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RESEARCH

Sensitivity of Real Time –PCR in comparison with conventional methods for detection of AI virus

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ABSTRACT

Background: *Avian influenza virus* (AIV) infection, an important but difficult pathogen to control in poultry. Vaccination might reduce the risk of bird infection virus transmission by reducing virus shedding. Challenge of vaccinated birds with AI vaccines under strictly controlled condition with virulent HPAI virus may also be used to predict flock response to AI infection. The reduction in challenge virus replication can be quantified using classical virus isolation (VI) and titration methods in (ECE) or by molecular assay for detection AI virus specific nucleic acids such as (rRT-PCR).

Methods: Titration of the HPAI virus for infection of SPF chicken. The tracheal swabs were collected from diseased chicken for virus isolation and quantification by ECE inoculation and rRT-PCR.

Results: the titer of original HPAI virus was $10^{10.3}$ EID50/ml. The AI titers after experimental infection of chicken and viral re-isolation were $10^{10.3}$ EID50/ml in ECE and 1.6×10^{7} copies/ml by rRT-PCR to sample (1), $10^{5.4}$ EID50/ml in ECE and 1.2×10^{6} copies/ml by rRT-PCR to sample (2), and $10^{6.2}$ EID50/ml in ECE and 4×10^{5} copies/ml by rRT-PCR to sample (3). The egg inoculation method detect a gradual decrease in percentage of positive egg for AI infection which were 100, 80, 80, 60,20 and 0 %for the viral dilutions 6,7,8,9,10 and11 respectively. While the rRT-PCR give 100% positive results with all the sample dilutions. The reduction in viral shedding with values of 3.4, 2.9 and 2.4 when measured by ECE from the vaccinated chicken with inactivated H5N1, H5N2 and inactivated recombinant vaccines respectively. While, the ranges from 10^{2}, 10^{2} and 10 when measured by rRT-PCR respectively. Also, the live recombinant vaccine could not reduce the viral shedding when measured by both egg inoculation and rRT-PCR.

Conclusion: Depending on the previous phenomena this study showed that despite the expensive and latency of VI, it is more sensitive and accurate for detection of AI infection and titer of viral shedding in vaccinated chicken.

Keywords: AIV; comparison; detection; ECE; rRT-PCR

BACKGROUND

Avian influenza virus (AIV) infection, in particular the highly pathogenic H5 and H7 subtypes, leads to high economic losses for poultry production worldwide. These losses are due to elimination of marketable birds, either from the disease itself or from culling of potentially exposed birds, and trade restrictions imposed on those countries where infections occurred. All these factors make AIV an important but difficult pathogen to control in poultry. Vaccination might reduce the risk of bird infection virus transmission by reducing virus shedding. Vaccination against AI has proven to be a successful additional controlled measure implemented alongside controlled culling (Capua and Alexander, 2004).

Inactivated whole virus vaccines were considered the main type used for protection against AI infection. They were licensed widely by several countries and have proven efficacy. Other types of vaccines have been developed for AI using alternative recombinant live vectored constructs and can provide some of the immunological advantages of live vaccines but without the reassortant risk of live AI virus (Swayne, 2008).

Challenge of vaccinated birds with AI vaccines under strictly controlled condition with virulent HPAI virus may also be used to predict flock response to AI infection. Also, this method

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can add considerable significance to test the immune efficacy of AI vaccines depending on the vaccine ability to prevent the disease and death (Ernesto, *et al*., 2010). The prevention of infection or the qualitative and quantitation reduction in virus replication in respiratory and digestive tract, is essential protective criteria that indirectly assess the role of the vaccine to limit field virus spread (Beard, 1992; Swayne, *et al*., 1997; Swayne, *et al*., 1999; Capua, *et al*., 2004 and Swayne, 2008). The reduction in challenge virus replication can be quantified using classical virus isolation (VI) and titration methods in Embryonated chicken eggs (ECE) (Stone, 1987 and Swayne, *et al*., 1997) or by molecular assay for detection AI virus specific nucleic acids such as rRT-PCR (Lee and Suarez, 2004).

So, this study aimed to compare between VI and rRT-PCR efficacies for the detection and quantitation of the AI viruses or reduction of viral shedding from the vaccinated birds.

