Original Article

Dynamic Changes in Infectious Bronchitis H120 Vaccine Virus Load Evaluated by Quantitative Real-time Reverse Transcription–polymerase Chain Reaction

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ABSTRACT

Background: Avian infectious bronchitis (IB) is a highly contagious and important respiratory disease that causes significant economic losses in the poultry industry. So far, several serotypes and genotypes of IB virus (IBV) have been identified, posing challenges to disease prevention and control. Vaccination is the most effective method for controlling the virus. The live attenuated H120 vaccine is one of the common vaccines used in the country against this disease. Today, the determination of the 50% infectious dose in SPF embryonated eggs (EID_{50}%) is used to quantitatively evaluate and determine the titer of the virus for vaccine formulation against IB.

Objectives: This study was conducted to establish a rapid and sensitive real-time polymerase chain reaction (PCR) method for quantitatively evaluating changes in virus titer and the rate of virus replication in embryonated eggs.

Methods: The assay was developed using a universal primer probe set, and the viral load was assessed at 12-hour intervals, beginning immediately after inoculation of embryonated chicken eggs (ECEs) with the H120 vaccine virus and continuing up to 60 hours postinoculation. Harvested allantoic fluids were investigated using the classical EID_{50} method and a molecular assay.

Results: Viral load dynamics were comparable between the two methods. The lowest titer was observed at 12 hours, and the highest load was detected at 24 and 36 hpi (h post-infection).

Conclusion: The results of this study demonstrate promising potential for using a molecular method instead of the classical chicken embryo infectious dose 50% (EID_{50}) method, which is expensive and time-consuming and has high variability. Nevertheless real-time PCR has not been approved for vaccine production. It is suggested that this method be applied as an in-process control test to reduce the time and expenses associated with manufacturing the H120 IB vaccine while ensuring accurate and precise analysis.

Keywords: Dynamic, EID_{50}, Infectious bronchitis (IB), Real-time polymerase chain reaction (PCR), Vaccine

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Introduction

Infectious bronchitis (IB) is a contagious viral disease that causes drastic economic losses to the poultry industry worldwide. The causative agent is an enveloped, single-stranded, positive-sense RNA virus belonging to the gammacoronaviruses. Since the first report of the disease by Schalk & Hawn in 1931, multiple variants have been emerged worldwide. For the first time, Beaudette & Hudson (1937) showed the growth of IB viruses (IBVs) on the chorioallantoic membrane of chicken eggs (Bijlenga et al., 2004).

In addition to biosecurity and proper management, vaccination is currently the most effective tool for controlling the disease. One of the eldest vaccine strains, with a history of more than 60 years, is H120, which originated from a broiler farm in the province of Brabant in the southern Netherlands. "Huyben" was the name of the flock owner. Several egg-adopted IBV strains have been used satisfactory as vaccine candidates to date. The H120 vaccine is a Massachusetts-type strain. Bijlenga (1956) identified this virus isolate and used it to prepare a vaccine through 52 serial passages in embryonated chicken eggs (ECEs). The resulting vaccine strain was named H52, with the "H" representing the initial letter of the farmer's name; however, the letter "H" has often mistakenly been thought to represent the initial letter of "Holland". After four years of using H52, Hoekstra and Rispens (1960) determined that H52 was too virulent for chickens. Therefore, further passages were required, resulting in the H120 strain (Jones, 2010). The virus has the ability to propagate in various systems, such as chicken kidney cells, chicken embryo kidney cells, and tracheal rings (Motamed & Bashashati, 2022).

The Razi Vaccine and Serum Research Institute has been producing vaccines against IB for more than 40 years. The conventional 50% egg infectious dose (EID_{50}) method is the classical method for estimating IBV titers. Optimizing the harvesting time after inoculation of the vaccine seeds into ECE is a critical consideration, in addition to other quality-control evaluations (Legnardi et al., 2020).

Viral titration is time-consuming, and the process can be subjective, have low sensitivity, and require substantial labor, thereby delaying vaccine production. First, the traditional method for calculating the 50% infectious dose requires at least 7 days of incubation after inoculation with the harvested vaccine (Lothert et al., 2023). Second, other methods, such as cell culture and plaque-forming unit (PFU) counting, including potential bias during the evaluation of cytopathic effects (CPEs), as well as time-consuming procedures that can result in considerable variation. Third, IBV are rarely adopted to cell cultures, especially continuous cell lines, which complicates IBV experiments. On the other hand, primary cells have their own complications, ranging from cell-culture preparation and media requirements to the infection and 50% infectious-dose estimation processes.

