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Comparing real-time RT-PCR to the conventional method for titration of live attenuated avian infectious bronchitis vaccine

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ABSTRACT

Infectious bronchitis (IB) is a highly contagious viral disease that affect poultry flocks all over the world. Inoculation of specific pathogen free (SPF) embryonated chicken eggs (ECE) is the current protocol for evaluating live infectious bronchitis virus vaccines (either variants or classical virus vaccines). Some variants of IBV vaccines do not cause obvious lesions in the inoculated embryos, which hinder the determination of the titer of those vaccines. The current study was designed to standardize a real-time RT-PCR (RT- qPCR) protocol for quantification of IBV vaccines that will provide accuracy, speed, and ease of use. A total of ten IBV vaccines were used, including both variant and classical virus vaccines. After traditional titration with SPF ECE, a classical IBV vaccine (MA5) was used to develop the standard curve for the RT- qPCR, and the end point titer was expressed as EID50. The optimal harvest time for all IBV vaccinal strains was determined to be 42 hours post inoculation (PI). All the IBV vaccines were titrated in SPF ECE and the titer of each vaccine was recorded as EID_{50}. our The results indicate that using RT-qPCR for quantification of IBV in live IBV vaccines offers accuracy, speed and ease of use compared to the traditional titration methods.

INTRODUCTION:

Infectious bronchitis (IB) is a highly contagious viral disease that affects chickens of all ages' upper respiratory tracts (Mo et al. 2019). It was first described in the United States in 1931 and has since spread throughout the world; it is caused by an avian coronavirus, infectious bronchitis virus (IBV) with a global economic impact (Sid et al. 2015, Shehata et al. 2019). This virus has the ability to multiply in the epithelia of a variety of tissues throughout the body, most notably the respiratory system. It can also proliferate in the epithelia of the Harderian gland, kidney, gastrointestinal tract, and oviduct (Hester. 2018) .

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It has several serotypes that cause a variety of clinical forms, primarily affecting the respiratory system (respiratory form), but also the renal system (nephropathogenic form) and the reproductive system (King Amqleamjceb 2012, Cook. 2012).

Secondary bacterial infections have been reported, and other respiratory pathogens may be linked to IBV that lead to high mortality rates (Hassan et al. 2017). IBV is a lipid-enveloped positive-sense single-stranded RNA virus of the genus *Gammacoronavirus* in the family *Coronaviridae* (Jackwood. 2013, Masters. 2006). IBV has a high mutation rate, which causes changes in viral genotype, antigenic properties, tissue tropism, pathogenicity, and, eventually, disease course (Cook 2012). The spike protein, which contains epitopes for serotype-specific antibodies and is the most important protein for virus identification, is the major determinant of IBV serotype specificity (Jackwood 2013, Dhama et al. 2010). Several IBV serotypes or antigenic variant strains have emerged as a result of point mutations in the IBV genome, and these variants are frequently responsible for IB outbreaks in vaccinated chicken flocks (Abdel-Moneim et al. 2012, Liu et al. 2007). As a result, pathogenic variants such as D274 and D1466, 793B, and Israel variants 1 and 2 (Gough et al. 1996, Callison et al. 2001) have emerged in recent decades. IBV was categorized into six main genotypes (from GI to GVI) and 32 sub-genotypic lineages (Valastro et al. 2016). Vaccines are crucial in the control and prevention of IBV in poultry, and vaccination against multiple IBV serotypes is routinely practiced in commercial poultry operations. This virus has been officially classified into more than 50 antigenic and genetic types (Jackwood. 2013, Jackwood et al. 2005). The degree of cross protection between IBV strains can range from very poor to moderate (Lim et al. 2012). The IB is a major problem in the global poultry industry, despite the existence of multiple vaccines due to the various serotypes. Despite the widespread use of live and inactivated vaccines (Oade et al. 2019), IB remains a significant financial problem for the global commercial egg industry (Hester. 2018). In Egypt, multiple IBV genotypes (GI-1, GI-13, GI-16, and GI-23) have been identified, each with unique genetic and pathogenic characteristics. Vaccines of classical and/or variant IBV strains are used in Egypt to control the spread of the disease through various vaccination programs (Ali et al. 2018, Abozeid and Naguib. 2020). Despite widespread vaccination, its epidemiology in poultry flocks with continuous evolution, spread, and evolution has been described, with multiple IBV variants and recombination evidence. Vaccination is still the most effective way to prevent IBV infection. (Kint et al. 2015) reported that the infectious particle quantity in a sample can only be detected by virus propagation in biological systems such as (embryonating chicken egg) ECE or cell culture techniques, and this is referred to as cytopathic effects (CPE) and can be seen by the naked eye or microscope. However, because some virus strains do not induce visible CPE, other assays are required to determine the presence or absence of a live virus and to quantify its titer. According to Wong and Medrano (Wong and Medrano. 2005), quantitative real-time reverse transcriptase polymerase chain reaction assays that quickly identify specific IBV types were developed for accurate and rapid diagnosis of IBV in the field. It has proven to be effective in detecting viral agents of infectious diseases (Mackay et al. 2002).