MATERIALS AND METHODS

Experimental host:

a) Embryonated chicken eggs:

Specific pathogen free embryonated chicken eggs (SPF ECEs), 9-11 day old were obtained from Kom Oshim farm for SPF-eggs, El-Fayoum, Egypt. They were used for virus re-isolation and titration and for performing titration of live vector vaccine.

b) Experimental birds:

SPF chickens were obtained from Kom Oshim farm as one day old. They were maintained at Central Laboratory for Evaluation of Veterinary Biologics (CLEVB), Abassia, Cairo and housed in positive pressure stainless steel isolation cabinets with continuous light exposure till used.

Virus:

Local HPAI field isolate was used in experimental infection and challenge test. It was isolated and identified by National Lab for Quality Control of Poultry (NLQP) as A/ch/Egypt/1709-6/2008 (H5N1). It was identified as escape mutant serotype of Egyptian HPAI H5N1 and related to class 2.2.1.1. Many samples of that virus were used in the infection of chicken either control or vaccinated different type of AI vaccines.

Vaccines

a- Inactivated H5N1 AI Vaccine:

Inactivated Egyptian H5N1 AI Vaccine; the imported inactivated oil emulsion reassortant Avian Influenza vaccine, EgyFlu-1 was produced by Harbin Veterinary Research Institute (HVRI), China. The vaccinal strain is A/chicken/Egypt/A-18-H/09. It was administrated subcutaneously at the lower third of the neck in a dose 0.3ml /bird.

b- Inactivated H5N2 AI Vaccine:

The inactivated oil emulsion LPAI H5N2 vaccine was produced by Boehringher Ingelheim VetMedica, Guadalajara, Mexico. The vaccinal Strain is A/Chicken/Mexico/232/94/CPA. It was administrated by the subcutaneous (s/c) at the lower third of the neck in a dose of 0.5ml/bird.

C- Recombinant AI vaccines:

a- Live recombinant fowl pox-AI vaccine:

It is a lyophilized vaccine containing a suspension of live recombinant fowl pox virus used as a vector containing an insert of HA subtype H5 gene of AI virus. It was produced by Boehringer Inglhiem Vet Medica S.A de C.V. Guadalajara, Merial, France. The H5 insert is

derived from Al vaccinal strains A/chicken/Ireland/83 (rFP-AI-H5)**.** The vaccine was administrated at one day old chicks subcutaneously in a dose of 0.2ml/bird.

b- Killed recombinant ND-AI vaccine:

It is an oil emulsion inactivated recombinant ND-AI vaccine. It was provided by Avimex Animal Health, Mexico. The rNDV-H5 vaccine is an NDV (LaSota strain) vector expressing a modified Egyptian heamagglutinin (H5) gene ectodomain from an HPAI A/Chicken/Egypt/1063/2010. The vaccine was administrated S/C in a dose of 0.5ml/bird.

Culture media for swabs processing:

The used media was tryptose phosphate broth code No.0060-01-Difco laboratories, Detrial, Michigan, USA. It was used in cultivation of fecal and oropharyngeal swabs for viral shedding detection or viral reisolation.

Titration of original AI virus:

It was done according to OIE (2017). The HPAI virus was serially diluted as 10 fold starting from 10^{-1} till 10^{-10}. Five SPF ECEs were inoculated with each viral dilution. Dead eggs were collected and chilled to the end of titration. Living as well as dead embryos were tested using rapid HA test and the virus titer was calculated according to Reed and Muench (1938).

Experimental infection of SPF chicken by Egyptian HPAI virus:

It was done according to OIE (2017). Ten SPF chicken, 4 weeks old for each viral sample strain were infected experimentally by injection of 0.1ml of the virus intranasal. The infected chickens were examined daily for any deaths or clinical signs of AI through the observation period (10 days). Tracheal swabs were collected from diseased chicken for virus isolation and quantification by ECE inoculation and rRT-PCR.

Estimation of viral shedding:

It was performed according to Spackman, *et al*. (2002). Two groups of SPF chicken one day and 4weeks old were vaccinated with the recommended dose according the manufacture companies of recombinant and inactivated vaccines respectively through the subcutaneous route. The vaccinated and control groups were challenged intranassaly with the HPAI virus 4 weeks post vaccination. The challenge dose was adjusted to be 10^5 EID_{50}/0.1ml. All bird was observed daily for 10 days post challenge for any AI signs. The viral shedding assessments were recorded during observation period second day post challenge, through collection of oropharyngeal and tracheal swabs in tryptose media. Estimation of the viral shedding was performed either by virus re-isolation in ECE or by rRT-PCR as follows:

Virus re-isolation:

a. **Inoculation of embryonated chicken eggs:**

Ten-fold dilution (10^{-1}-10^{-7}) for each tracheal swab sample was done. A volume of 0.2 ml from each dilution was inoculated into five,10-day-old, SPF ECE via the allantoic sac route according to the methods previously described by (OIE, 2017), incubated at 37^{o}C for 5 days with daily candling. Allantoic fluids (AF) from both dead and live embryos were collected, purified and tested for the presence of haemagglutinating (HA) activity at end of incubation period using rapid HA test.

b. **HA test:**

It was done according to (Swayne, *et al*., 1998). This method is based on the reaction between HA activity of virus and RBCs. If viral replication has occurred in the SPF-ECE, the allantoic fluid will contain virus particles with HA activity. The later can be visualized by adding a drop of allantoic fluid (AF) to a drop of 10 % RBCs suspension. The resulting reaction is macroscopically visible.

Quantitative real-time RT-PCR

For detection of viral shedding, oropharyngeal swabs were collected at 2 days post-challenge (DPC) and were processed for rRT-PCR according to (Das, *et al*., 2009). The viral RNA was extracted using RNA extraction kit (QiAamp viral RNA mini kit, Qiagen # 52904). Then the rRT-PCR was conducted according to the rRT-PCR kit instructions (Quanti Tech prabe RT-PCR kit,Qiagen # 204443) using 2 pairs of H5-HA specific primers and probes as shown in Table (1).

The RT-PCR reaction scheme was one cycle at 50°C for 30min, one cycle at 95°C for 15min and 40cycles (95°C for 10 seconds, 54°C for 30 seconds and 72°C for10 seconds).

Table (1): Oligonucleotide sequences of primer and probes.

Virus	Primer/Probe Sequences(5 ′ -3 ′)			Reference
AI (Hb)	Primers	H5LHI	5'- ACATATGACTACCCACARTATTCAG 3'	LÖndt.*et al*.2008
		H5RHI	5'- AGACCAGCTAYCATGATTGC 3'	
	Probe	H5PRO	(FAM)TCWACAGTGGCGAGTTCCCTAGCA(TAMRA)	

Experimental design:

110 SPF chickens were used in a comparative study between VI and rRT-PCR method for detection of AI virus either after AI infection or challenge of vaccinated birds with different types of AI vaccines. The chickens were divided into 2 experimental groups (A and B). The 1st group (A) was divided into 3 subgroups (10birds/each) and used for experimental infection with 3 samples of HPAI virus. The 2end group (B) was divided into 5 subgroups. Four subgroups (15 birds/each) were used for the vaccination with different AI vaccines then challenged with HPAI virus, while the 5^{th} subgroup (10 birds) was left as a control unvaccinated infected groups, as shown in table (2).

Table (2): Experimental design.

Group	subgroup	Birds No.	Treatment
A	1	10	Infected with viral sample (1) collected for VI
	2	10	Infected with viral sample (2) collected for VI
	3	10	Infected with viral sample (3) collected for VI
B	4	15	Vaccinated with Inactivated Egyptian H5N1 AI Vaccine
	5	15	Vaccinated with Inactivated oil emulsion LPAI H5N2
	6	15	Vaccinated with Live recombinant fowl pox-AI vaccine
	7	15	Vaccinated with Killed recombinant ND-AI vaccine
	8	10	Control unvaccinated and challenged with HPAI virus

RESULTS

Results of original AI virus titer

It was observed that the titer of original HPAI virus was $10^{10.3}$ EID50/ml.

Results of viral titer after experimental infection

The results of AI titers after experimental infection of chicken and viral re-isolation were showed in Table **(3).**

The titers of sample (1) were$10^{10.3}$ EID_{50}/ml and 1.6×10^{7} copies/ml when examined by egg inoculation and rRT-PCR respectively. Meanwhile, the original titer before infection was 10^{10} EID_{50}. Also, it was found that the viral titer of sample No. (2) was $10^{5.4}$ EID_{50}/ml in ECE

and1.2×10^6 copies/ml by rRT-PCR in comparison to its original titer which was10^5 EID_{50}. On the other hand, the titer of sample (3) after estimation by ECE inoculation or by rRT-PCR was $10^{6.2}$ EID_{50}/ml and 4×10^5 copies/ml respectively, although its original titer was10^6 EID_{50}.

