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Validation of qPCR for the Detection and Quantification of Attenuated Laryngotracheitis Virus Isolated from Allantoic Fluid by Determination of Yield Parameters

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Abstract

The production of efficient and safe vaccines against the infectious laryngotracheitis virus (ILTV) represents an important biological process for the health of millions of poultry birds. In this context, the validation of quantitative methodologies is important to guarantee that the results show the actual state of vaccines, allowing an adequate dosage. In this study, we developed, standardized and validated a methodology following Good Laboratory Practices (GLP) and the guidelines of the Food and Drug Administration (FDA) to evaluate and measure the titer of vaccines produced in allantoic fluid (AF). Therefore, a real-time PCR (qPCR) methodology was designed for the detection and quantification of the glycoprotein B gene (gB) of ILTV using SYBR Green I. For this purpose, an internal reference material for ILTV (IRM-ILTV), along with a plasmid with part of the gene encoding the gB protein of ILTV and lacking the rest of the ILTV genome were produced. The validation criteria showed that the qPCR assay has a Limit of Detection (LoD) of 1.017×10^5 genome copies/µL of AF which guarantees high precision, and a Limit of Quantification (LoQ) of 3.39×10^5 genome copies/µL of AF. The confidence limit for confirming the presence of ILTV with a conventional specific PCR obtained a LoD of 2.034×10^3 genome copies/µL of AF. These parameters demonstrated the safety and accuracy of the correct detection and quantification of the ILTV viral load in vaccines produced in AF. Hence, this procedure constitutes an important complementary tool for the quality assurance of vaccines for birds and for diagnostic, virus load on samples that are positive.

Keywords: ILTV; Real-time PCR; Validation; Limit of quantification; Limit of detection; Poultry; Vaccine

Introduction

Gallid herpesvirus 1 or infectious laryngotracheitis virus (GaHV-1 or ILTV) is a member of family Herpesviridae, subfamily Alphaherpesvirinae, genus *Iltovirus* [1] and causes the disease called avian infectious laryngotracheitis. ILTV has a genome characterized as linear double-stranded DNA of approximately 150 Kbp [2]. The natural infection can last six days, causing localized infection in the upper respiratory tract and decreased egg production in egg-laying birds [3]. Complementary to biosafety measures, vaccination controls the spread of the infection [4]. Therefore, this control measure has been adopted by the poultry industry to prevent different infectious diseases [5]. The biopharmaceutical industry has applied strict requirements for the validation of assays used in the characterization of drugs for animals and recombinant biological products with ILTV genes [6,7]. These measures could be the result of the observation of different ILTV vaccines (attenuated or recombinants) that present different degrees of protection when evaluated on field trials [8]. Furthermore, several additional procedure guidelines have been established to ensure the efficiency and safety of vaccines [9]. The efficacy of vaccines depends on the optimum number of virus particles in the vaccine dose. To estimate the titer of ILTV-attenuated virus in the different stages of production of the vaccine, is usually performed by laborious time-consuming processes such as the viral titration by inoculation of embryonated eggs [10], or poorly sensitive methods as immunoassay or serum neutralization [9,11], thereby hindering vaccine production.

The PCR method has been carried out by amplifying specific gene sequences of ILTV and viral titers, with the advantages of rapidity and sensitivity [9,12-21]. Also, many of these procedures have demonstrating specificity [20].

The validation processes based on analytical performance, evaluating parameters such as precision, diagnostic specificity, Limit of Blank (LoB), Limit of Detection (LoD) and Limit of Quantification (LoQ), have been previously published [22-24].

Development of a rapid and precise virus titration method based on PCR is essential for vaccine manufacturing in timely and cost-effective manner. By applying the guidelines of the International Conference on Harmonisation (ICH), we address the validation process based on the analytical performance of a PCR assay for the detection and quantification of ILTV. Precision was defined as the reproducibility of ILTV gene copy number; diagnostic specificity as the absence of impurities (closely related structural components); LoB as the highest fluorescence apparent signal of the ILTV gene found in replicates of a DNA material without target sequences of the ILTV gene; LoD was defined as the minimum amount of ILTV gene copies that could be detected in allantoic fluid (AF); and LoQ as the minimal number of ILTV gene copies that is acceptable for quantification purposes in AF [24]. Based on these parameters that reflect analytical performance, this study has validated a SYBR Green I-based method for the quantification of ILTV sequences in AF and has elaborated an

appropriate algorithm for its detection and quantification. The importance of the development of this validated strategy based on analytical performance parameters could be used for implementation of guidelines in the biopharmaceutical industry.

