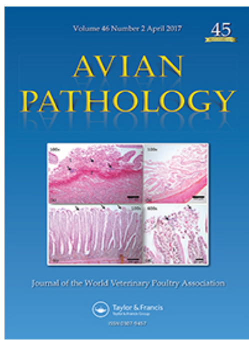

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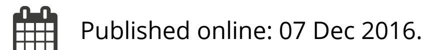

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ORIGINAL ARTICLE

Development of an RT-qPCR assay for the specific detection of a distinct genetic lineage of the infectious bursal disease virus

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ABSTRACT
The infectious bursal disease virus (IBDV) is a major health threat to the world's poultry industry despite intensive controls including proper biosafety practices and vaccination. IBDV (*Avibirnavirus, Birnaviridae*) is a non-enveloped virus with a bisegmented double-stranded RNA genome. The virus is traditionally classified into classic, variant and very virulent strains, each with different epidemiological relevance and clinical implications. Recently, a novel worldwide spread genetic lineage was described and denoted as distinct (d) IBDV. Here, we report the development and validation of a reverse transcription-quantitative polymerase chain reaction (RT-qPCR) assay for the specific detection of dIBDVs in the global poultry industry. The assay employs a TaqMan-MGB probe that hybridizes with a unique molecular signature of dIBDV. The assay successfully detected all the assessed strains belonging to the dIBDV genetic lineage, showing high specificity and absence of cross-reactivity with non-dIBDVs, IBDV-negative samples and other common avian viruses. Using serial dilutions of *in vitro*-transcribed RNA we obtained acceptable PCR efficiencies and determination coefficients, and relatively small intra- and inter-assay variability. The assay demonstrated a wide dynamic range between 10^3 and 10^8 RNA copies/reaction. This rapid, specific and quantitative assay is expected to improve IBDV surveillance and control worldwide and to increase our understanding of the molecular epidemiology of this economically detrimental poultry pathogen.

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KEYWORDS
IBDV; RT-qPCR; TaqMan-MGB probe; distinct IBDV; IBD

Introduction

Infectious bursal or Gumboro disease is a highly contagious viral affection that causes major economic losses in the global poultry industry. The aetiologic agent is the infectious bursal disease virus (IBDV) belonging to the genus *Avibirnavirus* within the family *Birnaviridae*. The virus replicates in the B lymphocytes of the bursa of Fabricius and affects the immune system of immature chickens, leading to an increased susceptibility to other infectious diseases and a poor antibody response to vaccines (Rosenberger & Gelb, 1978).

IBDV is a non-enveloped icosahedral virus with a bisegmented, double-stranded RNA genome (Müller *et al.*, 1979). Segment A (3.3 kpb) has two partially overlapped open reading frames (ORFs). ORF A-1 encodes a precursor polyprotein that is autocatalytically cleaved into the immature outer capsid protein pVP2, the viral protease VP4 and the ribonucleoprotein VP3 (Sánchez *et al.*, 1999; Lejal *et al.*, 2000; Da Costa *et al.*, 2002; Luque *et al.*, 2009). The pVP2 protein is further processed to yield the mature VP2, the major host-protective antigen of IBDV (Fahey *et al.*, 1991). ORF A-2 encodes VP5, a non-structural protein involved in virion release from infected cells (Wu *et al.*, 2009). Genomic segment B (2.9 kpb) encodes the RNA-dependent RNA polymerase VP1 (Spies *et al.*, 1987).

There are two IBDV serotypes (1 and 2), but only serotype 1 comprises pathogenic viruses (Ismail *et al.*, 1988). This serotype was traditionally divided into classic (c), variant (va) and very virulent (vv) strains using antigenic and pathogenic criteria. Classic strains can be further classified into classic virulent (cv) and attenuated vaccine strains, usually referred to as "classic attenuated" (ca) IBDVs.

Recently, we described the existence of a worldwide spread genetic lineage denoted as distinct (d) IBDV (Hernández *et al.*, 2015). This lineage is resolved in a well-supported clade and has a unique four-amino-acid signature (T272, P289, I290, F296). Most isolates that are now classified as dIBDV were initially considered atypical classic or variant strains that harboured unique nucleotide and amino acid changes as a consequence of local differentiation (Kwon *et al.*, 2000; Ikuta *et al.*, 2001; Domanska *et al.*, 2004; Remorini *et al.*, 2006; Jackwood & Sommer-Wagner, 2007; Ojkic *et al.*, 2007). Some dIBDV isolates have exhibited mild clinical signs and antigenic differences (Ikuta

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Table 1. Description of primers and probe used in this study.