Table (3): AI titers after experimental infection of chicken and viral re-isolation

AI virus	Original Titer (EID50)	Titer in ECE (EID50/ml)	Titer by rRT-PCR (copies/ml)
Viral sample (1)	10^{10}	$10^{10.3}$	1.6×10^7
Viral sample (2)	10^5	$10^{5.4}$	1.2×10^6
Viral sample (3)	10^6	$10^{6.2}$	4×10^5

The assessment of sensitivity VI and rRT-PCR methods

The assessment of the ability of the egg inoculation and rRT-PCR methods in estimation or detection of very small amount of AI viral shedding was cleared in table **(4).**

Table (4): The assessment of sensitivity

Sample no.	Virus titer	Serial dil. Of virus	Assessment of viral samples					
			ECE			rRT-PCR		
			No. of egg	No. of +ve/total	+ve %	No. of sample	No of +ve/total	+ve %
1	$10^{10.3}$	Dil. 6	5	5/5	100	3	3/3	100
2		Dil.7	5	4/5	80	3	3/3	100
3		Dil.8	5	4/5	80	3	3/3	100
4		Dil.9	5	3/5	60	3	3/3	100
5		Dil.10	5	1/5	20	3	3/3	100
6		Dil.11	5	0/5	0	3	3/3	100

It was observed from table (4) that the egg inoculation method can detect a gradual decrease in percentage of positive egg for AI infection which were 100,80,80,60,20 and 0 %for the viral dilutions 6,7,8,9,10 and11 respectively. While the rRT-PCR give 100% positive results with all the sample dilutions.

Detection of viral shedding of vaccinated birds:

The data from table (5) showed that, there was a reduction in viral shedding with values of 3.4, 2.9 and 2.4 when measured by egg inoculation method from the vaccinated chicken with inactivated H5N1, H5N2 and inactivated recombinant vaccines respectively. While, the previous vaccines cause a reduction in viral shedding with low values ranges from 10^2, 10^2 and 10 when measured by rRT-PCR respectively. On the other hand, it was observed that live recumbent vaccine could not reduce the viral shedding when measured by both egg inoculation and rRT-PCR methods (0.6 EID_{50} and 0 copies, respectively).

Table (5): Detection of viral shedding.

Sample no.	Vaccine type	No. of swabs	Viral shedding in 2ndDPC			
			By ECE		rRT-PCR	
			Viral titer (EID50)	Reduction of viral shedding	Viral titer (copies)	Reduction of viral shedding
1	H5N1	5	10^{2}	$10^{3.4}$	1.1×10^{4}	10^{2}
2	H5N2	5	$10^{2.5}$	$10^{2.9}$	4×10^{4}	$\sim10^{2}$
3	Live recombinant	5	$10^{4.8}$	$10^{0.6}$	1.1×10^{6}	0
4	Inactivated recombinant	5	10^{3}	$10^{2.4}$	4.3×10^{5}	~10
5	Control+ unvaccinated	5	$10^{5.4}$	—	1.2×10^{6}	—

DISCUSSION

Avian influenza virus infection is a major cause of poultry morbidity and rapid identification of the virus has important clinical, economical and epidemiological implications (Ng, *et al*., 2006). The detection of AI virus infection or viral shedding may be accomplished by isolation the virus in eggs or cell cultures or through the detection of viral protein, viral nucleic acid or detection of antibody to AI virus (Fedorko, *et al*., 2006). Diagnostic test for AI virus can be classified as type A specific, meaning they can identify any type A influenza virus of avian and mammalian origin (Shafer, *et al*., 1998) , or the test can subtype specific meaning will detect specific subtype and most of them target H5 and H7 haemagglutation subtype (Munch, *et al*., 2001 and Spackman, *et al*., 2002).

For poultry, many tests have been described for virus detection as virus isolation, real time RT-PCR, heamagalutination-inhibition HI and NI tests (Meulemans, *et al*., 1987 and Swayne, *et al*., 1998). The selection and use of diagnostic tests depend on application as well as other factors as cost, sensitivity, specificity, speed, complexity and the availability of human resources (Rimmelzwaan, *et al*., 1998).

This study outlines the comparison between the virus isolation (VI) and rRT-PCR methods for detection of AI virus. It is clear that, the reference standard for the detection of AI virus is VI. Also, the ECE (at 9-11 days) is considered the most widely used system and most sensitive for isolation of AI virus and can be used with all sample types as tissue homogenates, tracheal swabs, fecal swabs and environmental samples (Swayne, *et al*., 1998). In recent years, the application of molecular methods for viral detection has become an important tool for the detection of AI virus and identification of HA and Neuraminidase (NA) subtypes (Collins, *et al*., (2003a,b) and Moore, *et al*., 2004). RT-PCR based test are the most widely used molecular method either rRT-PCR (Spackman, *et al*., 2002) or conventional RT-PCR (Founchier, *et al*., 2000; Podder, 2002 and Starck, *et al*., 2000).

