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Molecular identification of H5N1 isolated from Egypt by sequencing of cleavage site

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Abstract

Avian influenza (AI) is caused by specified viruses that are members of the family Orthomyxoviridae and placed in the genus influenza virus A. There are three influenza genera – A, B and C; only influenza A viruses are known to infect birds. Diagnosis is by isolation of the virus or by detection and characterization of fragments of its genome. Infection in chickens with HPAI H5N1 is similar to infection with other HPAI viruses. In this study, we will explain how to make accurate identification for avian influenza strains isolated from many localities in Egypt by sequencing of the cleavage site.

Keywords: Avian influenza, chickens

Introduction

Avian influenza (AI) is caused by specified viruses that are members of the family Orthomyxoviridae and placed in the genus influenza virus A. There are three influenza genera – A, B and C; only influenza A viruses are known to infect birds. Diagnosis is by isolation of the virus or by detection and characterization of fragments of its genome. This is because infections in birds can give rise to a wide variety of clinical signs that may vary according to the host, strain of virus, the host's immune status, presence of any secondary exacerbating organisms and environmental conditions (OIE 2012).

Infection in chickens with HPAI H5N1 is similar to infection with other HPAI viruses (Perkins and Swayne 2001). Described clinical signs include anorexia, ruffled feathers, swollen hemorrhagic necrotic wattle and comb, congested legs, cyanosis, dermal hemorrhage, coma and can even include acute death without clinical signs (Kobayashi *et al*., 1996 and Swayne 1997).

Identification of avian influenza requires many different types of techniques and tests such as **Plate Haemagglutination test**, **Haemagglutination inhibition (HI) test**, **Polymerase chain reaction** and sequencing of cleavage site of HA.

Material and Methods

Samples

Tracheas were taken from recently dead chicken from farms at (Mansoura, Qalubia, Sharkia and Minia) with high mortality rates and transferred on ice to be processed within six hours. The samples were collected during the year 2017- 2018.

Virus Isolation

Virus isolation and titration on SPF Embryonated Chicken eggs:

Specific pathogen free (SPF) Embryonated chicken eggs (9-11 day) old were used for virus isolation (Nile-SPF-eggs farm, Koom Oshiem, Fayom, Egypt).

Buffers and solutions used for virus isolation and titration according to Hudson and Hay (1980)

Methods

Virus isolation on SPF Embryonated Chicken eggs

Tracheas were opened and the mucosa surfaces were scratches with sterile swaps and stirred in sterile normal saline. After centrifugation for ten minutes at 5000 rpm at 4˚C, the viral suspension was filtered through 22 µ Millipore syringe filter and then 0.2ml were injected in nine days old SPF Embryonated chicken eggs and incubated at 37 ˚C in humid incubator, the eggs were candled daily till the embryos were died and chilled for 2hrs at 4 ˚C. The chorioallantoic fluid was harvested and purified by centrifugation at 13.000 rpm at 4 ˚C for 10 minutes and stored at -20 till use.

Identification of virus

Virus identification by Haemagglutination (HA) and Haemagglutination Inhibition (HI) assays

Preparation of the RBCs used in HA

Three mL of chicken blood were taken and mixed with sod Citrate solution .The blood was centrifuged at 1500 rpm for 5 min and supernatant was decanted. The packed RBCs was washed three times with normal physiological saline and stored at 4 ˚C till used within 48 h.

Plate Haemagglutination test

The Haemagglutination (HA) and Haemagglutination inhibition (HI) tests were carried out following the recommendation of OIE Manual, (2009), Katz *et al*., 2009 and Potter and Oxford 1979. A volume of 50 µl of PBS at pH 7.2 was dispensed into the individual wells of a double raw in a U – shaped bottomed micro titer plate then 50 µl of the chorioallantoic fluid was added to the first well and serial double fold dilution was performed using a multi –channel micropipette. A volume of 50 µl of 1% washed RBCs suspended in PBS were added to all wells, the virus – RBCs mixture was allowed to stand at room temperature for 30 min. Three wells containing only PBS with RBCs were included in the test and served as negative controls. The HA titer was calculated as the reciprocal of the highest dilution of the virus giving a complete HA pattern.

