Journal of Agricultural Science and Technology A 8 (2018) 230-238
doi: 10.17265/2161-6256/2018.04.006

Diagnosis of Clinical Cases of Infectious Bursal Disease Using a Modified Rapid Taq Man-MGB Real-Time RT-PCR Assay

Maryame Cheggag[1], Khalil Zro[2], Ghizlane Sebbar[2], Naoufal Rahmatallah[3], Mohamed Mouahid[3], Mohammed EL Houadfi[1] and Faouzi Kichou[1]

1. Unit of Histology and Pathological Anatomy, Department of Veterinary Pathology and Public Health, Institute of Agronomy and Veterinary Medicine Hassan II, B.P. 6202, Rabat, Morocco

2. Society of Veterinary Pharmaceutical and Biological Productions (Biopharma), B.P. 4569, Rabat, Morocco

3. Veterinary Clinic Temara, Morocco

Abstract: Infectious bursal disease (IBD) is an important contagious viral infection of immune system of poultry. This infection possesses a permanent threat to the profitability of poultry industry worldwide. The aim of this work was to modify the Taq Man-MGB real-time reverse transcription-polymerase chain reaction (rRT-PCR) in one step involving two fluorogenic Taq Man labeled probe and using this protocol for detection of infectious bursal disease virus (IBDV) collected from suspected cases distributed in different regions of the country during the period 2013-2016. The intralaboratory validation of modified method was realized for specificity, linearity, repeatability, sensitivity and reproducibility. It allowed reducing the test running time by six folds. This method was applied on 102 pools of bursa of fabricius (BF) samples collected from affected broiler farms suspected to be infected by IBDV. Birds showing macroscopic lesions including muscle petechial hemorrhages, hypertrophy and hemorrhage of BF, were subjected to molecular analysis using modified protocol "Taq Man-MGB rRT-PCR". The validation satisfied all criteria and the assay developed could be a useful tool for a very rapid diagnosis of IBDV and permit to detect and to discriminate in one-step very virulent (vv) from non-vv (classic and variant) IBDV strains. Out of 84 IBDV positive samples, a prevalence of 39% for vv strains and 61% for classical strains was noted. These results indicate that despite the vaccination against IBDV, the vv form of this pathologie continues to cause serious problems for Moroccan broiler chickens. The obtained results indicate the successfully detection of IBDV and differentiated all vvIBDV strains from non-vvIBDV strains; Avian infectious agent RNA viruses tested are negative, demonstrating great specificity of the assay. The results obtained indicate that this method is suitable as a routine laboratory test for the rapid detection and differentiation of IBDV strains in samples of avian origin.

Key words: IBDV, one-step rRT-PCR, Taq Man-MGB, macroscopic lesions.

1. Introduction

Infectious bursal disease (IBD) first described in 1962 [1], is a highly contagious [2] and immunosuppressive disease of young chickens [3] caused by infectious bursal disease virus (IBDV) which belongs to the genus *Avibirnavirus* of the Birnaviridae family. There are two serotypes of IBDV (serotypes 1 and 2) [4]. IBDV replicates in differentiating lymphocytes of the bursa of fabricius, causing the immunosuppressive and often fatal condition [5]. The strains of serotype 1 IBDV are infectious for chickens [5], and are further classified as classical virulent IBDV (cvIBDV), antigenic variant IBDV (avIBDV), attenuated IBDV (atIBDV) and very virulent IBDV (vvIBDV) [6]. These later vvIBDV strains were reported to break through high levels of maternal antibodies in commercial flocks, causing up to 60%-100% mortality rates in chickens

Corresponding author: Maryame Cheggag, Ph.D., research fields: histology and pathology anatomy veterinary and molecular biology.

and producing IBDV typical lesions [7].