Primer/probe name	Sequence 5′→ 3′	Polarity	Position[a]	Amplicon size
F964rt	AAACAATGGGCTRACGGC	+	964–981	72
R1035rt	GTTATCTCGYTGGTCGGRAA	–	1035–1016	
P1011rt	NED-AGRTTGAATGGAAYAGGA-MGB-NFQ	–	1011–994	

[a]Sequences numbering according to segment A of the vvIBDV strain D6948 (AF240686).

et al., 2001; Domanska *et al.*, 2004; Vera *et al.*, 2015) but further phenotypic studies are needed to understand the epidemiological and sanitary relevance of this lineage that contains part of the genetic variability of the virus (Hernández *et al.*, 2015).

Global surveillance and research programmes require reliable assays for the diagnosis of IBDV variants to understand virus spreading and evolution, and to provide strain-specific treatments (Van den Berg *et al.*, 2000). Strain classification can be performed by the phylogenetic analysis of the VP2 hypervariable region (hvVP2), which recovers all IBDV strains (cv, ca, va, vv and d) (Martin *et al.*, 2007; Wu *et al.*, 2007; Xia *et al.*, 2008; Kim *et al.*, 2010; Hernández *et al.*, 2015). However, this methodology is time-consuming, expensive and requires trained staff, not being suitable to be widely applied in clinical settings. A good alternative is the application of molecular typing techniques (e.g. restriction fragment length polymorphisms and allele-specific PCR) that allow the straightforward virus classification by identifying nucleotide or amino acid residues specific for each IBDV strain. Several useful assays have been described for ca, va and vv strains (Peters *et al.*, 2005; Kong *et al.*, 2009; Ghorashi *et al.*, 2011; Hernández *et al.*, 2011; Tomás *et al.*, 2012), but no specific typing technique has been reported for the dIBDVs. Here, we present the development of a reverse transcription-quantitative PCR (RT-qPCR) assay for the specific detection of dIBDVs in the poultry industry worldwide.

Materials and methods

Sequence analysis and primer/probe design

Multiple sequence alignments were carried out with most of the hvVP2 sequences available in the GenBank database ($n = 955$) using the MUSCLE algorithm implemented in *MEGA* 5.0 (Tamura *et al.*, 2011). The dIBDV sequences were identified by phylogenetic clustering and amino acid markers (T272, P289, I290 and F296) following Hernández *et al.* (2015). Based on the nucleotide variants linked to the dIBDVs, specific primers and TaqMan-minor groove binding (TaqMan-MGB) probe were designed and synthesized by IDT DNA (Coralville, IA, USA) and Applied Biosystems (Foster City, CA, USA), respectively (Table 1). A BLAST search was also performed to predict in silico primer and probe sequence specificity in order to evaluate the occurrence of non-specific homology with other IBDV genome regions or with the chicken genome.

dIBDV samples

The Uruguayan dIBDV field strain UY-221201 was used to generate the standard RNA transcripts for the standardization and testing of the analytical

Table 2. IBDV field samples and vaccine strains used in this study.

