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A simple and sensitive isothermal reverse transcription–thermophilic helicase-dependent amplification (RT-tHDA) assay for screening of infectious bursal disease virus in resource-limited settings

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Abstract

Infectious bursal disease (IBD), caused by infectious bursal disease virus (IBDV), remains a major threat to poultry production due to its immunosuppressive effects and economic losses. Rapid and reliable detection of IBDV is essential for disease surveillance and outbreak management, particularly in field and resource-limited laboratory settings. In this study, an isothermal reverse transcription–thermophilic helicase-dependent amplification (RT-tHDA) assay targeting a conserved region of VP1 gene of IBDV was developed and evaluated, amplifying a 99-bp fragment at 65°C within 90 min without the need for a thermocycler. Analytical sensitivity testing using ten-fold serial dilutions of viral RNA demonstrated a detection limit of 440 fg, representing a 100-fold improvement over conventional RT-PCR and sensitivity comparable to real-time RT-PCR. The assay showed high analytical specificity, with no cross-reactivity against other avian viral or bacterial pathogens. Diagnostic evaluation of 180 suspected field bursal samples showed IBDV detection rates of 35.0% by RT-tHDA, 35.6–36.1% by RT-PCR (VP1/VP2), 31.7% by virus isolation, and 45.0% by real-time RT-PCR. Comparing with World Organization for Animal Health (WOAH) reference methods, RT-tHDA demonstrated excellent performance, with 100% sensitivity to virus isolation, ≥ 96.9% sensitivity to RT-PCR, high specificity (≥ 95%), and almost perfect agreement (κ > 0.93). Although real-time RT-PCR detected more positive samples due to its ability to identify very low viral loads, RT-tHDA consistently detected all clinically relevant infections confirmed by reference methods. Overall, RT-tHDA offers high sensitivity, specificity, and strong agreement with WOAH methods, supporting its use as a rapid screening tool for IBDV in field and resource-limited laboratories.

Key Words: Helicase-dependent amplification, Infectious bursal disease virus, Isothermal amplification, Molecular diagnostics, VP1 gene.

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Introduction

Infectious bursal disease (IBD), also known as Gumboro disease, is a highly contagious and immunosuppressive viral disease of young chickens caused by infectious bursal disease virus (IBDV), a member of the genus *Avibirnavirus* within the family *Birnaviridae*[8,23]. The disease predominantly affects chickens between 2 and 8 weeks of age, a critical period when maternally derived antibodies decline and immature B lymphocytes actively populate the bursa of Fabricius. Infection during this window leads to extensive lymphoid depletion, resulting in severe immunosuppression, vaccination failure, secondary infections, poor growth performance, and substantial economic losses to the global poultry industry[2,20,21].

IBDV possesses a non-enveloped, icosahedral capsid enclosing a bi-segmented double-stranded RNA genome. Segment A encodes the structural proteins VP2 and VP3, the viral protease VP4, and the non-structural protein VP5, while segment B encodes VP1, the RNA-dependent RNA polymerase responsible for viral replication[8,31]. Two serotypes of IBDV have been identified, of which only serotype I is pathogenic to chickens, whereas serotype II strains are avirulent. Serotype I viruses are further classified into classical virulent, antigenic variant, novel variant, very virulent (vvIBDV), and attenuated strains based on virulence, antigenicity, and genomic characteristics[16,22].

Although the hypervariable region of VP2 has traditionally been used for strain differentiation and molecular epidemiology, recent studies have emphasized the role of VP1 and genomic reassortment between segments A and B in shaping viral fitness, replication efficiency, and virulence[7,19]. The emergence and global predominance of reassortant IBDV strains, particularly A3B1 genotypes, have been reported in several regions despite routine vaccination, highlighting the ongoing evolution of the virus under immune pressure[7,32]. In India, multiple studies have documented the circulation of genetically diverse vvIBDV and variant strains in both vaccinated and unvaccinated flocks, underscoring the need for continuous molecular surveillance and rapid field-level diagnostics[1,6,24,27,29].

