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Development of a Fast and Affordable Diagnostic System for RNA Viruses Using Loop-Mediated Isothermal Amplification and DNA-Nanoprobe Detection

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Abstract

Airbone viral pathogens can rapidly spread and become a global menace, including both human and animal viruses which can trigger important socioeconomical and health effects. Therefore, to prevent and contain potential epidemic outbreaks, accurate, fast, and affordable diagnostic point-of-care tests (POCT) are required. As a proof of concept, we have developed a molecular detection system based in Loop-mediated isothermal AMPlification technique (LAMP) for two different RNA airborne viruses: the well-known human SARS-CoV-2, and the avian metapneumovirus (aMPV), the aetiologic agent of a communicable disease infecting mainly turkeys and chickens. To obtain a POC diagnostic system, we coupled the LAMP technique to DNA-functionalized gold nanoparticles detection. Validation of this system was carried out in 140 pharyngeal swabs from COVID-19 symptomatic and asymptomatic patients, and in 50 different samples (pharyngeal swabs and tracheal tissue samples) collected from aMPV infected chickens and turkeys. The system allows viral detection in about 60 minutes by the naked eye with 100% specificity, and 97.22% and 87.88% sensitivity for SARS-CoV-2 and aMPV, respectively. In summary, this novel detection system based on the coupling of RT-LAMP to DNA-nanoprobes allows suitable virus testing in the field, with accurate levels very close to conventional qRT-PCR based diagnosis.

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Cea-Callejo, Pablo[1,2]+; Arca-Lafuente, Sonia[1]+; Gomez-Lucia, Esperanza[2,3]; Doménech, Ana[2,3], Biarnés, Mar[4]; Blanco, Angela[4]; Benítez, Laura[2,5] and Madrid, Ricardo[1,2,5].

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Keywords: molecular detection, RT-LAMP, nanoprobes, SARS-CoV-2, aMPV, POC-test.

Abstract

Airbone viral pathogens can rapidly spread and become a global menace, including both human and animal viruses which can trigger important socioeconomical and health effects. Therefore, to prevent and contain potential epidemic outbreaks, accurate, fast, and affordable diagnostic point-of-care tests (POCT) are required. As a proof of concept, we have developed a molecular detection system based in Loop-mediated isothermal AMPlification technique (LAMP) for two different RNA airborne viruses: the well-known human SARS-CoV-2, and the avian metapneumovirus (aMPV), the aetiologic agent of a communicable disease infecting mainly turkeys and chickens. To obtain a POC diagnostic system, we coupled the LAMP technique to DNA-functionalized gold nanoparticles detection. Validation of this system was carried out in 140 pharyngeal swabs from COVID-19 symptomatic and asymptomatic patients, and in 50 different samples (pharyngeal swabs and tracheal tissue samples) collected from aMPV infected chickens and turkeys. The system allows viral detection in about 60 minutes by the naked eye with 100% specificity, and 97.22% and 87.88% sensitivity for SARS-CoV-2 and aMPV, respectively. In summary, this novel detection system based on the coupling of RT-LAMP to DNA-nanoprobes allows suitable virus testing in the field, with accurate levels very close to conventional qRT-PCR based diagnosis.

Introduction

Emerging infections account for at least 15% of all human pathogens (Petersen et al., 2018). To prevent the rapid spread of viruses, particularly airborne transmitted viruses, rapid and accurate diagnostic systems are essential. Point-of-care (POC) diagnostic platforms that meet the ASSURED criteria from the WHO (Affordable, Sensitive, Specific, User-friendly, Rapid and robust, Equipment-free, and Delivered) are of special interest in this regard. These platforms can be performed entirely *in situ* , from sample collection to final reading, in an integrated manner (Iliescu et al., 2021).

According to the One Health approaches, SARS-CoV-2 and avian metapneumovirus (aMPV) are two examples of RNA viruses of interest for people and animals, respectively. Portable antigen tests can detect an active infection, but they are less reliable than molecular tests (qPCR or qRT-PCR as gold standard), especially in asymptomatic patients (Schuit et al., 2021). Thus, to prevent silent SARS-CoV-2 outbreaks and transmission, accurate and highly sensitive molecular systems are needed. aMPV infection is considered one of the most economically important upper respiratory tract diseases in poultry (Kabouni and Lachhbeb, 2021). Early described as Turkey Rhinotracheitis (TRT) virus, it mainly affects turkeys but causes the Swollen Head Syndrome (SHS) disease in chickens as well. Different avian respiratory diseases have very similar clinical signs to TRT, such as avian infectious laryngotracheitis (ILT), avian infectious bronchitis (IB) or Newcastle disease (ND). Therefore, a specific and sensitive diagnostic system is essential to investigate its actual epidemiology in ongoing respiratory outbreaks in farms.