The effectiveness of TaqMan^{TM}-based quantitative real-time PCR methods for rapidly detection IBV was investigated. The evaluation of amplification efficiency is sensitive, precise, and has a number of advantages, including the absence of contamination and the rarity of qualitative misinterpretations of experimental results (Moriya et al. 2006, Callison et al. 2006). Traditional titration of live IBV vaccines in SPF ECE had many disadvantages including the absence of obvious lesions in the inoculated embryos for some variant vaccines, in addition to the difference in reading and consequently titer calculation from one person to other considering the experience and other issues. So, the purpose of this study is to develop a protocol for the titration of live IBV vaccines for both classical and variant vaccines using quantitative Real-time RT-PCR and to compare it with the conventional method.

MATERIALS and METHODS:

Ethical approval

All methods in the study were performed ac-cord-ing to relevant guidelines and regulations. All exper-iments were carried out according to ARRIVE 2.0 guidelines and were approved by the Institutional Animal Care and Use Com-mittee (IACUC) in the Faculty of Veterinary Medicine, Cairo University (Code: VetCU01102020217)."

Vaccines and Viruses

In this study, ten different IBV vaccine batches representing five different IBV strains were used, as shown in the table (1).

Table 1. Ten IBV vaccine batches, five IBV strains

Code	Vaccine name	Strain
A	IB primer	IB (H120+ D274)
B	Nobilis IB 4/91	4/91
C	IB birde	D274
D	Nobilis IB 4/91	4/91
E	IB birde	D274
F	Mevac IB H120	H120
G	MA5	Cloned H120 (MA5)
H	MA5	Cloned H120 (MA5)
I	Mevac IB H120	H120
J	MA5	Cloned H120 (MA5)

Five variant IBV vaccine from different batches were coded as A, B, C, D, and E, and five classical IBV vaccine from different batches were coded as F, G, H, I, and J.
These vaccine batches were delivered to the Central Laboratory for the Evaluation of Veterinary Biologics (CLEVB) in Abbasia, Cairo, and were evaluated in 2020.

Embryonated chicken eggs

The SPF Production Farm in Koum Osheim, El-Fayoum, Egypt, provided the pathogen-free (SPF) eggs. The eggs were incubated at 37 °C with a humidity level of 40–60%. According to CFR (2017) and OIE, they were used for IBV vaccine titration.

Titration of IBV vaccines in SPF ECEs

Ten-fold serial dilutions of the vaccine ranging from 10^{-1} to 10^{-7} were prepared in ster-ile PBS, pH 7.2, and containing antibiotics .the dilutions 10^{-4} to 10^{-7} were inoculated into 9-11-day-old SPF ECEs using CAS (5 eggs per dilu-tion, 0.1 ml/egg). The eggs were incubated for 7 days at 37 ^{o}C with daily inspections. The em-bryonic deaths that occurred in inoculated ECEs during the first 24 hours were discarded, at the 7th day PI, the inoculated eggs were chilled, and the embryos were removed and examined for the presence of the specific IBV lesions, namely dwarfing, curling, and stunting of the embryo; a dwarfed embryo is defined as one that weighs less than 75% of the normal embryo weight at the corresponding age. IBV is thought to be present in dead embryos. Ac-

cording to **(Reed and Muench. 1938)**, CFR (2017), and OIE, the titer will be expressed as the number of EID_{50}/ml (**OIE. 2018**).