Materials and Methods

Biological material

An Internal Reference Material (IRM) was prepared from an isolated virulent strain of ILTV. Briefly, the strain was propagated in specific pathogen-free (SPF) eggs to collect enough AF. Total viral DNA extraction (internal reference material or IRM) was performed using the QIAamp®MinElute Virus Spin kit (QIAgen, Germany) according to the manufacturer's instructions. The characterization of this IRM was performed by fluorometry using the Qubit 1.0 fluorometer (Invitrogen, USA), its integrity was confirmed using the 2100 Bioanalyzer (Agilent, USA), and its purity was confirmed using the BioPhotometer™ plus UV/VIS photometer (Eppendorf, USA). IRM aliquots of 1 pg/µL were stored at -80°C and used to standardize both qualitative (cPCR) and quantitative (qPCR) amplification conditions. Serial dilutions of the IRM (100 fg/µL, 10 fg/µL, 1 fg/µL, 100 ag/µL and 10 ag/µL) in DNA from DF-1 cell line from chicken simulated the matrix effect [25] of the chicken genome. Viral nucleic acids from other avian pathogens were used to determine diagnostic specificity (Table 1).

Biological material	Code
FAdV, *Fowl adenovirus*	X1
HVT, *Herpesvirus* of turkey	X2
GPV, *Goose parvovirus*	X3
EDS, Egg drop syndrome	X4
CAV, Chicken anaemia virus	X5
MDV, Marek's disease virus	X6
FPV, Fowl pox virus	X7
Coriza, *Avibacterium gallinarum*	X8
ORT, *Ornithobacterium rhinotracheale*	X9
Salmonella gallinarum, 9R strain	X10
Gallibacterium anatis	X11
DF-1 Chicken Fibroblast Cell	DF-1
ILTV, Infectious Laryngotracheitis virus	IRM-ILTV

Table 1: List of most common avian pathogens used in this study.

Primer design

A pair of primers for ILTV Glycoprotein B gene (gB) were designed using the software Primer3 [26]. The forward primer ILTF_2 (5´-ggatagcggtggtggtga-3') and the reverse primer ILTR_2 (5´-tagagggtcgtggaggagtt-3´) specifically targeted the gB of ILTV (amplicon of 132 bp). These proved to have no overlapping polymorphisms specific in the binding sites of each primer, and specific to the target sequence of interest. We also included the primer pairs gB-S (5´-cagtatctggcatcgcctcat-3´) and gB-A (5´-cctgggaacagaacctgaact-3´) [19] to specify genomic location (amplicon of 148 bp). The sequences were evaluated with the GenBank database using BLAST and with the OligoAnalyzer Tool [27]. No significant cross-reaction with other sequences or sufficient free energy was found to identify dimers and secondary structures.

Construction of plasmids for the preparation of standards

To detect and quantify copies of ILTV, we created a standard curve. Briefly, a plasmid containing the gB sequence was synthesized (GenScript, China), and quantified by fluorometry (Qubit 1.0 fluorometer, Invitrogen, USA). Then, we prepared subsequent serial dilutions of the plasmid with PCR-grade water comprising from 1×10^8 to 1×10^1 copies per reaction of the gene of interest. The number of plasmids/µL was calculated using the following formula: [Plasmid concentration (g/µL) × (6.022×10^{23})] / [plasmid size (bp) × 660] [12].

cPCR optimization

A conventional PCR was optimized in total volume of 20 µL containing 5 µL DNA (standard dilutions or samples), 0.25 µM of both primers ILTF_2 and ILTR_2 or gB-S and gB-A, and 1X of Q5 High-Fidelity 2X Master (0.25 µM each), 200 µM deoxynucleotide triphosphates (dNTPs), 2.0 mM $MgCl_2$, and 0.02 U/µL Q5 High-Fidelity DNA polymerase (New England Biolabs, USA). The following amplification conditions were used in a Mastercycler® Pro-S thermocycler (Eppendorf, USA). Initial denaturation at 95°C for 20 min; 40 cycles of 90°C for 20 sec, 62°C for 20 sec and 72°C for 20 sec; and a final extension step at 72°C for 2 min. The products were separated in 2% agarose gel and visualized using SYBR® Safe DNA Gel Stain (Invitrogen, USA) or QIAxcel® (QIAgen, Germany). The identities of the PCR products were confirmed by sequencing (Macrogen, Korea).

Qualitative analysis

Diagnostic specificity of the conventional PCR: To evaluate the diagnostic specificity of the conventional PCR, we used a panel of the most common poultry pathogens that elicit pathologies that are closely related to the one caused by ILTV (Table 1). The diagnostic specificity was calculated in terms of the presence or absence of a band of 148 bp using the following formula: % false positives=(false positives/total negatives known) × 100 [23] (Figure 1).

