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Full Length Article

Development and Validation of a Two-Step TaqMan Real-Time RT-PCR for the Detection of Infectious Bursal Disease Virus in Infected Chickens

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Abstract

Context: Infectious bursal disease (IBD) is a major health threat to the world's poultry industry. This infectious outbreak in Morocco and many other countries throughout the world occurs despite intensive controls including proper biosafety practices and vaccination. The current research work was conducted to develop a rapid, sensitive, and specific TaqMan real-time reverse transcription-polymerase chain reaction (RT-PCR) method for rapid diagnosis of infectious bursal disease virus (IBDV). The intralaboratory validation of the conceptualized method was carried out with increased specificity, sensitivity, linearity, repeatability, and reproducibility. The diagnostic efficacy of the new assay was tested on 125 bursal samples collected from suspected cases distributed in different Moroccon regions and compared to the SYBR Green and conventional RT-PCR assay in terms of sensitivity and specificity. The new conceptualized method was more sensitive and less expensive compared to both conventional RT-PCR and Green method assays. The results obtained indicate that the standard curve exhibited a dynamic linear range ($1 –1\times10^8$ copies/μL) with a linear correlation (R2) of 0.999 between the cycle threshold (Ct) values *vs.* template concentration. The assay showed high sensitivity and ability in detecting 2×10^1 RNA copies per PCR reaction without any cross-reaction with other potential viruses belong avian type. The developed approach in the current research could be used to rapidly distinguish IBDV belongs to other pathogens. The developed test has been shown to be highly sensitive, specific, and reproducible and can be accurately used to detect avian IBDV RNA in clinical samples with high specificity within a short time compared to both conventional RT-PCR and Green method assays.

Keywords: IBDV; Tow-step TaqMan rRT-PCR; SYBR Green -RT-PCR; Diagnosis; Assay validation

Introduction

Infectious bursal disease (IBD) is a contagious viral disease with immunosuppressive effects on young chickens (Snyder 1990; Müller *et al.* 2003). The etiologic agent is a virus with a bi-segmented RNA genome among the family Birnaviridae (Kibenge *et al.* 1988; Berg 2000). Two serotypes are known for IBDV: serotype 1 viruses are responsible for diseases in poultry, while serotype 2 viruses are not pathogenic (McFerran *et al.* 1980; Becht *et al.* 1988). Gumboro disease virus-induced immunosuppression can be considered as one of the main health threats to poultry production in all industrialized poultry countries. Due to high cost of prophelaxtic agent to migtaigte infectious bursal disease, vaccination measures are requitred to achieve this goal (Sharma *et al.* 1994).

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Traditional diagnostic methods have been mainly based on the isolation of infectious bursal disease virus from cell culture,embryonated chicken eggs (Hitchner 1970; Fitzgerald *et al.* 1999), young specific-pathogen-free (SPF) chickens, detection of viral antigens in infected tissues using the agar-gel immunodiffusion test (Hirai *et al.* 1974; Snyder *et al.* 1992; Takase *et al.* 1993), immunohistochemistry, antigen-capture enzyme-linked immunosorbent assay (ELISA) (Marquardt *et al.* 1980; Meulemans *et al.* 1987) or fluorescence assay. Due to lack of sensitivity of conventional assays, various diagnostic methods targeting the viral genome using conventional reverse transcription-polymerase chain reaction (RT-PCR) were performed to be alternative for traditional diagnostic tools and to replace animal experiments (Stram *et al.* 1994; Liu 2000). As a result, pre-existing approaches are qualitative and do not allow to quantify IBD viral RNA in infected tissues. Recently the use of real-time RT-PCR (rRT-PCR), using a Taq-Man hydrolysis probe or SYBR Green, as a diagnostic tool for IBDV infections has many advantages, including quantification of viral RNA, short time of detection, low contamination, high sensitivity and specificity (Tomás *et al.* 2012, 2017).

The objective of the present study was to develop and validate a rapid, sensitive, and specific tow-step TaqMan rRT-PCR test for the detection and the quantification of IBDV viral RNA directly from clinical samples collected from chickens.

Material and Methods

Reference virus

IBDV strains Faragher 1970 (F 52/70) was used as a reference strain in this study (Bygrave and Faragher 1970).