Conventional diagnostic methods for IBDV include agar gel precipitation test, virus neutralization test, enzyme-linked immunosorbent assay, immunofluorescence, and electron microscopy[13,14,39]. While these techniques are useful for serological surveillance and confirmatory diagnosis, they are limited by lower sensitivity, longer turnaround times, or the requirement for specialized reagents and expertise. Virus isolation in embryonated eggs or cell culture remains a gold standard for confirmatory diagnosis but is labor-intensive and time-consuming[33].

Molecular diagnostic assays such as conventional RT-PCR and real-time RT-PCR have become the methods of choice for IBDV detection due to their high sensitivity and specificity[16,18,36]. However, these assays require sophisticated thermal cyclers, uninterrupted power supply, and skilled personnel, limiting their applicability in field investigations and resource-constrained laboratories. Moreover, conventional RT-PCR involves multiple reaction steps, and its sensitivity may be compromised by RNA degradation or low viral loads commonly encountered during early or subclinical infections.

To address these limitations, several isothermal nucleic acid amplification techniques have been developed, including strand displacement amplification, transcription-mediated amplification, rolling circle amplification, and loop-mediated isothermal amplification[10,12,25,38]. Despite eliminating the need for thermal cycling, many of these methods involve complex primer designs, multiple enzymes, or are unsuitable for certain target regions, restricting their broader diagnostic adoption.

Thermophilic helicase-dependent amplification (tHDA) represents a PCR-like isothermal amplification strategy that utilizes a DNA helicase to separate double-stranded nucleic acids, replacing the heat denaturation step used in PCR. The reaction proceeds under constant temperatures (60–65°C) using minimal equipment

and yields high levels of amplified product[3,37]. When coupled with reverse transcription, RT-tHDA enables direct amplification of RNA viruses in a single-temperature reaction, making it particularly suitable for rapid molecular screening in field and low-resource diagnostic settings.

Although tHDA has been evaluated for the detection of several viral pathogens, limited data are available on its application for IBDV detection using field samples and comprehensive diagnostic validation. Given the continued emergence of genetically diverse and reassortant IBDV strains, and the constraints associated with real-time RT-PCR in field settings, there is a clear need for simple, rapid, and reliable molecular screening tools that can complement existing laboratory-based diagnostics.

The present study was undertaken to develop and validate an isothermal reverse transcription–thermophilic helicase-dependent amplification (RT-tHDA) assay targeting a conserved region of the VP1 gene of IBDV. The VP1 gene, encoded by genome segment B, was selected because of its essential role in viral replication and its growing importance in contemporary molecular classification of IBDV. Although VP2 is widely used for antigenic and virulence-associated characterization, increasing evidence indicates that segment B contributes significantly to viral pathogenicity, replication efficiency, and genetic reassortment[27]. Furthermore, phylogeny-based bi-segment genotyping systems now incorporate partial VP1 sequences to assign B genogroups and to identify reassortant strains that cannot be detected using VP2 alone. Accordingly, targeting VP1 enables broad detection of genetically diverse IBDV strains and supports downstream genotype and pathotype interpretation when combined with VP2-based analyses. The analytical sensitivity, specificity, and diagnostic performance of the RT-tHDA assay were evaluated and compared with World Organization for Animal Health (WOAH) recommended method conventional RT-PCR, virus isolation by embryonated egg inoculation andVP1-based real-time RT-PCR, with particular emphasis on its suitability as a rapid screening assay for IBDV detection in field conditions and resource-limited laboratories.

Materials and Methods

Sample collection and processing

A total of 180 bursal tissue samples were collected during infectious bursal disease outbreaks from thirteen epidemiologically distinct poultry farms across southern India. Samples were obtained from chickens exhibiting typical clinical signs and characteristic post-mortem lesions suggestive of infectious bursal disease. Tissues were transported to the laboratory under cold-chain conditions. Approximately 1 g of each bursal tissue was homogenized in 1 mL of sterile phosphate-buffered saline (PBS, pH 7.2), clarified by centrifugation at 10,000 × g for 5 min at 4°C, and the supernatants were stored at –80°C until RNA extraction.