Currently, the gold standard for molecular detection of many infections, such as SARS-CoV-2 or aMPV, is based on qPCR techniques. However, this method requires specialized training and expensive equipment, which can limit its implementation in the field. Therefore, there is a growing interest in developing robust and reliable molecular diagnostics platforms that utilize the Reverse Transcriptase coupled to Loop-mediated isothermal amplification (RT-LAMP) technique, as an attractive and alternative point-of-care (POC) technique for the detection of RNA viruses. LAMP protocols are based on nucleic acid amplification under isothermal conditions, facilitated by a DNA polymerase with strand displacement activity and a set of four to six specific primers (Notomi et al., 2015). Detection can be achieved using RT-LAMP procedures, as an endpoint by visualizing the amplification products by DNA-agarose gel electrophoresis, or with the na-

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ked eye by colorimetric detection. For example, detection of SARS-CoV-2 by RT-LAMP can be achieved by using pH indicators, such as hydroxynaphthol blue (Thompson and Lei, 2020; Juscamayta-López et al., 2021; Nawattanapaiboon et al., 2021; Trassante et al., 2021; Raddatz et al., 2022), or metal indicators that change colour depending on the concentration of free Mg^{2+} ions (Goto et al., 2009). While these methods have performance limitations, they have been already approved as useful*in vitro* diagnostic (IVD) tools for large-scale screening.

Colloidal gold nanoparticles (AuNPs) have recently gained popularity as an alternative POC-test based on colorimetric assays, which can use either unlabelled AuNPs or DNA-functionalized AuNPs (DNA-nanoprobes) in combination with a portable device (Sivakumar, 2021; Raddatz, 2022). AuNPs possess unique Plasmon Resonance properties, which have already demonstrated promising applications for colorimetric detection of pathogens (Liu and Liu, 2017). Specific molecular detection with DNA-nanoprobes relies on RNA or DNA hybridization, which causes nanoprobe instability and a marked colour shift visible within 15 minutes (Carter et al., 2013; Sabela et al., 2017). In summary, AuNPs-based colorimetric assays offer an attractive POC alternative for rapid and sensitive molecular detection of pathogens.

In this study, we validate a POC tool based on RT-LAMP coupled to specific DNA-nanoprobes for viral detection as a proof of concept. This approach provides high sensitivity and accuracy levels close to RT-PCR based diagnosis for SARS-CoV-2 or aMPV. Moreover, the combination of these techniques makes our colorimetric system doubly reliable: RT-LAMP amplification of the viral target with a set of 6 specific primers, followed by its detection by specific-sequence DNA-nanoprobes.

Materials and Methods

Sample collection

Retrospective 140 pharyngeal swabs SARS-CoV-2 samples (denoted as S1 to S140) from both symptomatic and asymptomatic patients were provided in inactivating transport media by different Spanish healthcare agents. The sample collection was conducted during the sixth wave of COVID-19 in December 2021 in Spain, which was associated with the Omicron variant spread. Out of the collected samples, 72 tested positive for SARS-CoV-2, while 68 tested negatives. For this study, the sample processing was performed under BSL2 standard operating procedures at BioAssays (Certification n. CS17817 for health centers, services, and establishments by the Health Department of the Autonomous Community of Madrid). TaqPath COVID-19 CE-IVD RT-PCR Kit (ThermoFisher Scientific, USA) was used for confirmation. As a negative control, purified RNA from the human coronavirus 229E (hCoV-229E, GenBank Access Number: NC_002645.1) was used.

For aMPV, the "Centro de Sanidad Avicola de Cataluna y Aragon" (CESAC) kindly provided viral RNA from 50 samples (15 upper respiratory tract swabs and 35 tracheal tissue samples), denoted as M1 to M50, collected from 34 chickens and 16 turkeys (Table S1). Among the 50 samples, 33 were diagnosed as aMPV-positive using a qRT-PCR screening test targeting the Small Hydrophobic (SH) gene (Mescolini et al., 2021). Additionally, 17 samples that tested negative for aMPV but positive for other respiratory viruses affecting poultry, such as Infectious Bronchitis Virus (IBV) and Infectious Laryngotracheitis Virus (ILTV), were also analysed. Live attenuated vaccine NOBILIS(r) ND CLONE 30 (Merck Sharp and Dohme) was used as positive control for Newcastle Disease Virus (NDV). To optimize the diagnostic system for aMPV, the live-attenuated vaccine HIPRAVIAR SHS (strain 1062 from HIPRA S.A., Spain) available in vials with lyophilized suspension ranging from $10^{2.4}$ to $10^{4.4}$ $TCID_{50}$ in 1 mL, was used as a positive control.