Strain name	Genetic lineage	Sample type	Origin	Ct value[d]
UY-221201	Distinct	Bursa	Uruguay	22.3
UY-42/07	Distinct	Bursa	Uruguay	30.3
UY-04/09	Distinct	Bursa	Uruguay	22.9
UY-04/10	Distinct	Bursa	Uruguay	23.8
UY-06/10	Distinct	Bursa	Uruguay	23.6
UY-07/10A	Distinct	Bursa	Uruguay	24.0
UY-07/10B	Distinct	Bursa	Uruguay	16.6
UY-07/10C	Distinct	Bursa	Uruguay	18.8
UY-07/10D	Distinct	Bursa	Uruguay	19.5
UY-171101	Distinct	Bursa	Uruguay	17.8
UY-421101	Distinct	Bursa	Uruguay	16.1
UY-421102	Distinct	Bursa	Uruguay	21.7
UY-421103	Distinct	Bursa	Uruguay	20.7
UY-221201	Distinct	Bursa	Uruguay	22.3
UY-271201	Distinct	Bursa	Uruguay	17.1
UY-301201	Distinct	Bursa	Uruguay	28.8
UY-341201	Distinct	Bursa	Uruguay	26.6
UY-351201	Distinct	Bursa	Uruguay	31.3
UY-141403	Distinct	Bursa	Uruguay	28.1
UY-171401	Distinct	Bursa	Uruguay	25.7
dIBDV/UY/2014/2202	Distinct	Bursa	Uruguay	25.6
UY-221401	Classic virulent	Bursa	Uruguay	40.0
Winterfield 2512[a]	Classic virulent	Vaccine	–	40.0
Lukert[b]	Classic virulent	Vaccine	–	40.0
D78[c]	Classic attenuated	Vaccine	–	40.0
UY-281301	Classic attenuated	Bursa	Uruguay	40.0
UY-291301	Classic attenuated	Bursa	Uruguay	40.0
UY-251501	Classic attenuated	Bursa	Uruguay	40.0
1355	Variant (Del-E)	Bursa	United States	40.0
2564	Variant (Del-E)	Bursa	United States	40.0
2566	Variant (Del-E)	Bursa	United States	40.0
2567	Variant (Del-E)	Bursa	United States	40.0
Uy-1	Very virulent	Bursa	Uruguay	40.0
Uy-2	Very virulent	Bursa	Uruguay	40.0
Uy-3	Very virulent	Bursa	Uruguay	40.0
Uy-4	Very virulent	Bursa	Uruguay	40.0
Uy-5	Very virulent	Bursa	Uruguay	40.0

[a]Obtained from the CEVAC-IBD-L vaccine, Ceva-Phylaxia, Budapest, Hungary.
[b]Obtained from the Bursine-2 vaccine, Fort Dodge Animal Health, Iowa, United States.
[c]Obtained from the NobilisGumboro D78 vaccine, Intervet International B.V., Boxmeer, Holland.
[d]Mean Ct value of two replicas. Threshold value = 0.05ΔRn.

performance of the developed RT-qPCR assay (Table 2). Twenty Uruguayan dIBDV outbreaks were employed to test the clinical sensitivity of the developed RT-qPCR assay (Table 2). Molecular diagnosis of the strains were performed by quantitative PCR (Tomás *et al*., 2012), and assigned to the dIBDV lineage by hvVP2 sequence analysis (Hernández *et al*., 2015).

IBDV-negative samples

Thirty IBDV-negative field samples, diagnosed by quantitative PCR (Tomás *et al*., 2012), were used for testing cross-reactivity (specificity).

Avian viruses for specificity testing

Representatives of all IBDV strains (Table 2), and the following avian viruses were employed to assess the cross-reactivity of the assay: infectious bronchitis virus (Bronchitis vaccine Mass. Type, Fort Dodge Animal Health, IA, USA), avian reovirus (Tenosynovitis vaccine, Fort Dodge Animal Health, IA, USA), chicken infectious anaemia virus (Nobilis CAV P4 vaccine, Intervet International B.V., Boxmeer, Holland) and Newcastle diseases virus (Nobilis ND Hitchner vaccine, from Intervet International B.V).

RNA extraction from vaccine virus and field samples

Total RNA was extracted using the Quick-RNA™ MiniPrep kit (Zymo Research, Irvine, CA, USA). RNA from vaccines was extracted using 200 µl of a phosphate-buffered saline resuspension of lyophilized vaccine. Tissue-infected samples were processed starting with 50 mg of bursae internal folds. The extracted RNA was eluted in 35 µl of RNase-free water.

Reverse transcription and quantitative PCR assay

For the RT step, we used the RevertAid™ H Minus First Strand cDNA Synthesis kit (Fermentas Life Sciences Inc., Hanover, MD, USA) with 10 µl of extracted RNA. The whole IBDV genome was reverse transcribed to complementary DNA (cDNA) using random hexamer primers.

Quantitative PCR was carried out in a 20 µl reaction volume containing 1 × Hot Rox Master Mix (Bioron, Ludwigshafen, Germany), 300 nM each primer, 400 nM probe and 1 µl of cDNA. Thermocycling was performed on the ABI Prism 7500 (Applied Biosystems) and consisted of a 5 min hold stage at 50°C, followed by a 10 min denaturation at 95°C, 40 cycles of 15 s at 95°C and 1 min at 60°C, ending with a 5 min at 70°C final step. Fluorescent measurements were collected at the hold stage, at the 60°C step of each cycle, and at the end of the run.