LoD of conventional PCR: The LoD was established to confirm that the method does not produce false positives due to the matrix components used [22]. To determine the LoD, the minimum concentration of the analyte that could be detected with confidence (≥ 95%) was used in nine conventional PCR runs (in three days) using the different dilutions of the IRM sample as templates (Figure 2).

qPCR optimization using SYBR Green I

The SYBR® Green I real-time PCR assay (qPCR) was developed and optimized for the ILTF_2 and ILTR_2 primers in a 20 µL reaction volume using the LightCycler®480 SYBR Green I Master kit (Roche, Germany), with the purpose of using it to quantify the titer of ILTV produced in AF. The reaction mix contained 1X of LightCycler®480 SYBR Green I Master Mix, 0.125 µM of each primer and 5 µL of sample DNA (5 pg per assay). The amplification was performed in a LightCycler® 480 Real-Time PCR System under the following

conditions: initial denaturation step at 95°C for 5 min followed by 40 cycles at 95°C for 10 sec, 64°C for 10 sec and a final extension step at 72°C for 10 sec.

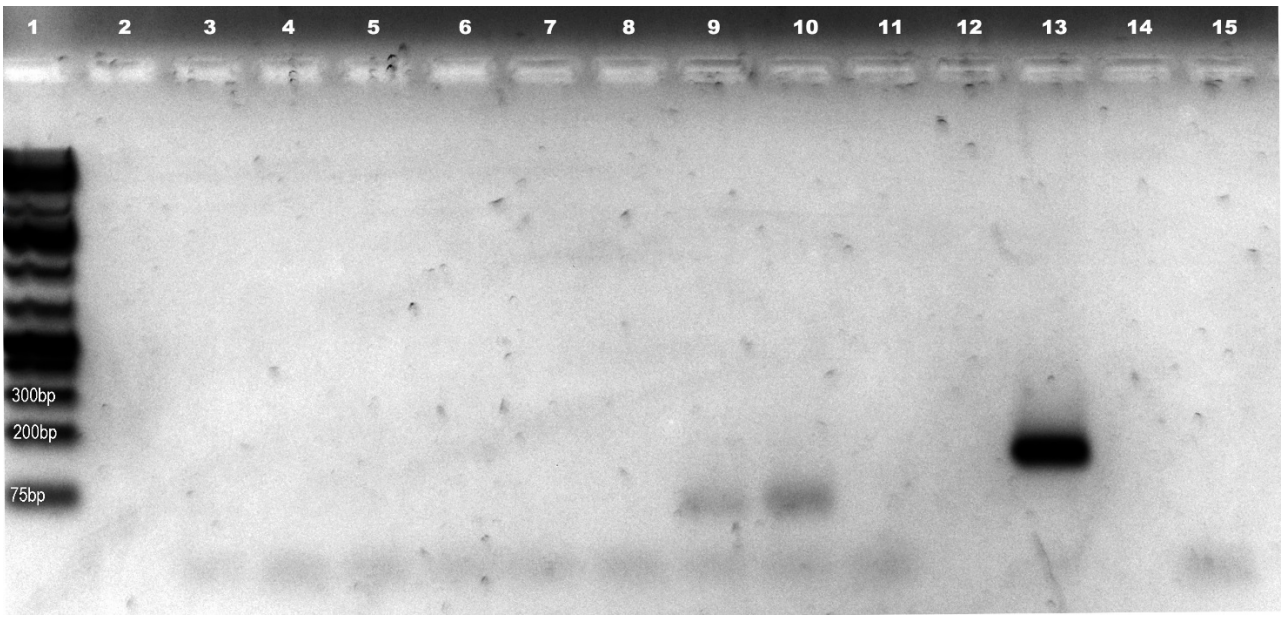

Figure 1: Specificity Test of the Conventional PCR. The PCR product is 148 bp with primers gb-S and gb-A in a 2% Agarose gel. Lane 1: O´GeneRuler 1k Plus DNA Ladder, ready-to-use (Fermentas), Lane 2: Empty, Lane 3: *Avibacterium paragallinarum*, Lane 4: *Ornithobacterium rhinotracheale*, Lane 5: *Salmonella* spp., Lane 6: *Gallibacterium anatis*, Lane 7: *Fowl Adenovirus* 4, Lane 8: Egg drop syndrome virus, Lane 9: *Herpesvirus* of turkey, Lane 10: Chicken Anemia virus, Lane 11: Negative Control (DNA of uninfected DF-1 cells), Lane 12: Empty, Lane 13: Positive control of ILTV, Lane 14: Empty, Lane 15: Blank (PCR-grade water).