IBDV is a non-enveloped icosahedral virus with a diameter of 60 nm and its genome consists of two segments of double stranded RNA (segments A and B) [2]. The larger RNA segment (3.2 kb) contains two partially overlapping open reading frames (ORFs), A1 and A2 [3]. The small ORF encodes a 17 kDa protein (VP5) that is not essential for viral replication in cell culture but may be related to pathogenicity. The large ORF encodes a 110 kDa polyprotein that is processed into VP2, VP4 and VP3 proteins. VP4 protein is involved in the processing of polyprotein and considered as a protease. VP2 and VP3 are the major capsid proteins of IBDV [8]. While the small RNA segment encodes VP1, a RNA dependent RNA polymerase [9]. The variable VP2 gene of IBDV has been used for most molecular epidemiology and phylogenic studies because this part of the genome contains relatively conserved sequence regions unique to vvIBDV. In mostvv viruses, fourtypical amino acids are present (A222, I256, I294 and S299) [10].

Various methods have been developed for the diagnosis of IBD, such as virus isolation in cell culture, embryonated chicken eggs, or young specific-pathogen-free (SPF) chickens and localization of the virus in infected tissues by electron microscopy, fluorescence assay, agar immunodiffusion, antigene-capture enzyme-linked immunosorbent assay (ELISA), or immunohistochemistry [11]. However, all these methods have disadvantages, such as being time consuming, labor intensive, expensive, or non-specific [12]. Recently, various diagnostic methods targeting viral nucleic acids have been elaborated such as conventional reverse transcription-polymerase chain reaction (RT-PCR), real time RT-PCR (rRT-PCR) and loop-mediated isothermal amplification (LAMP) [13]. The application of rRT-PCR as a tool for the diagnosis of IBDV infections has been used [14] because of its simplicity, high sensitivity and specificity [15]. The assay is usually carried out with the use of hydrolysis probe (Taq-Man) [16].

In order to discriminate geno-groups of IBDV strains, an rRT-PCR run in two stages has been developed by Tomás *et al*. [17]. In the first stage, RT was performed on RNA producing complementary DNA (cDNA) by the activity of reverse transcriptase and in the second one, the cDNA obtained was amplified by PCR under the activity of the DNA polymerase using primers and probes which included out with the VP5/VP2 overlying region of segment A of IBDV.

The purpose of this work was to establish the situation of the disease in broiler farms in Morocco using modifying the RT-PCR developed by Tomás *et al*., 102 IBDV outbreaks were investigated in order to detect and to differentiate geno-groups of field strains of IBDVs.

2. Materials and Methods

2.1 Viral Strains

Selected avian viruses were used to test the specificity and sensitivity of rRT-PCR assay (Table 1).

2.2 RNA Extraction

Viral RNA was extracted from 150 µL of vaccine virus, IBDV challenge solutions and suspension of organs using the NucleoSpin RNA Virus Extraction kit (Machery-Nagel, Germany) according to the manufacturer's instructions. The extracted RNA was eluted in 50 µL of nuclease free water and stored at -80 °C until use.

2.3 Primer and Probe Design

The primers and probes used for rRT-PCR amplification of IBDV were designed by Bioneer, Korea and targeting the VP5/VP2 overlapping region of segment A as described by Tomás *et al.* [17]. The forward primer F 178 matched positions 178-198 (5'-GAGCCTTCTGATGCCAACAAC-3'); the probes positions 222-236 (FAM-5'-ACACCCTAGAGAAGC-3'-MGB) for

detection of vvIBDV, (VIC-5'-ACACCCTGGAGAAGC-3'-MGB) for detection of non-vvIBDVs and the reverse primer was located at positions 272-248 (5'-TCAAATTGTAGGTCGAGGTCTCTGA-3').

2.4 Real-Time RT-PCR Assay

Real time RT-PCR amplification and detection was performed using Smart-cycler real time PCR instrument (Cephied, Sunnyvale, California, USA) with the SensiFast^{TM} Probe No-ROX One-Step Kit (Bioline, United Kingdom). Briefly, each 20 reactions contained 4 µL extracted RNA, 10 µL 2× Sensifast Probe No-Rox Mix, 0.2 µL reverse transcriptase enzyme, 0.1 µL probe (50 nM), 0.4 µL forward and reverse primers (200 nM) and 4.5 µL nuclease free water.

The modified protocol conditions based on running transcription and amplification in one step were as follow: reverse transcriptase at 48 °C for 10 min; reverse transcriptase inactivation and activation Taq at 95 °C for 10 min, followed by 40 cycles of amplification (10 s at 95 °C, 10 s at 63 °C and 10 s at 72 °C).