EID_{50} determination is the conventionally used assay for IBV because of its simplicity and ease of use. Nevertheless virus titration in ECE is an expensive, time-, and labor-intensive method that may produce substantial variation. Additionally, it is not suitable for conducting large-scale testing or multiple tests within a short period (Zhang et al., 2014). Therefore, developing a rapid, sensitive, and reliable quantitative method, such as quantitative real-time polymerase chain reaction (qRT-PCR) assay, is essential for vaccine manufacturing processes.

The purpose of this study was to investigate the proliferation trend of the H120 virus after inoculation into embryonated eggs, determine the optimum time for vaccine harvesting using qRT-PCR, evaluate the suitability of this method for vaccine titration, harvest the vaccine fluid from the eggs, and compare the results with the harvesting time determined using the EID_{50} test in specific pathogen free (SPF) (ECEs).

Materials and Methods

H120 virus cultivation

The working seed was prepared according to the vaccine dossier. Then, 0.1 mL of the virus was inoculated into the allantoic cavity of 450 SPF embryonated chicken eggs on three separate occasions. Allantoic fluid was harvested at 12-hour intervals, including 12, 24, 36, 48, and 60 hours postinoculation.

The allantoic fluids collected at each time point were pooled, and the pooled samples were divided into two portions. One portion was used for molecular analysis, and the second portion was used to determine the EID_{50} in specific-pathogen-free eggs.

qRT-PCR

Primers and probe

The primers and TaqMan probes used for the RT-qPCR assays were described previously. Callison et al.

reported a primer pair that included the forward primer IBV5′GU391 (5′-GCT TTT GAGCCT AGC GTT-3′) targeting nucleotide positions 391–408; the reverse primer IBV5′GL533 (5′-GCCATG TTG TCA CTG TCT ATT G-3′), flanking nucleotide positions 533–512; and a TaqMan® dual-labeled probe, IBV5′G probe (5′-FAM CACCAC CAG AAC CTG TCA CCT C-BHQ1-3′), targeting nucleotide positions 494–473 of the IBV M41 strain genome sequence. These primers and probe were used to amplify a 143-bp fragment of the 5′-UTR gene (Callison et al., 2006).

To quantify the viral load, a standard curve was required. The standard curve was constructed by designing another primer pair to amplify a 560-bp amplicon based on a conserved region of the 5′-UTR gene sequence of the reported H120 vaccine strain in the NCBI GenBank database (Table 1). Primer properties were investigated using the Beacon Designer online tool.

Viral RNA extraction and cDNA synthesis

Viral RNA was extracted from 300 µL of H120 IBV-infected allantoic fluid samples using the Ribospin^{vD} kit (Gene All, Germany), according to the manufacturer's instructions (Gene All, 2016). The extracted RNA was stored at −70 ˚C until further use.

For cDNA synthesis, a reverse transcriptase reaction was performed conducted using a Biotech Rabbit RT kit, Germany, consisting of 1 µL of Random Hexamer, 0.5 µL of RNase inhibitor, 1 µL of Revert Tm transcriptase, 2 µL of dNTP mix, 4 µL of 5X buffer, and 10 µL of extracted RNA. Nuclease-free water was added to a final volume of 20 µL. The temperature program for the reverse-transcription reaction consisted of 30 ˚C for 10 min, followed by 50 ˚C for 60 min and enzyme inactivation at 99 ˚C for 5 min.

Standard plasmid construction

The synthesized cDNA was mixed with 5 mL of 10X PCR Buffer, 1 mL of $MgCl_2$, 2 mL of dNTP mix, 1 mL of 2.5 u/mL Accupol DNA polymerase, and 2 mL of each forward and reverse primers in a final volume of 50 µL to amplify a blunt-ended 560-bp PCR product. The thermal conditions consisted of an initial step at 95 ˚C for 2 min, followed by 35 cycles of denaturation at 95 ˚C for 30 sec, annealing at 52 ˚C for 30 sec, and elongation at 72 ˚C for 2 min, followed by an additional elongation step at 72 ˚C for 2 min.

