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METHOD

Development of a Novel Loop-Mediated Isothermal Amplification (LAMP) for Rapid Diagnosis of Newcastle Disease in Field and Resource-Limited Areas

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Background: Newcastle Disease (ND) is a highly contagious and fatal poultry disease caused by Newcastle disease virus (NDV) and has a global distribution. To control this disease, a rapid method for diagnosing it is needed. PCR-based molecular diagnostics such as real-time reverse transcription-PCR (RRT-PCR) for detecting NDV genes have been conducted worldwide. However, because PCR methods are time-consuming and require a well-equipped laboratory, they are not well-suited for use in field and resource limited areas.

Methods: In this study, we established a field-friendly loop-mediated isothermal amplification (LAMP) assay for NDV diagnosis using primers targeting the highly conserved L gene, which bypasses the need for RNA extraction and makes it more suitable for field application than previously reported LAMP methods.

Results: Our LAMP method is capable of detecting a broad range of NDV genotypes and showed no cross-reactivity with other avian viral diseases or the host genome. The reaction is completed within 35 minutes of incubation at 65°C. NDV was successfully detected directly from swab and tissue samples without the need for conventional RNA extraction.

Conclusion: The LAMP method developed in this study offers a rapid, affordable, and field-friendly diagnostic tool for NDV detection.

Keywords: loop mediated isothermal amplification, Newcastle disease, RNA preparation

Introduction

Newcastle disease (ND) is a highly contagious and fatal disease of poultry caused by Newcastle disease virus (NDV), a negative-sense single-stranded RNA virus. NDV has six genes that encode six structural proteins: nucleocapsid protein (NP), phosphoprotein (P), matrix protein (M), fusion protein (F), hemagglutinin-neuraminidase protein (HN), and large protein (L), in the 3′ to 5′ direction.[1] NDV is classified into two major classes: Class I and Class II. Class I NDVs are genetically less diverse and consist of a single genotype, whereas Class II NDVs are more diverse and have been classified into 21 genotypes based on the fusion (F) gene sequences.[2] Based on the clinical signs observed in infected chickens, NDV strains are further classified into five pathotypes: Viscerotropic velogenic: Highly virulent, causing intestinal hemorrhages, Neurotropic velogenic: Highly virulent, associated with respiratory and neurological symptoms, Mesogenic: Moderately virulent, causing respiratory signs and occasional nervous symptoms, Lentogenic: Low virulence, result in mild respiratory infections and subclinical: infections without apparent symptoms.[3]

Rapid diagnosis of NDV is important to prevent the spread of the disease. Real-time reverse transcription polymerase chain reaction (RRT-PCR), targeting the M gene,[4] is the most commonly used diagnostic method. However, this

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technique is labor-intensive, expensive, and requires well-equipped laboratories. An excellent alternative to RRT-PCR is loop-mediated isothermal amplification (LAMP), a method based on strand displacement reaction developed by Notomi et al.[5] LAMP is performed at a constant temperature and does not require a thermal cycler. It utilizes four to six primers, allowing for highly specific and sensitive amplification of the target gene.[5,6] LAMP results can be obtained in a short time, and interpretation can be made visually, without the need for additional equipment or complex procedures.[7]

LAMP-based methods have been developed for NDV diagnosis.[8–12] With the exception of Song et al,[12] all of these methods were based on the F gene. World Organization for Animal Health (WOAH),[3] recommends that molecular screening of NDV be based on conserved genes such as the matrix (M) and large polymerase (L) genes. Since the F gene is one of the most diverse NDV genes used for genotyping,[2] F gene-based LAMP primers may not effectively detect the broad range of NDV genotypes currently circulating worldwide. Song et al[12] developed a LAMP method targeting the F (for virulent NDVs) and HN gene (for all NDV strains). However, the HN is also less conserved,[3] which may limit the assay's specificity and broad-range detection capability. Moreover, all of these approaches depend on RNA extracted using conventional RNA extraction kits, which can be expensive, particularly in resource-limited settings. In recent years, following the global COVID-19 pandemic, several new techniques have been reported that enable the preparation of RNA for the detection of viral genes from clinical materials in a simple manner without the use of special kits or equipment.[13–15] To enable NDV testing in the field and in resource-limited settings, a more affordable and easily applicable method is needed. Therefore, the objective of this study was to develop a user and field-friendly LAMP assay by eliminating the RNA extraction step and to design primers targeting the L gene, one of the most conserved regions of the NDV genome,[3] to allow the detection of a broad range of NDV genotypes.