Cycle threshold (CT) is defined as the number of cycles required for the fluorescent signal to cross the threshold (i.e., exceeds background level of fluorescence). Samples were considered positive when they showed a CT.

2.5 Analytical Specificity and Sensitivity

The specificity of the primer/probe sets was tested on nucleic acids extracted from a diverse array of virus that may be present in samples of avian origin (infectious bronchitis virus (IBV), Newcastle disease virus (NDV) and avian influenza virus (AIV)) (Table 1).

In the present study, the term "sensitivity of the method" reflects the efficacy of the entire method applied to recover the target organism in the specimens, including the RNA extraction procedure and the rRT-PCR protocol [18]. For this reason, the solution of D78 RNA strain genome was serially diluted from 10^{4} to 10^{-2} $CCID_{50}$ dose and used as templates, and the RNA was extracted and then used for the sensitivity test. The CT values at each dilution were collected in five repetitions and used to prepare a standard curve.

Evaluation of the analytical sensitivity of the method was done by testing each dilution in five replicates. The sensitivity of the method was determined as the last dilution at which at least four of five replicates of each dilution was positive [17-19].

2.6 Detection Limit

It's defined as the estimation of the standard deviation of smallest amount of an agent detected by the assay. In order to determine the detection limit of the assay, 10 independent runs using the 10^{-1} $CCID_{50}$ dose dilution of the vaccine D78 virus were performed [19, 20].

2.7 Repeatability and Reproducibility

The Repeatability of IBDV assay was measured by analyzing 10 positives samples of Gumboro in two replicates on the same conditions. However, the reproducibility of the assay was validated by analyzing 10 positive IBDV in two replicates by two different analysts on two different days (conditions of reproducibility [19, 20].

2.8 Detection of Virus RNA in Field Cases

The method developed was applied on 102 samples pool of bursi of fabricius and spleens from suspected affected broilers from different field cases in Morocco that occurred between 2013 and 2016 and kept at -80 °C. Frozen samples of bursa tissue were homogenized in chilled sterile phosphate buffered saline (PBS). The homogenate was clarified by centrifugation at 2,000× g for 5 min, and the obtained supernatant was processed accordingly as described above for RNA extraction and rRT-PCR assay.

3. Results and Discussion

Different diagnostic assays for IBD include the enzyme-linked immunosorbent assay, monoclonal antibody assay, the virus neutralization assay, virus isolation, electron microscopy, immunodiffusion, the agar-gel precipitin test and the immunofluorescence (IF) assay. All these assays suffer several disadvantages, such as being labor intensive, expensive, time-consuming, non-specific, or insensitive. More importantly, these methods lack the ability to detect low levels of IBDV antigens in tissues. Thus, in order to control the disease, a quickly, specific and sensitive method such as rRT-PCR for identifying IBDV is very essential [11, 21, 22]. This assay is rapidly becoming one of the most promising methods for improving epidemiological surveillance programs despite being incorporated more recently in the IBDV analysis [23]. Though various rRT-PCR assays have been advanced for IBDV, some of them can simultaneously identify and characterize field strains using non-specific dyes or either fluorogenic probes [17, 24-29] like the assay using in this paper.

Validation of laboratory results is essential for molecular detection of pathogens and diagnosis of infectious diseases to ensure accurate, repeatable and reliable results. This paper describes the qualitative intra-Laborataory validation of a Taq Man-MGB rRT-PCR one-step assay. The Taq Man-MGB rRT-PCR assay performances were assessed by determining its specific characteristics, such as specificity, linearity, accuracy (repeatability), sensitivity and detection limit. The major advantage of this protocol, compared to that described by Tomás *et al*. in 2012 consist to developed a more rapid one step rRT-PCR reducing cycling time from 6 h to 47 min in order to detect and to differentiate geno-groups of field strains of vvIBDVs.

3.1 Analytical Specificity and Sensitivity

The specificity of the real-time RT-PCR method depends mainly on the primer sequence, which is designated to be specific to the target virus. No positive results were obtained with any of the other organisms listed in Table 1.

The exclusive IBDV specificity has been confirmed in this study against other common avian viruses, such as IBV, NDV and AIV.

