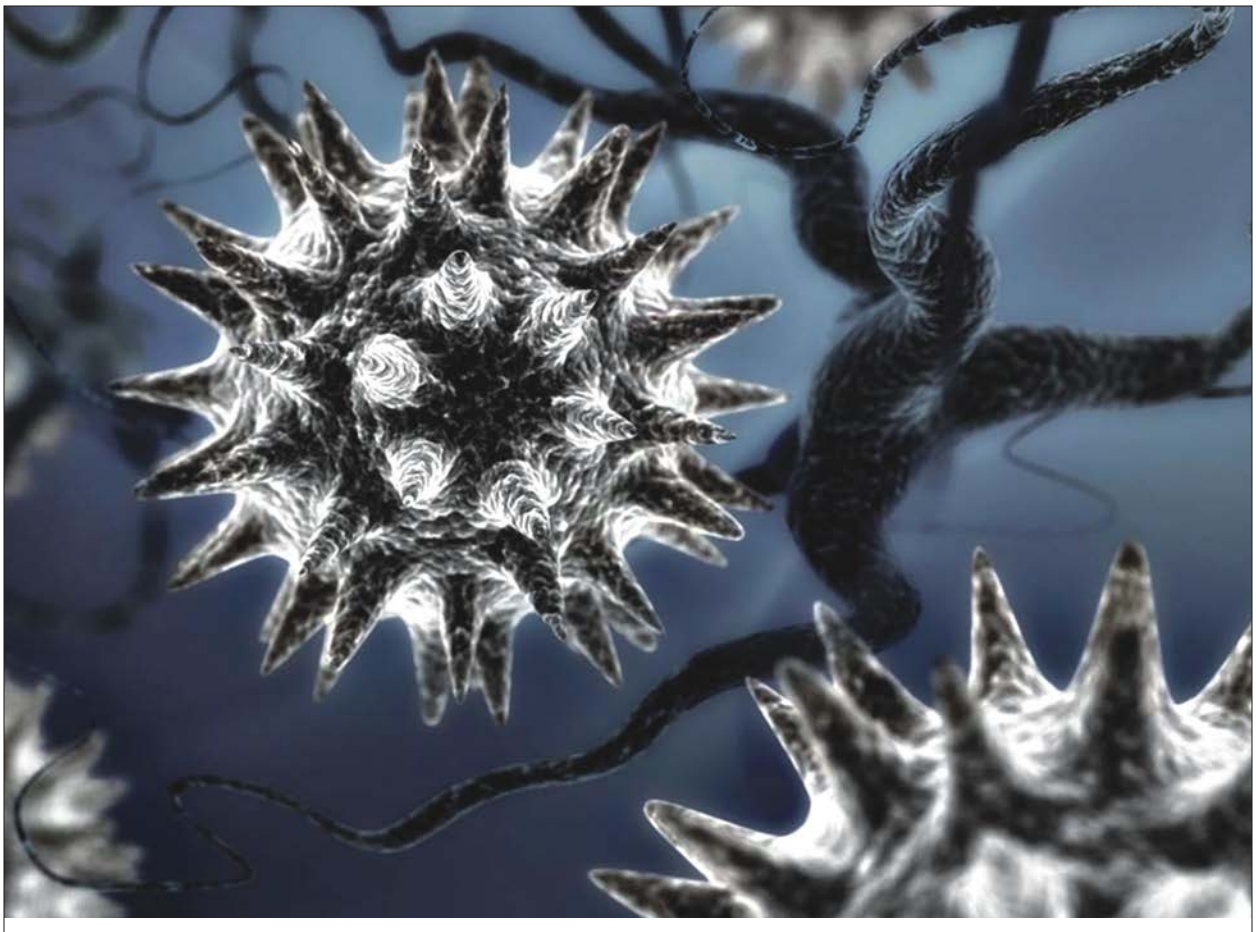

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Comparative Detection of H5N1 Avian Influenza Virus Using Conventional Rt-PCR and Real Time Rt-PCR