Haemagglutination inhibition (HI) test

Twenty five µl of PBS pH 7.2 were Dispensed into each well of a plastic U-bottomed micro-titer plate, then 25µl of mono-specific antisera against either avian influenza or Newcastle disease viruses was placed into the first well of a separate plates and then 2 fold serially diluted across the plate. Four HA units of each virus isolate suspended in 25µlPBS were then added to each well in both plates and left for 30 minutes at room temperature (i.e. about 25°C). Chicken RBCs was then added (25µl of 1% concentration) to each well and after gentle mixing the RBCs was allowed to settle for about 30 minutes at room temperature till the control RBCs settled to a distinct button. The HI titer calculated as the highest dilution of serum causing complete inhibition of 4 HAU of virus. The agglutination is assessed by tilting the plates. Only those wells in which the RBCs stream at the same rate as the

control wells (containing 25µl RBCs and 50µl PBS only) considered showing inhibition.

The validity of results assessed against a negative control serum, which should not give a titer >1/4 (>22 or >log2 when expressed as the reciprocal).

Virus identification by Polymerase chain reaction

The virus isolated were identified as Influenza A virus by amplification of a conservative region in matrix gene and then subjected to amplification of the cleavage site of HA gene of HPAI.

RNA extraction

The RNA of the isolated virus was extracted using Simply P total RNA extraction kit (BioFlux). According to the manufacture instruction, 100µl of chorioallantoic fluid was lysed with 100µl of R1 solution at room temperature for not more than one minute. The solution was then neutralized for 5 min using 600 µl of R2 solution and then placed in the spin column provided with the kit. The spin columns were centrifuged at 6000rpm/2min/4°C and washed twice with 600µl of washing solution. After cleaning the spin columns from the residual of washing buffer by centrifugation at 1000rpm/2min, the RNA bounded to the silica membrane was eluted off in 50µl of the elution buffer and stored at -20°C till used.

RNA quantification

The RNA concentration was measured using Qubit 2 Fluorometer; one µl of RNA was mixed with 199µl of the RNA dilution buffer and kept for 3 min at room temp. The absorbance of the mixture was measured and the results were plotted against a standard curve created with standard concentration of RNA provided with the kit.

One step RT-PCR

It was done using one step RT-PCR kit (Bioer, China) according to Sambrook *et al*. (1989).

Agarose gel electrophoresis

According to Sambrook *et al*., (1989), 4 mm thickness of 1% agarose containing 0.5µg/ml Eth-Br was poured in mini-gel and left till solidify before submarine loading of 5µl of PCR-product mixed with 2µl of DNA loading buffer. Solis BioDyne 100 bp

DNA ladder was used as DNA marker (three µl in 1µl loading buffer). DNA was electrophoresed at 80 v/15 minutes and finally examined using UV trans-illuminator.

Virus identification by sequencing of the cleavage site

Preparation of low melting agarose gel 1%

According to Sambrook, *et al*., (1989), Four mm thickness of 1% low melting agarose containing a 5 µg/ml EBr was poured in medi-gel and left till solidify at 4°C/2 hours before submarine loading of 16 µl of the PCR product (cleavage site amplicon) mixed with 4 µl DNA loading buffer. Electrophoresis was done at 30 v/60 minutes at 4°C. The gel was then transferred to the UV trans-illuminator and the 300 bp of insert (the amplified cleavage site) were sliced out and placed separately in 1.5 ml nuclease free tubes.