Viral RNA isolation and cDNA synthesis

Total RNA was extracted from 250 µL of clarified bursal tissue supernatant using TRIzol™ reagent (Takara Bio Inc., Japan) according to the manufacturer's instructions, with minor modifications. RNA was precipitated with isopropanol and incubated at –80°C overnight to enhance recovery, followed by washing with 70% ethanol and resuspension in nuclease-free water. First-strand cDNA was subsequently synthesized using RevertAid™ H Minus M-MuLV reverse transcriptase (Thermo Scientific™, USA) using random hexamer primers following the manufacturer's protocol. The synthesized cDNA was stored at –80°C until further molecular analysis.

tHDA primer design and in-silico screening

Primers for tHDA were designed to target a conserved region of the VP1 gene (segment B) of IBDV using Primer3 software. Primer design parameters were optimized for isothermal amplification, with amplicon size restricted to 80–120 bp, primer length of 24–30 nucleotides, GC content of 30–60%, and predicted melting temperatures compatible with thermophilic

Table 1. Primers used for detection of infectious bursal disease virus

S.No	Primer Name	Primer sequences (5'-3')	Nucleotide position	Product size	Reference
1.	HDAVP1 FP	TTG AAC AGG ATC GTC GAG TGG ATA TTG	1255-1282	99 bp	This study
2.	HDAVP1 RP	TTG AGT ACC ACG TGT TTG AGT GGA CAA	1327-1354		
3.	qRT-VP1 FP	TAG TCC AGA CAG CGA AGA G	1573-1592	194 bp	This study
4.	qRT-VP1 RP	GCA GAC CAT CCG AGT AGG	1749-1767		
5.	IBDV VP1 FP	CTA CGG GAG TGG GAC CTA CA	600-619	749 bp	5
6.	IBDV VP1 RP	ACC ACG TGT TGG AGT GAA CA	1329-1348		
7.	IBDV VP2 FP	GCC CAG AGT CTA CAC CAT	737 to 754	743 bp	17
8.	IBDV VP2 RP	CCC GGA TTA TGT CTT TGA	1462-1489		

helicase-dependent amplification. Primer specificity was verified in silico against available IBDV sequences and non-target avian pathogens.

tHDA assay

tHDA amplification was performed using the IsoAmp™ HDA kit (BioHelix Corporation, USA) following the manufacturer's recommendations with optimization for IBDV detection. The assay was conducted as a two-step isothermal reaction involving two reaction mixtures (A and B). Reaction mixture A (12.5 µL) consisted of 3 µL of cDNA (10–40 ng/µL), 5 pmol/µL of each primer, 1.25 µL of 10X annealing buffer, 2 µL of DMSO, and nuclease-free water to volume. Reaction mixture B (12.5 µL) contained 1X annealing buffer, 1 µL of 100 mM $MgSO_4$, 2 µL of 500 mM NaCl, 1.75 µL of IsoAmp™ dNTPs, 1 µL of IsoAmp™ enzyme mix, and nuclease-free water.

For the two-step tHDA procedure, reaction mixture A was initially incubated at 65°C for 2 min to reduce nonspecific amplification and then rapidly cooled on ice. Reaction mixture B was subsequently added to mixture A, and the combined reaction was incubated at 60–65°C for 30–120 min in a heating block. The amplified products were resolved by electrophoresis on 2% agarose gels stained with ethidium bromide and visualized under UV illumination.

Analytical sensitivity and specificity of tHDA

Analytical sensitivity of the tHDA assay was determined using ten-fold serial dilutions of IBDV RNA starting from an initial concentration of 44 ng and compared with conventional RT-PCR and VP1-based real-time RT-PCR. The detection limit was defined as the lowest RNA concentration consistently producing a visible amplification product.

Analytical specificity was evaluated using nucleic acids from heterologous avian viral pathogens, including Newcastle disease virus (velogenic and lentogenic strains), infectious bronchitis virus, and Marek's disease virus, as well as bacterial pathogens (*Salmonella typhi*, *Brucella abortus*, and *Escherichia coli*).