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ABSTRACT

Highly Pathogenic Avian Influenza (HPAI) caused by influenza A H5N1 virus, poses a significant threat to the poultry industry and humans in Egypt. Since it was first recognized in 2006, the disease has become enzootic in poultry throughout Egypt and still circulates in the poultry population, so the ability to rapidly recognize AIVs in biological specimens is critical for limiting further spread of the disease in poultry. Application of molecular methods such as Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) and Real time RT-PCR (RRT-PCR) as a rapid, specific and sensitive detection methods currently used in national and reference laboratories worldwide. In this study a comparison of the specificity and sensitivity between 2 different formats of conventional RT-PCR (one and two steps) and 3 different formats of RRT-PCR (one step using TaqMan® probe, two steps using TaqMan® probe and two steps using hybridization® probe) were performed and compared as a diagnostic tools for H5N1 virus detection. All these formats of PCRs appeared within the same specificity for H5 gene detection, while they showed difference in sensitivity as the one step conventional RT-PCR showed to be more sensitive than two steps conventional RT-PCR by 10 folds, one step RRT-PCR TaqMan® probe was more sensitive than two steps RRT-PCR TaqMan® probe by 10 folds, two steps RRT-PCR hyberidization® probe is more sensitive than two steps RRT-PCR TaqMan® probe by 100 folds, finally two step RRT-PCR using hyperidization® probe is more sensitive than conventional RT-PCR two steps by 1000 folds. Fifty one field samples were further tested by all mentioned PCR formats the results were the same of that obtained in the sensitivity experiment and agreed with them in placing hybridization probe system of higher sensitivity than TaqMan one step than TaqMan two steps and conventional PCR were of the lowest sensitivity although one step showed higher sensitivity than two steps.

Key words: Avian influenza, highly pathogenic avian influenza, polymerase chain reaction, reverse transcriptase (conventional PCR), real time-RT-PCR, embryo infective dose fifty

INTRODUCTION

The rapid diagnosis of Avian Influenza Virus (AIV) during a poultry outbreak is critical for a timely control program (Pelzel *et al*., 2006) Any delays in diagnosis or response to an outbreak allow the virus to spread, making eradication more difficult. Diagnosis of avian influenza can be made by a variety of methods, including clinical signs, serologic methods and direct virus detection methods. Clinical signs with highly pathogenic avian influenza can be a valuable tool for

presumptive diagnosis in chickens and turkeys, but none of the lesions are pathognomonic and the etiology must be confirmed by diagnostic tests (Swayne and Halvorson, 2003).

For some species, including ducks and wild birds, disease expression is extremely variable and clinical disease is a less reliable indicator of infection. Serologic diagnostic tests are widely used for trade purposes to show freedom of infection from mainly low-pathogenic avian influenza. However, serology is of little value for Highly Pathogenic Avian Influenza (HPAI) because most birds die before producing antibody. Even in surviving birds the time for an antibody response to develop causes a considerable delay of diagnosis that allows the virus to continue to spread. Currently the most useful diagnostic tests are ones that can directly detect the virus, either live virus, antigen, or nucleic acid. Three common direct diagnostic tests for avian influenza are virus isolation, antigen capture immunoassays, and molecular diagnostic tests (Cattoli *et al*., 2004). Virus isolation remains a valuable tool for the diagnosis of avian influenza, especially for the diagnosis of avian influenza on the index case (Cattoli and Capua, 2006).

Virus isolation allows for the biological characterization of the virus as well as allowing for full sequence analysis of the isolate. For countries like the United States, the isolation of AIV is a necessary step before reporting an outbreak to the World Organization of Animal Health (OIE), even a presumptive diagnosis will trigger animal health authorities to initiate quarantines and other control measures until a definitive diagnosis can be made. However, virus isolation has several important drawbacks (Cattoli and Capua, 2006). The most important is the time necessary for diagnosis which can be several days to weeks. Additionally, virus isolation using embryonating chicken eggs requires a readily available supply of eggs. Finally, since virus isolation amplifies live virus to high levels in the laboratory, higher levels of biosecurity need to be maintained if highly pathogenic avian influenza is suspected. In general, biosafety level 3 agriculture (BSL-3 ag) facilities are recommended. Virus isolation remains a performance or benchmark standard for other diagnostic tests and virus isolation remains a critical part of the initial diagnosis of AIV during an outbreak.

Methods used for influenza A identification in birds should be specific enough to allow detection of antigenically and genetically different influenza subtypes. Among them, the RT-PCR technique is widely used to detect influenza viruses directly in specimens collected from animal species susceptible to influenza virus infection and from humans (Fouchier *et al*., 2000).