Samples

During the period 1991–2018, 125 bursae of Fabricius were collected during clinical episodes of Gumboro disease in broiler farms located in different regions of Morocco (Table 1). All samples possessed lesions of congestion, edema, hemorrhage, or atrophy. The stock exchanges were stored at -20°C until their analysis. Table 1

The procedures used in the current research work were in accordance with the internationally accepted guidelines for care and use laboratory animals. Ethics committee at the National Laboratory of Veterinary Drug Control has reviewed and approved the present study.

Preparation and processing of the homogenate of Fabricius bursa

The bursa of Fabricius was washed with PBS and then treated with 1, 1, 2-Trichloro-1, 2, 2 Trifluoroethane to remove cellular debris. The viral suspensions obtained were treated with chloroform overnight at 4°C to inactivate any enveloped viral contaminant.

RNA extraction

The extraction of RNA virus was done using the QIAamp RNA Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The RNA obtained was eluted in 50 μL of nuclease-free water and stored at -70°C until further use.

Design of primers and probe

The primers and probe (Table 2) were obtained using the Gene Runner 3.05 software by aligning several sequences of different genotypes from GenBank (National Center for Biotechnology Information [NCBI]). Probes and primers were synthesized by Eurofins MWG Operon (Ebersberg, Germany) then purified by high-performance liquid chromatography (HPLC). The TaqMan probe was labelled using 6-carboxyfluorescein (FAM) at the 5'end by the time at the 3'end the TaqMan probe was labelled using 6-carboxytetramethylrhodamine (TAMRA). Table 2

Reverse transcription

Reverse transcription (RT) was performed using the Taq-Man Reverse Transcriptase Reagent kit. Synthesis of cDNA was done at 42°C for 30 min succeeded by a denaturation step at 99°C for 5 min. RNA sample from a reference strains F52/70 was used as a standard for every RT reaction. The conservation of the cDNA at−20°C allowed further use with better reproducibility (Bygrave and Faragher 1970).

Generation of standard RNA

The validation of the assay reliability, the determination of the detection limit, the performance of the assay precision, the correlation square (R^2), the efficiency as well as the standard curves were performed using the genomic RNAc of the IBDV reference strain F 52/70. Thus, the *in vitro* transcription was performed using the RNA production system according to the manufacturer's instructions. After treatment with DNase, the synthesized RNA was quantified by NanoDrop 1000S spectrophotometer (Thermo Scientific, Wilmington, U.S.A.) at 260 nm, which allowed the evaluation according to the molecular weight of the transcript. Successive dilutions of RNA transcripts were then made in water treated with DEPC (Applied Biosystems, U.S.A.) to obtain a range of 2×10^7 to 2×10^1 copies/mL (Bygrave and Faragher 1970).

TaqMan real-time PCR assay

The amplification and the detection of real-time RT-PCR were done by the Smart-cycler real-time PCR instrument (Cephied, California, USA) with the QuantiTect Probe RT-

Table 1: IBDV field samples and vaccine strains used in the study

Pathogen[a]	Sample type	Ct Value [b]	TaqManr RT-PCR	Conventional RT-PCR
IBDV	Bursa	35.18	positive	positive
IBDV	Bursa	34.26	positive	negative
IBDV	Bursa	38.41	positive	positive
IBDV	Bursa	37.23	positive	positive
IBDV	Bursa	39.50	positive	positive
IBDV	Bursa	30.15	positive	negative
IBDV	Bursa	37.31	positive	positive
IBDV	Bursa	39.60	positive	positive
IBDV	Bursa	35.81	positive	positive
IBDV	Bursa	31.40	positive	negative
IBDV	Bursa	30.53	positive	negative
IBDV	Bursa	31.39	positive	positive
IBDV	Bursa	30.52	positive	negative
IBDV	Bursa	11.29	negative	negative
IBDV	Bursa	32.38	positive	negative
IBDV	Bursa	39.82	positive	negative
IBDV	Bursa	28.91	positive	Negative
IBDV	Bursa	31.32	positive	positive
IBDV	Bursa	35.30	positive	positive
IBDV	Bursa	32.22	positive	positive
IBDV	Bursa	27.95	positive	negative
IBDV	Bursa	35.80	positive	negative
IBDV	Bursa	39.51	positive	negative
IBDV	Bursa	10.42	negative	negative
IBDV	Bursa	32.90	positive	negative
IBDV	Bursa	34.82	positive	negative
IBDV	Bursa	35.84	positive	negative
IBDV	Bursa	37.90	positive	negative
IBDV	Bursa	32.45	positive	positive
IBDV	Bursa	28.97	positive	negative
IBDV	Bursa	35.91	Positive	negative
F52/70	Bursa	31.60	positive	positive
ILTV	Vaccine	11.25	negative	negative
NDV	Vaccine	12.20	negative	negative
IBV	Vaccine	10.23	Negative	negative