Determination of optimal virus harvest time

Three ECEs were infected with different commercial IBV vaccines and the chorioallantoic fluid of three inoculated ECEs was collected separately at different times (6, 12, 18, 24, 30, 36, 42, 48, 60, 72, and 96 hours post inoculation) for each tested vaccine to determine the optimal time at which the maximum infectious titer of five live attenuated infectious bronchitis vaccines can be obtained.

The samples were then processed for viral quantification using the RT-qPCR assay with specific primers and probes. The Ct values were compared to the standard curve. The time at which the maximum IBV titer was achieved was used to determine the virus's optimal harvest time **(Hitchner et al. 1955, Chomiak. 1958)**.

Experimental design

To compare the quantity of IBV EID_{50} determined using the conventional titration method and the real-time RT-PCR method, ten distinct IBV vaccines with different lots were titrated separately using both EID_{50} and qRT-qPCR. In all vaccines, the traditional EID_{50} was determined using tenfold serial dilutions from 10^{-4} to 10^{-7} and inoculation of five SPF ECE per dilution. After a 7-day period, the vaccine's end point titer was determined by the presence of death or CPE in chicken embryos; the virus titre is expressed as EID_{50}/dose. For each vaccine dilution , three SPF embryonated chicken eggs at 9 days old were inoculated with a reconstituted (0.1ml) of each vaccine, and the allantoic fluid was collected from the incubated eggs after only 42 hours of incubation and quantified using RT-qPCR.on the other hand, for quantification of IBV vaccinal strains directly from the vaccine vials Each vaccine was reconstituted to contain 1000 doses per milliliter, and total RNA was extracted from each vaccine before proceeding to RT-qPCR.

Total RNA extraction

QIAamp® Viral RNA Mini Extraction Kit (Qiagen, Valencia, Calif., and USA) Cat. No. 52906 was used to extract total RNA from allantoic fluid samples.

IBV RT-qPCR assay

The One Step SuperScript III Platinum kit was used for RT-PCR. Reverse transcription step was at 50 C for 20 min then Initial denaturation at 95 C for 3 min Then 40 cycle of denaturation at 95C for 30 sec and finally annealing and extension at 58 C for 1 min. The titer of each vaccine was calculated using the BioRad CFX 96 software and expressed as RNA copies/dose which will be expressed as EID_{50}/ dose when applied on the standard curvemand the test were conducted according to **Callison et al. (2006)** .

Table 2. Universal primers and probes for IBV detection

Primer	Sequence (5′ - 3′)
Forward	GCCATGTTGTCACTGTCTATTG
Reverse	GCTTTTGAGCCTAGCGTT
Probe	FAM - CACCACCAGAACCTGTCCCTC -BHQ

Setting up the standard curve:

Two vials of the used IB vaccine strains virus in this study of known titer were reconstituted in 1 ml of sterile PBS and thoroughly mixed together. One ml of the mixture was serially diluted in sterile PBS to form 10 fold serial dilutions ranging from 10^{-1} to 10^{-8}. After extracting RNA from each dilution for conducting the standard curve, real-time RT-PCR was performed, and the curve was produced using the Biorad CFX 96 software.

Test validation

The RT-qPCR test was validated using known titers of classical IBV strains.

RESULTS :

Determination of optimal virus harvest time

Inoculated eggs' allantoic fluid was collected and quantified using real-time RT-PCR. Table 3 shows that the optimal IB virus harvest time for achieving the maximum titer level for all vaccines is 42 hours PI. After that, the PI begins to drop sharply, and the best time for harvesting is 24 to 48 HPI.

