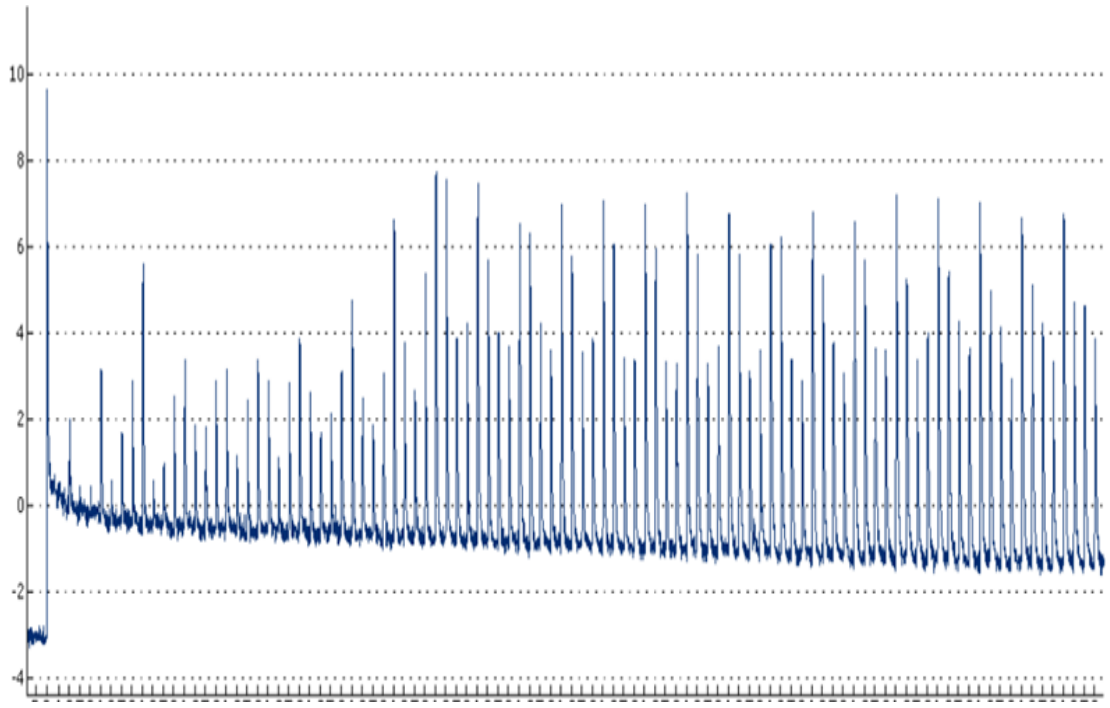

Fig. (1): Pyro-mark SQA analysis of H9N2

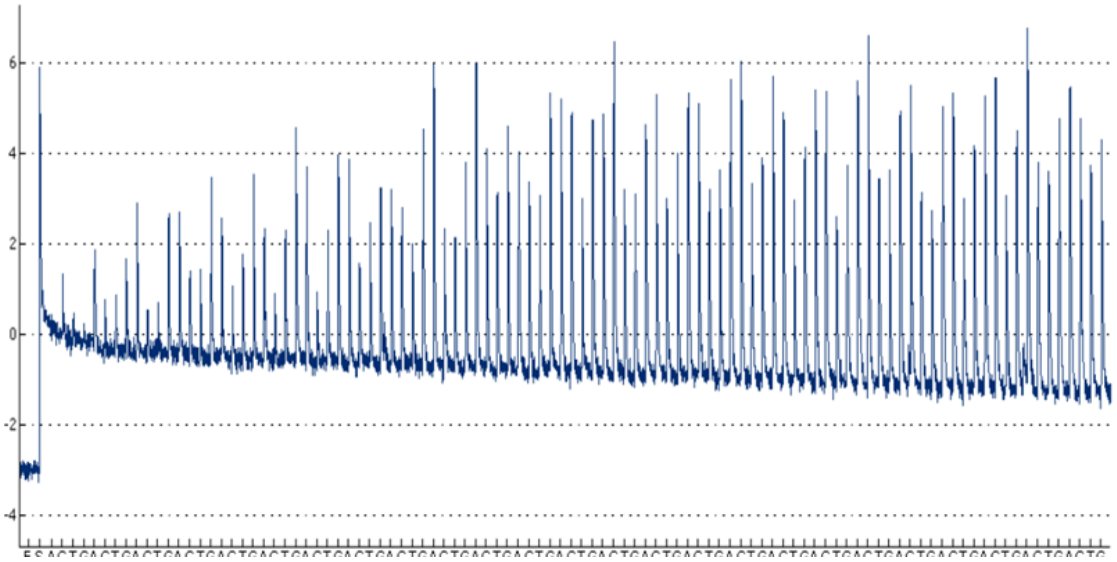

Fig. (2): Pyro-mark SQA analysis of H5N1

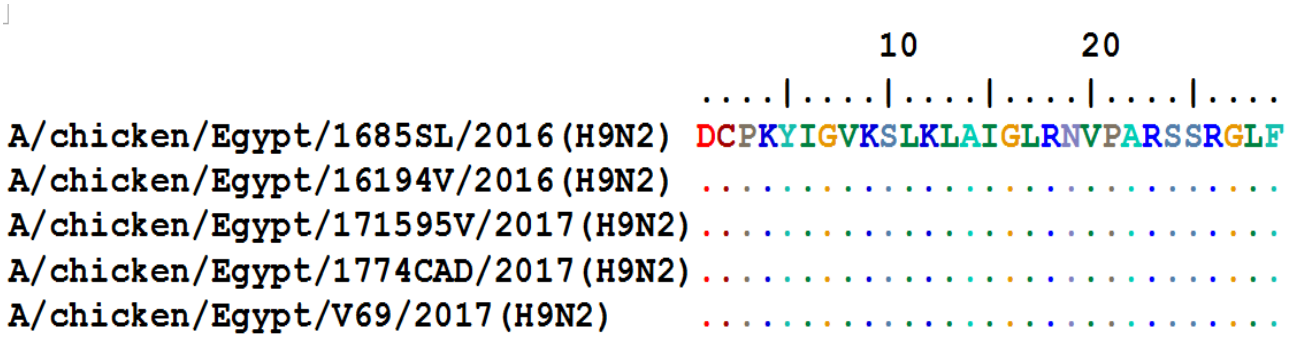

Fig. (3): Partial Amino Acid Sequence of H9N2 cleavage site region of HA protein using Sanger method

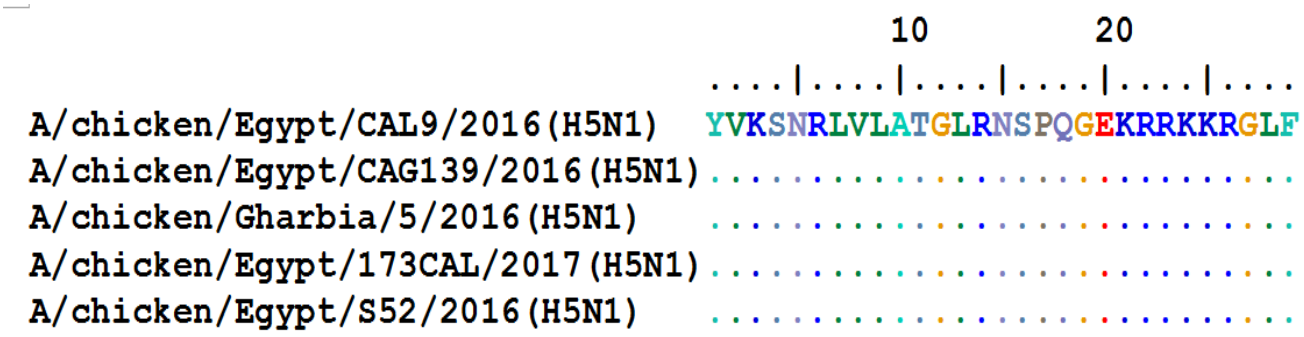

Fig. (4): Partial Amino Acid Sequence of H5N1 cleavage site region of HA protein using Sanger method

Discussion

Unlike the traditional Sanger approach which takes more than 5hr, pyrosequencing provides data in real time without the requirement for gel processing, therefore 96 samples can be analyzed within 2 hours after RT-PCR, and in certain circumstances may be less than 1 hour **(Chantratita *et al.,* 2011).** Furthermore, pyrosequencing has been described as a cost-effective method for diagnosing the avian influenza virus A (H5N1-H9N2) infection **(Ellis *et al.,* 2007).** Pyrosequencing is thus easier, faster, and less expensive than Sanger sequencing **(Bao *et al.,* 2011; Chantratita *et al.,* 2011 and Deng *et al.,* 2011).**

Pyrosequencing was also quite effective in determining cleavage site mutations, with high sensitivity and specificity when compared to Sanger sequencing, moreover, the pyrosequencer built-in system making it easier in handling than a traditional sequencer. In this study pyrosequencing was found to be highly sensitive. However, due to the presence of repetition of the same nucleotide three or more times in some sequences (homopolymeric) that could not be distinguished by pyrosequencing, so the sequence process success is dependent on the sequence to be analyzed **(Parameswaran *et al.*,2007).**