RNA extraction from clinical samples

Human pharyngeal swabs were extracted using the automatized robot Chemagic 360/96 RodHead and Chemagic Viral DNA/RNA 300 Kit H96 purification kit (Chemagen Technology, Perkin Elmer).

HIPRAVIAR-SHS vial was reconstituted in 1 mL of phosphate buffer saline (PBS) and RNA was isolated using GeneJET RNA Purification Kit (ThermoScientific), following the manufacture's recommendations.

RT-LAMP and qRT-LAMP amplifications

Purified viral RNA was amplified by one-step RT-LAMP or qRT-LAMP using WarmStart RTx Reverse Transcriptase and Bst 2.0 WarmStart(r) DNA Polymerase (New England Biolabs). Optimization of RT-LAMP reaction was performed in 25 µL by mixing 12.5 µL of WarmStart MasterMix (WarmStart® Fluorescent LAMP/RT-LAMP Kit with Uracil-DNA Glycosylase (UDG) (New England Biolabs, USA), 1 µL of each primer set (optimization from 0.8 to 1.6 µM internal FIP/BIP primers; 0.1 to 0.4 µM outer F3/B3 primers, and 0.2 to 0.6 µM loop LF/LB primers), 20 U Ribonuclease Inhibitor (NZYtech, Portugal) and 5 µL of purified RNA. Additionally, for qRT-LAMP, 0.5 µL LAMP Fluorescent Dye were added. Reaction mix was prepared at room temperature to allow UDG activity, then incubated at 60-65°C for 15 to 60 min for retrotranscription and Bst 2.0 amplification, followed by enzyme inactivation at 80°C for 5 min. Fluorescence signal was monitored along incubation in a QuantStudio 5 Real-Time PCR System (ThermoScientific), adding an end-point melt curve step to verify the specificity of the amplification. Alternatively, end point amplification was visualized in a 2% TAE-agarose gel electrophoresis.

To assess the specificity of the method, hCoV-229E RNA was used. Similarly, IBV and ILTV-positive samples, were underwent same procedures as the aMPV samples. The primer sets employed for the detection of SARS-CoV-2, targeting the nucleocapsid (N) and envelope (E), as well as the primer set for aMPV, targeting the fusion protein gene (F), are detailed in Table S2.

Oligonucleotide probes

To design the specific pangenotypic oligonucleotide probes for either SARS-CoV-2 or aMPV, we conducted CLUSTAL Omega alignments (*https://www.ebi.ac.uk/Tools/msa/clustalo/*) using the reference sequences (GenBank Accession Number: MT121215.1) and (GenBank Accession Number: NC_039231.1), respectively. To check system specificity, hCoV-229E genome was used. Then, 20 nt-long sequences were selected from the conserved regions of N and E genes (SARS-CoV-2) or F gene (aMPV). The designed probes were analysed for their secondary structure using the online RNAfold server (Vienna RNA Web Service; Gruber et al., 2008). Oligonucleotide probes with higher Gibbs free energy (ΔG_0) values were chosen and synthetised. All oligonucleotide probes used in this study were synthesized by Sigma-Aldrich (Merck, USA) and included a 5'-Thiol modification.

Gold nanoparticles functionalization

Gold nanoparticles (AuNPs), 20nm, in citrate buffer solution ($6.8x10^{11}$nanoparticles/mL) were purchased from Nanovex Biotechnologies (Spain). A solution of 6-mercapto-1-hexanol (MCH) and DTT-reduced thiol SARS-CoV-2 or aMPV probe-oligonucleotides (ratio 10:90, final probe concentration 2.5 µM) were incubated with 1 mL of AuNPs for 16h. Then, the mixture was salt aged up to 1.3 M NaCl, as previously described by Hurst et al. (2008), with a few modifications. Briefly, oligonucleotide probe/AuNPs mixture was incubated with 0.2 M NaCl for 2 h at room temperature, then 0.01% Tween-20 was added, and finally subjected to vacuum centrifugation to increase final salt concentration. Functionalized AuNPs with probe-oligonucleotides (DNA-nanoprobes) were washed twice with 10 mM PBS (pH 7.5) by centrifugation at 10,000 rpm for 15 min (MiniSpin Plus G, Eppendorf, Germany) at RT to remove unbound oligonucleotides. Finally, DNA-nanoprobes were resuspended in 10 mM PBS and conserved at 4°C until further use.