[a]IBDV: Infectious Bursal Disease Virus, NDV: Newcastle Disease Virus, ILTV: Infectious Laryngotracheitis Virus, IBV: Infectious Bronchitis Virus and IBDV, F 52/70 strain: Faragher 52/70 reference strain, [b]Ct value: Threshold value

Table 2: Sequences for primers and probe design

Primer/probe	Sequence 5′→ 3′	Accession no [a]
IBDVProbe	(FAM) –TCCCCTGAAGATTGCAGGAGCATTTG– (TAMRA)	D00869
IBDVF[b]	GAGGTGGCCGACCTCAACT	
IBDVR[c]	AGCCCGGATTATGTCTTTGAAG	

FAM, 6carboxyfluorescein; TAMRA, 6 carboxytetramethylrhodamine, [a]Genomic DNA sequence, [b]Forad, [c]Revere

PCR Master Mix two-step kit (Applied Biosystems, California, U.S.A.). The concentrations of the mix, as well as those of the primers and probe, were performed according to the Applied Biosystems kit recommendations. The amplification program was adapted to the RT-PCR enzyme. The denaturation step was shortened due to the performance of the Smart Cycler (Cephied, California, USA). The PCR cycles were performed under the following conditions; 5-min of incubation at 50°C, 5-min of denaturation at 99°C, 40 cycles of 15 sec at 95°C and 1 min at 60°C, and the final step was done within a time of 5 min at 70°C. Each experiment comprising a negative extraction control (diethyl pyrocarbonate-treated water), a negative PCR control (NDV, IBV, ILTV) and 4 range points corresponding to 2×10^5, 2×10^4, 2×10^3 and 2×10^2 copies/ml to establish a calibration line. Each used strain was tested in triplicate. The Smart Cycler interpretation software gave the quantification of each sample by comparing the obtained threshold cycle (or Ct) with the calibration line calculated from the range points. The positivity threshold used in the assay was 27.95 fluorescence units.

Amplification efficiency (E)

Considering the calibration line representing the threshold cycle as a function of the number of virus copies present in the sample, E can be performed according to the following Formula: E=10 (-1/slope). In theory, the repetition of n PCR cycles results in a theoretical exponential accumulation of 2n per DNA molecule because each PCR product was duplicated at each cycle. We should then be as close as possible to 2, *i.e.*, each PCR product was duplicated with each cycle (Wilkening and Bader 2004).

A range of 7 points: 2×10^7, 2×10^6, 2×10^5, 2×10^4, 2×10^3, 2×10^2 and 2×10^1copies/mL, tested in triplicate in three different experiments allows to estimate this efficiency.

Linearity and detection limit

The average linear regression coefficient (R^2) was obtained from a range of 7 points: 2×10^7, 2×10^6, 2×10^5, 2×10^4, 2×10^3, 2×10^2 and 2×10^1 copies/mL, tested in triplicate in 3 different experiments. R^2 must be greater than or equal to 0.99 to conclude that there is a correlation between 2 parameters. The loss of linearity was used to define the quantification threshold. The detection limit corresponds to the minimum detectable quantity with a probability of more than 95%.

Sensitivity of tow-stepTaqMan real-time RT-PCR

The sensitivity of the method that represents the slope of the calibration straight line; when the calibration curve is not a straight line, the sensitivity to a given concentration will be defined as the slope of the tangent to the curve at that concentration. The higher sensitivity will be used to distinguish 2 samples of similar concentration. It also appears that any increase in the sensitivity will induce a decrease in detection or quantification limits.