Polymerase Chain Reaction (PCR) methods have been described that is up to 100 fold more sensitive than virus isolation procedures. This technology promises to revolutionize influenza diagnosis and monitoring (Fouchier *et al*., 2000; Swayne and Halvorson, 2003). Also It was found that PCR-based methods of higher sensitivity than commercial Antigen Capture Enzyme Immunoassay (AC-EIA) (Cattoli *et al*., 2004) in detection of AI. Also the sensitivity of RT-PCR has been reported to be in the range of 90% to 100% when compared with cell culture; however, several researchers have reported significantly higher numbers of total positive specimens with RT-PCR, possibly reflecting its ability to detect nonviable virions (Hayden and Palese, 2002; Pachucki *et al*., 2004). Molecular diagnostics all share the same basic goal of amplifying nucleic acid to high levels to allow easy identification of the sample. Several different types of molecular diagnostic tests are available; the most commonly used are traditional Reverse transcription-Polymerase Chain Reaction (RT-PCR) (Lee *et al*., 2001) and real-time RT-PCR RRT-PCR that were developed in the last decade for rapid detection of influenza viral RNA in clinical and laboratory specimens. They are generally very sensitive, specific and adaptable to high throughputs.

PCR-based and sequencing protocols are available to detect subtype and pathotype of the virus directly on clinical materials, thus allowing a rapid turnaround time and faster characterization (Cattoli and Capua, 2006) RRT-PCR has been described to be 100 fold more sensitive than virus isolation procedures. This technology promises to revolutionize influenza diagnosis and monitoring (Fouchier *et al*., 2000).

The aim of this study was to compare the specificity and sensitivity of 2 different formats of conventional RT-PCR (one and two steps) and 3 different formats of RRT-PCR (one step using TaqMan probe, two steps using TaqMan probe and two steps using hybridization probe) and make a comparison between these types of PCR formats as a diagnostic tools for H5N1 virus detection.

MATERIALS AND METHODS

Egyptian strain of Avian influenza (H5N1) that has been isolated in the National laboratory for Veterinary Quality Control on Poultry Production (NLQP) (A/chicken/Egypt/06553-NLQP/2006 (H5N1) of gene bank accession no. EU496383, it has 5.71×10^5 numbers of DNA copies in relation to standard of Roche system, this strain was used in specificity and sensitivity testing. The standard of Roche system is in the form of a row with 6 different DNA concentrations from 101 to 106 copies of lyophilized cloned and purified DNA of AI (H5N1) subtype Asia and this row reconstituted with 40 µL PCR grade water, then use 5 µL from each concentration for a 20 µL PCR reaction to make the standard curve, then make 10 fold serial dilution to this isolate to detect its concentration in relation to this standard curve (Fig. 1).