The results of sensitivity testing are summarized in Table 2. CT values for each of the D78 RNA, 10^4 $CCID_{50}$ dose, dilutions match very well at the five rRT-PCR repetition testing.

The regression curve (Fig. 1) with a slop of 2.826 was obtained by integrating the results of sensitivity testing specifically the median of five tests rRT-PCR for serial dilution of D78 viral suspension.

From the results obtained, it can be concluded that there is a correlation in the linearity values.

Table 1 Viral strain used in this study.

Pathogen	Strain	Source
Infectious bursal disease virus (IBDV)	D78	Nobilis Gumboro D78, MSD Animal Health, Netherlands
	LC75	AviPro PRECISE, Lohman Animal Health, Germany
	H2512	Gallivac IBD H2512 Merial, France
	GM97	Hipra, Spain
	Moroccan field and challenge strains	Biopharma, Morocco
Infectious bronchitis virus (IBV)	H120	Biopharma (Rabat), vaccine strain
Newcastle disease virus (NDV)	Lasota	Biopharma (Rabat), vaccine strain
	TexasGB	Biopharma (Rabat), vaccine strain
Avian influenza virus (AIV)	H5N1	Biopharma (Rabat), vaccine strain

Table 2 Cycle threshold (CT) values of D78 RNA strain serial dilution in five RT-PCR repetitions of linearity testing.

$CCID_{50}$ dose	CT (non-vvIBDV)					Median CT
	Test 1	Test 2	Test 3	Test 4	Test 5	
10^4	25.54	27.55	27.5	27.2	28.38	27.5
10^3	30.24	29.6	33.2	30.55	33.18	30.55
10^2	32.29	33.47	36.16	35.15	34.19	34.19
10^1	35.96	36.73	37.85	37.54	36.48	36.73
10^{-1}	38.55	38.44	38.16	38.54	39.43	38.54
10^{-2}	0	0	0	0	0	0

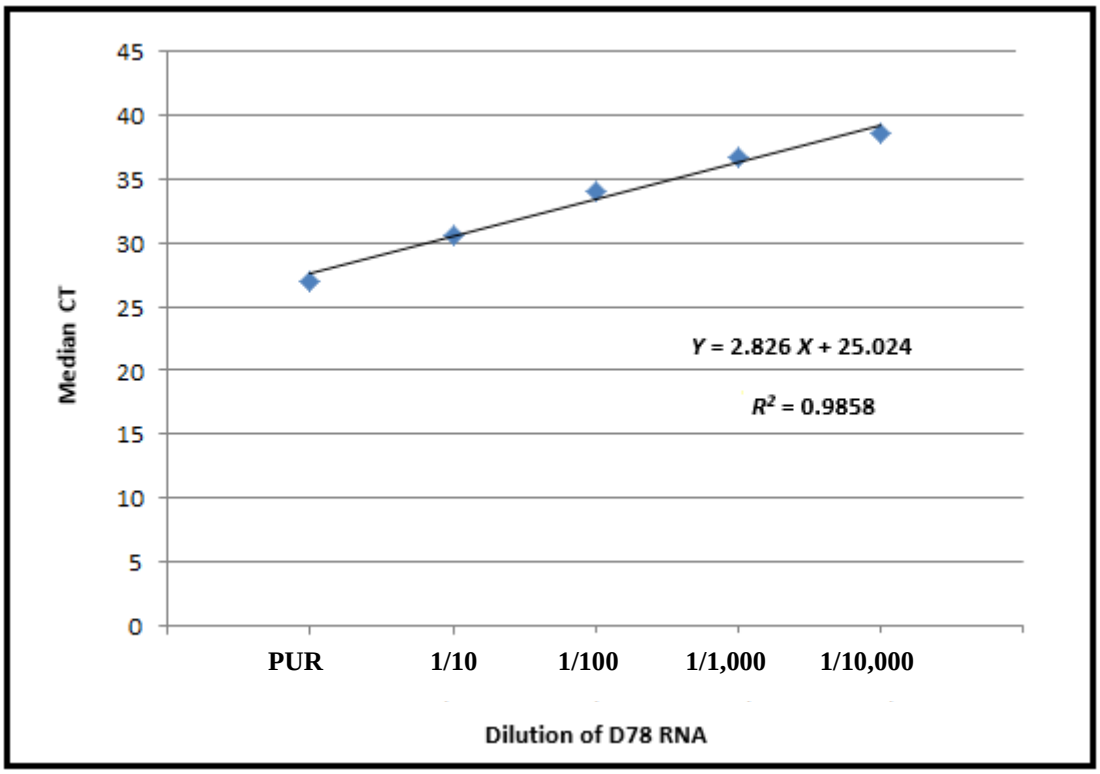

Fig. 1 Regression curve of sensitivity testing.