Detection by DNA-nanoprobes

To optimize colorimetric detection, 1.5 µL SARS-CoV-2- or aMPV-nanoprobes were incubated with 5 µL of RT-LAMP products in a 25 mM pH7.5 Tris-buffered reaction solution. For SARS-N nanoprobe, reaction

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buffer was supplemented with 3 M NaCl, 10 mM MgCl2, and 0.01% Tween-20, whereas for aMPV-F2 nanoprobe, it contained 2 M NaCl, 22 mM MgCl2, and 0.01% Tween-20. Sterile water was included as a negative control. Reaction was carried out on a thermal block at 37°C for 90 min. Absorbance intensity at 400-800 nm wavelength range was measured at different time points (15, 30, 45, 60 and 90 min) using a NanoDrop 2000 Spectrophotmeter (ThermoFisher Scientific), or at 90 minutes using a Multiskan FC plate reader equipped with a 540 nm filter (ThermoFisher Scientific). Based on visual screening of detection assays, we found that the most distinct contrast between the positive and negative samples was determined at 1:3 (v/v) ratio of LAMP products to DNA-nanoprobe.

Results

Optimization of RT-LAMP settings for SARS-CoV-2 amplification

To determine the optimal RT-LAMP primer sets for SARS-CoV-2 we used isolated RNA from 30 clinical samples previously confirmed positive by qRT-PCR. A gradient of temperatures ranging from 60 to 65°C were assayed for end-point RT-LAMP reactions using RNA from viral hCoV-229E as negative control.

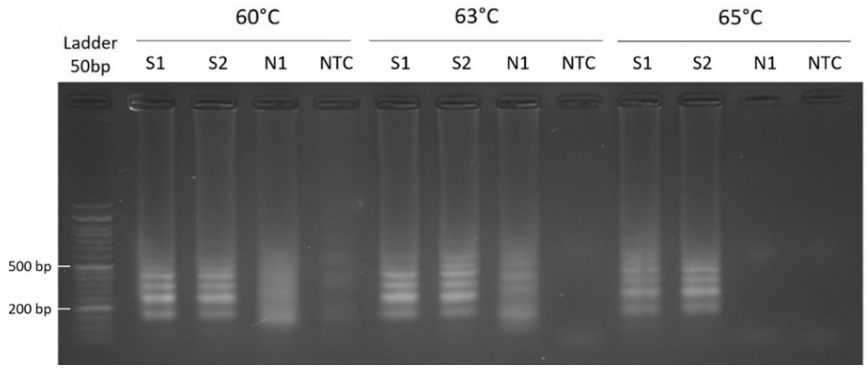

Figure 1. Representative end-point electrophoresis showing RT-LAMP products after 1 hour reaction at temperatures of 60°C, 63°C, or 65°C using the SARS-N primer set. S1 and S2 represent isolated RNA from positive SARS-CoV-2 samples; N1 corresponds to isolated viral RNA from hCoV-229E as a negative control. A non-template control (NTC) was also included.

Specific amplification was observed using the SARS-N primers (Figure 1) or the SARS-E primers (Figure S1) at 65ºC in a panel of 6 positive and four negative samples. In contrast, nonspecific amplification of RNA from hCoV-229E at either 60ºC or 63ºC was observed (Figure 1). To optimize the RT-LAMP reaction, the addition of dUTP/UDG was assayed. Since this enzyme effectively catalyzes the removal of any uracil nucleotides that may be present prior to the amplification reaction, it would prevent carryover contamination during the RT-LAMP reaction. The results obtained here-in validate the effectiveness of this enzyme, as no nonspecific amplification was observed under any of the tested conditions (Figure S2)

Subsequently, to establish the optimal time conditions, end-point RT-LAMP was performed at 65ºC for 15, 30, 45, or 60 minutes, maintaining an equivalent concentration of viral RNA as template. After evaluating the fluorescence intensity of LAMP products, we observed that the reaction yield was the highest after 30 minutes or more (Figure S3). Consequently, an optimal reaction time of 30 minutes was set for RT-LAMP assays aimed at analyzing SARS-CoV-2. Besides, further optimization of primer concentration ranging from 0.025 to 2 μM each was also tested. According to our results, outer FIP/BIP primers at 1.6 μM, F3/B3 primers at 0.2 μM, and loop LF/LB primers at 0.4 μM exhibited optimal performance in LAMP reactions (Figure S2)

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Validation of qRT-LAMP assays for SARS-CoV-2 detection

Sample validation was carried out by qRT-LAMP with end-point melt curve step to verify the specificity according to primer annealing temperature. To establish the suitability of N and E genes as detection targets for SARS-CoV-2, a preliminary sensitivity test was performed on purified viral RNA from confirmed 50 positive and 10 negative samples. The results demonstrated that out of the 50 positive samples examined, 48 were detected using the N-gene primer set, and 43 were detected using the E-gene primer one. No false positives were obseved with either primer set. Indeed, they show a 100% specificity, indicating their high accuracy in identifying SARS-CoV-2. These results indicate that N-gene primer set is the most appropriate for SARS-CoV-2 detection by RT-LAMP.