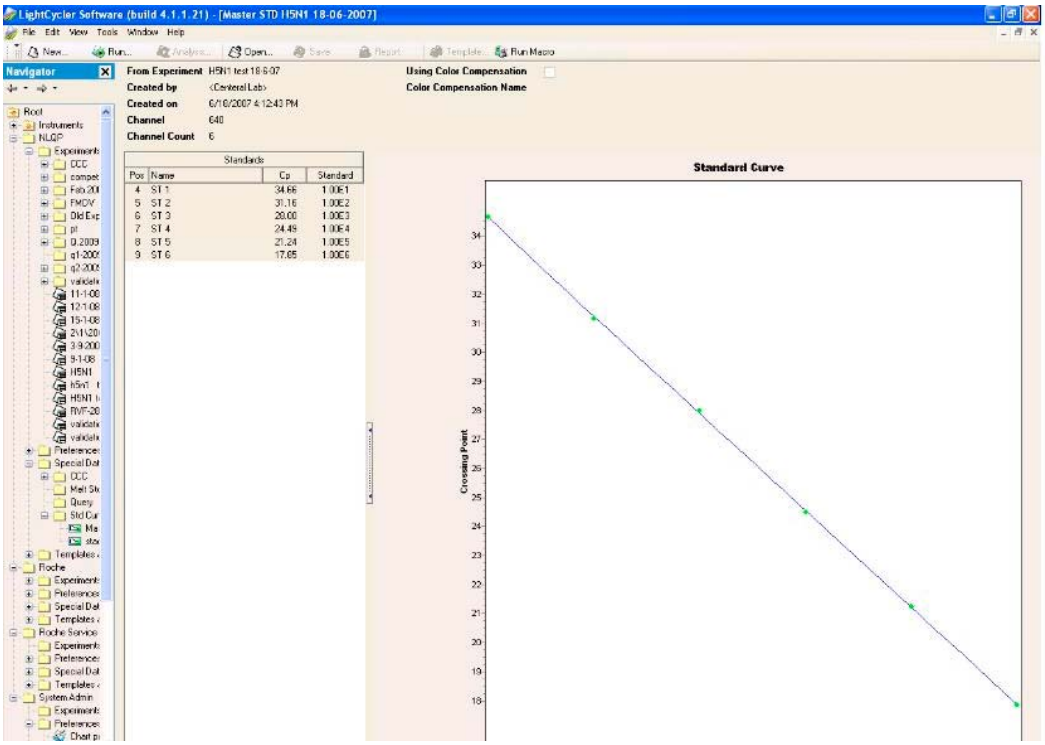

Fig. 1: Standard curve using panel of standard concentration of DNA

Three avian influenza strains which are H9N2, H7N3, H7N1, other avian vaccine viruses (*Infectious laryngeotrachitis virus ILTV*), *Infectious bronchitis* (*IBV*), *Newcastle disease virus* (*NDV*) and reference strains of (*Mycoplasma gallisepticum* and *Staph. aureus*). One step was conducted in (strategen) machine while two steps were conducted in both Strategen and Roche thermal cyclers.

The examined 51 field Samples included tracheal and cloacal swabs were tested for the presence of avian influenza from different avian species which included broiler breeders, layers, broilers, ducks, geese, turkey and quail flocks.

Conventional RT-PCR: Primers used were according to (Spackman *et al*., 2002) H5-Kha-1: CCTCCAGARTATGCMTAYAA AATTGTC H5-Kha-3: TACCAACCGTCTACCATKCCYTG The primers used were specific for the cleavage site of H5 gene and manufactured by METABION (Germany) and delivered in a lyophilized form. Reconstitution of the primers was carried out in nuclease free water buffer to prepare concentrated stocks. Working solutions of 20 pmol were prepared by individual dilution of the primer stocks in nuclease free water.

- Uni-12:5-AGC AAA AGC AGG-3

H5 Primers and probe used for real time PCR:

- H5LH1: ACA TAT GAC TAC CCA CAR TAT TCA
- H5RH1: AGA CCA GCT AYC ATG ATT GC
- H5 Probe: FAM-TCW ACA GTG GCG AGT TCC CTA GCA- TAMRA

According to (Spackman *et al*., 2002) One step Reverse Transcriptase- Polymerase Chain Reaction (RT-PCR). The samples were tested by RT-PCR for subtype H5 avian influenza virus. Briefly, RNA was extracted from pools of cloacal and tracheal swabs by using virus RNA Extraction Kit (QIAGEN, Valencia, Calif., USA). Samples were amplified using a One-Step reverse transcription-PCR(RT-PCR)kit (Quantitect Probe RT-PCR Kit (Cat. No. 204443) (Qiagen) with Rt-enzyme ACCESS Quick RT- PCR SYSTEM (RT-PCR kit). Cat.No #A1702 (Promega) in a 25 μL reaction mixture containing 12.5 μL of kit-supplied mix and 20 pmol of each primer, 0.1 μL from Access quick RT- Enzyme, 4.5 μL DEPC water and five microliters of each sample and control RNAs were amplified using the Thermocycler (T3 Biometra). The RT-PCR program consisted of 30 min at 50°C and 15 min at 95°C and a three- step cycling protocol was used as 95°C for 30 s, 56°C for 45 s and 72°C for 2 min for 40 cycles and final extention at 72°C for 10 min.

Two step Reverse Transcriptase-Polymerase Chain Reaction (RT- PCR): Using ACCESS Quick RT-PCR SYSTEM (RT-PCR kit). Cat. No #A1702 (Promega) FOR First Strand cDNA Synthesis.

This kit is composed from all the reagents required for first strand cDNA synthesis and act by using 15 μL from mix supplied with the kit with 100 pm primer, 0.6 AMV RT-enzyme, 0.4 Nuclease free water and 4 μL from RNA giving 30 μL from cDNA using the Thermocycler (T3 Biometra) The RT program consisted of 45 min at 45°C and 5 min at 92°C and then make amplification of this cDNA by PCR in a 25 μL reaction mixture containing 12.5 μL of kit-supplied mix and 20 pmol of

each primer, 5.5 μL DEPC water and five microliters of cDNA that amplified using the Thermocycler (T3 Biometra) The PCR program consisted of 15 min at 95°C and a three-step cycling protocol was used as 95°C for 30 s, 56°C for 45 s and 72°C for 2 min for 40 cycles and final extention at 72°C for 10 min.

One step real time RT-PCR (RRT-PCR) using taqMan probe: Real Time PCR kit used is Quantitict probe RT-PCRF or quantitative, real time, one step, RT-PCR using sequence Specific probe .with cat no.204443 (Qiagen) in a 25 μL reaction mixture containing 12.5 μL of kit-supplied mix and 0.2 μL from 30 pmol of each primer, 0.25 μL from H5 probe 50 pm, 0.25 μL from Access Quick RT-Enzyme and 6.6 μL DEPC water and five microliters of RNA that amplified using Stratagen PCR machine The RT-PCR program consisted of 30 min at 50°C and 15 min at 95°C and a three-step cycling protocol was used as 95°C for 10 s, 54°C for 30 s and 72°C for 10 sec for 40 cycles.