Table 3. Determination of virus optimal harvest time

V. C.	T EID_{50} /dose	Titter by RT-qPCR/dose										
		6 h	12 h	18 h	24 h	30 h	36h	42 h	48 h	60 h	72 h	96 h
A	4.0	0.966	3.467	4.198	4.229	4.293	4.311	4.763	4.326	4.249	3.589	3.250
B	4.0	1.235	3.621	4.579	4.658	4.676	4.698	4.732	4.716	4.544	3.798	3.344
C	4.5	1.075	3.552	4.451	4.630	4.646	4.907	5.065	5.062	5.009	3.990	3.546
F	4.5	1.164	3.548	4.538	4.772	4.837	4.880	4.978	4.900	4.898	3.807	3.349
G	4.5	1.370	3.347	4.449	4.453	4.477	4.512	4.598	4.512	3.883	3.123	3.046

V.C. = Vaccine Code.
F and G = IB mass vaccines (Classical)
h = hours post inoculation in ECE.

A, B and C= IB vaccines variant strain
T= Traditional method (Log_{10}/ dose)

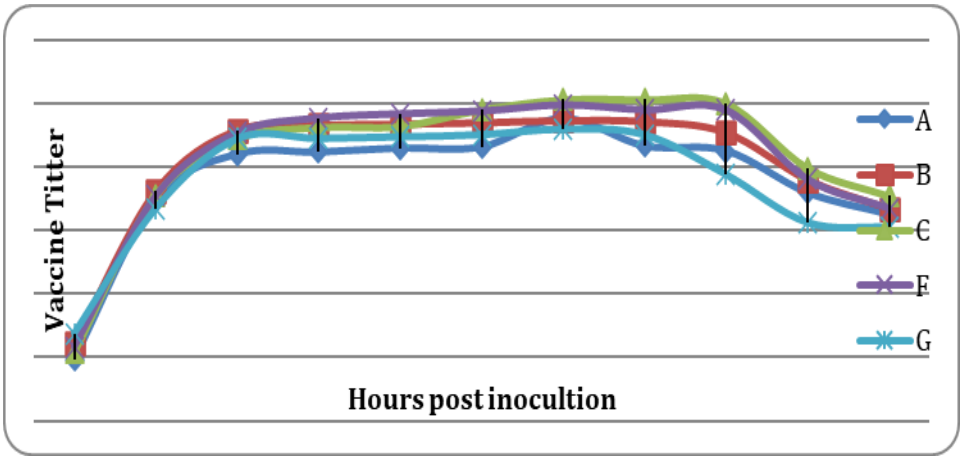

Figure (1): Determination of the virus's optimal harvest time

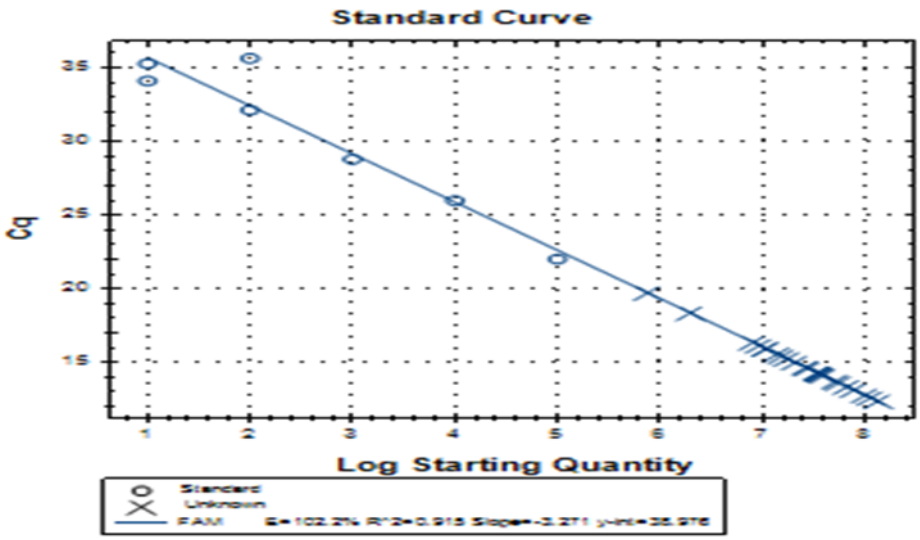

Figure (2): Control standard curve for IBV

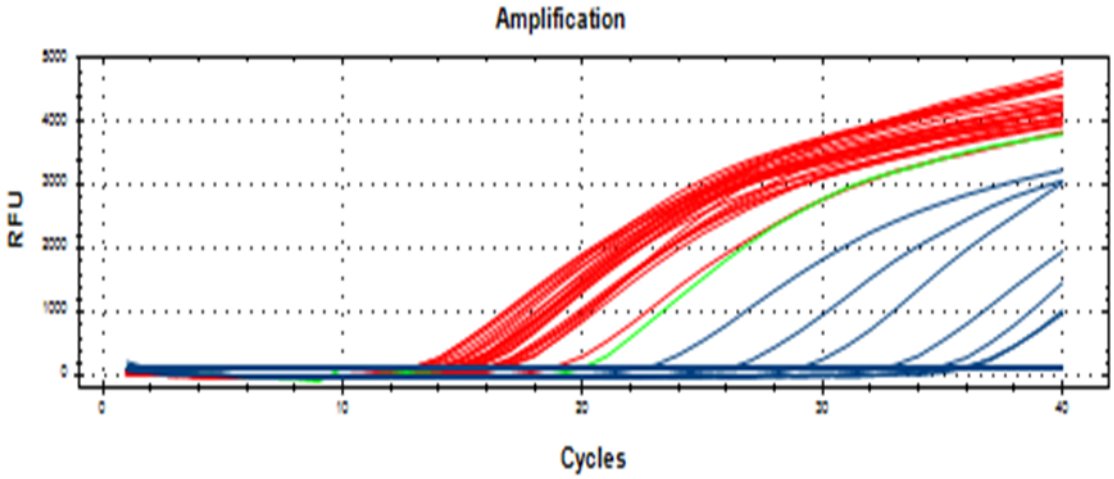

Figure (3): Amplification curve for IBV vaccine samples using RT-qPCR

EID_{50} versus RT-qPCR comparison

Table 3 shows that the titer of the ten vaccines measured by inoculation of chicken embryos and expressed as a number of EID_{50}/dose is lower than the titer of the same vaccine measured by RT-qPCR either directly from the vaccine vial or after 42 hours post inoculation (HPI) of three SPF ECE. Because the latter vaccine (D) was not detected using traditional titration methods, the RT-qPCR is highly sensitive and time consuming.

Table 4. Comparative titration reading of different IBV vaccines by different methods

Vaccine code	EID_{50} Titter	RT-qPCR log(10)	
		Before inoculation	42 HPI
A	4.0	4.043	4.763
B	4.0	4.036	4.732
C	4.5	4.520	5.065
D	ND	4.023	4.086
E	4.9	5.047	5.267
F	4.5	4.52	4.978
G	4.5	4.53	4.598
H	3.9	3.976	4.213
I	5.1	5.000	5.173
J	5.3	5.410	5.480

Variant IB vaccine batches were coded as A, B, C, D and E, and classical IB vaccine batches were coded as F, G, H, I and J.

DISCUSSION :