Materials and Methods

Designing Primers

LAMP primers were designed from the large polymerase (L) gene of NDVs. In order to design a primer that can detect a wide range of NDV pathotypes, consensus sequences were made from 19 NDV genotypes downloaded from GenBank (Table 1). The sequences were aligned by MUltiple Sequence Comparison by Log-Expectation (MUSCLE) using

Table 1 NDV Sequences Used for Designing LAMP Primers

S/N	Accession Number	Host	Country	Year	NDV Genotype
1.	AY935495	Chicken	Australia	1999	I.1.1
2.	AF077761	Chicken	USA	1946	II
3.	EF201805	Avian	India	1940	III
4.	AY741404	Fowl	UK	1933	IV
5.	MK046917	Pigeon	Mexico	2017	V.2
6.	FJ410145	Pigeon	USA	1984	VI.1
7.	LC874210	Chicken	Ethiopia	2023	VII.1.1
8.	FJ751918	Chicken	China	1979	VIII
9.	FJ436303	Chicken	China	1986	IX
10.	HQ266602	Chicken	Madagascar	2008	XI
11.	KC152048	Goose	China	2011	XII.2
12.	MF409241	Chicken	Zambia	2015	XIII.1.1

(*Continued*)

Table 1 (Continued).

S/N	Accession Number	Host	Country	Year	NDV Genotype
13.	MH996919	Turkey	Nigeria	2009	XIV.2
14.	JX119193	Chicken	Dominican Republic	2008	XVI
15.	MH996961	Chicken	Nigeria	2002	XVII
16.	MH392227	Chicken	Nigeria	2009	XVIII.2
17.	MK673141	Double crested cormorant	USA	2010	XIX
18.	AB853928	Chicken	Japan	1987	XX
19.	KY042132	Pigeon	Egypt	2015	XXI.1.1

MEGA11.[16] The consensus sequences were viewed by Jalview[17] and six LAMP primers were designed using the NEB LAMP primer design tool (https://lamp.neb.com/#!/). The designed primers included: Forward inner primer (FIP), Backward inner primer (BIP), Forward outer primer (F3), Backward outer primer (B3), Forward loop primer (FL) and Backward loop primer (LB).

RNA Preparation

RNAs were prepared using two different methods. The first involved a conventional RNA extraction method using the QIAamp viral RNA mini kit (Qiagen, Hilden, Germany, Cat. No. 52906). The RNA was extracted from oropharyngeal and cloacal swab samples, as well as tissue samples of chickens, confirmed to be NDV positive by RRT-PCR using M gene primer[4] following the manufacturer's instructions. The extracted RNA was stored at –70°C until use.

The second method was a modified version of the RNA extraction-free protocol described by Guan et al[14] for SARS-CoV-2. In addition, we incorporated 40 mM guanidine hydrochloride, which has been previously reported to enhance LAMP sensitivity for SARS-CoV-2 detection.[18] Briefly, swabs or homogenized tissue samples were centrifuged at 6000 rpm for 5 minutes, and the supernatant was transferred to a new tube. A 5% Chelex 100 resin (Bio-Rad, California, USA, Cat. No. 142-1253) was then added directly to the supernatant. The mixture was heated at 98°C for 5 minutes using a Dry Bath Plus (Nichiryo, Saitama, Japan, Product code 00-NDB+), followed by centrifugation at 6000 rpm for 2 minutes. The supernatant was used directly for the LAMP reaction.