Two step Real Time RT-PCR (QRT-PCR) using TaqMan probe: Two step real time PCR kit used is Quantitict probe RT-PCR For quantitative, real time, two step, RT- PCR using sequence Specific probe. with cat no.204443 (Qiagen) in a 25 μL reaction mixture containing 12.5 μL of kit-supplied mix and 0.2 μL from 30 pmol of each primer, 0.25 μL from H5 probe 50 pm and 6.76 μL DEPC water and five microliters of cDNA that amplified using Stratagen PCR machine and PCR program consisted of 15 min at 95°C and a three-step cycling protocol was used as 95°C for 10 s, 54°C for 30 s and 72°C for 10 sec for 40 cycles.

Two step Real Time RT-PCR (RRT-PCR) using hybridization probe:
Roche system was used for detection of H5N1 gene as follow: cDNA synthesis test was done by using Transcriptor First Strand cDNA Synthesis Kit (Roche, Germany). 20 μL reaction mixture containing 10 μL Total RNA or poly (A) +mRNA, 2 μL Primer (random Hexamer Primer) 4 μL Water, PCR- grade, 1 μL Transcriptor Reverse (AMV) Transcriptase Transcriptase, 0 The RT reaction was incubated 10 min at 25°C, followed by 30 min at 55°C. Transcriptor Reverse Transcriptase was inactivated by heating to 85°C for 5 min. The reaction was stopped by placing the tube on ice., then the cDNA amplified using Light Cycler 2.0 Instrument (Roche, Germany) and Light Cycler Capillaries in 20 μL reaction mixture contain 7 μL Water PCR-grade, 4 L HybProbe mix, 4 μL Light Cycler Fast Start DNA Master HybProbe and 5 μL cDNA using this thermal profile 10 min at 95°C and a three-step cycling protocol was used as 95°C for 10 s, 55°C for 15 s and 72°C for 15 sec for 54 cycles.

RESULTS

Specificity testing for AI H5 gene of one and two steps conventional RT-PCR: The specificity of the one step and two steps conventional PCR was verified by testing RNA OR DNA extracted from different pathogens. One and two steps conventional RT- PCR yielded specific band at 300 base pair in gel electrophoresis only for H5 gene of the Egyptian field strain (A/chicken/Egypt/06553- NLQP/2006(H5N1)) and didn't amplify DNA from other tested pathogens.

Specificity testing for AI H5 gene of one and two steps real time RT-PCR using Taq Man and hybridization probes: The specificity of the one step and two steps real time PCR was verified by testing the same bacterial and viral agents as in conventional PCR method. All real-time RT-PCRs either one or two steps were successful to amplify target H5 gene of avian influenza only

with no amplification detected in samples from other AI strains, bacterial and viral agents tested in this study.

Sensitivity test for H5 gene of one step and two steps conventional PCR: The test was carried out on avian influenza H5N1 isolate (A/chicken/Egypt/06553- NLQP/2006 H5N1). The concentration of this isolate is 5.71×10^{5} in relation to Roche standard curve, this type of quantification is absolute quantification as also the concentration of the isolate in relation to this curve was estimated.

One step and two steps real time RT-PCR Sensitivity test for H5 gene using Taq Man probe: The result of the *avian influenza H5N1* one step real-time PCR assay showed positive amplification signals with FAM dye for the original isolate and the first four dilutions From 10-^{1} to 10^{-4}, while the result of the avian influenza H5N1 Two steps real-time PCR (using TaqMan probe) assay showed positive amplification signals with FAM dye for the original isolate and the first three dilutions 10^{-1} to 10^{-3}, this may be due to using of gene specific primer in RT-step in one step real time PCR which may affect the sensitivity of the technique. Woolcock and Cardona, (2005) indicate that gene specific primers used in the one step kit may have been more efficient at generating full-length cDNA than the random hexamers and oligo dT primers in the two-step kits providing the use of the one-step method for increased sensitivity of detection of certain genes than two steps. From this result it was found that the sensitivity of one step RRT-PCR was 10 folds higher than two steps RRT-PCR using the same kit.

Two steps real time RT-PCR Sensitivity testing for H5 gene using both Taq Man probe and hybridization probe: The result using (TaqMan probe) showed positive amplification signals with FAM dye for the original isolate and the first three dilutions 10^{-1} to 10^{-3}, while The result of using (hybridization probe) showed positive amplification signals with FAM dye for the original isolate and the first four dilutions 10^{-1} to 10^{-5}.