To further assess the specificity and sensitivity of the N-gene primer set, we conducted a comparative analysis of a panel of 140 different pharyngeal swabs using qRT-PCR and qRT-LAMP methods (Figure 2). This primer set successfully detected viral RNA with a qRT-PCR cycle threshold (CT) of up to 30, with a sensitivity of 97.22% (70/72 positive samples) and 100% specificity of (68/48 negative samples). Notably, our results showed that qRT-LAMP using SARS-N primers was sensitive enough to detect viral RNA after just 30 min reaction (Figure 2A). Indeed, its limit of detection (LOD) was about 50 copies per reaction (equivalent to qPCR C_T = 30 according to manufacturer specifications). Moreover, we observed a nice correlation between the RNA copies and the reaction time, revealing that 400 RNA copies per reaction can be detected as early as 13 minutes. Considering that retro-transcription step lasts for 15 minutes in qRT-PCR based methods, we found that qRT-LAMP was faster as amplification were actually evident even 11 minutes earlier.

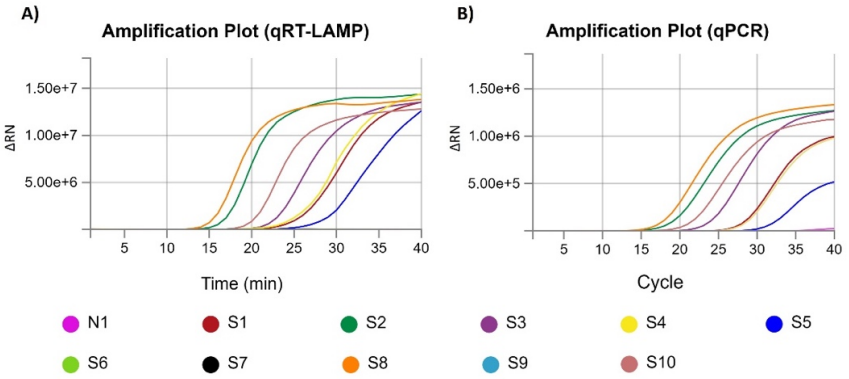

Figure 2. Representative comparison of qRT-LAMP vs qRT-PCR amplification plots in 10 samples from COVID-19 patients (S1 to S10) using either (A) qRT-LAMP with the SARS-N primers set or (B) commercial qRT-PCR. N1 corresponds to isolated viral RNA from hCoV-229E as a negative control.

Validation of qRT-LAMP assays for aMPV detection

Compared to SARS-CoV-2 assays, a panel of only 50 RNA different samples from both turkeys and chickens was analyzed. The panel consisted of various positive/negative samples and included a reference sample of a commercial live-attenuated vaccine for aMPV (Figure 3). Our findings indicate that qRT-LAMP amplification of aMPV exhibited a high level of sensitivity (87.9%, n=29/33) and specificity (100%, n=17/17) after 30 minutes. Remarkably, we found a 100% coincidence of results by qRT-PCR or qRT-LAMP for turkey samples detection. However, coincidence values for chicken samples were lower than expected (80%). In fact, only 16 out of 20 chicken positive samples by qRT-PCR, were detected positive by qRT-LAMP (8/12 tracheal tissue and 8/8 in upper respiratory tract swabs). Of note, the four chicken samples (M8, M9, M39 and M41) negative by qRT-LAMP were viral RNA from tracheal tissue (Table S1), probably due to repeated freezing and thawing of the extracts.

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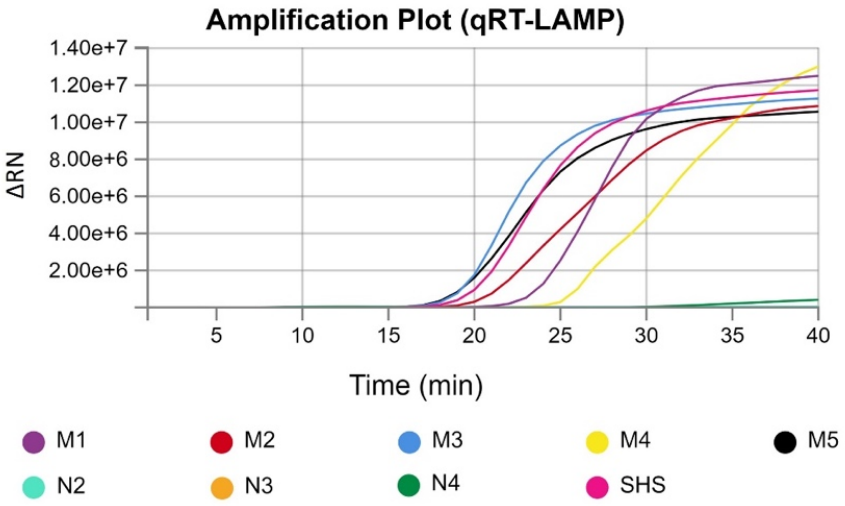

