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A closed-tube, single-step, real time, reverse transcription-loop-mediated isothermal amplification assay for infectious bronchitis virus detection in chickens

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ABSTRACT

Infectious bronchitis (IB) is a viral infection of the chicken respiratory tract that causes substantial economic burden on the industry. Simple, specific and rapid diagnosis of this disease is critical for the initiation of appropriate control measures. Conventional molecular diagnostic methods require a relatively sophisticated equipment and skilled staff. Here we describe a rapid, simple, semi-quantitative, closed-tube, single-step, real-time- reverse transcription-loop-mediated isothermal amplification (RT-LAMP) assay for IB and compare our assay with quantitative, reverse transcription- polymerase chain reaction (RT-qPCR). The limit of detection (LOD) of our RT-LAMP assay is 1 EID₅₀/ ml. Clinical evaluation of samples from diseased chickens with our RT-LAMP showed a very good concordance with RT-qPCR. Our assay enables simple, specific, rapid molecular detection and semi-quantification of the infectious bronchitis virus (IBV) in veterinary diagnostic laboratories. Furthermore, our RT-LAMP detection is carried out in a sealed tube, eliminating the risk of false-positive results in subsequent tests because of any contamination of the work area as in the case of lateral flow strip or gel electrophoresis-based amplicon detection.

1. Introduction

The infectious bronchitis virus (IBV) is a common cause of respiratory disease in chickens with considerable economic impact on the poultry industry. The disease is caused by a single-stranded RNA coronavirus. The disease clinical signs include respiratory manifestations and decline in egg quality and production rate. There are several IBV serotypes and genotypes globally (De Wit et al., 2011; Cook et al., 2012). Recently, a new IBV classification scheme was introduced based on phylogenetic analysis of the S1 gene. This scheme identifies six genotypes, which are further divided into 32 lineages (Valastro et al., 2016). In the last decade, an increasing number of IBV strains have been reported to induce nephritis (Cavanagh, 2005, 2007; Cook et al., 2012; Jackwood and de Wit, 2013; Gelb et al., 2013; Leghari et al., 2016).

Rapid identification of IBV would enable the application of suitable control measures, and consequently, decrease production losses. Current field diagnosis of IB relies on clinical signs and post-mortem lesions examination that are not pathognomonic and require

confirmatory laboratory testing. Confirmative virus detection is carried out in centralized laboratories by virus isolation (VI), virus neutralization (VN) test, enzyme-linked immunosorbent assay (ELISA), haemagglutination inhibition (HI) (Cooke et al., 1987; Cavanagh, 2007; Jackwood and de Wit, 2013; Pradhan et al., 2014; Ding et al., 2015), conventional RT-PCR, and quantitative RT-PCR (RT-qPCR) (Callison et al., 2006; Meir et al., 2010; Fraga et al., 2016; Marandino et al., 2016; Fellahi et al., 2016; Laamiri et al., 2018; Molenaar et al., 2020). However, the aforementioned diagnostic methods pose challenges for implementing rapid, specific and simple detection in rudimentary veterinary diagnostic laboratories, particularly in resource-poor settings. VI and VN are time-consuming and require skilled personnel and adequate laboratory infrastructure. ELISA and HI are simpler and faster than VI and VN but with less sensitivity and specificity (Karaca and Naqi, 1993; De Wit et al., 1997). While PCR tests are specific, sensitive, and commonly used in virus detection, they require expensive equipment and skilled personnel.

Loop-mediated isothermal amplification (LAMP) of nucleic acids is an innovative technology for the detection of viruses infecting

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respiratory tract of chickens with sensitivity on par with PCR (Pham et al., 2005; Chen et al., 2008; Li et al., 2009; Chen et al., 2010; Ou et al., 2012). LAMP utilizes a strand-displacing DNA polymerase and four primers along with two additional loop primers that recognize different sequences of the target nucleic acid (Nagamine et al., 2002).

The performance of LAMP assay-based detection relies not only on DNA amplification efficiency but also on amplicon detection. Various methods have been described for amplicon detection, including by naked eye (color change or turbidity), gel electrophoresis, fluorescence (Zhang et al., 2014), and bioluminescence (Gandelman et al., 2010; Kiddle et al., 2012; Yang et al., 2016). Fluorescence and bioluminescence-based detection are often carried out in real time, facilitating target quantification (Li et al., 2018; Song et al., 2018a and 2018b).