Comparison of different types of RT-PCRs for field samples: Samples were collected from different avian species including tracheal and Cloacal swabs as 51 pooled samples. These samples were examined by one and two steps real time RT-PCR TaqMan probe, also real time RT-PCR using hybridization probes shown in Table 1 and Fig. 2 and 3.

Table 1: Comparison of different types of Real time RT-PCRs on field samples

Results	Two steps RRT- PCR hybridization probe	One step RRT- PCR Taq Man probe	Two steps RRT-PCR Taq Man probe
Negative (no Ct)	0	5	13
Positive (Ct 10-15)	10	6	2
Positive (Ct 15-20)	11	13	10
Positive (Ct 20-25)	6	5	7
Positive (Ct 25-30)	11	9	6
Positive (Ct 30-35)	13	10	10
Suspected (Ct 35-40)	0	3	3
Total	51	51	51

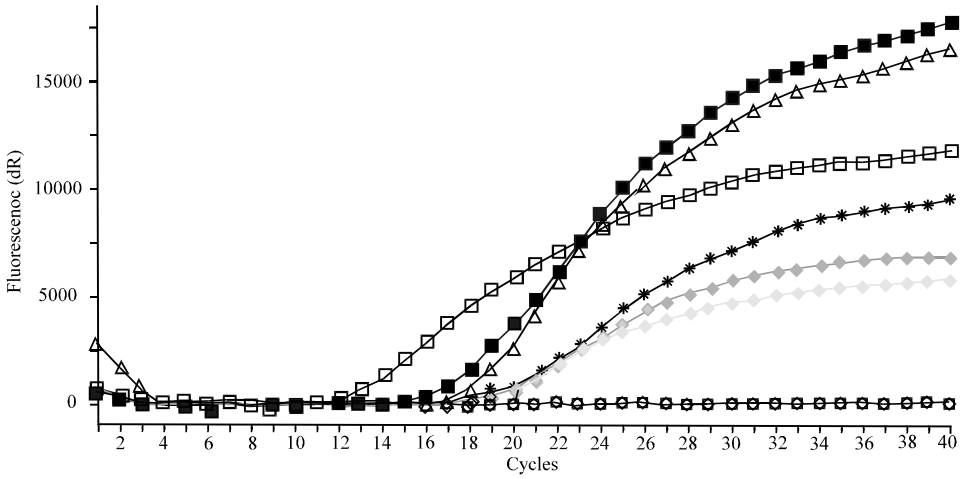

Fig. 2: Amplification curves of some positive H5 field samples and for positive control while there is no amplification result for the negative control using Taqman probe on Stratagen machine

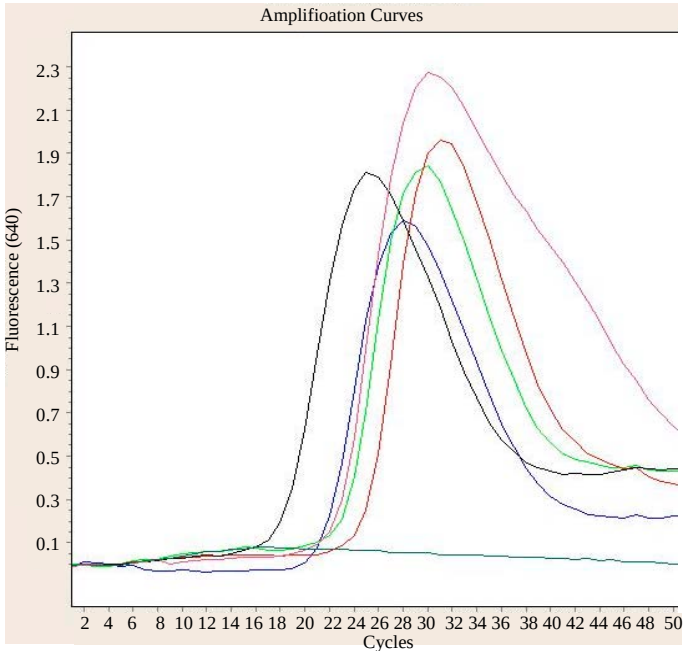

Fig. 3: Amplification curves of some H5 positive field samples and for positive control while there is no amplification detected for negative control, using, Hybridization probe on Lightcycler, Roche

Comparison of one and two steps conventional PCR upon field samples: We find that by using one step conventional RT-PCR 29 samples from 51 give positive results, while by using two step conventional PCR only 22 samples give positive results as shown in Fig. 4.