Figure 3. Representative qRT-LAMP amplification plot using aMPV-F primer set. RNAs isolated from aMPV-positive samples (M1 to M5) were analyzed. SHS is a commercial live attenuated vaccine used as positive control. To assess potential cross-reactivity, viral RNAs from positive samples of IBV-, ILTV-infected animals, and live-attenuated vaccine for NDV were used (referred as N2, N3, and N4, respectively)

Validation of DNA-nanoprobe detection system coupled to RT-LAMP

To assess the usefulness of the DNA-nanoprobes as a colorimetric tool for the molecular detection, the RT-LAMP products for SARS-CoV-2 (140 samples) or aMPV (50 samples) were incubated with the corresponding SARS-N- or aMPV-F2-nanoprobes, for 90 minutes at 37^{o}C (Figure 4A, left panels, and right panels). qRT-LAMP for SARS-CoV-2 or aMPV were carried out in parallel to confirm RT-LAMP amplification (Figure 4B, lower panels). During the incubation period, spectrophotometric measurements at UV-Visible wavelengths were acquired at 10-minute intervals. Significantly, we observed that a 30-minute incubation period proved sufficient for identifying and distinguishing positive samples. In fact, at this time, in presence of RT-LAMP products, the initially red-color reaction persisted due to the stabilization of nanoprobe molecules upon recognizing the target. Conversely, reactions turned colorless in the absence of target due to nanoprobe destabilization in the incubation buffer.

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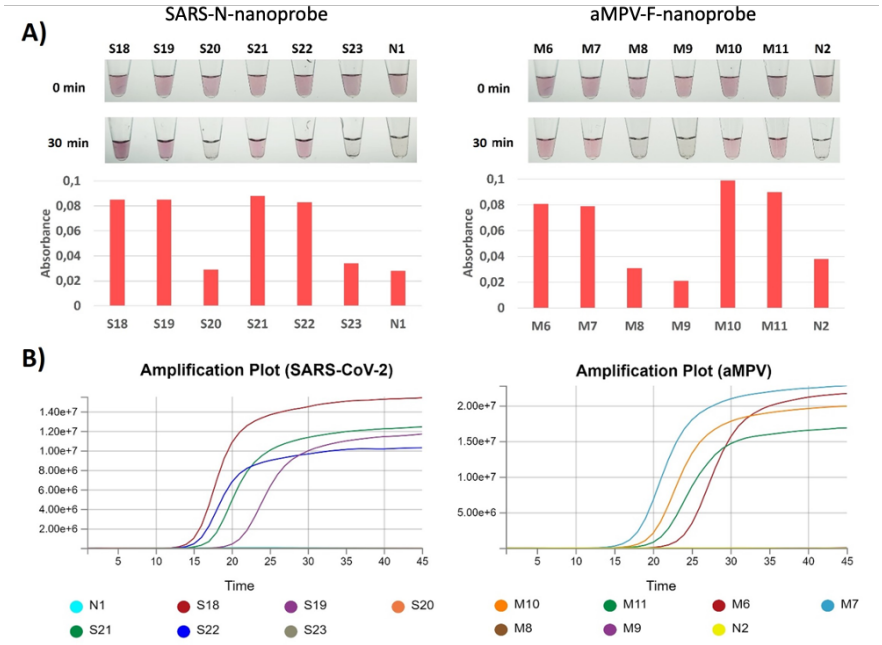

Figure 4. Representative validation of the RT-LAMP/DNA-nanoprobes detection system for SARS-CoV-2 (samples S18 to S23, left panels) or aMPV (samples M6 to M11, right panels). A) Representative digital pictures acquired after 0 min or 30 min incubation at 37°C of respective RT-LAMP products with either SARS-N- (left panels) or aMPV-F2-nanoprobes (right panels) are shown. Histograms represent their maximum absorbance intensity at λ= 540 nm at 30 min incubation. B) qRT-LAMP amplification plots after 45 min incubation at 65°C. Isolated RNA from hCoV-229E (N1) or from IBV (N2) was used as negative control for SARS-CoV-2 and aMPV, respectively.

The percentages of sensitivity and specificity obtained by coupling RT-LAMP to DNA-nanoprobes for molecular detection were calculated using a conventional confusion matrix (Table 1). It is worth noting that only two out of 72 positive SARS-CoV-2 were determined as false negatives (98.57% accuracy; 97.18% Matthews Correlation Coefficient (M.C.C.)). Regarding aMPV, detection values were slightly lower, since 4 out of 33 positive aMPV samples were determined as false negatives (92.00% accuracy; 84.34% M.C.C.)