Since PCR-based assays are not available in many resource-poor settings, this study aims to develop a closed-tube-single-step real time-RT-LAMP assay that facilitates rapid, molecular detection and semi-quantification of IBV infection in veterinary diagnostic laboratories with minimal instrumentation.

2. Materials and methods

2.1. IBV and clinical samples

For analytical characterization of our assay, we used Nobilis® IBV Ma5 attenuated vaccine strain (Intervet, Boxmeer, Netherlands). The vaccine's EID₅₀ was determined as previously described (Reed and Muench, 1938) and then diluted in IBV-negative nasal swab from pathogen-free (SPF) chicken to 10³ EID₅₀/mL.

For clinical characterization, we used thirty-five nasal swabs from diseased chickens that presented respiratory distress (provided by the Molecular Section of the Pennsylvania Animal Diagnostic Laboratory System, School of Veterinary Medicine, University of Pennsylvania, USA).

2.2. RNA extraction

RNA was extracted with MagMax™ -96 Viral RNA Isolation kit (Thermo Fischer Scientific, Waltham, MA) according to manufacturer's instructions.

2.3. LAMP primers design

Complete genome sequences of different IBV genotypes retrieved from the GenBank were aligned and analyzed to identify conserved sequences using MEGA X software (<http://www.megasoftware.net/>). A 286 nt sequence in the nucleocapsid protein encoding gene of IBV was selected as a template because of its high homology among the examined viruses. The LAMP primers (Fig. 1) were designed with the PrimerExplorer V5 software (Eiken Chemical Co. Ltd.). The primers used in this study were checked on the NCBI database BLAST (<http://www.ncbi.nlm.nih.gov>) for cross-reactivity with other viruses infecting respiratory tract of chicken, including avian influenza viruses (H5N1, H9N2, and H5N8), Newcastle disease virus, and infectious laryngotracheitis virus (ILT). No cross reactivity was identified. The LAMP primers were synthesized (IDT Company, Coralville, IA), supplied in lyophilized form, and suspended to the concentration of 100 μM in nuclease free water.

2.4. RT-LAMP reaction conditions

RT-LAMP assay was carried out to detect nucleocapsid gene of IBV. The RT-LAMP reaction mix (10 μL) contained 0.2 μM each of primers F3 and B3; 1.6 μM each of FIP and BIP primers; 0.8 μM each of LoopF and LoopB primers; 6 μL of Isothermal MasterMix (ISO-001, OptiGene, USA); 0.2 μL of AMV reverse transcriptase (200 U/ μL) (Promega, Madison, WI, USA); 0.4 μL 1 × EvaGreen® dye (Biotium, Inc.), and

0.6 μL of viral RNA template and nuclease free water (Invitrogen, Carlsbad, CA, USA). We added the reverse transcriptase enzyme to the reaction mix since we found in earlier work that it enhances assay performance (Song et al., 2016). Fluorescence emissions of DNA amplicons was monitored with the 7500-Fast Real Time PCR system (Applied Biosystems) at 63 °C for 40 min, then melting curves were obtained in the temperature range from 50 °C to 95 °C with 1.0 °C increment per second. Non template samples (negative control) were included in each run.

2.5. Quantitative RT-PCR (RT-qPCR) amplification

Quantitative RT-PCR (RT-qPCR) was carried out to detect IBV using forward (F3) and backward (B3) outer primers. Each reaction used 0.6 μL of viral RNA, 10 μM of primers F3 and B3, 5 μL of SsoFast EvaGreen Supermix (Bio-Rad), 0.2 μL of AMV reverse transcriptase (200 U/ μL) (Promega, Madison, WI, USA) and nuclease free water (Invitrogen, Carlsbad, CA, USA) to a total volume of 10 μL. The number of cycles and the cycling times were as follows: incubation at 50 °C for 30 min followed by incubation at 95 °C for 10 min, and then 45 cycles of 95 °C for 10 s and 60 °C for 30 s for annealing and extension, followed by melting curve analysis from 50 °C to 95 °C with 1.0 °C increment per second. Fluorescence emissions due to DNAs amplifications were monitored with the 7500-Fast Real Time PCR system (Applied Biosystems). Samples lacking templates (negative control) were included in each run.

2.6. Specificity of our assays

RNAs extracts of IBV vaccines Poulvac® IB-Ark (Zoetis, USA), IB-VAR2 (ME VAC, Egypt), Nobilis® IB 4/91, Nobilis® IB Ma5, and Nobilis® IB H120 (Intervet, Boxmeer, Netherlands) were tested with our assays. Extracted nucleic acids from Newcastle disease virus (NDV) vaccine, LaSota strain (Intervet, Boxmeer, Netherlands), infectious laryngotracheitis virus (ILT) isolate and *Escherichia coli* isolate available in our lab were used as negative controls to confirm the specificity of our assays.