Accurate evaluation is required to detect vaccine effectiveness in infectious disease control strategies. A real-time Taqman®-based RT -PCR assay was used and evaluated for the detection and quantification of IBV genomic RNA directly from vaccine samples in order to achieve a suitable rapid and specific vaccine titration assay instead of traditional EID50 from IBV vaccines, especially since some IB virus strain effects on embryonated chicken eggs cannot be detected in traditional vaccine evaluation methods. Dwarfism became more common as chicken embryos lived longer. The vaccine strain is highly pathogenic in chicken embryos, resulting in embryo death within 2-4 DPI, which is insufficient time for the embryos to show dwarfism (Tsai and Chywch. 2016), Feng 2015) . The dwarfing effect of embryos in highly adapted IBV disappeared at higher passages, as reported b Bijlenga et al. 2004) and some virus strains did not induce visible CPE (Kint et al. 2015). Our findings revealed that the different vaccine titers detected by RT-qPCR at 18 HPI were nearly identical to those detected by the traditional method, and that they continued to increase to 42 HPI before decreasing, but that they did not decrease as much as those detected by the traditional method until 60 HPI, after which they decreased. As a result, the best time to collect chorioallantoic fluid for RT -qPCR vaccine titration is 42 hours after ECE inoculation. This contradicts the findings of Chomiak et al. (1958) who reported that the titer appeared in 16 hours, peaked at 40 hours post inoculation, and then declined, and (Tsai and Chywch. 2016) who reported that the growth kinetics curves of IBVs peaked in one to two days after ECE inoculation and then sharply declined after four DPI. Oade et al. (2019) also found that the titer level peaked at 24 hours, remained stable for 48 hours, and then declined, as well as collecting allantoic fluid and using it for IBV detection PCR.

Although there are only two methods, RT-qPCR was used in two different ways. The titer of nine vaccines measured by inoculation of chicken embryos and expressed as numbers of EID_{50}/dose is lower than the titer of the same

vaccine measured by RT-qPCR either directly from the vaccine vial or after 42 hours post inoculation (HPI) of three SPF ECE. The variant IB vaccine (D) was not detected using traditional titration methods, necessitating the use of RT-qPCR, which has high sensitivity. The traditional method of serial dilution of the vaccine followed by egg inoculation and reading after seven days has the advantage of detecting only live virus titres, which is required in the evaluation of live attenuated virus vaccines, but its disadvantages are seen with some IBV vaccinal strains (some variant IBV vaccines) that do not have a very clear effect on embryos, making egg titration visually difficult to read. This result was consistent with the findings of Bijlenga et al. (2004), who reported that the dwarfing effect of the adapted IBV disappeared completely at higher passages. However, for some IBV vaccines for which we could not read the titer visually, we used conventional RT-PCR to detect the IBV in the allantoic fluid of each inoculated egg and then used the Read and Muench calculation to determine the titer (unpublished data). The advantages of RT-qPCR method include high specificity, sensitivity, and being less time consuming, and this result is consistent with that reported in OIE (2018), which stated that the most common method for IBV detection and identification is real-time RT-PCR in less than a few hours. However, the disadvantages emerge primarily from the quantification of both dead and live viruses, so the titration method using RT-qPCR for testing the allantoic fluids is highly suitable; the results are inconsistent with those of Geerlys et al. (2013).

But the titers measured by this method are significantly higher than those measured by other methods. We believe that the percentages of difference between the two main titers ranged from 5 to 20%, and these percentages will have a significant negative effect on the accuracy of the evaluation of IBV vaccines. For example, if an IBV vaccine has a titer of 3 $\log^{10}EID_{50}$/dose (by traditional method), it should be rejected. However, when using RT-qPCR after 42 hours, the titer will exceed 3.5 $\log^{10}$ virus copies/dose, which is greater than the IBV vaccine's permissible titer (3.5 $\log^{10}EID_{50}$/dose).

CONCLUSION :

The following conclusions are made from the examinations described in this study:

Titration of IBV vaccine using conventional RT-PCR of allantoic fluid from each egg and calculation by Reed and Muench is the best and most accurate method for all IBV vaccine strains (unpublished data).

Titration of IBV vaccines using real-time RT-PCR directly from the vaccine vial remains inaccurate.

Titration of IBV vaccines using RT-qPCR on allantoic fluid of three inoculated eggs 42 hours after inoculation is promising. More research is needed to determine the exact correction factor of the increased titer using repeatability and reproducibility with more IBV vaccine batches including both variant and classical vaccines to calculate the accurate IBV titer.

RT-qPCR technology has many advantages, such as reduced time, sensitivity, and cost, as well as reduced human errors.

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