Generation of standard RNA for analytical testing

Standard RNA generation procedure was carried out as described by Tomás *et al.* (2012), with minor modifications. Briefly, a 491 bp genomic fragment encompassing the RT-qPCR amplicon was obtained from the UY-221201 dIBDV strain using P3- and P4-specific primers (Liu *et al*., 1998). This amplicon was gel-purified and cloned into a pJET1.2 vector (Fermentas Life Sciences Inc.). Recovered plasmids were linearized and used for *in vitro* transcription with the TranscriptAid™ T7 High Yield Transcription Kit (Fermentas Life Sciences Inc.). Generated RNA transcripts were purified and the concentrations of the products were quantified by spectrophotometry using a Nanodrop 1000 (Thermo Scientific, Waltham, MA), determining the average concentration after five measures. The copy number of RNA molecules was obtained by the following formula: Y (RNA copies/µl) = [X (g/µl) RNA/(nt transcript length × 340)] × 6.022×10^{23} (Ummul Haninah *et al*., 2010). RNA transcripts were diluted to obtain a 10^9 copies/µl stock solution and stored at −80°C.

Standard curve generation for analytical testing

A standard curve was generated using 10-fold serial dilutions containing 10^0–10^8 RNA copies/µl. Each dilution was spiked in total RNA extracts (750 ng/µl) from IBDV-negative bursae to simulate a field sample environment, and analysed in triplicates in three independent runs by RT-qPCR. A standard curve was obtained by plotting threshold cycle (Ct) values per three replicates per standard dilution versus the logarithm of the RNA copy. Efficiency (E), coefficient of determination (R^2) and coefficients of variation (CV) were calculated from the resulting standard curves.

Results

Identification of dIBDVs in public databases

We detected 110 sequences in the hvVP2 dataset (n = 955) that cluster within the dIBDV lineage and have the typical amino acidic signature T272, P289, I290 and F296. These dIBDV sequences came from 11 different countries (Argentina, Brazil, Canada, Colombia, Hungary, Poland, Puerto Rico, Russia, South Korea, the United States and Uruguay), and had been collected during 1977–2014 (Table S1, supplemental data).

One Argentine and six Brazilian sequences cluster within the dIBDV lineage but have a valine (V) instead of an isoleucine (I) in the residue 290 used as a marker;

```
                             Forward primer (5'- 3')   Probe (3'- 5')       Reverse primer (3'- 5')
                             AAACAATGGGCTRACGGC        TCCTRTTCCATTCAAYCT   TTYCCGACCARCGAGATAAC

vv  D6948                    .G................        C.T...G.........A.   A....A..............
    UK661                    .G................        C.T...G.........A.   A....A..............
    849VB                    .G................        C.T...G.........A.   A....A..............

cv  Edgar                    .G.............T..        ..T...G...........   A...................
    STC                      .G................        ..T...G...........   A....A..............
    Faragher_52/70           .G.T..............        ..T...G...........   A....A.....T........

ca  Nobilis Gumboro D78      ................A.        C.T.T.G...........   A....A..A...........
    CT                       ................A.        C.T...G...........   A....A..A...........
    Cevac-Gumbo-L            ................A.        C.T.T.G...........   A....A..A...........

va  DEL/E                    ..................        ..T...G...........   A....A.....T........
    DEL/A                    ..................        ..T...G...........   A....A..............
    GLS                      ..................        ..T...G...........   A....A..............
```

Figure 1. Alignment of primers and probe-target sites including sequences of different IBDV representative strains. Nucleotide residues equal to dIBDVs' specific primers and probe are indicated with dots. vv: very virulent; cv: classic virulent; ca: classic attenuated; va: variant.

this change is not present in any other IBDV sequence. We considered these sequences as belonging to dIBDVs with a slightly different amino acidic signature (T272, P289, *V290*, F296).