Samples tested here by pyrosequencing analysis were correctly identified as being either highly pathogenic (H5N1) or low pathogenic (H9N2) AI strains depending on the sequence of cleavage site in relation to Sanger based sequencing (Table 3, Fig 3 & 4). This method may quickly deduce an amino acid sequencing result, which complies with one of the official pathotyping methods authorized by the international OIE organization. Rapid tests based on real-time PCR, RT-PCR, and other probe hybridization-based assays cannot satisfy this capability of pyrosequencing. Furthermore, as terminator enzymes and time-consuming purification processes are not required, the cost of pyrosequencing analysis is reduced. **(Dudley *et al.,* 2012; Pizzuto *et al.,* 2010; Timbs *et al.,* 2012).** Another essential practical advantage of the technique is the short fragment amplified, which enables the generation of PCR products adequate for pathotyping in samples with low quality or diminished or even degraded DNA integrity (**Rasmussen and Morrissey, 2008).** However, confirmation of the preliminary RT-PCR reaction phase is critical for obtaining the best amplified products and producing high-quality pyrosequences.

In conclusion, all pyrosequencing results were in perfect agreement with those obtained using the Sanger sequencing method, indicating that both methods are more efficient when they are used to distinguish between HPAI (H5N1) and LPAI (H9N2) strains, but the pyrosequencing is rapid and cost effective than the Sanger method.

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