Gel purification

According to the manufacture instruction, the gel slices were weighted and 3 volumes of extraction buffer were added to 1 volume of gel slice (10mg=100µl), then Incubated at 50°C until the gel melts in a heating water bath and the tube vortexes every 2-3 min during the incubation. The sample were applied to the spin column, and centrifuged for 1min at 6.000xg, and discard the flow through; 500µl of extraction buffer were added to the spin column and centrifuged for 60 sec at 6000 rpm, the flow through were discarded, after that 750µl of washing buffer was added to the spin column and centrifuged as before. Additional Centrifugation for 1min was done to clean up the spin column from the residual washing buffer. Elution was done using 100µl of elution buffer and let it stand for 1min at room temperature before centrifugation at 12000rpm/2min. the eluted DNA stored at -20°C till used.

Sequencing

Sequencing reactions were performed in a MJ Research PTC-225 Peltier Thermal Cycler using ABI PRISM 3730XL Analyzer BigDyeTM Terminator Cycle Sequencing Kits with AmpliTaq DNA polymerase (FS enzyme Applied Biosystems), following the protocols supplied by the manufacture. Single-pass sequencing was performed on each template using the primer used for PCR amplification. The fluorescent-labeled fragments were purified from the unincorporated terminators with an ethanol precipitation protocol. The samples were resuspended in distilled water and subjected to electrophoresis in an ABI 3730xl sequencer (Applied Biosystems).

Results

Virus isolation on SPF Embryonated Chicken eggs

Recently died chickens were subjected to extensive PM examination. Most of chicken were died without prominent signs, yet some of them showed the classical PM lesions such as hemorrhagic spots on the chunk typical for H5N1, hemorrhage on the coronary fat and severely hemorrhagic tracheas and sometimes serous exudates were seen. The SPF Embryonated chicken eggs inoculated with the tracheal swaps showed embryonic deaths at about 18 - 24 hours post inoculation. The embryos were either sever hemorrhagic or even macerated (typical for the HPAI). The Chorioallantoic fluid was slightly turbid and was collected clarified by centrifugation and stored at -20°C till used.

Identification of virus

Identification of virus by Haemagglutination test (HA)

Chorioallantoic fluids obtained from all inoculated eggs were subjected first for rapid (slide) HA assay to test for the presence of Haemagglutination agents. Those gave negative results were excluded from the study (usually no embryonic deaths were observed with these samples till 72 h post inoculation).

Identification of virus by Haemagglutination inhibition test (HI)

The Chorioallantoic fluid of the samples was diluted to obtain 4HA unit which will be used for HI assay using standard anti H5N1 mono-specific antisera, NDV antisera. As shown in table (1) all the tested samples gave no reaction with the mono-specific antisera against NDV, on the other hand, all samples gave positive inhibition to the agglutination using the mono-specific antisera against H5N1. The titer vary from 7-9.

Identification of virus by reverse transcriptase polymerase chain reaction (RT-PCR)

Samples that gave positive HA and HI were further analyzed by one step RT-PCR to amplify the HA cleavage site. All samples gave a positive amplicon migrating at ~ 300 bp (Fig 1). These amplicons were varied in the intensity denoting variation in the concentration of the virus titer in the original sample (chorioallantoic fluid) used in the RNA extraction.

Sequencing of the cleavage site of HPAI H5 gene

The nucleotide sequence of selected 12 isolates and the deduced amino acid sequence were aligned using CLC bio software. As seen in fig 2, several point mutations were seen between the isolates these mutations were mostly substitution mutations and resulted in changes in the amino acid sequence as seen in fig 3. At least 10 areas of amino acid substitutions were detected in the deduced amino acid but no more than 4 substitutions were detected for any analyzed strain. The poly basic amino acid sequence determinant for the HPAI was mainly GERRRKKR with the exception of strain 5, 7 and 10 where the first R residue was substituted with G.

Discussion

All samples taken from different localities all over Egypt from farms showed high rate of morbidity and mortalities characteristics to avian influenza major outbreaks during 2010 and 2011. Viral isolations and identification procedures were in accordance with the OIE guidelines (OIE 2012).