2.7. Limit of detection (LOD)

To determine the minimum EID₅₀ that can still be detected with our real time-RT- LAMP assay and our RT-qPCR assay, we carried out ten-fold serial dilutions of IBV Ma5 vaccine strain (10³ EID₅₀/mL) using IBV-negative nasal swab from SPF chickens, and extracted RNA from each dilution. Each dilution was tested in three replicates.

2.8. Clinical performance of our assays for IBV detection

Nucleic acids were extracted from thirty-five clinical samples from diseased chicken. These samples were first tested for IBV with RT-PCR (Callison et al., 2006) that is routinely used as the confirmatory assay for IB in chicken in many laboratories, including the Molecular Section of the Pennsylvania Animal Diagnostic Laboratory System, School of Veterinary Medicine, University of Pennsylvania, USA. We carried out our RT-PCR and RT-LAMP tests four weeks after Callison et al., 2006 RT-PCR. Positive (IBV-Ark vaccine strain) and negative controls were included in each test. A linear regression analysis of real time-RT-LAMP threshold times (Tt) and RT- qPCR threshold cycles (Ct) was performed.

3. Results

3.1. Detection of IBV – analytical performance

Fig. 2A and Fig. 2D depict, respectively, the fluorescence emission intensity (arbitrary units) of our IBV LAMP and IBV RT-PCR amplicons as functions of time for various template concentrations. As the

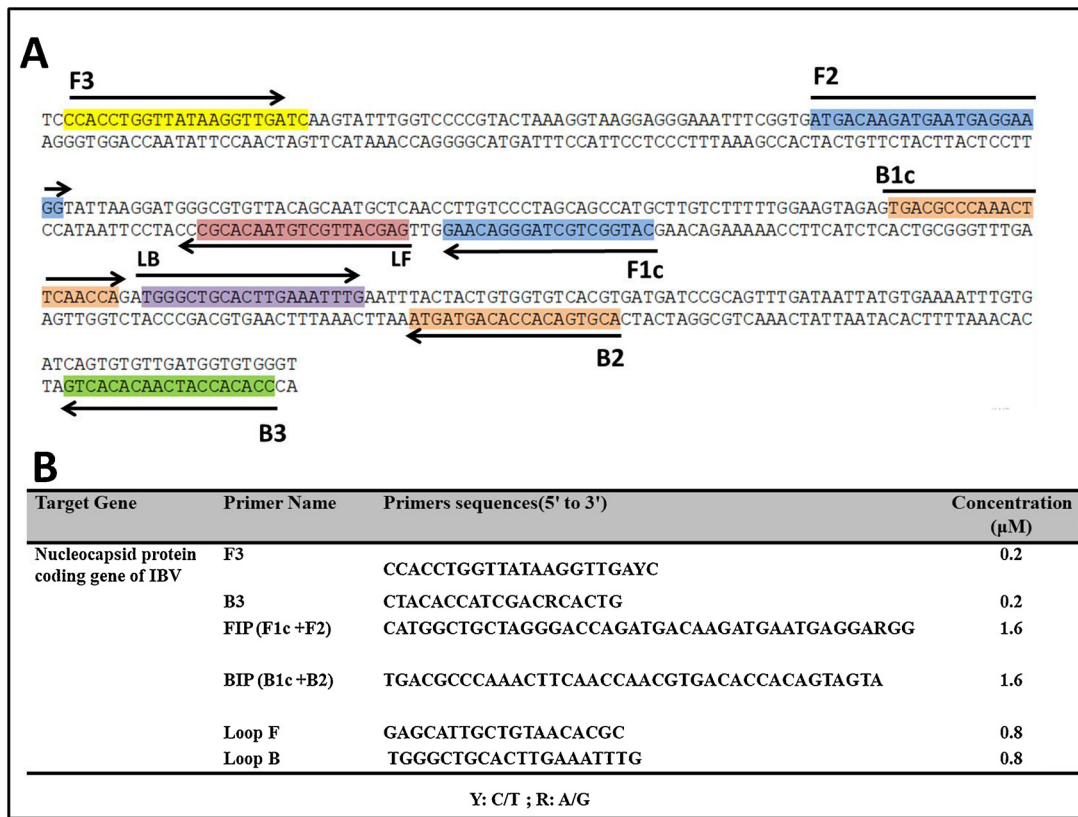


Fig. 1. Sequences and targeted regions of LAMP primers for IBV detection: (A) IBV nucleocapsid protein gene sequence (accession number: GQ504721.2, from 26573 nt to 26840 nt) with locations of the six primers: B3, F3, BIP (B1c + B2), FIP (F1c + F2), LB and LF indicated, arrows show the extension direction. (B) Sequences of primers for IBV LAMP reaction.