LAMP Assay

LAMP reactions were conducted in a total volume of 12.5 µL containing 6.25 µL of Warmstart Colorimetric LAMP 2X master mix (NEB, Ipswich, MA, USA, Product No. M100S), 1.25 µL of 10X primer mix containing all 6 LAMP primers (16 µM FIP, 16 µM BIP, 2 µM F3, 2 µM B3, 4 µM LF and 4 µM LB), 0.5 µL of 1M guanidine hydrochloride (40 mM), 3.75 µL of nuclease free water and 0.75 µL of viral RNA. The reactions were carried out at 65°C for 35 minutes in Dry Bath Plus (Nichiryo, Saitama, Japan). Phenol red, a pH-sensitive dye included in the Warmstart Colorimetric LAMP 2X master mix, changes color from pink to yellow at low pH, which indicates a positive reaction.

Specificity and Sensitivity of the LAMP Assay

For a specificity test, RNA was extracted using a QIAamp viral RNA mini kit (Qiagen, Hilden, Germany) from avian paramyxovirus 3 (APMV-3), avian paramyxovirus 4 (APMV-4), avian paramyxovirus 6 (APMV-6) and H5N8 avian influenza virus (AIV) isolated in 9–11 day-old embryonated chicken eggs (ECE). The infectious bursal disease virus (IBDV) RNA was extracted from cloacal swab collected in viral transport medium. To assess the assay's ability to detect a broad range of NDV genotypes, RNA extracted from genotype I (Ishii), genotype II (Hitchner B1 (HB1)), and genotype IV (Herts) isolated in 9–11 day-old ECEs were tested. In addition, RNAs extracted from five chicken swabs (three tracheal, two oropharyngeal, and

five cloacal) and tissues (brain and lung-trachea) of NDV-free chickens by RRT-PCR, were examined to check for any non-specific reactions with the chicken genome. To evaluate the sensitivity of the LAMP assay, 16 oropharyngeal and 10 cloacal swabs confirmed to be NDV-positive by RRT-PCR were used. Detailed profiles of these samples are provided in the previous study.[19] LAMP sensitivity was assessed by comparing the LAMP results with the RRT-PCR results for each tested sample.

Evaluation of LAMP Assay Using Tissue Samples

To evaluate the LAMP protocol under field conditions, all reagents required for the test were transported from Japan to Ethiopia and stored at the optimal temperature upon arrival at the Animal Health Institute (AHI), Sebeta, Ethiopia. The LAMP reagents were examined using RNA prepared from NDV-positive brain and lung-trachea tissue to verify whether the reagents remained unaffected by transportation at ambient temperature. These tissue samples were previously stored at –80°C at the AHI. The master mix, comprising WarmStart Colorimetric LAMP 2X master mix, 10X primer mix, guanidine hydrochloride, and nuclease-free water, was prepared in PCR tubes. For the positive control, RNA extracted from NDV antigen (Antigen NDV pigeon Batch 2/13, Inactivated, Expiry 02/2025) provided by the WOAH was added to a designated positive control tube.

Field Application of RNA Extraction-Free LAMP Assay

The prepared master mix was transported on ice to a poultry farm in Sebeta, Ethiopia. Supporting equipment including a Dry Bath Plus, a portable vortex mixer, and a centrifuge were also brought to the site. Oropharyngeal and cloacal swab samples were collected from two chickens and tested on-site using the LAMP assay at 65°C for 35 minutes.

Results

Optimization of LAMP Method

LAMP primers targeting conserved regions of the NDV L gene were designed and screened for their ability to specifically detect NDV RNA without non-specific amplification. To optimize assay conditions, the LAMP reaction was tested at temperatures ranging from 60°C to 67°C. Initial screening was conducted using RNA extracted from the lentogenic HB1 vaccine strain and two velogenic genotype VII.1.1 NDV isolates from Ethiopian chickens (NDV/chicken/Eth_HT9C15/2023 and NDV/chicken/Eth_SLBM12C1/2023,[19] all propagated in 9–11 day-old ECEs. The LAMP primers shown in Table 2 were selected for our LAMP method. The optimal reaction condition was established as incubation at 65°C for 35 minutes.