3.2 Detection Limit

The result of 10 tests of rRT-PCR using the 10 $CCID_{50}$ dose dilution of the vaccine D78 virus are shown in the Table 3 with a standard deviation of the order of 0.40.

The detection limit of the Taq Man-MGB rRT-PCR one-step assay was evaluated from dilutions of the vaccine Nobilis Gumboro D78. It was determined to be10^{-1} $CCID_{50}$ dose.

3.3 Repeatability and Reproducibility

After the two replicates of the assay rRT-PCR for 10 positives samples of Gumboro, the CT in the repeatability testing were repeatable (Table 4).

For the reproducibility testing, even by changing the running parameters of the test, the repeatable CT values were obtain for all samples (Table 5).

The evaluations of the assay repeatability and reproducibility indicated that the Taq Man-MGB rRT-PCR one-step assay could be used with high precision.

3.4 Detection of Virus RNA in Samples Collected from Field Cases

The IBDV represents a major threat to the poultry sector in Morocco as it does in other poultry producing

Table 3 CT values at RT-PCR of 1/10^4 dilution D78 RNA strain solution.

$CCID_{50}$ dose	CT value (non-vvIBDV)									
	Test 1	Test 2	Test 3	Test 4	Test 5	Test 6	Test 7	Test 8	Test 9	Test 10
10^{-1}	38.55	38.44	38.16	38.54	39.43	38.4	39.4	38.5	39.1	38.6

Table 4 CT values at RT-PCR of IBDV strains in repeatability testing.

Identification	CT value			
	Test 1		Test 2	
	non-vvIBDV	vvIBDV	non-vvIBDV	vvIBDV
Challenge strain	0	25.13	0	25.7
H2512	33.64	0	29.08	0
GM97	28.6	0	30.78	0
LC75	28.98	0	29.5	0
Field strain	23.95	0	22.69	0
Field strain	25.94	0	26.84	0
D78	24.99	0	25.71	0
1/10 dilution of D78	27.65	0	28.88	0
1/100 dilution of D78	31.5	0	32.3	0
1/1,000 dilution of D78	36.4	0	35.91	0

Table 5 CT values at RT-PCR of IBDV strains in reproducibility testing.

Identification	CT value			
	Test 1		Test 2	
	non-vvIBDV	vvIBDV	non-vvIBDV	vvIBDV
Challenge strain	0	26.47	0	27.90
H2512	29.87	0	27.67	0
GM97	30.93	0	30.13	0
LC75	29.33	0	29.66	0
Field strain	24.41	0	24.22	0
Field strain	25.78	0	26.24	0
D78	24.78	0	25.79	0
1/10 dilution of D78	29.44	0	28.33	0
1/100 dilution of D78	32.72	0	31.5	0
1/1,000 dilution of D78	36.37	0	35.13	0

Table 6 RT-PCR of broiler field cases suspected to be infected with IBDV.

Year	Flock size	No. of flocks	Range of daily mortality	Age (day)	RT-PCR results		
					No. of classical/variant IBDV	No. of vv IBDV	Négative
2013	8,000-25,000	8	157-165	19-30	6	2	0
2014	3,000-35,000	18	20-600	16-34	6	8	4
2015	5,000-65,000	33	20-1,200	12-42	12	15	6
2016	3,500-40,000	43	10-90	18-38	27	8	8

countries all over the world [30]. The severe form of Gumboro disease has been reported in Morocco since 1992 [31] and it has affected 56.20% of IBD vaccinated farms located in Kenitra-Casablanca regions [32]. The majority of positive poultry farms to IBDV were located in the same regions.