In this study, the sensitivity assessment of the test was performed using various concentrations of IBDV standard RNA as a model. RNA was quantified using NanoDrop 1000 S pectrophotometer (Thermo Scientific, Wilmington, U.S.A.) and was diluted 10 times in series from 2×10^7 to 2×10^1copies/μL as a model (Tomás *et al.* 2012).

Specificity of tow-stepTaqMan real-time RT-PCR

In the framework of specificity determination of the assay, RNA or DNA was extracted from three avian viruses including NDV, IBV and ILTV then tested. Avian IBDV and ultrapure water were used as positive and negative controls respectively. The assay was conducted in triplicate.

Repeatability of tow-stepTaqMan real-time RT-PCR

The repeatability test (or intra-assay variability) was done by analyzing the same sample several times to characterize the best performance of the technique. Thus, the repeatability of our technique was evaluated by analyzing 5 samples of different concentrations (10^{-1} to 10^{-5} copies/mL) of standard RNA, which were tested 3 times in the same experiment.

The mean and the standard deviation (SD) for the intra-assay test were performed separately using Excel software.

Reproducibility of tow-stepTaqMan real-time RT-PCR

The reproducibility test (or inter-test variability) was performed by analyzing the same sample under different conditions (variations concerning the operator, the batches of reagents, the places, and times of realization). The extracts used were the same as for repeatability. Each extract was run 3 times in 5 different series with 2 different operators and 2 different batches of amplification kit.

The mean and coefficient of variation (COV) of the inter-test variability test were calculated separately for each standard dilution of cRNA according to their Ct values using Microsoft Excel software.

Detection of virus RNA in field cases

The collection of clinical samples was conducted in different chicken farms in different Moroccan regions during the period 1991–2018, and then were screened using a conventional RT-PCR assay. The conventional RT-PCR assay was conducted using a thermal cycler (Applied Biosystem, California and U.S.A.) with some modifications (Gerber *et al.* 2014).

The one-step real-time RT-PCR test based on the SYBR Green was used to perform comparison with the newly developed method in terms of sensitivity and specificity. Primer Express TM version 3.0 software (Applied Biosystems) was used to establish specific primers for the SYBR® Green quantitative PCR. The composition of the reaction mixture (per well) was: 12.5 μL of 2x SYBR® Green Master Mix, containing Taq DNA polymerase, dNTPs, reaction buffer, SYBR Green 1 and 5 m*M* $MgCl_2$ (Bio-Rad), 2.5 μL of each primer (500 n*M* final concentration), 2.5 μL of diluted transfer RNA and 5 μL of extracted RNA. Parallelly, each 96-well plate includes 6 dilutions of the genomic RNAc of the IBDV reference strain F 52/70 (10 to 106 copies of DNA). After addition of RNA, the 96-well plates were sealed in clear film and inserted into the 5700 Sequence detector (Perkin-Elmer Applied Byosystems) thermal cycler; the thermal cycling program consists of 40 cycles [denaturation at 95°C - hybridization - elongation at 60°C], followed by a single dissociation cycle, consisting of a gradual temperature increase from 60 to 95°C, to establish the dissociation curve [denaturation at 95°C - hybridization - elongation at 60°C], followed by a single dissociation cycle, consisting of a gradual temperature increase from 60 to 95°C to establish the dissociation curve.

Statistical analysis

In this research, the data were analyzed using Stata V. 11.1 software (Stata Corp 4905 Lakeway Drive, Texas, U.S.A.). The variables were analysed by the Chi-2 test or the exact Fisher test. The significant values of difference were considered at P value < 0.05. Logistic regression was used to confirm whether epidemiological data predicts the presence of the desired event (Greenland *et al.* 2016).