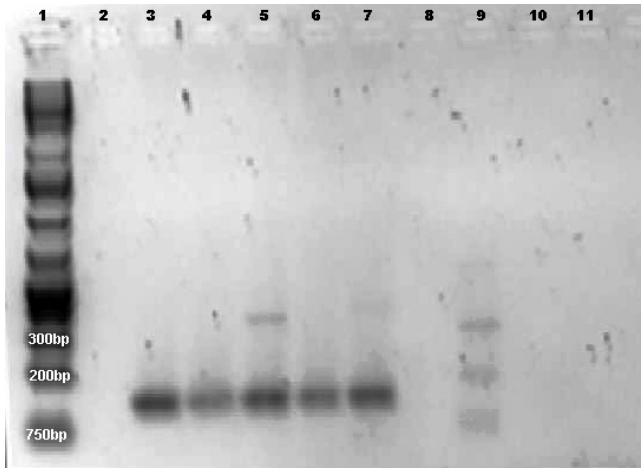

Figure 2: LoD of the conventional PCR in 2% agarose. The PCR product is 148 bp with primers gb-S and gb-A. Lane 1: O´GeneRuler 1k Plus DNA Ladder, ready-to-use (Fermentas, USA), Lane 2: Empty, Lane 3: C (1.0 pg/µL), Lane 4: D (100 fg/µL), Lane 5: E (10 fg/µL), Lane 6: F (1 fg/µL), Lane 7: G (100 ag/µL), Lane 8: H (10 ag/µL), Lane 9: DF-1 (Dilutor), Lane 10: Empty, Lane 11: Water.

The denaturation curve was performed from 50°C to 90°C with a ramp of 0.11°C/sec and 5 readings/°C. The derivative of the fluorescence peaks -d(RFU)/dT was used to determine the LoB, LoD and LoQ. The number of ILTV copies was determined with the following formula: [A/C] × [D/E] × F, where A corresponds to the copy number of the gB gene calculated by the thermocycler, C consists in the total of microliters of the eluted DNA sample used for the PCR procedure, D constitutes the total microliters of elution buffer used to elute the extracted viral DNA, E represents the total of microliters of initial sample used for the viral DNA extraction, and F corresponds to the dilution factor of the sample used in the PCR procedure. This formula corresponds to the one specified in a previous publication [28]. However, we decided to express the final results in number of gB copies/µL of AF.

qPCR precision: In order to demonstrate the accuracy of the procedure if it is preformed daily, IRM dilutions with DNA from DF-1 cell line were prepared, and the precision was operatively defined as repeatability of the mean of the gB copy number/reaction between assays in nine repetitions in three days (three repeats each day).

Diagnostic specificity of qPCR: The qPCR diagnostic specificity was evaluated nine times by taking positive DNA samples from 11 most common poultry pathogens, DF-1 cells, IRM-ILTV (Table 1). This parameter was measured based on the presence or absence of a specific denaturation peak (descriptive data of -d(d/dT) for each of these pathogens. DNA extracted from DF-1 cells was used as a negative control (Ct-) and PCR water as a blank.

LoB of qPCR: Aliquots of DNA from DF-1 cells were prepared as negative controls for testing in duplicate in three runs. The LoB was

operatively calculated with the following formula (1) [22], where, 1.645 times the standard deviation of -d(d/dT) is added to mean of -d(d/dT) signal of the Ct-.

$$LoB=Mean_{NC}+1.645(SD_{NC}) \quad (1)$$

LoD and LoQ of qPCR: IRM dilutions with DNA from DF-1 cell line were (Table 2) tested in duplicates in three runs. The LoD was operatively calculated (2) [22] where, the LoB is added to 1.645 times the standard deviation of the -d(d/dT) signals of the dilution with a confidence level ≥ 95%. These dilutions also allowed calculate the LoD as ILTV gene copies/µL of AF. The LoQ was operatively derived with the following formula (3), where, the LoD is expressed as the number of ILTV gene copies/µL of AF.

$$LoD=LoB+1.645(SD_{low \geq 95\%}) \quad (2)$$

$$LoQ=10/3 \times LoD \quad (3)$$

	C	D	E	E1	F	G	H
Theoretical Viral Genome Copies	6.083×10^3	6.083×10^2	6.083×10^1	3.042×10^1	6.083×10^{-1}	6.083×10^{-2}	6.083×10^{-3}
[DNA]	1.0 pg/µL	100 fg/µL	10 fg/µL	5.0 fg/µL	1.0 fg/µL	100 ag/µL	10 ag/µL
[gB/rxn]	2.2607×10^4	2.2607×10^3	2.2607×10^2	1.13×10^2	>5% of the replicates produces values such as Cp (-)		
[copies/µl]	1.356×10^4	1.356×10^3	1.356×10^2	6.78×10^1			

Table 2: IRM-DNA concentrations and corresponding theoretical viral genome copies. Initial IRM-DNA of ILTV corresponds to the letter C.

Statistical analysis

The normality of the distribution of the IRM replicates was determined using the Shapiro-Wilk test. The PCR precision was assessed using the Mann-Whitney U test with a significance level of 95% (α=0.05), and the diagnostic specificity was analyzed using the Chi-squared test. Data analysis was performed using GraphPad Prism® 5 software program (San Diego, CA).