All the field isolates were tested for purity (devoid of other respiratory disease like NDV) and the properties of high pathogenicity before selecting a strain to amplify the full H5 gene from it to be cloned and used for the DNA vaccine production.

The isolates were subjected to further molecular investigations through amplification and sequencing of the cleavage site of the HA gene. All the tested strains gave a positive amplicon at 300bp for the CS denoting the H5N1 nature of all isolates.

Sequence analysis of the cleavage site of the twelve H5N1 isolates from Egypt revealed that all were belong to the highly pathogenic AI as they contained the polybasic amino acid sequence characteristic for the HPAI form of the H5N1subtype, PQGE (R/G/)RRKKR↓GLF at the cleavage site in the HA molecule, indicating their high virulence (Horimoto *et al*., 1995). This cleavage sequence was slightly differ from that of A/Hong Kong/156/97(H5N1) virus PQRERRRKKR↓G as the 3rd amino acid was substituted from R in Hong Kong strains to G in the Egyptian strains and also the 5th amino acid was R in all Hong Kong strains but it was either R, G or K in the Egyptian strains (Subbarao *et al*., 1998).

Cleavage of the HA molecule (HA0), by host-cell proteases, into two di-sulphide-linked HA1(that forms a globular head)and stalk consisting of HA2 subunits is

essential for viral infectivity as it a required step allowing the viral membrane to fuse with the host cell membrane (Wiley and Skehel 1987 and Webster *et al*.,1992,).

In fact, the cleavability of HA is a critical component determining the pathogenicity of AI viruses (Bosch *et al*., 1979). HA1 binds to the viral receptor, Sialic acid residues on host glycoproteins and glycolipids (Wiley and Skehel 1987). The virus enters the cytoplasm through fusion of the viral and host membranes via a conformation change of the HA2 due to low pH in the endosome (Skehel *et al*., 1982 and Wharton.1987)

Avian influenza viruses with high and low levels of pathogenicity differ in their cleavage sequence, the former possess multiple basic amino acid residues which is prerequisite factor for pathogenicity in chicken, while the latter do not. HA sequences with monobasic cleavage site (e.g. HA1-PSIQVR-GL-HA2) are cut by tryptase yielded from respiration and digestive tract epithelial (Whittaker 2001; Chen *et al*., 2004). HA sequences with polybasic cleavage site (e.g. HA1-KKREKR-GL-HA2), allow proteolysis process to be done by proteases such as furine and pro-protein-convertase6 (PC6) found in Golgi apparatus of all cells (Horimoto *et al*. 1994). AI Virus with polybasic cleavage site have unlimited distribution network and may cause fatal systemic infection.

Thus, a cleavage sequence containing several basic amino acids is more readily activated by cellular proteases present in a variety of cells distributed throughout the body compared with a cleavage sequence containing only a single basic amino acid, which can be cleaved by a limited range of cellular proteases. Our results were in agreement with Zambon, (2001) who found that influenza viruses containing multiple basic amino acids have multiple sites of virus replication and produce more severe infection in birds and mammals.

Blast search revealed that all the Egyptian isolates have polybasic amino acid sequence (PQGE(G/R)RRKKRGLF) which represent clade 2.2 other slightly different sequence have been seen in other clade (clade 2.3) where the sequence QRERRRKKR or QRESRRKKR which were isolated from 2003 -2007 in South East Asia (Susanti *et al*., 2008).

Conclusion

The present study explain how to make accurate identification for avian influenza strains isolated from many localities in Egypt by sequencing of the cleavage site.

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Table 1: The results of Haemagglutination and Haemagglutination inhibition assays using standard antisera against either H5N1 or NDV showing that most samples gave a very high HA and HI titer in comparison the negative control

Sample ID	HA titer	HI titer	
		H5N1	NDV
Strain No.1	11	7	0
Strain No.3	12	8	0
Strain No.4	11	8	0
Strain No.5	11	8	0
Strain No.6	11	7	0
Strain No.7	11	8	0
Strain No.9	9	7	0
Strain No.10	9	8	0
Strain No.12	10	7	0
Strain No.18	8	9	0
Strain No.22	12	9	0
Strain No.40	11	9	0
Negative control	0	0	0

Fig 1: A representative results of amplification of the cleavage site of HPAI strains. Notice the clear bands migrating ~ 300 bp.