Diagnostic validation and comparative evaluation

All 180 field samples were subjected to tHDA, conventional RT-PCR targeting VP1 and VP2 genes, VP1-based real-time RT-PCR, and virus isolation by embryonated chicken egg inoculation. Conventional RT-PCR assays were performed using previously published primers and cycling conditions[5,17]. Real-time RT-PCR was performed using SYBR Green chemistry (Takara Bio Inc., Japan) in an Eppendorf Mastercycler® ep realplex system, followed by melting curve analysis to confirm amplicon specificity.

Statistical analysis

The diagnostic performance of the tHDA assay was evaluated using WOAH-recommended reference methods, including virus isolation in embryonated eggs and conventional VP1- and VP2-based RT-PCR assays. VP1-based real-time RT-PCR was included as a high-sensitivity comparator

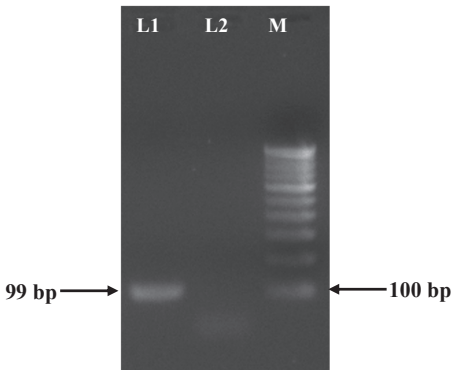

Fig. 1. Standardization of RT-tHDA assay targeting the VP1 gene of IBDV. Lane 1, 99 bp VP1 amplicon; Lane 2, negative control; M, 100 bp DNA ladder.

assay rather than a reference standard, as it may detect low levels of viral RNA not necessarily associated with viable virus or confirmed infection. Diagnostic sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) were calculated using 2 × 2 contingency tables, and agreement between tHDA and each comparator assay was assessed using Cohen's kappa (κ) statistic. Detection rates among diagnostic methods were compared using the chi-square (χ^2) test. All statistical analyses were performed using Microsoft Excel (Microsoft Corp., USA), and a p-value < 0.05 was considered statistically significant.

Results

Optimization of the tHDA assay

An isothermal tHDA assay targeting a conserved region of the VP1 gene of IBDV was successfully developed and optimized. Temperature optimization experiments revealed that specific amplification was obtained only at 65°C, while no specific products were observed at lower or higher temperatures tested. Accordingly, 65°C was selected as the optimal reaction temperature. To determine the optimal incubation time, the tHDA reaction was evaluated at 30, 45, 60, 90, and 120 min at 65°C. No detectable amplification was observed at incubation periods up to 60 min. In contrast, a clear and specific amplicon of the expected size (99 bp) was consistently observed at 90 min and 120 min. Based on the presence of a single, well-defined band and reduced nonspecific amplification, an incubation period of 90 min at 65°C was selected as the optimal condition for subsequent experiments (Fig.1). To confirm assay specificity, representative tHDA amplicons were sequenced, and BLAST analysis revealed 100% identity with the IBDV VP1 gene.

Analytical sensitivity and specificity of the tHDA assay

The analytical sensitivity of the tHDA assay was assessed using ten-fold serial dilutions of IBDV RNA and compared with conventional RT-PCR and VP1-based real-time RT-PCR. Conventional RT-PCR targeting the VP1 gene detected viral RNA up to a concentration of 44 pg, whereas both tHDA and real-time RT-PCR consistently detected viral RNA down to 440 fg. These findings indicate that the tHDA assay exhibited a 100-fold higher analytical sensitivity than conventional RT-PCR and a detection limit comparable to that of real-time RT-PCR. The analytical specificity of the tHDA assay was evaluated using nucleic acids from heterologous avian viral and bacterial pathogens. Amplification was observed exclusively with IBDV-positive samples, while no cross-reactivity was detected with Newcastle disease virus (velogenic and lentogenic strains), infectious bronchitis virus, Marek's disease virus, *Salmonella typhi*, *Brucella abortus*, or *Escherichia coli*. These results confirm the high analytical specificity of the tHDA assay for IBDV detection (Fig.2A-E).