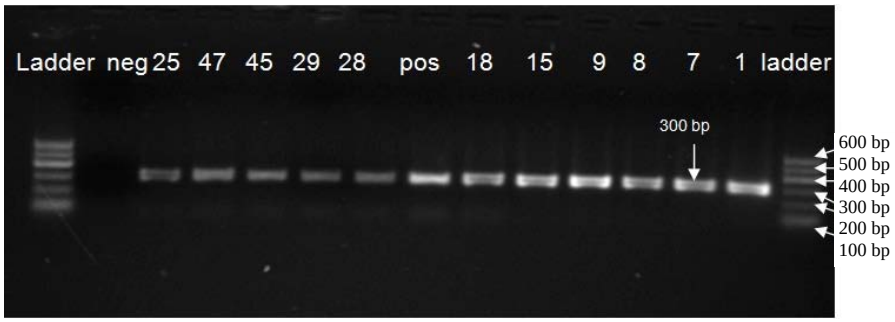

Fig. 4: The positive amplification of 300 bp bands of some field samples and for positive control while there is no band for negative control

DISCUSSION

The widespread occurrence of HPAI of subtype H5N1 in Egypt (Aly *et al*., 2007) and the potential of the virus to cross- species and infect humans pose major threats to human and animal health in the country. The best is to control the disease in birds, so that rapid diagnostic capability for H5N1 diagnosis is crucial for diagnosis, facilitating timely implementation of control measures. Standard RT- PCR has been previously applied to the detection of avian influenza virus and each of the 15 HA subtypes (Lee *et al*., 2001; Munch *et al*., 2001; Starick *et al*., 2000). Additionally, an RRT-PCR assay for influenza virus has been developed; such as a two-steps RT-PCR, multiplex assay based on human influenza virus sequences for the detection of influenza virus types A and B (Van Elden *et al*., 2001). Therefore rapid, highly specific and sensitive assays are required in avian influenza virus diagnosis. (Di Trani *et al*., 2006).The use of conventional RT PCR will continue to be used to diagnose avian influenza because the technology is widely available and the test can provide high sensitivity and specificity (Suarez *et al*., 2007). RRT-PCR is the technique of choice for AI diagnosis as it requires swab sample (cloacal or tracheal) completely machine dependant for preparation and reading of results, requires only 2.5 h, highly sensitive, risk of contamination is very low. Generally the real-time PCR system is based on the detection and quantitation of a fluorescent reporter product in a reaction (Lee *et al*., 1993; Livak *et al*., 1995). By recording the amount of fluorescence emission at each cycle, it is possible to monitor the PCR reaction during exponential phase where the first significant increase in the amount of PCR product correlates to the initial amount of target template. The higher the starting copy number of the nucleic acid target, the sooner a significant increase in fluorescence is observed.

A significant increase in fluorescence above the baseline value measured during the 3-15 cycles indicates the detection of accumulated PCR product. In general TaqMan® has been considered to be more sensitive when detecting low copy numbers (<10 copies) because of its ability to resolve the signal of a single copy of template (Wittwer *et al*., 1997).

One additional advantage of TaqMan® is that the probe offers an added layer of specificity in addition to the forward and reverse primers. The probe sequence must exactly match the target sequence to which it binds, as a single nucleotide difference in the probe sequence will prevent the cleavage event necessary to generate a reporter signal (Spackman *et al*., 2002).

In the present study we compare the sensitivity of one step or two steps Real time RT-PCR with conventional RT-PCR either one step or two steps in diagnosis of avian influenza. To validate RRT-

PCR assay, it was necessary to test the specificity of the RRT- PCR assays (either one or two step RRT-PCR using Taqman probe) and RRT-PCR using hybridization probe) in comparison to conventional RT-PCR assays (either one step or two steps).

The results showed positive amplification of RNA or DNA of H5 gene by RRT-PCR using one step and two steps and the RT-PCR using one steps and two steps techniques and no amplification was detected in other strains of AI as H9N2 and H7N1, H7N3, bacterial strains as (*Mycoplasma gallisepticum*, *Staph. aureus*) and other viruses as (NDV, IBV, ILT). So, the specificity of the RRT-PCR and RT-PCR was the same and these results coincide with that obtained by (Di Trani *et al*., 2006).