Results

Primer design and PCR optimization

The primers were designed to amplify part of the ILTV gB gene, and a search in the GenBank database did not reveal any hybridization between the primers and none of the known sequences belonging to any bacteria or virus of poultry, or other accession numbers. The optimum concentrations of the primers used for the conventional PCR and qPCR were 0.25 µM and 0.125 µM, respectively.

Quantitative analysis

qPCR precision: To test the hypothesis of the existence of precision (in three days), an assumption was made of the existence of repeatability in the gB copy number/reaction between two days. We applied Mann Whitney test and found a p-value of 0.9336, which rejected that our readings are different; therefore, at a significance level of 95% the replicates with the IRM-ILTV were homogeneous.

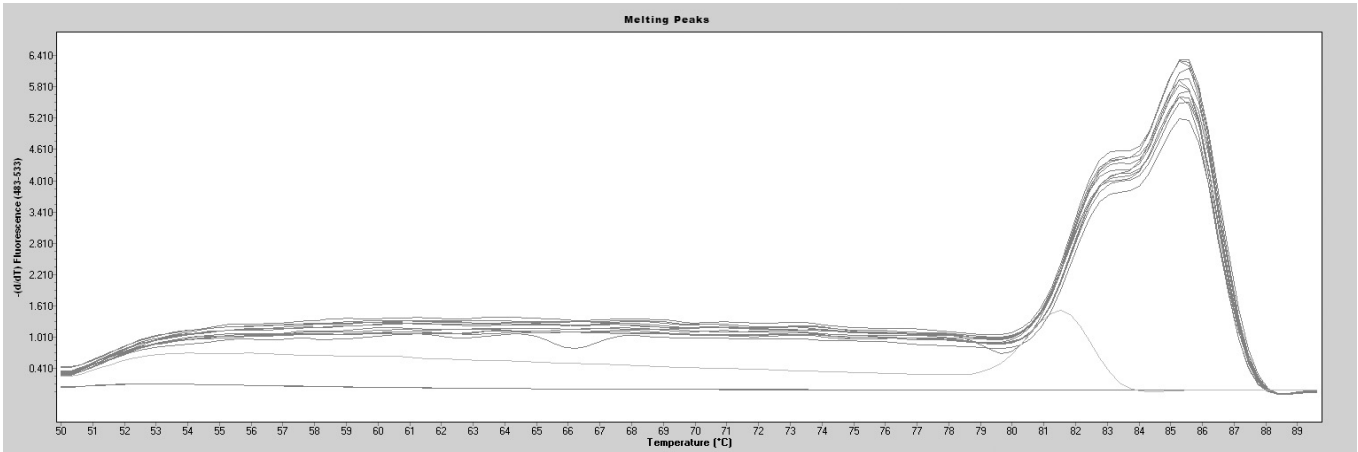

Figure 3: Melting peaks of qPCR products obtained using the LightCycler®480 (Roche). The PCR product is 132 bp with primers ILTF_2 and ILTR_2. The illustration shows the denaturation temperature (Tm) of the ILTV PCR products at 85.46°C and adjacent negative controls. The X-axis represents the temperature (°C), and the Y-axis represents the negative derivative of the fluorescence (488-533 nm) on the derivative of the temperature.

qPCR diagnostic specificity: We did not obtain any amplification in both PCR water and DNA from DF-1 cell line. When the specificity of the qPCR was assessed, we found that the seven samples of different viruses had no amplification (median signal, 0.03-d(RFU)/dT). We also included analysis of the amplification from IRM (median signal, 6.2-d(RFU)/dT) which differed significantly (p-value<0.0001).

Therefore, we conclude that at a significance level of 95%, there is no evidence of false positives in the development of the analytical procedure (Figure 3).

qPCR LoB, LoD and LoQ: When the nine replicates of a DNA of DF-1 cell line were tested, LoB or highest apparent signal of the ILTV gene by qPCR was 0.044 –d(RFU)/dT. LoD was distinguished from the limit of blank (1.56 –d(RFU)/dT) and will be able to reliably detect targets as low as 5.0 fg/µL or 6.78×10^2 gB copies/µL of AF (113×10^2 gB copies/reaction). The LoQ reliably quantify targets from 16.6 fg/µL or 2.226×10^1 gB copies/µL of AF (376.6×10^2 copies gB/reaction) (Table 3, Figures 4 and 5).

	C (IRM)	LoD	LoQ
[DNA]	1.0 pg/µL	5 fg/µL	16.6 fg/µL
[gB /rxn]	2.2607×10^4	1.13×10^2	3.766×10^2
[copies /µL]	1.356×10^4	6.78×10^1	2.226×10^1

Table 3: LoD and LoQ of qPCR (LightCycler®480) with primers ILTF_2 and ILTR_2. Limit of Detection (LoD), Limit of Quantification (LoQ) of the test and its equivalence in minimum DNA concentration, copies per reaction and copies per µL.