In summary, we propose the following reaction conditions for both SARS-CoV-2 or aMPV detection: the RT-LAMP should carried out in presence of UDG, 20 U of RNAse inhibitor, primers FIP/BIP at 1.6 µM, F3/B3 at 0.2 µM, and LF/BF at 0.4 µM, using 5 µL of purified RNA for 25 µL reactions, amplification for 30 minutes at 65ºC, and heat inactivation for 5 min at 80ºC. , amplicons are visualized by adding 1,5 µL of DNA-nanoprobes in the adequate buffer and allowing the reaction to happen for 30 min at 37ºC.

Table 1. Performance parameters of the RT-LAMP/nanoprobe detection system for SARS-CoV-2 or aMPV (N.P.V, Negative Predictive Value; F.D.R, False Discovery Rate; F.P.R, False Positive Rate; F.N.R, False Negative Rate; M.C.C., Matthews Correlation Coefficient)

	SARS-CoV-2	aMPV		SARS-CoV-2	aMPV
Sensitivity	97.22%	87.88%	**F.D.R.**	0%	0%
Specificity	100%	100%	F.N.R.	2.78%	12.12%
Precision	100%	100	Accuracy	98.57%	92%
N.P.V.	97.14%	80.95%	F1 Score	98.59%	93.55%
F.P.R.	0%	0%	**M.C.C.**	97.18%	84.34%

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Overall, our results clearly indicate that a system combining RT-LAMP and specific DNA-nanoprobes can be used as useful colorimetric method for molecular detection even with the naked eye in just 60 minutes: 30 minutes of RT-LAMP plus 30 minutes incubation with DNA-nanoprobes. These characteristics clearly suggest its usefulness as a POC diagnostic system for early, simple, cheap, fast and reliable screening of viral infections *in situ.*

Discussion

In this study, we have developed and fully validated a method based on RT-LAMP amplification combined with specific DNA-nanoprobes for the detection of two RNA viruses, using SARS-CoV-2 and aMPV samples as a proof of concept for POC diagnosis of emerging respiratory pathogens. We suggest that the combination for RT-LAMP procedures, using three pairs of specific oligonucleotides, and DNA-nanoprobes targeting discrete regions of the viral genome reach both sensitivity and specificity levels comparable to gold standard procedures based on qRT-PCR methods. Therefore, a RT-LAMP/DNA nanoprobe-based device could be easily implemented as a cost-effective and reliable system widely applied for the screening of many diseases and viruses *in situ* , including human and avian viruses (Padzil et al., 2022)

The high sensitivity levels for LAMP are one of its main virtues as a potential alternative for molecular detection as POC devices, notably in developing countries or rural areas without basic health care infrastructures. However, some drawbacks of this technique have been widely reported, including potential cross-contamination (Tomita et al., 2008; Morris et al., 2015; Bao et al., 2020). To avoid this, detection kits for RT-LAMP have been improved by adding an UDG enzyme (Lai et al., 2022). This enzyme promotes the degradation of uracil-nucleotides on pre-amplified products but without any effect on the original template. In our study, the usefulness of this strategy has been successfully attained since the number of false positive results was virtually reduced to 0% using field samples.

As a drawback of the LAMP technique as a potential POC device concerns the read-out procedure to detect and discriminate positive over negative samples. Various methods have been described so far that do not require any equipment, enabling diagnosis by the naked eye. One of the first approaches relies on the turbidity measurement at end-point LAMP amplification. In fact, as the reaction progresses, magnesium pyrophosphate is produced and precipitates in the amplification of positive samples (Mori et al., 2001). However, in order to achieve a noticeable precipitate, this strategy relies on a high amplification efficiency and a substantial product yield. Otherwise, samples with low viral loads may be categorized as false negatives. Other LAMP systems use colorimetric indicators, such as hydroxynaphthol blue, a metal indicator that turns from violet to blue as free Mg^{2+}concentration decreases when positive samples are successfully amplified. Nevertheless, this method has very low sensitivity levels, approximately 10-100ng of RNA (Hongjaisee et al., 2021), and low specificity values, around 80%, compared to gold standard qRT-PCR (Prakash et al., 2023). Phenol- or cresol-red are also pH indicators used as a read-out for LAMP-based diagnosis (Huang et al., 2020). These pH indicators can detect the release of protons during DNA synthesis, resulting in a color shift from pink to yellow in positive samples. While Raddatz et al. improved this system for SARS-CoV-2 detection, the slight pH changes registered during LAMP amplification may be one of the major limitations for its use in POC testing (Raddatz et al., 2022). This constraint can become particularly problematic when working with raw samples. In general, LAMP-based diagnostic technologies may exhibit lower specificity levels, leading to increased uncertainty in distinguishing false positive results. In contrast to these technologies, the colorimetric system validated in this study provides a highly sequence-specific read-out. This is because the DNA-nanoprobes we have designed for detection of either SARS-CoV-2 or aMPV can readily and specifically bind to those 100% identical viral RNA sequences upon undergoing RT-LAMP amplification.