Diagnostic validation and comparative performance

The diagnostic performance of the tHDA assay was evaluated using 180 bursal tissue samples collected from 13 suspected infectious bursal disease outbreaks across four southern Indian states. Among the assays evaluated, real-time RT-PCR detected the highest number of positives (81/180; 45.0%), followed by VP1-based RT-PCR (36.11%), VP2-based RT-PCR (35.56%), tHDA (35.0%), and virus isolation in embryonated eggs (31.67%), with a significant difference in detection

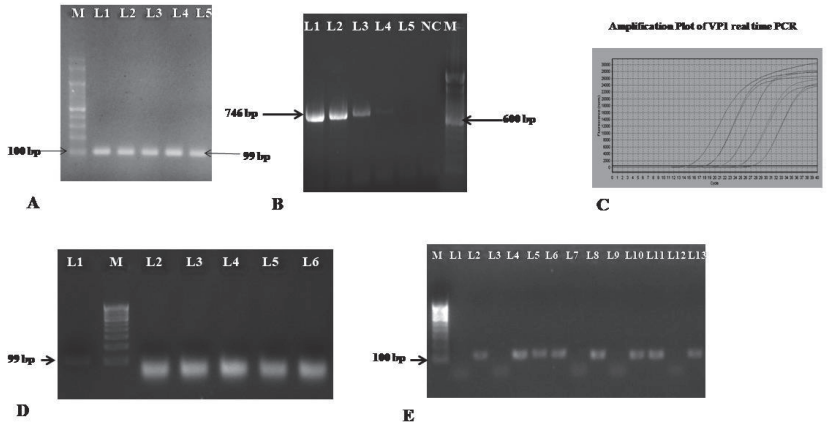

Fig. 2. Analytical sensitivity, specificity, and field applicability of VP1 gene–based assays for IBDV detection.
(A) Analytical sensitivity of the tHDA assay targeting the VP1 gene of IBDV. Lanes 1–5 represent ten-fold serial dilutions of IBDV RNA ranging from 44 ng to 440 fg. M, 100 bp DNA ladder, confirming amplification of the expected 99 bp product.
(B) Analytical sensitivity of VP1-based conventional RT-PCR using ten-fold serial dilutions of IBDV RNA. Lanes 1–5 correspond to 44 ng, 4.4 ng, 440 pg, 44 pg, and 440 fg of RNA, respectively. Lane 6 represents the negative control (no-template control). M, 100 bp DNA ladder.
(C) Analytical sensitivity of VP1-based real-time RT-PCR.Amplification plots generated from ten-fold serial dilutions of IBDV RNA showing a progressive increase in Ct values with decreasing RNA concentration.
(D) Analytical specificity of the RT-tHDA assay evaluated against related avian pathogens and bacterial species. Lane 1, IBDV (positive control); Lane 2, Newcastle disease virus; Lane 3, infectious bronchitis virus; Lane 4, Marek's disease virus; Lane 5, Escherichia coli; Lane 6, Brucella abortus. Specific amplification was observed only for IBDV. M, 100 bp DNA ladder.
(E) Screening of suspected IBDV field samples using the developed RT-tHDA assay. Representative gel image showing amplification of the VP1 gene in positive field samples, demonstrating the applicability of the assay for rapid detection of IBDV in clinical specimens. M, 100 bp DNA ladder.

rates (χ^2 = 16.22; P < 0.01). When compared with WOAH-recommended reference methods, tHDA showed excellent diagnostic performance, demonstrating 100% sensitivity and 95.12% specificity against virus isolation (κ = 0.93) and sensitivities of 96.92% and 98.44% against VP1 and VP2 based RT-PCR, respectively, with almost perfect agreement ($\kappa \geq 0.97$). Although real-time RT-PCR detected a higher number of positive samples, reflecting its high analytical sensitivity, rapid turnaround time, and routine use in diagnostic laboratories, tHDA showed substantial agreement with this assay (κ = 0.83). The reduced sensitivity of tHDA relative to real-time RT-PCR is attributable to the ability of real-time assays to detect low viral loads, including samples with minimal viral RNA that may be below the detection threshold of isothermal amplification methods. Importantly, the tHDA assay detected IBDV in samples that were also identified as positive by both VP1 and VP2 based conventional RT-PCR assays as well as by embryonated egg inoculation, demonstrating a high degree of consistency with these established diagnostic methods. (Table 2).