Specificity of the LAMP Method

Once positive results were obtained using RNA extracted from the HB1 strain and the two velogenic NDV isolates (Figure 1a), the specificity of the LAMP method was evaluated. RNAs from IBDV, APMV-3, APMV-4, APMV-6, and H5N8 AIV were tested, and no non-specific amplifications were observed (Figure 1b). The LAMP method developed in this study successfully detected all tested NDV genotypes: genotype I (Ishii), II (HB1), and IV (Herts) within 35 minutes of incubation (Figure 1c). To evaluate the specificity of the RNA extraction-free method, the LAMP assay was tested for

Table 2 LAMP Primer Set Based on NDV L Gene

Primer	Sequence (5′-3′)
FIP	AAGCATCTTCTCACTCAGGTTATCACTTATATGCAGGGAATAAGTACG
BIP	CTGATTTCCCGGTTATGCTGTGATAGGCCCCTTATTTTTGG
F3	GTTAGTCTCTTGCACTCGA
B3	GTACTGAGCATTTCTCTTCTG
LF	CTAAGACAGATGGGAACAGCAGAT
LB	CTGTACACAGTGCTCTTTGCTACA

potential non-specific amplification against chicken genomic material and swab samples. Tracheal, oropharyngeal, and cloacal swabs were collected from five NDV-free chickens and used in RNA extraction-free LAMP reactions. No non-specific amplification was observed in any of the samples.

Sensitivity of LAMP Method

Sensitivity of our LAMP method was compared with RRT-PCR by conducting LAMP reactions using RNA extracted with a QIAamp viral RNA mini kit (Qiagen, Hilden, Germany) (purified RNA). The results of the LAMP method were 100% consistent with those of RRT-PCR for all 16 oropharyngeal swabs (Ct values: 23.09–33.1) and 10 cloacal swabs (Ct values: 22.97–32.02) (Figures 2a, b and 3a), resulting in 100% sensitivity and positive predictive value. All samples prepared using the RNA extraction-free method with Ct values ≤ 31.3 were successfully detected by RRT-PCR (Figure 3b). Furthermore, samples prepared using an RNA extraction-free method (unpurified RNA) were tested by RRT-

Figure 1 Specificity of the LAMP method. (**a**) Successful amplification was observed using RNA extracted from two velogenic NDV strains (1 and 2) and the lentogenic HB1 strain (3); (**b**) No amplification was detected for other avian viral pathogens: IBDV (1), APMV-3 (2), APMV-4 (3), APMV-6 (4), and H5N8 AIV (5); (**c**) LAMP test results for NDV genotype I (1), II (2), and IV (3). Velogenic NDV strains used: NDV/chicken/Eth_HT9C15/2023 (Accession number: LC846631) and NDV/chicken/Eth_SLBM12C1/2023 (Accession number: LC846618). Negative control (NC): nuclease-free water. A positive reaction is indicated by a color change from pink to yellow.

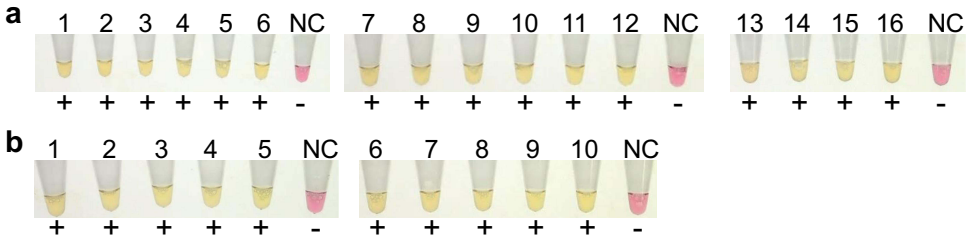

Figure 2 LAMP test results using extracted RNA for NDV-positive oropharyngeal and cloacal swab clinical samples. (**a**) Sixteen oropharyngeal swabs (1–16); (**b**) Ten cloacal swabs (1–10). A positive reaction is indicated by a color change from pink to yellow.
Abbreviation: NC, negative control.