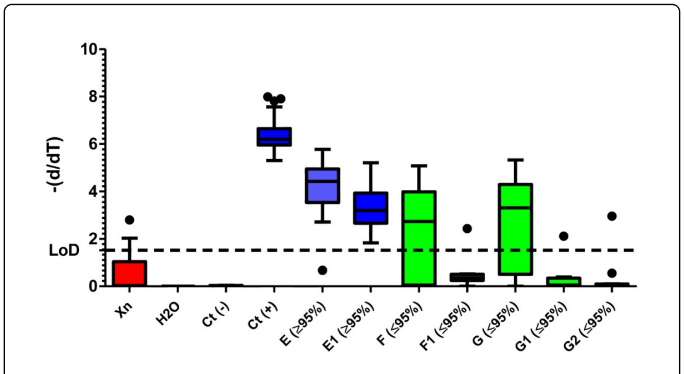

Figure 4: LoD of qPCR, in samples with low amount of analytes. The PCR product is 132 bp with primers ILTF_2 and ILTR_2. The trimmed line shows the LoD (1.56), Xn: Other avian pathogens, H_2O: Water, Ct(-): DF-1, Ct(+): IRM-ILTV, E: 10 fg/µL, E1: 5.0 fg/µL, F: 1.0 fg/µL, F: 0.5 fg/µL, G: 100 ag/µL, G1: 50 ag/µL, G2: 25 ag/µL. Data was obtained using the LightCycler®480 (Roche).

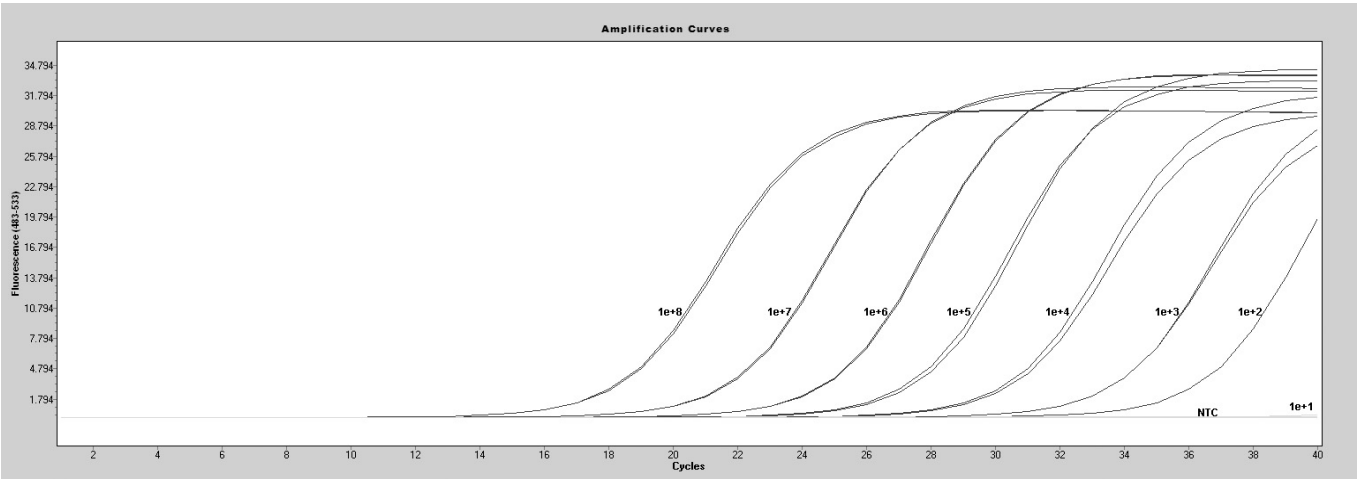

Figure 5: LoD and LoQ of qPCR using the LightCycler®480. The PCR product is 132 bp with primers ILTF_2 and ILTR_2.

Qualitative analysis

Diagnostic specificity of conventional PCR: Neither our primers nor the primers reported by Zhao et al. [19] produced any non-specific bands of the gB gene in any replicate (Figure 1). The identity of the IRM-ILTV specific band (1 pg/µL) was confirmed by sequencing.

LoD of conventional PCR: The LoD of the conventional PCR was determined from the presence of a single-intensity band in more than 95% of the nine replicates of samples containing small but known concentrations of the target gene (Figure 2). The LoD was found to be 100 ag/µL, indicating the limit of copies that can be distinguished with confidence (Table 2).

Discussion

PCR is considered to be a valid procedure for the detection of biological contaminants in avian vaccines due to its higher sensitivity compared with conventional techniques [9]. In this study, the results demonstrated the systematic methodology is robust, and fulfill the highest requirements to titer ILTV loads in the production of vaccines. It has not been used CAM (Chick Chorioallantoic Membrane) assay because it is used for angiogenesis levels [29] and would not be relevant in this study. Thus, it would be useful for the evaluation of the viral load in avian vaccines at different stages in the production process, at the time of vaccination and in epidemiological studies. Furthermore, to our knowledge, this is the first report validating a PCR-based methodology whose aim is to complement the existing tools for the quality control of ILTV vaccines produced in AF.