Summarized information about year of collection of the tested samples, as well as origin, flock size, daily mortality, age of birds and type of IBDV strains identified are depicted in Table 6.

Diagnosis of IBD in the country has been always based on the unique pathological findings. Necropsies were carried out on an average of 5-10 carcasses suspected to be affected with IBD. The main lesions detected included hypertrophy and hemorrhage of the bursa of fabricius and spleen. The presence of muscular petechial hemorrhages was also noted on some cases. Suspected cases originated from different regions of the country: Rabat-Sale-Kénitra (40% of total samples), Souss-Massa (22% of total samples), Fes-Meknes (16% of total samples), Casablanca-Settat (16% of total samples), Oriental (6% of total samples) and Draa Tafilalet (1% of total samples). The age of birds varied from 12 d to 42 d and daily mortality reached 1,200 birds/day in some flocks.

The rRT-PCR results indicate that 84 among the 102 samples tested were found positive with a prevalence of 61% for cvIBDV and 39% for vvIBDV. Both cvIBDV and vvIBDV were identified in all age groups with slight higher prevalence between the 3rd and 5th week of age. The 16% of flock cases had an age between 15 d and 21 d, 18% between 22 d and 25 d, 20% between 26 d and 35 d, and only 4% between 36 d and 42 d. For vv virus, 21% had an age between 15 d and 21 d, 26% between 22 d and 28 d, 15% between 29 d and 35 d and just 6% between 36 d and 42 d.

The vv strain were found in three broiler farms on 2013, eight on 2014, 15 on 2015 and eight on 2016; whereas the classical strain were detected in five broiler farms on 2013, six on 2014, 12 on 2015 and 27 on 2016.

The broiler farms in which the results of the rRT-PCR show that are infected by vv strains had high daily mortality compared to those who are infected by classical strains.

Characteristic lesions of IBD and the detection of IBDV were mainly observed in chickens between the 3rd and 5th week of age. These phases of occurrence seem to have the same trends of occurrence previously reported by different authors [12, 33, 34].

4. Conclusions

The protocol described in this paper can be used as a fast, simple, specific and highly sensitive molecular tool for the detection and discrimination the geno-group of the vvIBDVs from that of the non-vvIBDVs from suspect farms. The application of this protocol allowed to detect thus to make a first screening in the sampling by the discrimination between the classical/variant viruses and the vv viruses.

Acknowledgments

The authors present great thanks to sanitary mandated veterinarians supervising poultry farms in different regions in Morocco, who collaborated in samples collection and case submission.

References

[1] Cosgrove, A. S. 1962. “An Apparently New Disease of Chickens: Avian Nephrosis.” *Avian Diseases* 6 (3): 385-9.

[2] Müller, H., Mundt, E., Eterradossi, N., and Islam, M. R. 2012. “Current Status of Vaccines against Infectious Bursal Disease.” *Avian Pathology* 41 (2): 133-9.

[3] Vukea, P. R., Willows-Munro, S., Horner, R. F., and Coetzer, T. H. T. 2014. “Phylogenetic Analysis of the Polyprotein Coding Region of an Infectious South African Bursal Disease Virus (IBDV) Strain.” *Infection Genetics and Evolution* 21 (1): 279-86.

[4] Tsai, S. M., Liu, H. J., Shien, J. H., Lee, L. H., Chang, P. C., and Wang, C. Y. 2012. “Rapid and Sensitive Detection of Infectious Bursal Disease Virus by Reverse Transcription Loop-Mediated Isothermal Amplification Combined with a Lateral Flow Dipstick.” *Journal of Virological Methods* 181 (1): 117-24.

[5] Jenberie, S., Lynch, S. E., Kebede, F., Christley, R. M., Gelaye, E., Negussie, H., Asmare, K., and Ayelet, G. 2014. “Genetic Characterisation of Infectious Bursal Disease Virus Isolates in Ethiopia.” *Acta Tropica* 130: 39-43.

[6] Jeon, W. J., Lee, E. K., Joh, S. J., Kwon, J., Yang, C. B., Yoon, Y. S., and Choi, K. S. 2008. “Very Virulent Infectious Bursal Disease Virus Isolated from Wild Birds in Korea: Epidemiological Implications.” *Virus Research* 137: 153-6.