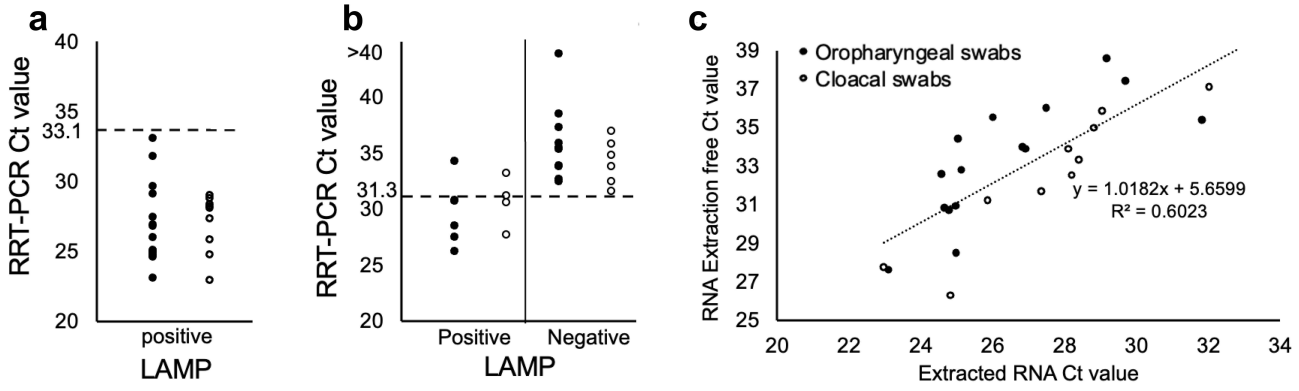

Figure 3 Comparison of LAMP and RRT-PCR assays. (**a**) Results of LAMP and RRT-PCR using RNA extracted from 16 oropharyngeal and 10 cloacal swab samples; (**b**) Results of LAMP and RRT-PCR using the same samples prepared by an RNA extraction-free method; (**c**) Comparison of Ct values obtained using extracted RNA and RNA extraction-free preparation methods. Filled circles (●): Oropharyngeal swabs, open circles (○): Cloacal swabs.

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PCR to assess their suitability. The unpurified RNA Ct values were increased by 2–9 cycles for oropharyngeal swabs and 4–6 cycles for cloacal swabs (Figure 3c).

LAMP Test for Clinical Samples Using RNA Extraction-Free Method

A total of 16 NDV-positive oropharyngeal swabs with cycle threshold (Ct) values ranging from 23.09 to 33.1 and 10 NDV-positive cloacal swabs with Ct values ranging from 22.97 to 32.02, as determined by RRT-PCR, were processed using an RNA extraction-free method (unpurified RNA). All of these sample were previously classified as sub-genotype VII.1.1 of genotype VII NDVs.[19] Among samples with Ct values ≤ 25, 83.3% (5/6) of oropharyngeal swabs (Or1-6) and 100% (2/2) of cloacal swabs (C1-2) were successfully detected using the rapid and user-friendly LAMP assay developed in this study, resulting in 87.5% sensitivity and 100% positive predictive value. In contrast, for samples with Ct values > 25, only 10% (1/10) of oropharyngeal swabs (Or7-16) and 25% (2/8) of cloacal swabs (C3-10) were detected by the LAMP method (Figures 3b and 4a, b).

Evaluation of LAMP Assay Using Tissue Samples

The LAMP method, tested at the AHI in Ethiopia using samples prepared with an RNA extraction-free method from NDV-positive brain and lung-trachea tissues, showed positive results. No amplification was detected in NDV-negative

Figure 4 LAMP test results using RNA extraction-free method for NDV-positive oropharyngeal and cloacal swab clinical samples. (**a**) Sixteen oropharyngeal swabs (1–16); (**b**) Ten cloacal swabs (1–10). A positive reaction is indicated by a color change from pink to yellow.
Abbreviation: NC, negative control.