The PCR product and the ability to amplify the targeted gene were analyzed by electrophoresis on a 1.5% agarose gel to visualize the predicted 560-bp product. After purification using the High Pure PCR Purification Kit (Roche, Germany), the amplicon was ligated into the pJET1.2/blunt vector according to the instructions for the CloneJET PCR Cloning Kit (Thermo Fisher), using T4 DNA ligase. Because the recombinant plasmids contained an ampicillin-resistance gene, the growth of bacterial colonies was assessed on ampicillin-containing LB agar medium after transformation of the amplicon into competent *Escherichia coli* Top10 cells. Recombinant plasmids and the target sequence in the clones were confirmed by colony PCR and restriction enzyme digestion.

The recombinant plasmids were sequenced and then used as standard DNA. The plasmid concentration was determined using a NanoDrop spectrophotometer by measuring absorbance at 260 nm. The concentration, initially measured in g/mL, was converted to copy numbers/mL using the Science Primer online calculator.

Based on the resulting copy numbers, log10 serial dilutions ranging from 10^{10} to 10^{1} DNA copy numbers were prepared in nuclease-free water.

Table 1. Oligonucleotide sequence of the standard curve primer set

Primer Name	Sequence (5'→3')	Length	Ref.
MB-IB-STF	AGCCTTGCGCTAGATTTCAA	20 nt	In-house
MB_IB-STR	TGTCTTTGGAAACAAGAATGACA	23 nt	In-house
IBV5′GU391	GCT TTTGAGCCT AGC GTT		Callison et al. (2006)
IBV5′GL533	GCCATGTTGTCACTG TCTATTG		Callison et al. (2006)
IBV5′G probe	FAMCACCACCAGAACCTGTCACCTCBHQ1		Callison et al. (2006)

Real-time PCR reaction

To study quantitative changes in virus titers at different time points following inoculation in SPF eggs, real-time PCR assays were performed using pooled samples from the allantoic cavities harvested at different times, serial dilutions of standard plasmids, and negative (containing no template) and positive controls. A standard curve was drown using serial dilutions (from 10^1-10^9) by plotting the cycle threshold (Ct) values against the log10 dilutions of the virus titer and performing linear regression analysis. All RT-PCR runs were conducted using an ABI3600 instrument and the Realtime Master Mix Kit (Biotechrabbit). First, 5 µL of 4X CapItase Master Mix, 1 µL of each primer, 0.5 µL of probe, and 2 µL of cDNA were added. Nuclease-free water was then added to reach a final volume of 20 µL.

The optimum thermal cycle conditions were as follows: 95 ˚C for 3 min, followed by 40 cycles at 95 ˚C for 15 s and 60 ˚C for 60 s.

EID_{50} assay

The EID_{50} assay was conducted to determine the infectious virus titer according to the World Organisation for Animal Health standard protocol (World Organisation for Animal Health, 2018). Five 9-10-day-old SPF embryonated eggs were inoculated with 100 µL of various tenfold serial dilutions of alantoic samples collected at different harvest times.

The eggs were incubated at 37 ˚C for up to 7 days. After this period, the eggs were checked for specific signs of IBV in the embryos, such as death, dwarfism, curling, and stunting. Any deaths occurring within 24 hours of infection were considered nonspecific and excluded from the titer calculations. The highest dilution that still produced observable IBV-related lesions in the embryos was noted. The EID_{50} titer per 0.1 mL was determined using the Spearman-Karber method (Figure 1).

Determination of the critical time for virus harvest

Data from the molecular assays and embryonated eggs were evaluated for at least 3 different vaccine-production batches to define the optimal harvest time for obtaining the highest virus loads in allantoic fluids for each assay separately.

Results

Specificity and sensitivity of universal primers and probes

The specificity of the primer and probe set was first examined in silico using the BLAST search function at NCBI. We further verified the specificity of the qRT-PCR assays using RNA extracted from known IBV viruses, including H120 and Variant 2,793B, as well as from avian pathogens, such as Newcastle and influenza viruses, *Mycoplasma* spp., and *Salmonella*. The universal IBV primers and probe successfully detected all IBV types, and none of the non-IBV poultry pathogens were detected by the primer and probe set (Figure 2). The qRT-PCR assays using the universal IBV primers and probe produced highly repeatable data, with an R2 ≥0.99 and an average calculated reaction efficiency of 98.7%.