The present work describes the usage of the two methods (VI and rRT-PCR) in detection of AI viral titer of 3 virus samples for experimental infection of chicken table (3). It was noticed that the virus titer were $10^{10.3}$, $10^{5.4}$ and$10^{6.2}$ EID_{50} when measured by VI respectively and approximately identical to the original titer before experimental Infection. While the 3viral samples titers were 10^{7}, 10^{6} and 10^{5} copies respectively when measured by rRT-PCR and noticed that these titers were different than original titer of AI viral samples before infection. These results agreed with Suarez *et al*. (2003) that reported that rRT-PCR method can detect both live and inactivated virus. So, this method may not be appropriate for viral titration where the goal is

to detect the live viruses. So, care must be taken to ensure that cross- contamination is prevented when use molecular methods (Wiedbrauk and Stoerker. 1995).

Moreover, the assessment of efficacy of VI and rRT-PCR methods in detection of very small amount of viral shedding of live AI virus was showed from table (4). It was cleared that VI in ECE can detect tenfold diluted live AI virus with 100% at dil. 6 and reached 20% of live virus at dil. 10. Also, it can't detect (0% of live virus) at dil.11. While, it was showed that rRT-PCR can detect the nucleic material of AI virus with 100% (all samples) in all dilutions starting from dil.6 till dil.11. The above results may be due to VI detect the infectivity titer of live virus while the rRT-PCR based on detection of genetic material of the AI virus which may be alive or dead. This result is also in parallel with that found by Spackman, *et al*., (2002) who said that the sensitivity of rRT-PCR test has been shown to be as low as10^{-1} 50% egg infective doses.

Also, in this study the two methods (VI and rRT-PCR) were used for detection of viral shedding after challenge in vaccinated chicken with different types of AI vaccine as shown in table (5). By using VI method, this study could detect the real reduction in viral shedding from vaccinated chicken after challenge with local HPAI virus. Where the reduction in viral shedding were $10^{3.4}$, $10^{2.9}$, $10^{0.6}$ and $10^{2.4}$ in case of chickens vaccinated with inactivated H5N1, inactivated H5N2, live recombinant and inactivated recombinant vaccines respectively. While rRT-PCR failed to detect real amount of reduction viral shedding from vaccinated birds. The previous data may be due to the rRT-PCR molecular method can affect with cross contamination in comparison with VI. So it gave high titer of viral shedding in samples of vaccinated birds as well as in control group and the reduction of viral shedding of vaccinated bird showed very low in comparison to that detect by VI (Cattoli, *et al*., 2004). While, the VI is more sensitive and accurate for detecting AI virus but there is some practical consideration that should be taken into account as it is relatively expensive and cause amplification of high level of virus leading to exposure of laboratory personal to infectious virus. So, it need to laboratory with enhanced biosecurity as biosafety level (3) BSL3.

Despite all the above disadvantage of VI method, it is used to detect AI virus during any stage of an active infection because of its high sensitivity. Depending on numerous host and virus related factors, AI virus may be detected within 24hrs of infection in an individual bird and for several weeks post exposure in a flock (Swayne and Halvorson, 2003). Although, the possibility false positive results obtained by rRT-PCR in detection of live AI virus, molecular methods can offer numerous advantages for AIV detection as ability to a accommodate any sample type with proper sample processing (Pregliasco, *et al*., 1998), minimization of contact with infectious materials, rapid where results can be obtained in less than 3hrs, reasonable cost, screening of specimens during routine surveillance or during an outbreaks (Atmar, *et al*., 1996; Spackman, *et al*., 2002 and Cattoli, *et al*., 2004). Thus, any positive results should be confirmed by VI and rRT-PCR provides initial information that can be used as the basis for an immediate response (Thayer and Beard, 1998). Also , one of the most critical aspects of implementing diagnostic and evaluation tests for any disease is fitness-for-purpose. A given test may have superior specificity like rRT-PCR, but the rapid and portable nature of Antigen detection kits make the medial for on-farm testing where rRT-PCR must be done in a laboratory (Lee and Suarez, 2004).

Finally, depending on the previous phenomena this study showed that despite the expensive and latency of VI, it is more sensitive and accurate for detection of AI infection and titer of viral shedding in vaccinated chicken.

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