Overall, these findings indicate that the tHDA assay is highly concordant with WOAH-recommended gold-standard diagnostic methods and performs comparably to conventional RT-PCR assays, while maintaining substantial agreement with real-time RT-PCR (Table 3). Given its simplicity, rapidity, and minimal equipment requirements, tHDA represents a reliable and practical molecular screening tool for IBDV detection, particularly in field-level and resource-limited diagnostic settings.

Discussion

In this study, an isothermal tHDA assay was developed for detection of IBDV by targeting a conserved 99-bp region of the VP1 gene located on genome segment B. The 99-bp fragment of the VP1 gene targeted in this study was selected for diagnostic screening and molecular surveillance

Table 2. Comparison of tHDA assay with embryonated egg inoculation, RT-PCR, and real-time RT-PCR for detection of IBDV in field samples

Reference / comparator assay	TP	FP	FN	TN	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Cohen's κ (agreement)
Virus isolation in embryonated eggs (WOAH gold standard)	57	6	0	117	100.00	95.12	90.48	100.00	0.93
VP1-based conventional RT-PCR (WOAH gold standard)	63	0	2	115	96.92	100.00	100.00	98.29	0.97
VP2-based conventional RT-PCR (WOAH gold standard)	63	0	1	116	98.44	100.00	100.00	99.15	0.98
VP1 real-time RT-PCR (high-sensitivity molecular assay)	63	0	18	99	77.78	100.00	100.00	84.62	0.83

Table 3. Diagnostic performance of tHDA and WOAH reference methods for IBDV detection in field samples

Diagnostic method	Sensitivity (%)	Specificity (%)	Agreement (κ)
tHDA	77.8	100.0	0.79
VP1 RT-PCR	80.2	100.0	0.82
VP2 RT-PCR	79.0	100.0	0.81
Virus isolation (egg inoculation)	70.4	100.0	0.73

Sensitivity, specificity, and Cohen's kappa (κ) values were calculated using WOAH-recommended reference methods (virus isolation in embryonated eggs and conventional RT-PCR) as comparators. κ values indicate substantial to almost perfect agreement.

purposes rather than for definitive pathogenicity classification. Targeting VP1 enables broad detection of genetically diverse IBDV strains, including both virulent and avirulent variants. In this context, the tHDA assay developed in this study is intended as a rapid molecular screening tool for IBDV detection, therefore, surveillance approaches that include VP1/segment B alongside VP2 improve detection and interpretation of outbreak strains[7,15,17,34].

Optimization experiments showed that specific amplification was obtained at 65°C, and time-course evaluation at this temperature demonstrated that the expected amplicon was detectable only after 90 min, remaining detectable at 120 min; consequently, 65°C for 90 min was selected as the optimal condition to maximize specificity while minimizing reaction time. These findings are consistent with previous reports describing optimal thermophilic HDA performance within a similar temperature window for diverse targets, reflecting the underlying enzymology of helicase-mediated strand separation and polymerase extension under isothermal conditions[3,11,26].

In the present study, tHDA demonstrated superior analytical sensitivity compared with conventional RT-PCR and a detection limit comparable to VP1 based real-time RT-PCR when evaluated using ten-fold serial dilutions of viral RNA. The assay consistently detected IBDV RNA down to 440 fg, representing an approximately 100-fold improvement over conventional VP1 RT-PCR (44 pg). This enhanced analytical sensitivity reflects the intrinsic efficiency of helicase-dependent isothermal amplification chemistry under controlled conditions, where high-quality RNA templates, absence of inhibitory substances, and optimized reaction kinetics promote efficient target amplification. Similar improvements in analytical sensitivity of helicase-based isothermal amplification platforms over conventional endpoint PCR have been reported for several viral and bacterial pathogens[3,11,26].