One of the first studies that used a similar approach was from Jang et al. [30], in which they validated a method for the detection and rapid titration of the Newcastle Disease Virus (NDV) that had been produced in cells to speed up the vaccine manufacturing process. They

evaluated parameters such as specificity, linearity, precision, LoD, LoQ and robustness.

To establish techniques that fulfill the quality requirements of the GLP/GMP and the FDA guidelines for the qualitative and quantitative detection of ILTV, we chose to use primers directed against the gB gene. We targeted this gene because it has been demonstrated that it is vital for the protection of chickens against this pathogen, allowing its extended use in recombinant vaccines [31]. The use of a different gene would limit the validation of our methodology in recombinant vaccines with an inserted gB gene. Therefore, we selected a pair of primers previously designed by Zhao et al. [19] to be used in conventional PCR, and a newly designed a new pair of primers for qPCR-based quantification of the ILTV load in vaccines produced in embryonated eggs.

Callison et al. [17] developed and validated a qPCR technique using TaqMan® probes to detect and quantify gC gene of the ILTV in tracheal swab and histopathological samples of sick birds. They achieved a LoD of 25 copies/reaction and a LoQ of 100 copies/reaction using a standard curve that maintained its linearity for five orders of magnitude (from 10^6 copies/µL to 10^2 copies/µL), with an R^2 value of 0.994 and an efficiency of 94.54%. Mahmoudian et al. [12] opted for the use of SYBR Green I-based qPCR method for detection of the UL15a gene in infected birds, using a standard curve that maintained its linearity for seven orders of magnitude (from 10^8 copies/µL to 10^2 copies/µL). The R^2 value obtained was of 0.994, and the efficiency was 96.36 (Figure 6). However, only three orders of magnitude were achieved with vaccine strains. Vagnozzi et al. [21] optimized a duplex PCR to quantify ILTV with primers directed against the gC gene, with collagen α2 gene from chicken as an internal control. They obtained an LoD similar to the one reported in 2007, with an analytical sensitivity of 5 to 50 copies/reaction, a linear range of six orders of magnitude (from 10^6 copies/µL to 10^1 copies/µL) and R^2 values of 0.9981 for gC gene and 0.9966 for α2 gene. Likewise, Zhao et al. [19] validated the use of a SYBR® Green I-based qPCR method directed towards infected birds and clinical samples using gB gene. They achieved a LoQ of 10 copies/reaction, and the method-maintained linearity for seven orders of magnitude (from 10^7 copies/µL to 10^1 copies/µL) with an R^2 value of 1.952 and an efficiency of 96.36%. Shil et al. [20] developed and validated a qPCR method using TaqMan® probes to quantify and differentiate wild ILTV from vaccine strains of ILTV by amplifying the gG gene in clinical samples. They achieved a LoD of 10 copies/reaction, and the standard curve maintained its linearity over seven orders of magnitude (from 10^7 copies/µL to 10^1 copies/µL) with an efficiency of 94.54% and an R^2 value of 0.994.