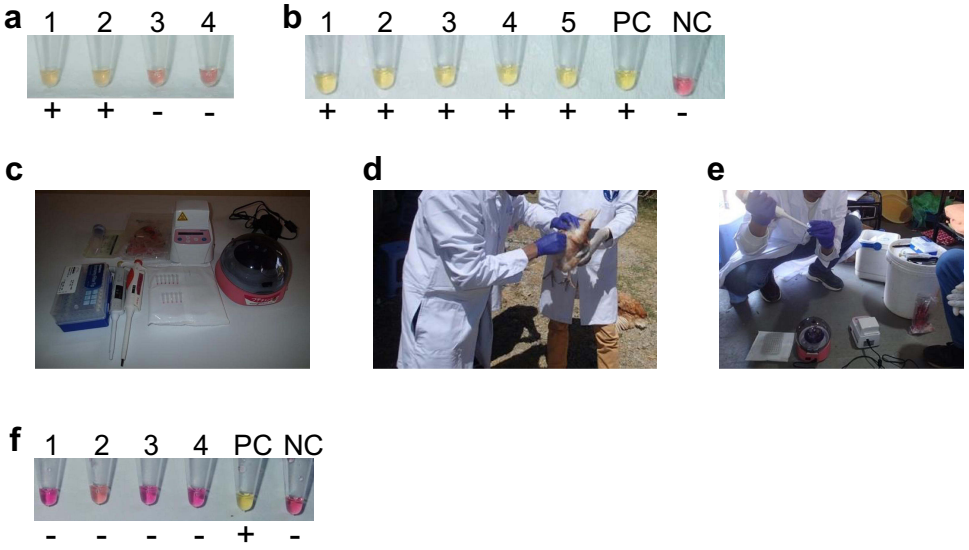

Figure 5 LAMP assay for field application: (**a**) LAMP test results using RNA extraction-free method from NDV-positive (1, 2) and NDV-negative (3, 4) brain and lung-trachea tissues; (**b**) LAMP test results using extracted RNA for NDV-positive brain (2, 4, 5) and lung-trachea (1, 3) tissues with Ct values ranging from 24.39 to 29.06; (**c**) Equipment and reagents transported to the field; (**d**) Sample collection in the field; (**e**) LAMP assay using an RNA extraction-free method conducted on-site; (**f**) LAMP test results using an RNA extraction-free method at the field, with oropharyngeal (1, 2) and cloacal (3, 4) swabs. PC: positive control (RNA from NDV antigen); NC: negative control (nuclease-free water).

brain and lung-trachea tissues, which were used as controls to evaluate any potential reactivity against host tissues (Figure 5a). Furthermore, the LAMP test on RNA extracted from six NDV-positive brain and lung samples, with Ct values ranging from 24.39 to 29.06, demonstrated 100% positivity, consistent with the results obtained using real-time RT-PCR (RRT-PCR) (Figure 5b).

Field Application of LAMP Assay

For the field application, the master mix prepared in the laboratory and transported to the poultry farm produced a positive result only for the NDV-positive control. The two oropharyngeal and two cloacal swabs collected and tested on-site were negative by the LAMP method (Figure 5c–f). These swab samples were also confirmed to be NDV-negative by RRT-PCR using RNA extracted from the same specimens.

Discussion

ND is a major problem in poultry production, particularly in developing countries where diagnostic laboratories and infrastructures are limited. Rapid diagnosis of NDV is essential to provide effective control measures. However, the most commonly used diagnostic method, RRT-PCR requires expensive equipment and well-established laboratory settings. In this study, we developed a rapid, cost-effective, and easily applicable diagnostic method for NDV that can be used in the field and in resource-limited settings. The LAMP assay, combined with an RNA extraction-free approach, enables direct detection of NDV from swab and tissue samples, without the need for conventional RNA extraction kits. Moreover, the reaction can be performed using a portable heat block, making it highly suitable for field diagnosis, with results obtained within 35 minutes of incubation at a constant temperature of 65°C. We have confirmed suitability of the method through field testing. This LAMP method effectively detects NDV in samples with Ct values ≤ 25, making it practical for field diagnosis in NDV outbreaks where the viral load is expected to be high. Samples prepared using the RNA extraction-free method can also be used for RRT-PCR in laboratories where RNA extraction kits are limited.