Assay design

After identification of the strains, we focused our analysis on finding regions with optimal conditions for primer and probe design. The probe was designed to target a nucleotide signature that was exclusively found in the dIBDVs (Figure 1). Since the probe included the codon of the amino acid position 290, a degenerated nucleotide was included in the probe to detect all dIBDVs, regardless of the 290 residue (I or V). The designed TaqMan-MGB probe contains a minimum of two mismatches with non-dIBDV sequences, avoiding cross-reaction (Figure 1).

Primers were designed to match perfectly with the dIBDVs and to maximize the divergence with non-IBDV viruses, including attenuated vaccine strains.

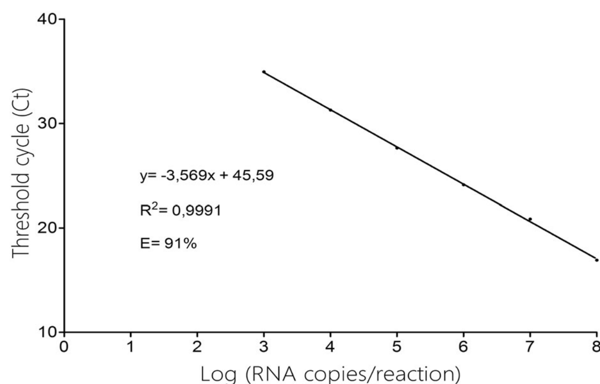

Figure 2. Standard curve of the developed RT-qPCR assay using the dIBDV-specific probe. Linear dynamic range was established between 10^3 and 10^8 RNA copies/reaction. Each point represents the mean Ct of nine different measures (three independent reactions, three replicates each). The coefficient of determination (R^2) and the efficiency (E) of the linear regression curve are indicated.

Both primers have some mismatches with other non-dIBDV strains, a 3′ mismatch in the reverse primer being the most relevant (Figure 1).

Nucleotide BLAST search of primers and probe showed only complete homology with dIBDV sequences. No cross-reaction signal was observed when a dIBDV-specific probe was tested with c, va and vvIBDV strains or with infectious bronchitis virus, avian reovirus, chicken infectious anaemia virus and Newcastle disease virus. No increase of fluorescent signal was detected in any case, resulting in Ct values equal to 40 (Table 2).

Analytical performance of the assay. A genomic fragment encompassing the RT-qPCR amplicon was successfully cloned and *in vitro* transcribed to obtain dIBDV RNA. Dilutions in total RNA extracts were analysed by the developed RT-qPCR. A standard curve was generated using 10-fold serial dilutions of RNA standards from 10^0 to 10^8 RNA copies/reaction. The linear dynamic range was established between 10^3 to 10^8 RNA copies/reaction, with an average R^2 value of 0.9991, and an efficiency of 91% (Figure 2).

The assay reproducibility assessed by the intra- and inter-assay CVs was lower than 3%.

Clinical sensitivity of the assay. All previously diagnosed and characterized dIBDV field strains were correctly diagnosed with the RT-qPCR assay. The fluorescent signal clearly increased above the threshold in all cases, showing Ct values ranging from 16.1 to 31.3 (Table 2). Probe and primer binding sites in these Uruguayan strains are completely conserved among global strains, indicating that the same clinical sensitivity would occur with the dIBDV viruses from different origins.

Discussion

Assessing the current global prevalence and relevance of the recently described dIBDV lineage is of crucial importance for improving disease control and

understanding viral dynamics. The dIBDVs have been collected over a period of almost 40 years from different continents, an indicative of its wide and persistent spreading (Table S1). By analysing the hvVP2 sequences available in the GenBank, we inferred that more than 10% of the IBDV sequences correspond to dIBDVs, suggesting a high frequency of this lineage in the global virus population. Countries such as Argentina, Canada and Uruguay have reported a high prevalence of this lineage circulating in the poultry production, while in countries such as Brazil, Colombia, Hungary, Poland, Puerto Rico, Russia, South Korea and the United States, there are only sporadic reports of dIBDVs (Shcherbakova *et al.*, 1998; Kwon *et al.*, 2000; Ikuta *et al.*, 2001; Jackwood *et al.*, 2001; Smiley & Jackwood, 2001; Domanska *et al.*, 2004; Remorini *et al.*, 2006; Jackwood & Sommer-Wagner, 2007; Ojkic *et al.*, 2007; Hernández *et al.*, 2015; Tomás *et al.*, 2015; Vera *et al.*, 2015). This uneven prevalence among different countries needs to be confirmed by performing more extensive studies with a specific diagnostic method, taking into consideration that dIBDVs can be easily ignored during routine surveillance due to the apparent lack of differential clinical signs (Ikuta *et al.*, 2001; Domanska *et al.*, 2004).