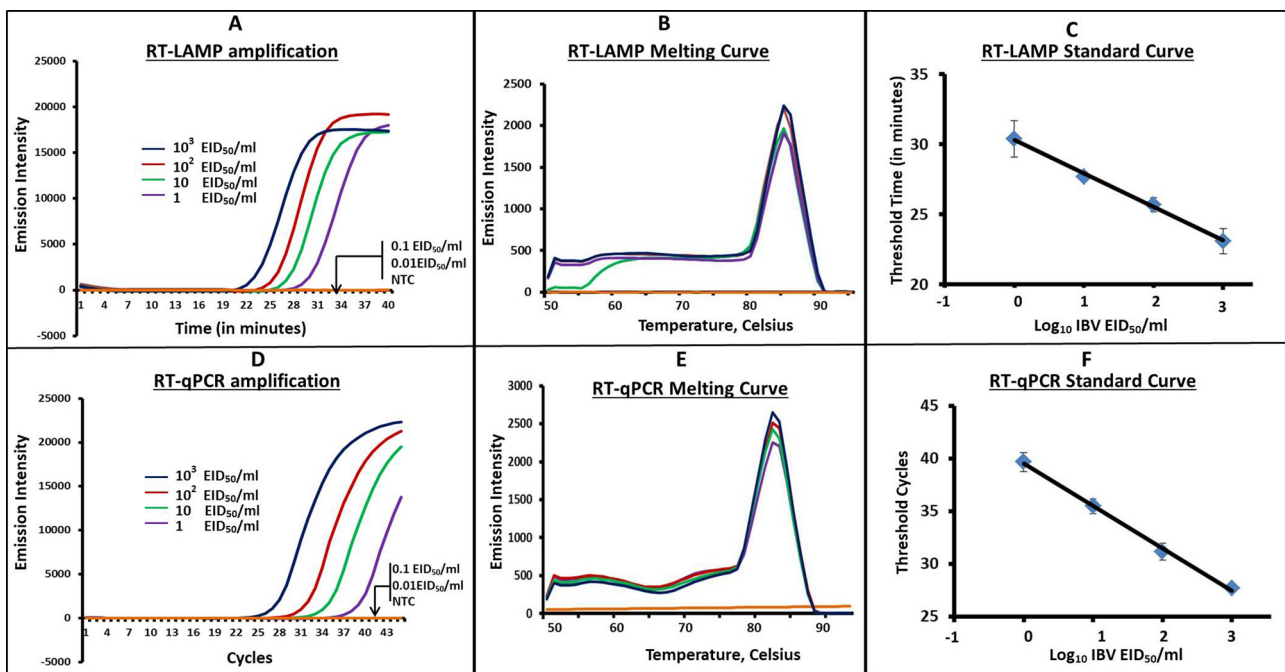


Fig. 2. Quantitative detection of IBV with real time-RT-LAMP and RT-qPCR: A) Real time monitoring of IBV RT- LAMP assay with 10^3 , 10^2 , 10, 1, 0.1, 0.01 EID₅₀ per ml. B) Melting curve of our RT-LAMP assay with a single peak. C) The threshold times (in minutes) of RT-LAMP assay as a function of IBV concentration (EID₅₀/mL). D) Real time monitoring of IBV RT- PCR assay with 10^3 , 10^2 , 10, 1, 0.1, 0.01 EID₅₀/mL. E) Melting curve of our RT-PCR assay with a single peak. F) The threshold cycles of RT-PCR assay as a function of IBV concentration (EID₅₀/mL).