Results

Evaluation of tow-step TaqMan real-time RT-PCR characteristics

The optimization and the evaluation of the investigated method were carried out using a strategy based on systematic experiments with different concentrations of primers (5, 10, 15, 20, 25 pmol) and probes (2, 4, 6, 8, 10 pmol) (data not shown). The optimal amount of each primer was determined and will then be used to evaluate the optimal amount of the probe. The amount of the other components of the reaction mixture (enzyme, buffer, dNTP, and Mg^{2+}) shouldn't vary. Following manufacturer recommendations, systematic aliquoting of PCR reagents (primers and probes) were implemented to limit the risks for contamination and improve the quality of the molecular test, as PCR reagents that were repeatedly opened and closed more likely to avoid any contamination by amplified DNA and multiple freeze/thaw detection systems.

The evaluation of the technique was carried out using ranges of transcripts of RNA of virus present in the standard sample (2 x 10^7 to 2 x 10^1copies/mL) A range of standard RNA transcripts was established on 7 points: 2×10^7, 2×10^6, 2×10^5, 2×10^4, 2×10^3, 2×10^2 and 2×10^1 copies/ml, tested in triplicate using 3 different experiments to estimate this efficiency. The cDNAs were then used to determine the detectability and linearity of the assay (Fig. 1). The standard curve produced a linear range of seven magnitudes as well as linearity over the whole quantitation range (slope = -3.605), it provided also an accurate measurement over a very wide range of starting target quantities. The coefficient of linear regression (R^2) was 0.999 and the PCR efficiency was around 89.260% Fig. 1.

Linearity and detection limit

The average linear regression coefficient, obtained for the 3 regression lines ranged from 2×10^7 to 2×10^1 copies/mL, was 0.99. The coefficients of variation of the Ct (or detection threshold cycles) established for each point of the 3 ranges, showed variations of less than 2% up to 2×10^1 copies/mL. The quantification threshold of the technique set at 2×10^1 copies/mL when there was a loss of linearity in the detection of the range points.

The detection limit of the tow-step TaqMan rRT-PCR was 2×10^1 copies/μL, whereas that of RT-PCR was 2×10^2 copies/ μL. Moreover, TaqMan rRT-PCR was 10-fold more sensitive than RT-PCR as reported in the current findings.

Specificity of the test

In the current research work, the specificity of the tow-step TaqMan rRT-PCR assay was assessed using other animal viruses. It was reported that strong fluorescent signals were registered due to the detection of IBDV meanwhile the signals of other samples of viruses and water used as controls were equivalent to levels of baseline (Fig. 2). The differentiation of IBDV from other viruses was done by comparing the signal strengths at different levels Fig. 2.

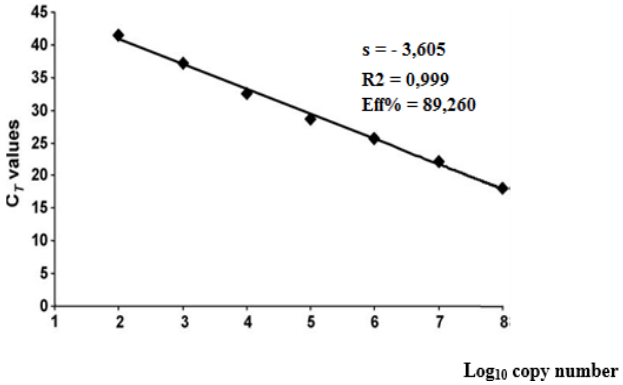

Fig. 1: Standard curve of the TaqMan rRT-PCR assay for avian IBDV. Ten-fold dilutions of standard RNA prior to amplification were used, as indicated in the x-axis, whereas the corresponding cycle threshold (CT) values are presented on the y-axis. The standard curve was linear, with a correlation (R2) of 0.999 between the cycle threshold (CT) value and template concentration, and a slope of -3.605

Intra- and Inter-assay variability

The determined of the fluorogenic reproducibility of the investigated assay was done by testing five 10-fold serial dilutions of standard cRNA (10^{-1} to 10^{-5}). Regarding the standard cRNA, dilutions from 10^{-1} to 10^{-5} were used. The results showed that the values of the intra-assay standard deviation (SD) as well as the coefficient of variation (CV) ranged from 0.02 to 0.55 and 0.02% to 0.7%, respectively as shown in Table 3. The obtained results of the inter-assay (SD) and (CV) ranged from 0.19 to 1.25 and 0.19% to 1.62%, respectively as reported in Table 4. Therefore, the obtained findings showed that the present assay is more repeatable and reproducible with negligible variation (Table 3–4; Fig. 3).