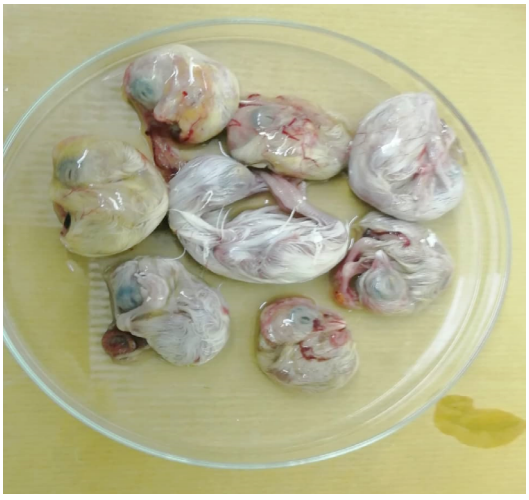

Figure 1. H120 IB vaccine pathogenicity in chicken embryos

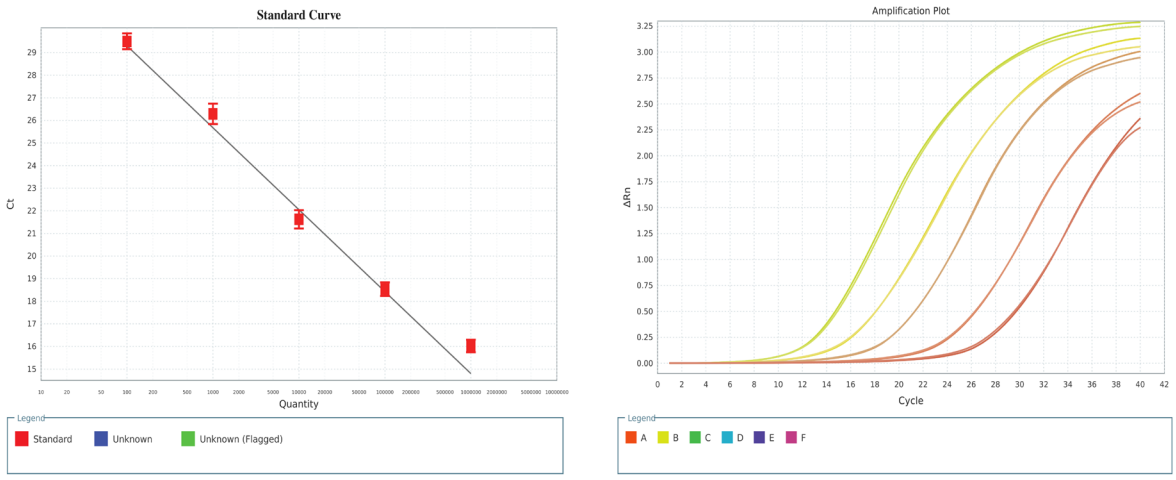

Figure 2. Standard curve and amplification plot constructed by the real-time PCR reaction

Dynamics of the H120 virus in real-time PCR reaction

According to molecular dynamic results, the viral load of the H120 IB vaccine strain showed an increasing trend beginning at 12 PI. The virus load reached its highest level at 36 hours and between 36 and 48 hours. Some variation in virus load was observed at 48 and 60 hours post-inoculation. The designed test was sensitive enough to detect 100 DNA copy number at 25 CT and specific enough not to detect other avian pathogens. All time intervals contained sufficient virus for detection, and there was 1 peak in virus load at 36 hours PI only, with a mean CT of ~9. A gradual decreasing trend then occurred until 60 hours.

This was the first time a quantitative method was developed to evaluate infectivity during the vaccine production process. Using the real-time PCR method, a peak was observed at 36 hours post-inoculation (Figures 3A and 3B), whereas in the embryonated chicken eggs, the peak was detected during two intervals, from 24 to 36 hours/PI. In both assays, after the virus load reached a peak, the titer continued to increase until the end of the experiment (Table 1).