(M) 100 DNA ladder

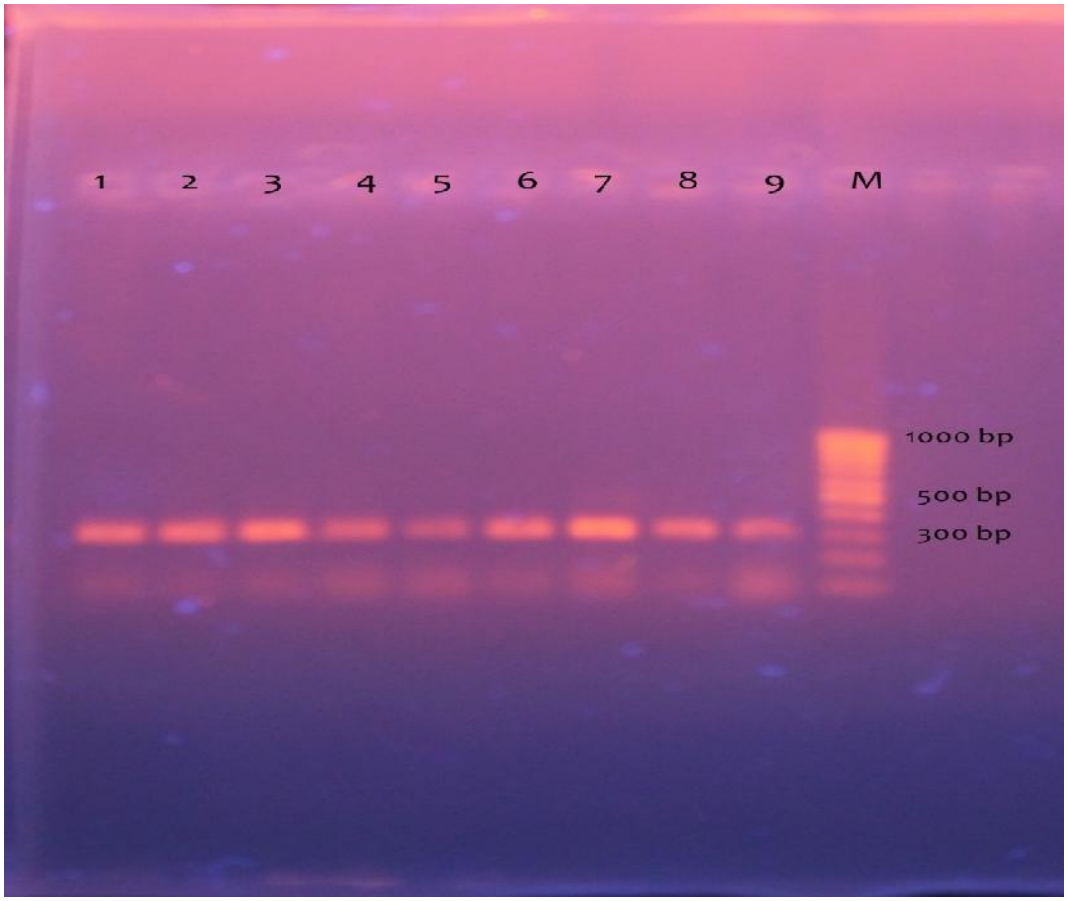
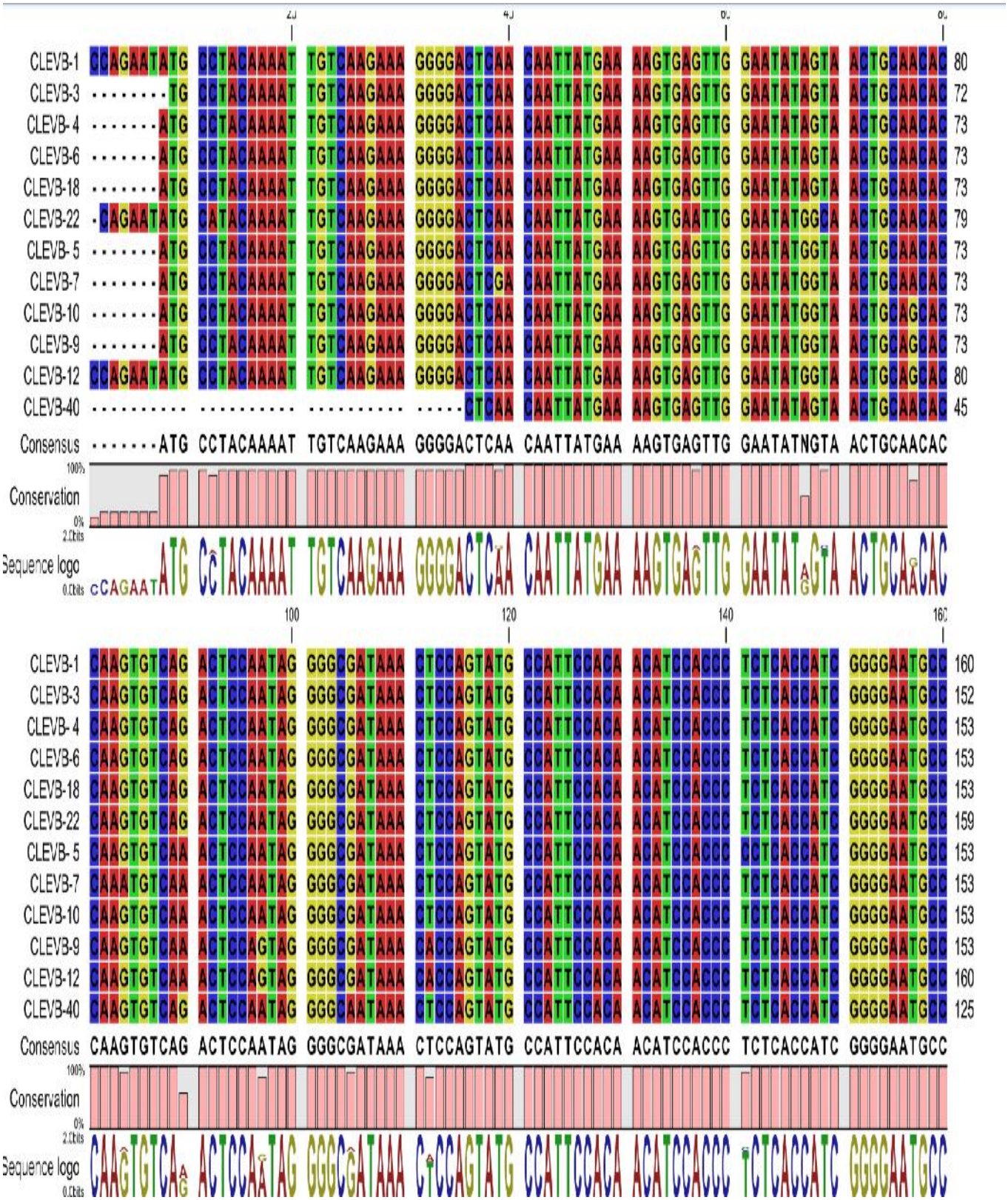


Fig 2: Alignment comparison of the nucleotide sequences of the cleavage site of the H5 gene showed the Egyptian AI viruses exhibited many point mutations.