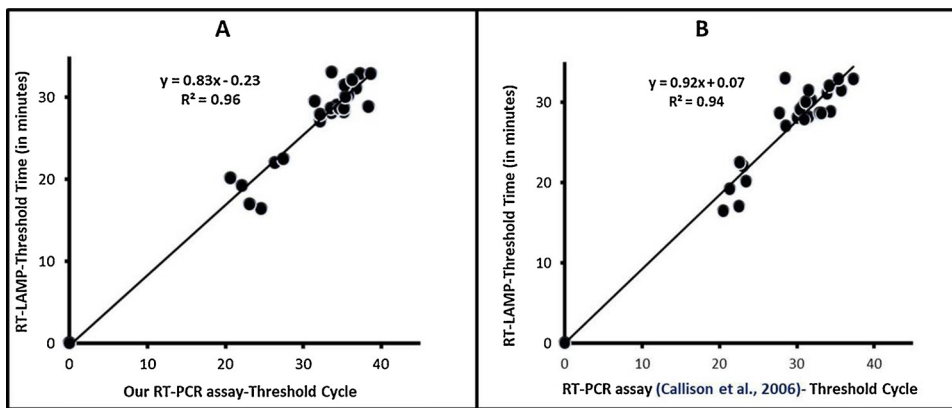


Fig. 3. Comparison of our RT-LAMP test results with our RT-qPCR (A) and Callison et al. (2006) RT-PCR assay (B) for detection of IBV in collected field samples. Thirty-Five RNA extracts of samples from suspected cases of IBV infections were screened. Linear regression analysis of RT-LAMP threshold time (Y axis) and RT-PCR threshold cycles values (X axis) were determined.

template concentration decreases, the threshold time (T_t) and the threshold number of cycles (C_t) increases. We identify the threshold time (T_t) and the threshold number of cycles (C_t) as the time needed for the normalized amplification curve to achieve half its saturation value. Both T_t (Fig. 2C) and C_t (Fig. 2F) are nearly linear functions of the log of the concentration. The lowest detectable EID_{50} of our RT-LAMP and RT-PCR assays as well as the Callison et al., 2006 RT-qPCR assay is 1 EID_{50}/mL . Melting curve analysis of both our RT-LAMP (Fig. 2B) and our RT-PCR (Fig. 2E) products revealed a single peak, indicating absence of non-specific products and primers dimers. Different IBV serotypes used in our study gave positive results. Non template controls and negative controls did not show any amplification signal (data not shown).

3.2. Performance of the assays with clinical samples

Our RT-LAMP test results of thirty five samples collected from diseased chicken flocks compared favorably with our RT-qPCR assay results and results previously obtained with the Callison et al. (2006) RT-PCR assay (Supplementary Table 1). Our IBV-RT-LAMP assay had 100 % sensitivity and 100 % selectivity when compared with both RT-qPCR assays. The LAMP threshold time correlated nearly linearly with our RT-PCR threshold cycle (Fig. 3A, $R^2 = 0.96$) and with the Callison et al. (2006) RT-PCR (Fig. 3B, $R^2 = 0.94$). Overall, our RT-LAMP assay was faster than our RT-qPCR assay. The number of threshold cycles of the Callison et al. (2006) RT-qPCR was smaller than that of our RT-PCR assay.

4. Discussion

Along with avian influenza and Newcastle disease, IB is a major threat to the poultry industry (Cavanagh, 2005). To rapidly enact appropriate control measures to contain the infection, real time, molecular detection methods are needed to test samples from chickens suspected of being infected in proximity to poultry farms. In resource-rich settings, this task is accomplished with single-step-real-time RT-PCR assays for IB detection, genotyping, and discrimination between vaccine and pathogenic strains (Fraga et al., 2016; Marandino et al., 2016; Domanska-Blicharz et al., 2017; Stenzel et al., 2017; Tucciarone et al., 2018).

PCR tests require, however, well-equipped laboratory facilities, sophisticated and expensive equipment, and trained personnel; all of which are in short supply in resource-poor settings. RT-LAMP offers an attractive alternative to RT-PCR. In contrast to RT-PCR that requires stringent sample preparation, incubation time exceeding an hour, sophisticated thermal cyclers and laboratory facilities, RT-LAMP is faster (less than an hour) and carried out at a fixed incubation temperature ($\sim 63^\circ C$), which reduces equipment complexity, cost, and power consumption. Although in our experiments, the LAMP reaction incubated

in a thermal cycler (operating at a fixed temperature), the RT-LAMP tube can be incubated in an instrument more easily accessible in developing countries like Genie II (Optigene, Horsham, UK) or an ESE-Quant TubeScanner (GmbH, Stockach, Germany) or a water bath, with a simple electrical heater, and even instrumentation-free with an exothermic chemical reaction of the type used in ready-to-eat meals and military rations, where temperature control is provided with a phase change material and fluorescent excitation and detection with a smartphone (Liao et al., 2016; Bau et al., 2017). Furthermore, LAMP assays are also more tolerant to inhibitors than PCR (Kaneko et al., 2007; Yang et al., 2014), reducing the need for extensive preparation of clinical samples. In summary, LAMP assays have proved to be highly sensitive, specific, robust, and tolerant of temperature variations ($60 - 65^\circ C$), making them suitable for use at the point of need (Mori and Notomi, 2009; Kokkinos et al., 2014).