Our LAMP method demonstrates high specificity and sensitivity for NDV. No cross-reaction was observed with other avian viral diseases tested in this study (IBDV, AMPV-3, APMV-4, APMV-6, and H5N8 AIV), and no non-specific amplification was detected against the host genome. Furthermore, our LAMP assay successfully detected a broad range of NDV genotypes, including both lentogenic and velogenic pathotypes. This makes it a viable alternative to the commonly used M-gene-based RRT-PCR[4] for diagnosing all NDV pathotypes. The sensitivity of our LAMP method is 100% consistent with RRT-PCR when using RNA extracted with a commercial RNA extraction kit. These results suggest that the LAMP method established in this study can be a reliable alternative to RRT-PCR for the broad detection of NDV genotypes across all pathotypes.

Previously established LAMP methods for NDV diagnosis have some limitations. The method developed by Pham et al[10] requires cDNA synthesis and a reaction time of 2 hours. Additionally, Li et al[9] reported that the primers designed by Pham et al[10] resulted in false-negative results. To address this, Li et al[9] developed a one-step reverse transcription (RT)-LAMP method based on F gene primers. However, the visualization method used in this assay requires the addition of magnesium ions or SYBR Green I, necessitating the opening of the reaction tube, which increases the risk of laboratory contamination. Kirunda et al[8] established an RT-LAMP assay that allows visual detection by the naked eye. However, the primers were designed based on a single NDV strain's F gene and were not tested against different genotypes. Similarly, Selim et al[11] developed an RT-LAMP assay specifically for genotype VII NDV, limiting its applicability to that single genotype. More recently, Song et al[12] developed two sets of RT-LAMP assays targeting the F and HN genes. However, since both genes are less conserved,[3] the long-term effectiveness of these primers may be reduced due to the ongoing evolution of NDVs.[20] Furthermore, the HN gene-based primers developed by Song et al[12] were found to be less effective when used with the RNA extraction-free method established in this study. The LAMP assay established in this study targets the highly conserved L gene, enabling detection of a wide range of NDV genotypes and pathotypes. To date, all published LAMP methods for NDV detection have been based on RNA prepared using conventional RNA extraction kits. Our method eliminates the need for RNA extraction kits, allowing the assay to be performed rapidly and at low cost, making it particularly suitable for field use and in resource-limited settings. The

limitation of this assay is its reduced sensitivity in low viral load samples and its evaluation on a limited number of field samples.

Potential Applications in Surveillance and Outbreak Control

The developed LAMP assay can be employed for routine surveillance of NDV in poultry and for rapid on-site outbreak investigations, particularly in resource-limited settings where conventional laboratory infrastructure is unavailable.

Conclusions

The LAMP method established in this study offers a rapid, cost-effective, and field-applicable diagnostic tool for the detection of a broad range of NDV genotypes. The RNA extraction-free approach developed in this study enhances the practicality of this method, enabling timely diagnosis and effective response to outbreaks in field and resource-limited settings.

Ethical Statement

Swab samples were collected from live chickens by a veterinarian in accordance with the World Organization for Animal Health (WOAH) guidelines for the collection and shipment of diagnostic specimens (OIE Terrestrial Manual, 2008) https://www.woah.org/fileadmin/Home/eng/Animal_Health_in_the_World/docs/pdf/1.1.01_COLLECTION.pdf.
Informed consent was also obtained from bird owners for the sample collection. Tissue samples used in this study were previously stored at –80°C at the Animal Health Institute in Ethiopia. These samples were originally collected following the same WOAH guidelines for the collection and shipment of diagnostic specimens.

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Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

Disclosure

The authors report no conflicts of interest in this work.

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