[7] Mohamed, M. A., Elzanaty, K. E. S., Bakhit, B. M., and Safwat, M. M. 2014. "Genetic Characterization of Infectious Bursal Disease Viruses Associated with Gumboro Outbreaks in Commercial Broilers from Asyut Province, Egypt." *ISRN Veterinary Science* 2014 (2): 916412.

[8] Chang, H. C., Lin, T. L., and Wu, C. C. 2001. "DNA-Mediated Vaccination against Infectious Bursal Disease in Chickens." *Vaccine* 20 (3-4): 328-35.

[9] Hsieh, M. K., Wu, C. C., and Lin, T. L. 2007. "Priming with DNA Vaccine and Boosting with Killed Vaccine Conferring Protection of Chickens against Infectious Bursal Disease." *Vaccine* 25 (29): 5417-27.

[10] Kim, H. R., Kwon, Y. K., Bae, Y. C., Oem, J. K., and Lee, O. S. 2010. "Genetic Characteristics of Virion Protein 2 Genes of Infectious Bursal Disease Viruses Isolated from Commercial Chickens with Clinical Disease in South Korea." *Poultry Science* 89 (8): 1642-6.

[11] OIE. 2008. "Infectious Bursal Disease (Gumboro Disease)." In *OIE Terrestrial Manual*, Chapter 2.3.12, 2008.

[12] Barlicmaganja, D., Zormanrojs, O., and Grom, J. 2002. "Detection of Infectious Bursal Disease Virus in Different Lymphoid Organs by Single-Step Reverse Transcription Polymerase Chain Reaction and Microplate Hybridization Assay." *J. Vet. Diagn. Invest.* 14 (3): 243-6.

[13] Xu, J., Zhang, Z., Yin, Y., Cui, S., Xu, S., Guo, Y., Li, J., Wang, J., Liu, X., and Han, L. 2009. "Development of Reverse-Transcription Loop-Mediated Isothermal Amplification for the Detection of Infectious Bursal Disease Virus." *Journal of Virological Methods* 162 (1-2): 267-71.

[14] Zierenberg, K., Raue, R., and Müller, H. 2001. "Rapid Identification of 'Very Virulent' Strains of Infectious Bursal Disease Virus by Reverse Transcription-Polymerase Chain Reaction Combined with Restriction Enzyme Analysis." *Avian Pathology* 30 (1): 55-62.

[15] Cavanagh, D. 2001. "Innovation and Discovery: The Application of Nucleic Acid-Based Technology to Avian Virus Detection and Characterization." *Avian Pathology* 30 (6): 581-98.

[16] Tomás, G., Hernández, M., Marandino, A., Techera, C., Grecco, S., Hernández, D., Banda, A., Panzera, Y., and Pérez, R. 2017. "Development of an RT-qPCR Assay for the Specific Detection of a Distinct Genetic Lineage of the Infectious Bursal Disease Virus." *Avian Pathology* 46 (2): 150-6.

[17] Tomás, G., Hernández, M., Marandino, A., Panzera, Y., Maya, L., Hernández, D., Pereda, A., Banda, A., Villegas, P., Aguirre, S., and Pérez, R. 2012. "Development and Validation of a Taq Man-MGB Real-Time RT-PCR Assay for Simultaneous Detection and Characterization of Infectious Bursal Disease Virus." *Journal of Virological Methods* 185 (1): 101-7.

[18] U.S. Environmental Protection Agency (USEPA). 2004. *Quality Assurance/Quality Control Guidance for Laboratories Performing PCR Analyses on Environmental Samples*, USEPA.

[19] NF T90-210 Mai. 2009. "Protocol for Initial Evaluation of the Performance of a Method in a Laboratory." Accessed November 7, 2018. https://www.boutique.afnor.org/norme/nf-t90-210/qualite-de-l-eau-protocole-d-evaluation-initiale-des-performances-d-une-methode-dans-un-laboratoire/article/771330/fa160221.

[20] NF V03-110 Mai. 2010 AFNOR. "Characterization Protocol for the Validation of a Quantitative Analysis Method by Construction of the Accuracy Profile." Accessed November 7, 2018. https://www.boutique.afnor.org/norme/nf-v03-110/analyse-des-produits-agricoles-et-alimentaires-protocole-de-caracterisation-en-vue-de-la-validation-d-une-methode-d-analyse-qu/article/707662/fa159944.