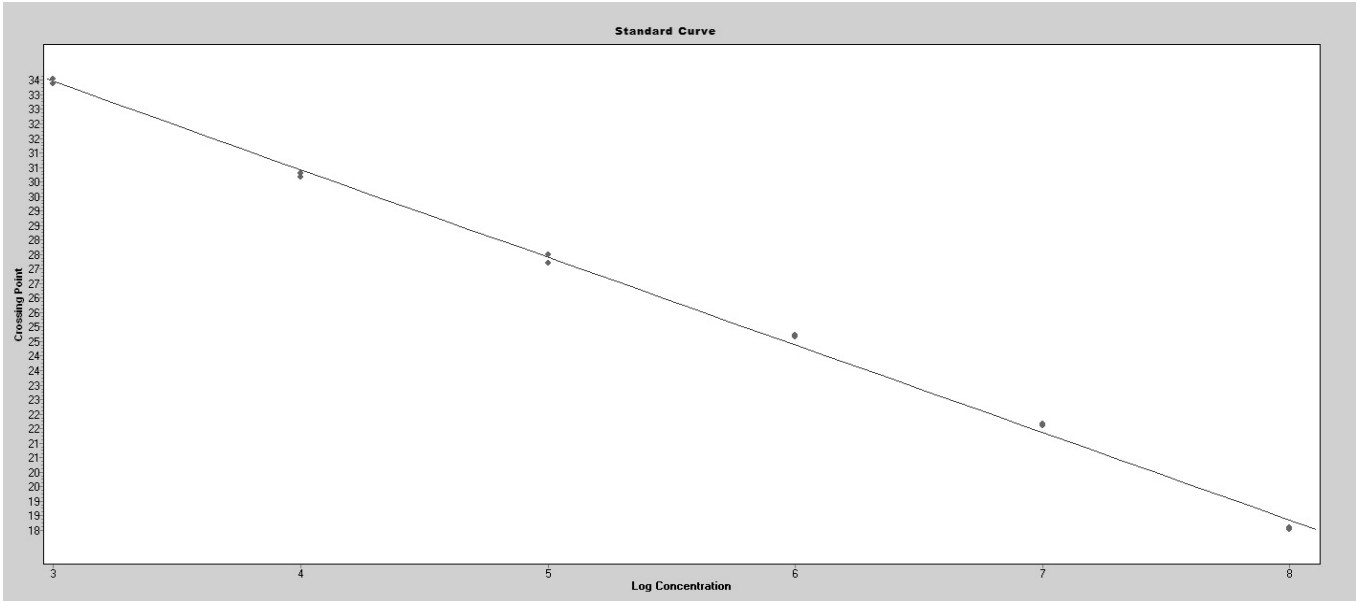

Figure 6: Linearity analysis of qPCR obtained with the LightCycler®480 (Roche). The PCR product is 132 bp with primers ILTF_2 and ILTR_2. The standard qPCR curve used to quantify ILTV was generated from serially prepared one-tenth dilutions of the plasmid containing a portion of the gB (from 1×10^8 to 1×10^1 partial gene copies per µL).

Compared to the previous studies, our study generated an IRM from an ILTV strain normalized in AF by performing serial dilutions of chicken DNA (from DF-1 cells) to evaluate the matrix effect [25] for both PCR systems.

The use of primers gB-S and gB-A produced an LoD of 1.017×10^5 copies/µL. However, our primers showed better observable definition at lower concentrations. Based on this, we opted to use them for qPCR with SYBR Green I because this nucleic acid stain is more accessible in terms of costs. These new primers achieved linearity over seven orders of magnitude (from 10^8 copies/µL to 10^2 copies/µL) with an R^2 of 0.9978, a LoD of 1.017×10^5 copies/µL of AF and a LoQ of 3.39×10^5 copies/µL of AF (Table 4). All these features proved that our newly designed PCR procedure constitute a useful laboratory technique to measure viral loads in industrially-produced vaccines for poultry. In addition to this, based on our experimental results, we established a decision-making algorithm (Figure 7) with two PCR techniques, allowing rapid and confident monitoring of AF to ensure the quality of vaccines produced in embryonated eggs (Table 4).

Characteristics of the test	Criteria of acceptance	Result of validation
Specificity	p-Value<α (α=0.05 a 95% confidence level)	Accepted (p-Value=0.0001)
Precision	p-Value>α (α=0.05 a 95% confidence level)	Accepted (p-Value=0.9336)
LoB	-(d/dT)=0.044	0 copies/µL
LoD	-(d/dT)=1.56	1.017×10^5 copies/µL (1.13×10^2 copies/rxn, 5 fg/µL)
LoQ	gB/rxn=336.6	3.39×10^5 copies/µL (3.366×10^2 copies/rxn, 1.66×10^1 fg/µL)
Linearity	R^2<1	Accepted: 0.0234<0.16 (0.9978)

Table 4: Summary of the statistical analyses with primers ILTF_2 and ILTR_2. Values found in the validation of the qPCR.

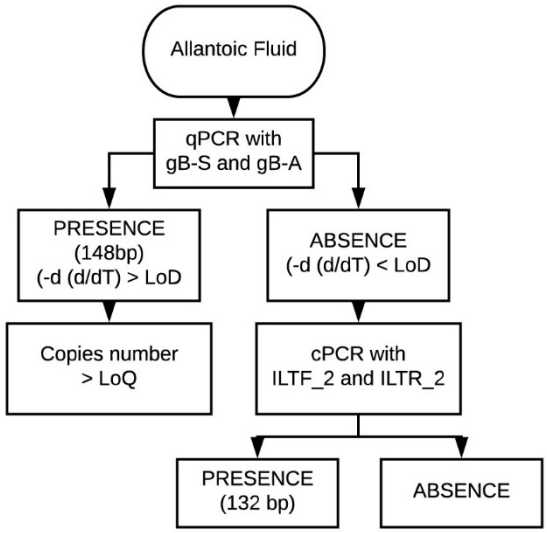

Figure 7: Flow chart for decision-making to apply it in the assessment of ILTV viral load in allantoic fluid.

Conclusion

In conclusion, we have validated a methodology based on both conventional PCR and qPCR for fast detection and quantification of ILTV produced in AF. This methodology considered yield parameters such as specificity, precision, LoB, LoD, LoQ and linearity. The study results would provide a systematic and robust methodology to achieve high demands on vaccine production. Therefore, it can be used as a guideline for the development and validation of other PCR-based systems that are needed for production and quality control of avian vaccines.

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