However, when applied to field-derived clinical samples, the diagnostic sensitivity of tHDA was comparable to or slightly lower than that of conventional RT-PCR and lower than real-time RT-PCR. This discrepancy between analytical and diagnostic performance can be attributed to factors inherent to clinical specimens. Bursal tissues collected from field outbreaks often exhibit variable viral loads, partial RNA degradation, co-purification of host-derived inhibitory substances,

and heterogeneous tissue matrices, all of which can adversely affect isothermal amplification reactions. In contrast, real-time RT-PCR benefits from thermal cycling, fluorescent probe-based detection, and advanced reaction chemistries that enable reliable amplification and detection of low-copy-number targets, including samples containing minimal or residual viral RNA. This explains the higher positivity rate observed with real-time RT-PCR, particularly in samples representing early, resolving, or subclinical infections that may fall below the practical detection threshold of isothermal assays.

Despite these limitations, tHDA exhibited excellent diagnostic concordance with WOAH-recommended gold-standard methods, including virus isolation and conventional VP1 and VP2 based RT-PCR, with almost perfect agreement ($\kappa > 0.93$) and high specificity ($\geq 95\%$). Notably, all virus isolation-positive samples were also detected by tHDA, indicating that the assay reliably identifies clinically relevant infections associated with active viral replication. Therefore, while real-time RT-PCR remains the most sensitive method for detecting very low viral loads, tHDA offers a robust, rapid, and field-adaptable alternative that balances sensitivity with operational simplicity, making it particularly suitable for outbreak investigations and resource-limited diagnostic settings.

The assay also demonstrated high analytical specificity, with amplification restricted to IBDV and no cross-reactivity with common avian viral pathogens or selected bacterial agents tested. High assay specificity is a recognized advantage of tHDA platforms when primer designs and reaction conditions are optimized, supporting their utility in routine diagnostic workflows where mixed infections and overlapping clinical syndromes are common[4].

The diagnostic importance of a rapid, simplified molecular assay is underscored by the current epidemiology of IBDV. Multiple studies including those from India and other regions highlight continued circulation of very virulent and variant strains, frequent outbreaks in vaccinated flocks, and ongoing evolution driven by antigenic drift and segment reassortment involving both genome segments[7,9,24,35].

In particular, recent large-scale surveillance work has shown that reassortant genotypes can predominate in field settings and that sequencing of both VP2 and VP1 improves epidemiological resolution[7]. Furthermore, complete-genome investigations from India demonstrate naturally occurring reassortment where segment A shows very virulent characteristics while segment B clusters with non-vv lineages, reinforcing the diagnostic value of VP1/segment B–based detection and monitoring[9,27,29]. Taken together, the present findings indicate that tHDA offers a practical balance of high analytical sensitivity, excellent specificity, and minimal equipment requirements, making it suitable for resource-limited laboratories and field-adjacent testing where uninterrupted power supply and real-time instrumentation may be limiting. While tHDA does not replace real-time RT-PCR for maximal diagnostic sensitivity, it can meaningfully strengthen early detection and outbreak response by enabling rapid screening and prioritization of samples for confirmatory testing and molecular epidemiology.

Conclusion

This study demonstrates that RT-tHDA targeting the conserved VP1 gene of infectious bursal disease virus is a rapid, sensitive, and highly specific isothermal molecular assay. The optimized assay operates at 65°C within 90 min and achieved an analytical detection limit of 440 fg of viral RNA, representing a 100-fold improvement over conventional RT-PCR and performance comparable to VP1-based real-time RT-PCR. Field evaluation showed high diagnostic specificity and substantial agreement with the reference assay, supporting the reliability of tHDA as a screening tool. Its simplified workflow and minimal equipment requirements make tHDA suitable for field surveillance and laboratories with limited infrastructure, providing an effective front-line approach for early detection and triage

of IBDV infections.

Conflict of Interest

The authors declare that there is no conflict of interest involved in this study

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