Fluorescent-based LAMP has been also reported as an alternative for qPCR-based detection systems. For instance, it has been recently described a multiplexed (mLAMP) that is able to identify three different pathogens in one single reaction tube (Fan et al., 2022). While the specificity, sensitivity, and accuracy of this approach are comparable to PCR-based procedures, its readout based on fluorescence at different

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wavelengths requires more complex and costly equipment compared to the colorimetric developed in our study. Furthermore, this fluorescent-based LAMP procedure exhibits a sensitivity that is 10-fold lower (LOD $>$ 500 copies/reaction) compared to our detection system. Nevertheless, a fluorescent-based LAMP for avian influenza virus detection has demonstrated impressive sensitivity (LOD$>$10 copies/reaction) (Padzil et al., 2022). Regardless, it is worth noting that, additionally, these systems require fluorescent-labeled primers, resulting in increased costs.

As far as the usefulness of AuNPs is concerned, although showing low LOD values, recent studies describe some detection systems based on its combination with the RT-LAMP technique in lateral flow biosensors (Zhu et al., 2020; Chen et al., 2021). Besides, a singular lab-on-a-chip system for SARS-CoV-2 detection based on the combination of LAMP reactions and colorimetric detection by AuNPs upon UV radiation has been recently developed (Sivakumar et al., 2021). In this study, the researchers employ unlabeled AuNPs that are synthesized *in situ*on a portable device, instead of DNA-nanoprobes, to detect LAMP amplicons. Of note, this promising system has been just assayed using a synthetic plasmid DNA encoding the envelope (E) gene from SARS-CoV-2, whereas our detection system has been validated using a substantial number of real patient samples, specifically 140 samples from individuals with SARS-CoV-2. Although, it is not possible to compare their performance in terms of specificity and accuracy, both systems are able to detect LAMP amplicons by the naked eye within 45 min after LAMP reaction. Nonetheless, the use of unlabeled AuNPs and biosensors designed to target labeled LAMP primers (Zhu et al., 2020; Chen et al., 2021) may lead to a reduction in the specificity of colorimetric detection. Our system is based on sequence-specific colorimetric detection, that not only practically ensures no false positive results upon LAMP reaction, but also avoids detection of nonspecific amplifications or artifacts due to the formation of primer dimers.

Regarding the diagnosis of aMPV infection in poultry, while previous studies have developed diagnostic methods for other avian viruses (Padzil et al., 2022), this is the first study describing the development of an RT-LAMP for its molecular detection. The clinical diagnosis of aMPV in field conditions is not easy due to the occurrence of co-infections in animals. Currently, RT-PCR and qRT-PCR tests have become the established gold standard methods in reference laboratories for diagnosing active infections. Indeed, primer sequences for the RT-PCR have been designed for specific detection of the F, M, N and G genes (Ferreira et al., 2009; Kariithi et al., 2022; Wang et al., 2022). For instance, since the gene G shows the highest variability between subtypes it is the region most widely used at aMPV genotyping.

In this study, the usefulness of the F gene as a target for aMPV detection by RT-LAMP is based on its high conservation degree in the reference strains of aMPV subtypes A and B. A recent study supports these subtypes, specially aMPV-B, as the most prevalent not only in Spain but also in many European countries (Mescolini et al., 2021). We also performed different LAMP primer designs targeting the gene M but with lower sensitivity and specificity values using field samples (not shown). A highly desirable approach would be the development of a multiplexed LAMP strategy for the detection of viral respiratory co-infections and/or those that exhibit similar clinical signs to aMPV infection, irrespective of the readout device used.

Conclusions

The combination of RT-LAMP and colorimetric detection with DNA-nanoprobes developed in this study has led to a final plasmonic biosensor easy to perform in resource-limited settings, and has proven to be fast, reliable, and accurate. Furthermore, the presented platform exhibits considerable potential for development as a Point-of-Care (POC) diagnostic system through the utilization of a partial lysis buffer to facilitate accessibility to viral RNA. Since it can be readily implemented for different pathogens as SARS-CoV-2 and aMPV, our approach will help to improve access to cost-effective diagnostic devices for the massive screening *in situ* of, at least, most of the RNA-based viruses of clinical or veterinary concern. Moreover, as colorimetric results can be acquired by a conventional plate reader equipped with a 540 nm filter, this system could be also implemented in clinical laboratories for routine molecular analysis.

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