Dynamics of H120 IB virus in SPF ECEs

During the first hours PI, the virus replicated rapidly, and its titer increased exponentially. Therefore, there was a slight decrease in virus titer at 24–36 hours, followed by another peak at 60 hpi (h post-infection). However, 60 hpi was not an appropriate time for harvesting because of increasing mortality and the presence of inhibitory materials that negatively affected virus viability. Therefore, according to the EID_{50} assay and considering the time and process-related expenses, the best time to harvest the allantoic fluid was 36 hours after vaccine seed inoculation (Table 2).

Discussion

It is important to replace old and classic methods with novel ones to reduce time and expenses and decrease cross-contaminations. Rapid and precise determination of infectious titers is highly important in viral vaccine production. Conventional methods for measuring infectious titers, such as PFU or EID_{50}, are time-consuming, monotonous, and subjective, which can lead to inconsistencies in vaccine quality and production timelines.

Table 2. H120 virus titre assessed by EID_{50} in the ECE system

Hours after Inoculation	1st Batch	2nd Batch	3rd Batch
12	10^{-6}	$10^{-5.5}$	10-6
24	$10^{-8.25}$	10^{-8}	10-8
36	$10^{-8.25}$	$10^{-8.5}$	10-8
48	$10^{-7.75}$	10^{-8}	10-7.75
60	10^{-8}	$10^{-7.75}$	10-8

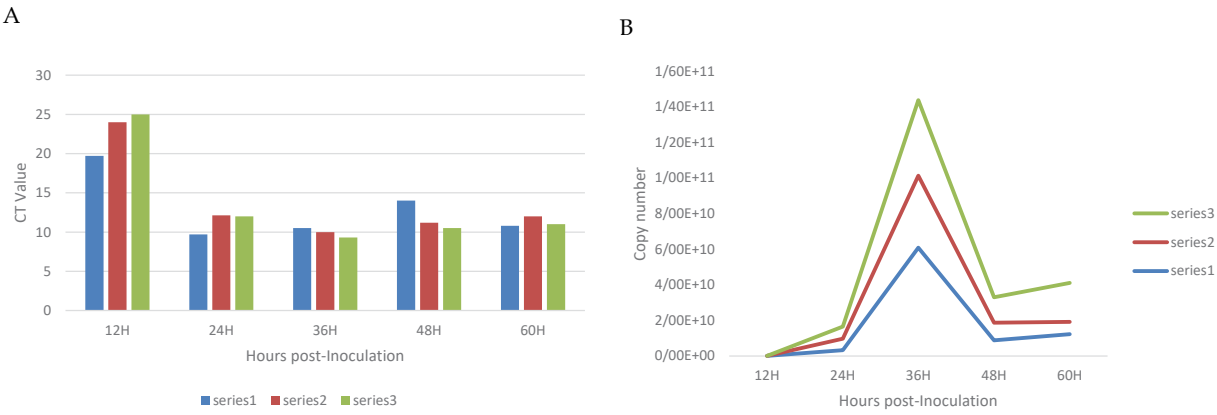

Figure 3. H120 virus load evaluation by RT-qPCR

A) Ct values of vaccine strain H120 virus at different time intervals, B) Nucleic acid copy number of the H120 vaccine strain virus at different time intervals

Note: Each color is related to one vaccine batch.

Other titration methods, including enzyme-linked immunosorbent assay, flow cytometry, and fluorescence-based virus detection, have been introduced to address these problems in the titration of various viruses. However, in addition to being less sensitive and more complicated, these methods are not as readily applicable to IBV (Lougovskaia et al., 2002; Bose et al., 2010). In recent decades, RT-qPCR assays have received increased attention as a means of overcoming the drawbacks of traditional methods (Schalk et al., 2004; Russell et al., 2011). Notably, the titration of noncytopathic viruses using RT-qPCR can be performed more accurately than with CPE-based methods (Azizi et al., 2013; Zhang et al., 2019).

On the other hand, the most common method, which uses SPF eggs, has its own difficulties. SPF eggs are an expensive source for virus culture, and there is an ongoing concern about potential shortages (Lougovskaia et al., 2002). In the current experiment, we studied the possibility of using a molecular test—qRT-PCR—to determine the optimal harvesting time and nucleic acid quantity for the H120 IBV vaccine and to assess its compatibility with the classical EID_{50} method in embryonated chicken eggs. qRT-PCR has been used in the research and diagnosis of many avian pathogens (Wen et al., 2024). In contrast to ECE and conventional PCR, real-time is quantitative, sensitive, rapid, and less expensive. In many studies, this test has been used to evaluate vaccine efficiency and in challenging experiments (Lougovskaia et al., 2002; Bose et al., 2010; Azizi et al., 2013). However, although the standard method for detecting the IBV titer is the 50% chicken embryo infectious dose, it was unclear whether the novel test could replace the classical method.