The development of a specific and rapid method for dIBDVs differentiation requires the detailed analysis of the genetic variability of the virus. The hvVP2 region has a level of variability that allows the proper classification of all strains and the dIBDV lineage (Islam *et al.*, 2001; Le Nouën *et al.*, 2005; Yamaguchi *et al.*, 2007; Kim *et al.*, 2010; Liu *et al.*, 2013; Amin & Jackwood, 2014; Hernández *et al.*, 2015). The phylogenetic clustering and the molecular signature T272, P289, I290 and F296 were strongly conserved among the sequences despite being collected over a period of almost four decades. A few dIBDV isolates show an amino acid substitution in the 290 residue of the signature (I→V). Considering that V290 is also unique for the dIBDVs, the amino acidic signature T272, P289, I/V290 and F296 should be regarded as the right marker for the dIBDV lineage.

Here we developed an RT-qPCR assay for the rapid detection and precise identification of dIBDVs. The method bases its detection capacity on the presence of various molecular markers within the hvVP2 of dIBDV. The designing of primers and probes in the hvVP2 is challenging by the occurrence of single nucleotide polymorphisms in the region, implying that the target has to be carefully selected to include strong molecular markers that persist in time. In the probe hybridization region, two nucleotides are strongly conserved in all dIBDVs and located near the 3′ end of the probe, improving its discrimination capability and reinforcing the specificity of the assay (Figure 1) (Kutyavin *et al.*, 2000). These nucleotides are part of the codons for the P289 and I/V290 residues that comprise the amino acid signature of the dIBDV linage. The 289 and 290 amino acids are important because they occur within the hvVP2 hydrophilic peak 2, which is antigenically relevant in IBDV (Berg *et al.*, 1996). As these two nucleotides are not present in any other IBDVs, the designed TaqMan-MGB probe is expected to be highly specific; TaqMan-MGB probes form extremely stable duplexes with complementary DNA, and a single mismatch in the probe-target duplex would result in a high Tm difference that prevents cross-hybridization to non-specific targets (Kumar *et al.*, 1998). There are also mismatches between primers and target sites of non-dIBDVs (Figure 1). The most significant change occurs in the reverse primer that differs in its 3′ end with all known non-dIBDVs; this change reduces significantly the primer hybridization with non-dIBDVs and thus increases the specificity of the assay (Kwok *et al.*, 1990).

The RT-qPCR assay was assessed by testing its analytical performance, specificity and clinical sensitivity. Analytical tests indicate that the assay has a broad linear dynamic range (10^3–10^8 RNA copies/reaction), and acceptable coefficient of determination and PCR efficiency (0.9991 and 91%, respectively) (Figure 2). The assay also shows high specificity and lack of cross-reactivity with non-dIBDV strains, IBDV-negative samples and other common avian viruses. All dIBDV samples tested positive with this assay, which in conjunction with the high detection capacity (10^3 genome copies/reaction) support its use as a good diagnostic method, even in samples with low viral titre.

Another main application of the assay is the precise quantification of viral genomes in field samples. This could be used for establishing the virus load in diverse tissues and to provide information about the intra-host circulation. In protection assays, the accurate identification and quantification of the field strain is essential and has to be distinguished from the vaccine virus, which might replicate and persist in the bursa of Fabricius for several days (Ashraf *et al.*, 2005; Iván *et al.*, 2005). It is also possible that different strains co-infect the same avian host, making mapping the quantification and distribution of the viruses relevant (Stoute *et al.*, 2013).

The possibility of screening a large number of samples in a rapid, sensitive and reproducible way makes this assay a suitable tool for dIBDV impact assessment in field samples. It could be used to simultaneously diagnose and characterize dIBDV samples with low titres, since the high sensitivity of the assay facilitates the detection of few genome copies, allowing the direct analysis of the virus without prior propagating in culture or in embryonated eggs. It is expected that these attributes improve the IBDV surveillance and control worldwide and increase our understanding

of the molecular epidemiology of this economically detrimental poultry pathogen.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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