Detection of virus RNA in field cases

In the framework of sensitivity assessment, the tow-step TaqMan rRT-PCR was compared to conventional RT-PCR assay. Both tow-step TaqMan rRT-PCR and conventional RT-PCR were simultaneously evaluated to test 125 clinical samples collected at different time intervals from bird flocks located in various parts of Morocco during the period 1991-2018. Genomic RNA was analysed in duplicate using TaqMan and conventional RT-PCR assays (Fig. 4). The real-time analysis forecasted that the number of starting RNA copy was 2×10^7 RNA copies/μL of the template. The limit of detection of TaqMan and conventional RT-PCR assays was 2×10^1 and 2×10^2 copies respectively. Belonging 125 sample's collection, 13 samples showed avian IBDV

Table 3: Intra-assay reproducibility of tow-step TaqMan rRT-PCR assay

Variation	Different dilutions	Ct values for the different dilution of standard cRNA				
		1	2	3	Mean Ct ± SD[a]	CV (%)
Intra-assay	$_{10}$−5	28.18	28.64	28.09	28.30 ± **0.55**	**0.7**
	10^{-4}	26.16	26.25	25.89	26.18 ± 0.18	0.20
	10^{-3}	23.17	23.13	23.10	23.13 ± **0.02**	**0.02**
	$_{10}$−2	18.18	17.92	18.15	18.08± 0.16	0.13
	10^{-1}	16.45	16.48	17.02	16.65 ± 0.27	0.21

[a] Means for three independent analyses, SD=Standard deviation, CT=cycle threshold, CV=coefficient of variation

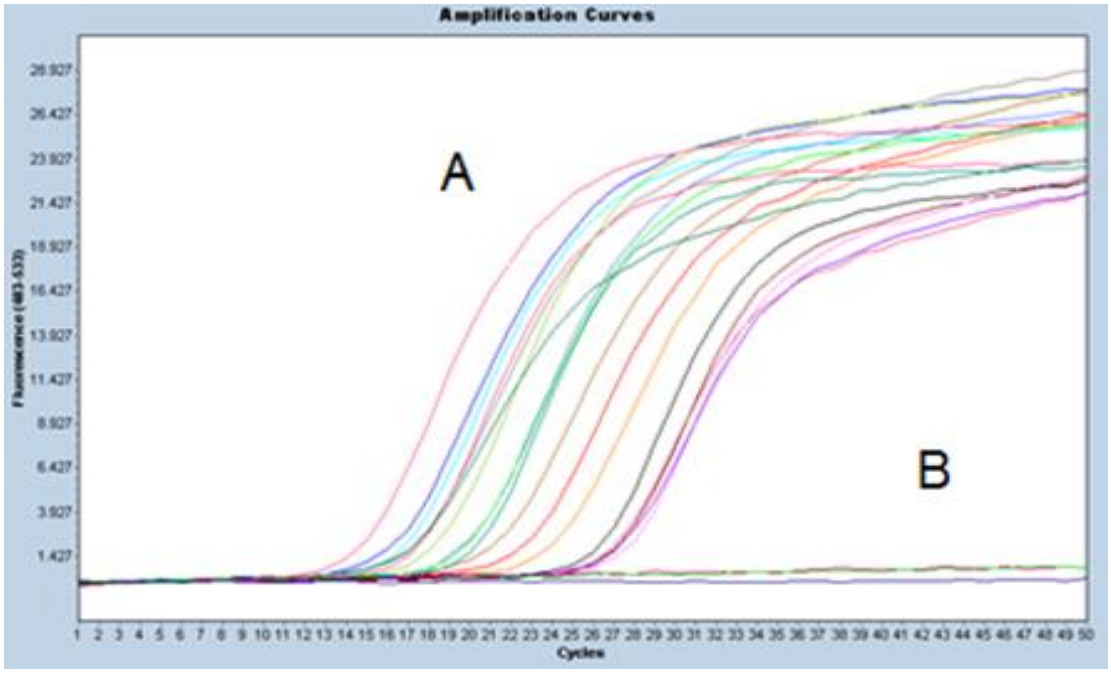

Fig. 2: Specificity evaluation of the tow-step TaqMan rRT-PCR assay for the detection of IBDV. Fluorescence intensity on the y-axis and number of PCR cycles on the x-axis. (**A**) Indicated IBDV specific amplification lines. Non-specific IBDV amplification lines for nucleic acids of other viruses such as ILTV, BIV, and NDV were indicated by (**B**)