In the present investigation, the sensitivity of real time RT-PCR in comparison with conventional RT-PCR was done by performing 10 fold serial dilution of a confirmed AI (H5N1) isolate and It was found that The sensitivity of avian influenza (H5N1) one step RRT- PCR using Taq Man® Probe was 10 folds higher than conventional one step RT-PCR, while The sensitivity of avian influenza (H5N1) one step RRT-PCR using Taq Man® Probe was 100 fold higher than conventional two steps RT-PCR, also The sensitivity of *avian influenza (H5N1) two steps R*RT- PCR using hybridization® probe was 100 folds higher than one step of the RT-PCR using TaqMan® Probe, The sensitivity of avian influenza (H5N1) two steps *RRT*- PCR using hybridization® probe was 1000 folds higher than two steps of the RT-PCR, finally the sensitivity of one step RRT-PCR using TaqMan® Probe was 10 fold higher than two steps RT-PCR using TaqMan ®Probe. In addition the sensitivity of two steps RRT- PCR using hybridization® probe was 100 fold higher than two steps RRT-PCR using TaqMan ®Probe with and 10 fold higher than one step RRT-PCR using TaqMan® Probe.

So in this study the two steps RRT-PCR (hybridization ®probe) was more sensitive than one step RRT-PCR and this agree with (Battaglia *et al*., 1998) that confirmed the RT step is critical for sensitive and accurate quantification and the amount of DNA produced by the reverse transcriptase must accurately represent RNA input amounts and using two tube/two enzyme based protocols is more sensitive than using one enzyme based protocols, also using hybridization® probes in this system play an important role in improvement of its sensitivity and specificity.

Also these results agreed with (Di Trani *et al*., 2006), who stated that The RT-PCR is capable to detect all tested influenza A viruses with analytical sensitivity of 10-100 times higher than conventional PCR. Also (Fouchier *et al*., 2000) reported that the RRT-PCR was higher in sensitivity than RT-PCR more than 1000 times.

In this study and after establishment of the sensitivity and specificity of RRT-PCR under experimental conditions, fifty one field samples were tested in order to confirm the result of the experimental work and to compare RRT-PCR with RT-PCR for diagnosis of avian influenza virus.

Sequence variation in the H5 gene may also explain why the RT- PCR or RRT-PCR tests failed to detect viral RNA in some of the virus-positive samples (Spackman *et al*., 2002). These results confirmed the results obtained in the sensitivity test of the validation step and this could be due to the use of florescent dye-labeled probe that increases the sensitivity of one step real time PCR as in this system we use Taqman probe also using of hybridization probes increase the sensitivity of Roche system than others, also due to the determination of the CT value within the logarithmic phase of the amplification reaction, instead of the end point determination used by conventional systems, also detection of result by a computerized system in RRT-PCR is much better than visual detection of bands in RT-PCR.

The turnaround time for data acquisition and data analysis by RRT-PCR is therefore short, it becomes quickly obvious to diagnosticians that the chemistry and platform system of RRT-PCR had

much to offer with respect to turn-around time, repeatability, sample throughput and in limiting contamination. Results of RRT-PCR are reliable, fast accurate although it is expensive it has superior sensitivity (Steininger *et al*., 2002). It can also be used to differentiate between subtypes and conduct phylogenetic analysis (Allwinn *et al*., 2002).

In conclusion, rapid and accurate diagnosis of Avian influenza (H5N1) in poultry is considered one of the most important tools used for the controlling the disease which considered one of the most important disease in the world. This study started by comparing the specificity of RT-PCR (one step and two steps) and RRT-PCR (one step and two steps) and we found that all tests had the same specificity as they showed positive results for Avian influenza (H5N1) strain, while they showed negative results for Avian influenza other than H5N1, viral and bacterial strains affecting respiratory tract of poultry.

In conclusion the results of testing of 51 field samples showed that 45 field samples were positive by using one step real time PCR TaqMan ®probe. However, when using two steps real time PCR TaqMan ®probe only 34 field samples were detected, also when using one step conventional PCR, there were 28 field samples detected and by using two steps conventional PCR, 23 field samples were detected.

The real time PCR using hybridization ®probe was the most sensitive for detection of Avian influenza H5N1 as it detected 51 positive samples.

From this work we found that real time PCR is more advantageous than conventional PCR due to the highest sensitivity of Real time PCR than conventional PCR by 10 to 1000 folds. Also, real time PCR is faster in diagnosis than conventional PCR and can be done within 2 h, while RT-PCR takes much more longer. Real-Time Amplification has also the advantage that the workload is minimized. beside it minimize use of ethidium bromide dye. The accuracy in the interpretation of the results of RRT-PCR than RT-PCR and the capability to make both a qualitative and quantitative detection of the target. The risk of contaminating the work environment is therefore strongly reduced as well as this makes data more safer and reliable.

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