Considering that RT can detect nucleic acids and, therefore, nonviable viruses, which may compromise the assay results, it is important to estimate its similarity and compatibility with in vivo methods. Callison et al. (2006) suggested a universal primer pair targeting the 5′-UTR gene, which was demonstrated to be effective for the detection and quantification of several IBV serotypes with high specificity and sensitivity. They reported that the developed assay was designed to detect a portion of the IBV RNA genome in the highly conserved 5`-UTR region, resulting in the amplification of all IBV strains except turkey coronavirus (TCoV), which is closely related to IBV and may have originated through a recombination event involving the spike gene (Callison et al., 2006). In this experiment, the universal primer and probes were successfully used as sensitive detectors of the virus genome. In addition, the in-house- designed primers used to construct the standard curve were sufficiently accurate to develop an IBV-specific detection assay. The specificity of the test was investigated using important bacterial and viral chicken pathogens, and the results were negative results. Another factor evaluated was the detection limit of the test, which was determined using different dilutions of the standard curve and was shown to be approximately 100 copies. According to the molecular investigations, the optimal time for harvesting the vaccine virus was 36 hours PI ($P<0.05$). Although the highest virus titer was detected at 36 hours PI, the virus quantity began increasing at 24 hours PI. The lowest virus quantity was observed at 12 hours PI, similar to the results obtained using ECE; however, the highest titer was observed at 36 hours PI ($P<0.05$).

The increasing virus titer began at 24 hours PI in the ECE test, reaching more than $10^{-7.5}$ EID_{50}. The highest titer was observed between 24 and 36 hours PI, followed by a slight decline and then an increase to the same titer at 60 hours PI.

The dynamic changes in the IBV-Sczy3 strain load in the supernatant on primary chicken embryo kidney cells were assessed by a SYBR Green I-based real-time PCR by Wei et al. (2015). Three CEK-adapted generations—20^{th}, 54^{th}, and 87^{th}—were used, and the nucleic acid copy numbers in the supernatant at 12, 24, 36, 48, 60, and 72 hours PI were determined. The results showed that the most rapid period of virus growth for all three generations was about 12-36 h post-infection (Wei et al., 2015).

Zang et al. (2014) evaluated a triplex TaqMan RT-qPCR assay to investigate the possibility of replacing the conventional 50% EID or plaque-forming-unit assay for the titration of a trivalent flu vaccine. In their experiment, the critical harvest times were reported to be 18 hpi for influenza A and 12 hpi for influenza B. There was not a significant difference between the 12 and 18 hpi titers for the B strain. They concluded that trivalent Attenuated Influenza Vaccines (LAIVs) inoculated into embryonated eggs could be simultaneously titrated within 24 h using a TaqMan RT-qPCR assay and that the titers obtained were comparable to those determined using the traditional EID_{50} assay. They said that the RT-qPCR assay could be used as a highly specific, precise, sensitive, and rapid substitute for the EID_{50} assay to calculate the infective dose of trivalent LAIVs. Estimating the titers of the 3 influenza vaccine viruses within 24 h using the RT-qPCR method was much faster than the 72 hours required for type B or the 48 hours required for type A in the EID_{50} assay. They also declared that the time and cost of vaccine manufacturing would be reduced because specific monoclonal antibodies for detecting the 3 subtypes would no longer be needed. The most notable benefit of the RT-qPCR method is that it is more reliable than the conventional EID_{50} method and produces results with lower CV% values.

Roh et al. (2014) studied the application of the qRT-PCR method using universal and type-specific primer and probe pairs for IBV and compared its results with challenge-virus detection in ECE to determine whether these 2 methods for evaluating vaccine efficacy were equivalent. In addition, they tested the assays on two different thermocyclers and found that the universal primer set produced results comparable to those obtained by detecting the virus in embryonated chicken eggs. Although, for some types (Conn and Mass41 on the SmartCycler II, and Mass41, Ark, GA98, and Conn on the ABI 7500), the molecular assay was more sensitive than the ECE assay, this could have occurred simply because molecular tests are more sensitive or because the RT-PCR assay detected nucleic acids from nonviable viruses. They concluded that the potential for RT-PCR to identify vaccinated, challenged birds as positive when they are negative in the ECE system must be considered in challenge studies in which the challenge virus is investigated in vaccinated chickens. They also found that universal primers and probes were more sensitive than the type-specific primers.