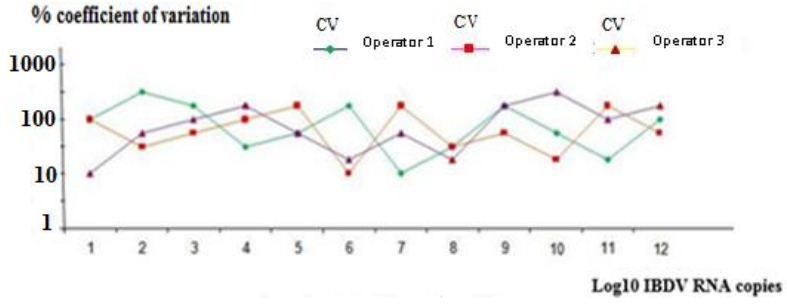

Fig. 3: Inter-assay reproducibility evaluation used for the detection of avian IBDV using tow-step TaqMan rRT-PCR

positive test according to conventional RT-PCR (Table 5) vs.16 showed positive test according to the tow-step TaqMan rRT-PCR (Table 5). Latter samples were confirmed using the analysis of the sequence. The findings obtained indicate that the tow-step TaqMan rRT-PCR could detect avian IBDV RNA in clinical samples with higher sensitivity compared to conventional RT-PCR (Table 5 and Fig 4).

Discussion

Infectious bursal disease constitutes a major burden to the poultry industry throughout the world despite the involvement of different assays to limit its inconveniences like vaccination (Berg 2000; Müller *et al.* 2003). Global surveillance programs need effective and rapid assays for the

Table 4: Inter-assay reproducibility of tow-step TaqMan rRT-PCR:assay

Variation	Dilutions	Ct values for the different dilution of standard cRNA				
		1	2	3	Mean Ct ± SD[a]	CV (%)
Intra-assay	$_{10}$−5	29.27	29.23	29.12	29.35 ± **1.25**	**1.62**
	10^{-4}	25.14	24.97	25.18	25.10 ± 0.53	0.59
	10^{-3}	23.29	22.89	23.25	23.14 ± **0.19**	**0.19**
	$_{10}$−2	19.23	17.32	19.20	18.58 ± 0.69	0.56
	10^{-1}	16.20	16.12	16.82	16.38 ± 0.74	0.53

[a] Means for three independent analyses, SD=Standard deviation, CT=cycle threshold, CV=coefficient of variation

Table 5: Sensitivity comparison of tow-step TaqMan rRT-PCR and conventional RT-PCR

Method	Number of positive samples	% of positive reactions	*P*-value*
Tow step TaqMan rRT-PCR	29/ 31	94%	0.019
Conventional RT-PCR	13/ 31	42%	0.028

The results were reported as % of positive reactions for each Method and analysed using ANOVA Test for statistical evaluation;*: *P*-value < 0.05 was considered significant

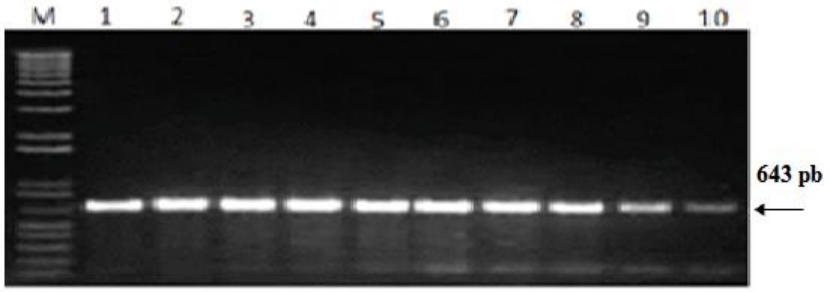

Fig. 4: Sensitivity of the conventional RT-PCR based on gene VP2 of avian IBDV. Lane M, molecular size marker Gene Ruler 100 bp DNA Ladder; lanes 1–10, 10-fold dilutions of avian infectious bursal disease virus-positive sample

diagnosis of this disease. Nowadays the selection of promising diagnostic tests relies on large parameters comprising specificity, sensitivity, cost, and other advantages.