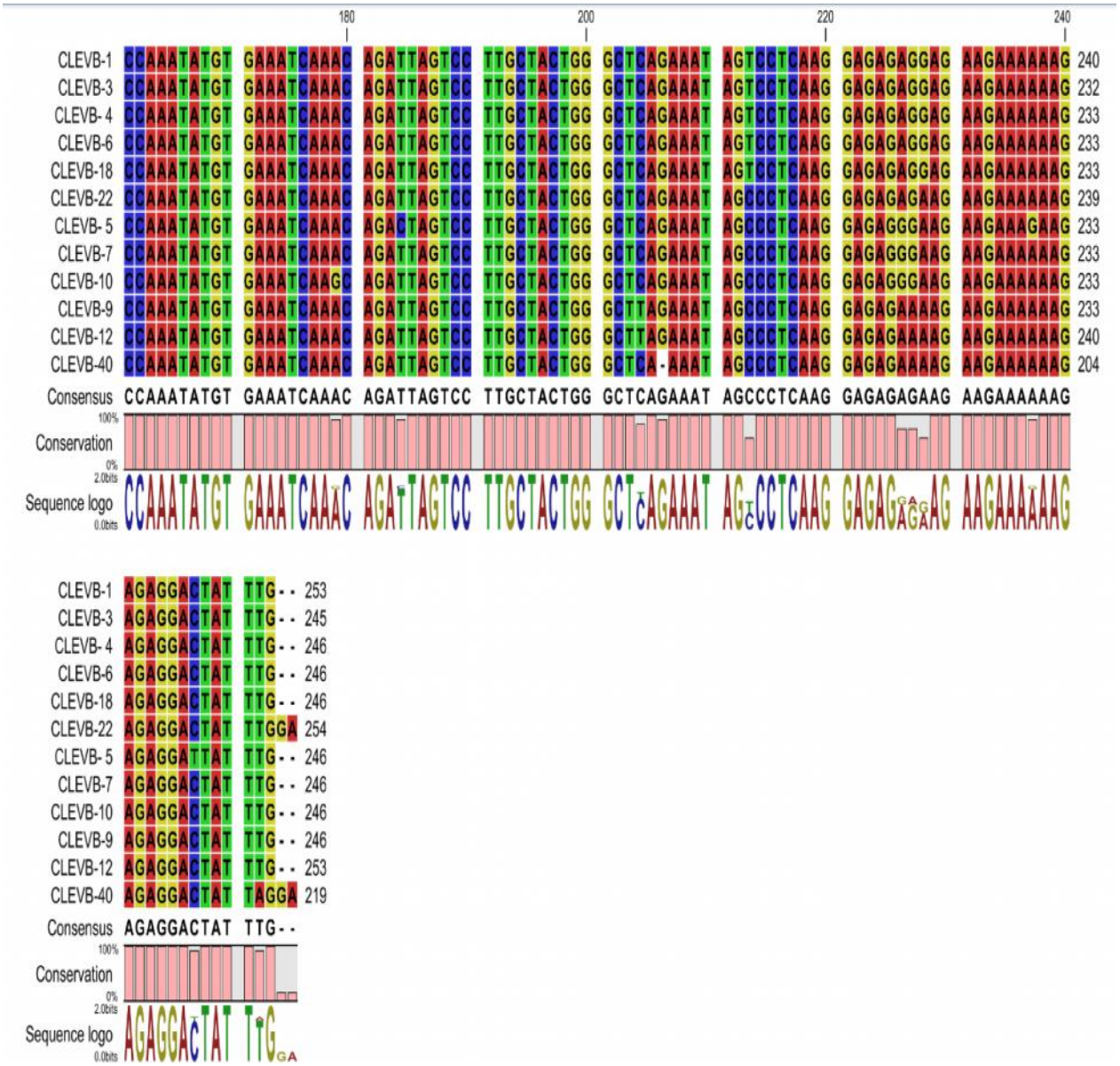

180
200
220
240
CLEVB-1 CCAAATATGT GAAATCAAAC AGATTAGTCC TTGCTACTGG GCTCAGAAAT AGTCCTCAAG GAGAGAGGAG AAGAAAAAAG 240
CLEVB-3 CCAAATATGT GAAATCAAAC AGATTAGTCC TTGCTACTGG GCTCAGAAAT AGTCCTCAAG GAGAGAGGAG AAGAAAAAAG 232
CLEVB-4 CCAAATATGT GAAATCAAAC AGATTAGTCC TTGCTACTGG GCTCAGAAAT AGTCCTCAAG GAGAGAGGAG AAGAAAAAAG 233
CLEVB-6 CCAAATATGT GAAATCAAAC AGATTAGTCC TTGCTACTGG GCTCAGAAAT AGTCCTCAAG GAGAGAGGAG AAGAAAAAAG 233
CLEVB-18 CCAAATATGT GAAATCAAAC AGATTAGTCC TTGCTACTGG GCTCAGAAAT AGTCCTCAAG GAGAGAGGAG AAGAAAAAAG 233
CLEVB-22 CCAAATATGT GAAATCAAAC AGATTAGTCC TTGCTACTGG GCTCAGAAAT AGCCCTCAAG GAGAGAGAAG AAGAAAAAAG 239
CLEVB-5 CCAAATATGT GAAATCAAAC AGACTAGTCC TTGCTACTGG GCTCAGAAAT AGCCCTCAAG GAGAGGGAAG AAGAAAGAAG 233
CLEVB-7 CCAAATATGT GAAATCAAAC AGATTAGTCC TTGCTACTGG GCTCAGAAAT AGCCCTCAAG GAGAGGGAAG AAGAAAAAAG 233
CLEVB-10 CCAAATATGT GAAATCAAGC AGATTAGTCC TTGCTACTGG GCTCAGAAAT AGCCCTCAAG GAGAGGGAAG AAGAAAAAAG 233
CLEVB-9 CCAAATATGT GAAATCAAAC AGATTAGTCC TTGCTACTGG GCTTAGAAAT AGCCCTCAAG GAGAGAAAAG AAGAAAAAAG 233