[21] Wu, C. C., Lin, T. L., Zhang, H. G., Davis, V. S., and Boyle, J. A. 1992. "Molecular Detection of Infectious Bursal Disease Virus by Polymerase Chain Reaction." *Avian Diseases* 36 (2): 221.

[22] Jackwood, D. J. 1990. "Development and Characterization of Nucleic Acid Probes to Infectious Bursal Disease Viruses." *Veterinary Microbiology* 24 (3): 253-60.

[23] Wu, C. C., Rubinelli, P., and Lin, T. L. 2007. "Molecular Detection and Differentiation of Infectious Bursal Disease Virus." *Avian Diseases* 51 (2): 515-26.

[24] Peters, M. A., Lin, T. L., and Wu, C. C. 2005. "Real-Time RT-PCR Differentiation and Quantitation of Infectious Bursal Disease Virus Strains Using Dual-Labeled Fluorescent Probes." *Journal of Virological Methods* 127 87-95.

[25] Jackwood, D. J., Spalding, B. D., and Sommer, S. E. 2003. "Real-Time Reverse Transcriptase Polymerase Chain Reaction Detection and Analysis of Nucleotide Sequences Coding for a Neutralizing Epitope on Infectious Bursal Disease Virus." *Avian Diseases* 47: 738-44.

[26] Mickael, C. S., and Jackwood, D. J. 2005. "Real-Time RT-PCR Analysis of Two Epitope Regions Encoded by the VP2 Gene of Infectious Bursal Disease Viruses." *Journal of Virological Methods* 128: 37-46.

[27] Moody, A., Sellers, S., and Bumstead, N. 2000. "Measuring Infectious Bursal Disease Virus RNA in Blood by Multiplex Real-Time Quantitative RT-PCR."

Journal of Virological Methods 85: 55-64.

[28] Ghorashi, S. A., O'Rourke, D., Ignjatovic, J., and Noormohammadi, A. H. 2011. "Differentiation of Infectious Bursal Disease Virus Strains Using Real-Time RT-PCR and High Resolution Melt Curve Analysis." *Journal of Virological Methods* 171 (1): 264-71.

[29] Kong, L. L., Omar, A. R., Hair, B. M., Ideris, A., and Tan, S. W. 2009. "Development of SYBR Green I Based One-Step Real-Time RT-PCR Assay for the Detection and Differentiation of Very Virulent and Classical Strains of Infectious Bursal Disease Virus." *Journal of Virological Methods* 161 (2): 271-9.

[30] Tahiri, F., Benaissa, A., Khadija, I. S. Y., and Driss, B. 2013. "Detection of Infectious Bursal Disease Virus from Clinical Samples in Morocco by Agar Gel Immunodiffusion Test and Reverse Transcription-Polymerase Chain Reaction." *Inter. J. Pharmaceut. Biol. Sci. Res. Developm.* 1 (03): 1-14.

[31] Bouzoubaa, K., Mouahid, M., Elhouadfi, M., Amara, A., Jaouzi, T., Bell, J., and Kichou, F. 1992. "Severe Outbreaks of Infectious Bursal Disease in Morocco." In *Proceedings of the 41st Western Poultry Disease Conference*, 3-4.

[32] Jaouzi, T. 1996. "Some Epidemiological Data on the Severe form of Gumboro Disease in Morocco, January 1991-April 1993." Ph.D. thesis, Institute of Agronomy and Veterinary Medicine Hassan II.

[33] Berg, T. P. 2000. "Acute Infectious Bursal Disease in Poultry: A Review." *Avian Pathology* 29: 175-94.

[34] Ezeibe, M. C. O., Okoye, J. O. A., Ogunniran, T. M., Animoke, P. C., Mbuko, I. J., Nwankwo, I. A., and Ngene, A. A. 2013. "Mortality Rates from a Nigerian Isolate of the Infectious Bursa Disease Virus and Passive Haemagglutination Antibody Titer That Protects Chicks against Challenge with the Virus Isolate." *Health* 5 (9): 1355-9.