Recently, many trials and assays have been adopted to develop an efficient diagnosis of diseases, mainly through the development of RT-PCR tests to improve effective *in vivo* pathogen detection in different biological samples (Stram *et al.* 1994; Liu 2000). Besides, RT-PCR is considered as one of the most rapid and specific methods compared to other conventional methods as well as immunofluorescence, ELISA and virus isolation from embryonated eggs (Rosenberger 1989; Wilkening and Bader 2004). However, The RT-PCR technique needs many requirements including a longtime of analysis (48 h) whether to attain a complete qualitative analysis in addition to post-PCR tests to detect PCR products. On the other hand, this technique has limited sensitivity as reported in earlier data (Espy *et al.* 2006).

In this current study, a new diagnostic test for IBDV has been developed. Validation of results of the tow-step TaqMan rRT-PCR assay was performed by estimating the following parameters: specificity, linearity, accuracy (repeatability), sensitivity, and detection limit. This new tow-step TaqMan rRT-PCR test developed has been compared to the conventional RT-PCR. According to our results, that the tow-step TaqMan rRT-PCR assay has several advantages over, mainly the reduction of analysis time (a time limit of 3 h instead of 48 h) and the processing of several samples simultaneously (Espy *et al.* 2006). Moreover, this essay does not require post-PCR tests since the reaction is achieved in a closed-tube system. This technique also guarantees a reduction in residual contamination during analysis especially that produced during separation between RT and fluorogenic PCR in conventional RT-PCR assay. On the other hand, the TaqMan rRT-PCR method in two stages was less expensive and more sensitive to detect and to quantify viral load in biological samples, (Stram *et al.* 1994; Liu 2000). Therefore, this new two-step TaqMan rRT-PCR assay is rapidly becoming one of the most promising methods to improve epidemiological surveillance programs due to its important results (Wu *et al.* 2007).

This new two-step TaqMan rRT-PCR assay was also compared to quantitative real-time PCR assay (qPCR), based on specific fluorophore in double-stranded DNA, SYBR Green. The chemistry of SYBR Green is based on the use of DNA intercalation molecules that emit fluorescence when incorporated into double-stranded DNA, even for small amounts of DNA. This technology has increased sensitivity, potentially allowing the detection of as little as 20 pg of genomic DNA initially present in a sample (Karlsen *et al.* 1995). The specificity of the reaction will also be ensured by the interpretation of the fusion curve, which provides information on the nitrogen base

composition of the amplified DNA fragment.

The choice between SYBR Green and Taqman chemistry will be made according to the specificity desired. A Taqman PCR will be preferred when a specific pattern without risk for variability. SYBR Green chemistry has the advantage of amplifying a potentially variable region, the stability of primer hybridization sites. The analysis of the melting curve offered by the SYBR Green technology allows the identification of the presence of relevant mutants or patterns. The use of Taqman probe considerably increases the specificity of the reaction. Therefore, this new two-step TaqMan rRT-PCR assay is rapidly becoming one of the most promising methods for improving epidemiological surveillance programs due to its significant results (Wu *et al.* 2007).

Conclusion

This work gives big data on intra-Laboratory validation of a tow-step TaqMan rRT-PCR assay. The present assay was conceptualized to detect infectious bursal viruses within a short time with high sensitivity and efficiency as well as to quantify the viral RNA in biological samples. The new assay could be used to characterize field samples and to assess the efficacy of antiviral drugs and experimental vaccines. Due to its promising results, the current assay is expected to improve IBDV surveillance worldwide.

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Author Contributions

Fatima Tahiri and Mohammed Bourhia wrote the original draft; EL Harrak Mehdi and Benaissa Attarassi visualized the work; Riaz Ullah, Hafiz Majid Mahmood, Ahmed bari, and Laila Benbacer reviewed and edited; Driss Belghyti supervised the work.

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