CLEVB-12 CCAAATATGT GAAATCAAAC AGATTAGTCC TTGCTACTGG GCTTAGAAAT AGCCCTCAAG GAGAGAAAAG AAGAAAAAAG 240
CLEVB-40 CCAAATATGT GAAATCAAAC AGATTAGTCC TTGCTACTGG GCTCA-AAAT AGCCCTCAAG GAGAGAAAAG AAGAAAAAAG 204
Consensus CCAAATATGT GAAATCAAAC AGATTAGTCC TTGCTACTGG GCTCAGAAAT AGCCCTCAAG GAGAGAGAAG AAGAAAAAAG
100%
Conservation
0%
2.0bits
Sequence logo
0.0bits
CLEVB-1 AGAGGACTAT TTG-- 253
CLEVB-3 AGAGGACTAT TTG-- 245
CLEVB-4 AGAGGACTAT TTG-- 246
CLEVB-6 AGAGGACTAT TTG-- 246
CLEVB-18 AGAGGACTAT TTG-- 246
CLEVB-22 AGAGGACTAT TTGGA 254
CLEVB-5 AGAGGATTAT TTG-- 246
CLEVB-7 AGAGGACTAT TTG-- 246
CLEVB-10 AGAGGACTAT TTG-- 246
CLEVB-9 AGAGGACTAT TTG-- 246
CLEVB-12 AGAGGACTAT TTG-- 253
CLEVB-40 AGAGGACTAT TAGGA 219
Consensus AGAGGACTAT TTG--
100%
Conservation
0%
2.0bits
Sequence logo
0.0bits

Fig 3: Alignment comparison of the deduced amino acid sequences of the cleavage site of the H5 gene showed the Egyptian AI viruses. All isolates had a cleavage site sequence PQGERRRKKRG with the exception of isolates 5,7 and 10 where the sequence was PQGEGRRKKRG