The prioritization of developing alternative in vitro potency assays for inactivated NDV vaccines to reduce the costs and time associated with in vivo batch-release testing in Egypt led to experimental work done by Safty et al., which aimed to establish a new protocol for assessing the in vitro potency of an inactivated Newcastle disease virus vaccine. They reported that real-time PCR could be used as an in vitro assay for Newcastle vaccine evaluation (Safty et al., 2023). Ct values ranged from 21.17 to 25.23, while hemagglutination inhibition (HI) titers ranged from 6.2 to 7.1 log2. A comparison of the Ct values of antigen extracts with challenge-test results and HI assays using sera from vaccinated birds showed a robust correlation between the in vitro and in vivo results.

In Shahkarami et al.'s study, the tissue culture infectious dose 50 (TCID50) and plaque formation unit (PFU) assays, which are traditionally used as gold-standard tests for assessing the heat stability of measles vaccines, were compared with real-time PCR, and three different stabilizers were assessed. The effects of the stabilizers and the vaccine degradation rate were evaluated by determining virus titers using TCID50 and viral RNA copy numbers using real-time PCR. The results revealed a significant correlation between the TCID50 and real-time PCR results ($P<0.05$), suggesting that real-time PCR is a valuable supplement to or replacement for these methods. They concluded that real-time PCR provides a faster, cheaper, and easier alternative to cell culture-based titration, which is the gold standard for viral vaccine titration and heat-stability assessment (Shahkarami et al., 2015).

IBV is one of the most concerning poultry pathogens, and vaccination is the best control tool. However, the vaccine production process and the need to evaluate the virus load after the production of each batch/lot remain extremely important challenges in vaccine quality control, especially from the perspectives of time and cost. RT-qPCR is receiving increased attention and is progressively being preferred to overcome the limitations of tra-

ditional assays. In particular, RT-qPCR-based virus load titration offers a faster alternative to in vivo and in vitro methods, even for noncytopathic or less embryo-adapted viruses.

Nevertheless, RT-qPCR assays also have some limitations. Considering the potential diversity of IBV, primers and/or probes must be redesigned according to changes in the virus. However, only minor modifications are required because the universal primers were designed based on the most conserved region of the viral genome. In addition, the highly sensitive nature of PCR may result in varying results if the assay is not performed using stringent operating procedures. Therefore, consistent and stringent practices are essential for this titration system. Moreover, because the qPCR technique has not been approved for vaccine-lot release, this assay can be used for in-process control during vaccine manufacturing. Mo et al. (2020) suggested that qRT-PCR assays can detect the challenge IBV. Nonetheless, each assay—including its assay conditions and thermocycler instrument—should be evaluated separately to determine whether its results are equivalent to those obtained by detecting the virus in embryonated chicken eggs (Mo et al., 2020).

Conclusion

The mean titers attained by the molecular method were comparable to those obtained using EID_{50} in our experiment; however, it is worth mentioning that the former assay can be performed in less time. In conclusion, this rapid, precise, sensitive, and specific method can be used as an alternative or complementary test, but not as an absolute substitute. Therefore, this RT-qPCR assay, with its advantages of high specificity, sensitivity, precision, and rapidity, may be used as a substitute for the EID_{50} method in H120 IBV vaccine production. It should be considered that numerous variables, such as the type of virus, primer and probe efficiency, test conditions, and instrument, affect the results of molecular tests. These variables must be considered when titrating a virus or comparing the results with those obtained using other techniques.

Ethical Considerations

Compliance with ethical guidelines

There were no ethical considerations to be considered in this research.

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Authors' contributions

Conceptualization and supervision: Najmeh Motamed; Methodology: Mohsen Bashashati and Najmeh Motamed; Investigation, Data collection and analysis: All authors; Writing: Mohsen Bashashati; Suyunov Rashid Uktamovich.

Conflict of interest

The authors declared no conflict of interest.

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