Given the above, it is not surprising that in recent years scientists have been developing RT-LAMP assays for IBV (Chen et al., 2010; Chandrasekar et al., 2015; Wu et al., 2019). These assays use naked eye, gel electrophoresis, and lateral flow strips for amplicon detection and are not amenable for quantification. Furthermore, opening a tube rich with amplicons to enable electrophoresis or lateral flow-based detection risks contamination of the work, potentially rendering future tests false positive. In our RT-LAMP assay, both incubation and detection are carried out in a closed tube without the need to open the tube or transfer products rich with amplicons to a lateral flow strip or gel electrophoresis.

Our RT-LAMP performs on par with the RT-PCR. Our RT-LAMP threshold times correlate linearly with the logarithm of template concentration, suggesting that our test is semi-quantitative and can be used to estimate viral load.

Our RT-LAMP assay's LOD is 1 EID_{50}/mL similar to the LOD of the Callison et al. (2006) RT-PCR. The LOD of previously developed IBV-RT-LAMP assays that used EID_{50} to evaluate the analytical sensitivity was 10 $EID_{50}/50 \mu L$ (Chen et al., 2010; Chandrasekar et al., 2015). The higher analytical sensitivity of our assay could be due to differences in LAMP amplicon detection method (naked eye or gel electrophoresis in Chen et al., 2010 and Chandrasekar et al., 2015 assays Vs. real time fluorescence detection in our assay) and usage of loop primers (LB and LF) in our assay. Typically, during early stages of infection, chicken respiratory tissues and secretions have IBV EID_{50} exceeding 10^2 copies/ μL (Hassan et al., 2017; Erfan et al., 2019). Therefore, our assay's detection limit is adequate for virus detection without a need to concentrate the sample.

To confirm clinical utility of our assays for IBV detection, we used thirty -five clinical samples from diseased chickens. The aims of this experiment are to demonstrate (A) that our RT-LAMP is not inhibited by the unpurified clinical samples and (B) the absence of cross-reactivity/interference of host nucleic acids. Clinical evaluation of samples from diseased chickens using our RT-LAMP assay showed 100 % sensitivity

in comparison with both our own RT-PCR assay and the previously reported Callison RT-qPCR assay (Callison et al., 2006) and nearly linear correlations between our RT-LAMP threshold time and both RT-qPCRs threshold cycle number. The threshold cycle numbers of our RT-qPCR were slightly higher than that of the Callison et al. (2006) RT-qPCR likely due to possible samples' RNAs degradation in the four weeks interval between the Callison et al. (2006) RT-qPCR and our RT-PCR and due to differences in template lengths (143 bp in the Callison et al. (2006) assay vs 268 bp in our assay).

During the production cycle, commercial chickens are vaccinated against IBV infection with live vaccines, which persist for a long time in the farm. Thus, our assay (targeting a conserved portion of the virus' genome) cannot distinguish between infection and vaccination. In other words, our assay can be used only as a screening test (+ve/-ve result) and semi-quantitative determination of IBV RNA load in clinical specimens. Further studies to improve assay's specificity to differentiate vaccine from field strains with specific primers or guided endonuclease enzymes are critical for improved control measures. Furthermore, it would be highly desirable to develop a rapid technique that integrates sample processing with amplification and detection (Li et al., 2019).

In conclusion, our closed tube, single-step-real-time-RT-LAMP assay can facilitate rapid, specific and simple molecular detection and semi-quantification of IBV without a need for sophisticated and expensive equipment. The least limit of detection is 1 EID₅₀/mL. Clinical evaluation of samples from diseased chickens using our assay shows a very good concordance with RT-qPCR assay.

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Ethics approval

Sampling from chickens and samples' nucleic acids transfer were performed in compliance with the University of Pennsylvania ethical guidelines.

CRedit authorship contribution statement

Mohamed El-Tholoth: Conceptualization, Funding acquisition, Methodology, Writing - review & editing. **Michael G. Mauk:** Methodology, Writing - review & editing. **Eman Anis:** Methodology, Writing - review & editing. **Haim H. Bau:** Conceptualization, Funding acquisition, Project administration, Resources, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.jviromet.2020.113940>.

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