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Use of the variplex™ SARS-CoV-2 RT-LAMP as a rapid molecular assay to complement RT-PCR for COVID-19 diagnosis

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ABSTRACT

Background: Molecular assays based on reverse transcription-loop-mediated isothermal amplification (RT-LAMP) may be useful for rapid diagnosis of the severe acute respiratory syndrome Coronavirus-2 (SARS-CoV-2) because of the easy performance and the option to bypass RNA extraction.

Objectives: This study was designed to evaluate the clinical performance of the CE-labeled variplex™ real time SARS-CoV-2 RT-LAMP assay in comparison to commercial RT-PCRs.

Study design: RNA extracted from pharyngeal swabs was tested by variplex™ RT-LAMP and Corman’s LightMix E gene RT-PCR as reference. Samples of respiratory secretions from Coronavirus infection disease (COVID-19) and negative control patients were analyzed by variplex™ without RNA extraction and tested in parallel with the Allplex™ and VIASURE BD MAX RT-PCRs.

Results: Using isolated RNA variplex™ RT-LAMP showed a sensitivity of 75 % compared to LightMix E gene RT-PCR but contrary to the latter it produced no false-positive results. For the evaluation of samples from respiratory secretions concordance analysis showed only a moderate agreement between the variplex™ RT-LAMP conducted on unprocessed samples and Allplex™ and VIASURE RT-PCRs (Cohen’s $\kappa$ ranging from 0.52–0.56). Using the approach to define a sample as true-positive when at least two assays gave a positive result the clinical sensitivities were as follows: 76.3 % for variplex™, 84.2 % for Allplex™ and 68.4 % for VIASURE. However, when results of RT-PCR and RT-LAMP were combined diagnostic sensitivity was increased to 92–100 %.

Conclusion: The variplex RT-LAMP may serve as a rapid test to be combined with a RT-PCR assay to increase the diagnostic accuracy in patients with suspected COVID-19 infection.

1. Background

The severe acute respiratory syndrome Coronavirus-2 (SARS-CoV-2) pandemic has already caused an enormous burden on healthcare systems worldwide [1]. Because the virus can be transmitted rapidly by direct contact and aerosols and it causes a severe life-threatening syndrome in a part of the patients timely and accurate diagnosis of Coronavirus infection disease (COVID-19) is essential to reduce virus spread and save patients’ lives by starting appropriate treatment and care as soon as possible [2–4]. Chest computed tomography (CT) imaging is a sensitive method to identify lung infection by SARS-CoV-2 in an early stage but it is not specific [1,3,5]. Reverse transcription (RT)-PCR actually serves as the gold standard laboratory test for confirmation of clinical infection and screening of contact persons [6]. A large number of assays adapted on commonly used technical platforms are currently flooding the market [4]. As viral targets of RT-PCR assays sequences of the envelope (E), nucleocapsid (N), spike (S), membrane protein (M), open reading frame 1ab (ORF1ab), and RNA-dependent RNA polymerase (RdRP) genes are used [2,6]. Although RT-PCR offers high analytical sensitivity several studies reported on false-negative as well as fluctuating results in patients whose clinical diagnosis using chest CT was in accordance with COVID-19 [2,7]. Problems with clinical sensitivity of nucleic acid amplification tests can be due to analytical errors of RNA isolation procedures and choose of inadequate primers. Other challenges in diagnostics are associated with the significantly increased requests for testing, resulting in time delays to generate diagnostic reports [8,9].
Moreover, mass testing has rapidly caused serious shortages in the supply of RNA purification kits in many countries [9,10]. For a rapid diagnosis of SARS-CoV-2 cost-effective methods with low hands-on time that circumvent limitations of RT-PCR may be helpful tools for a routine diagnostic workflow [9,11]. RT-loop-mediated isothermal amplification (LAMP) may offer the possibility to be established as an alternative diagnostic technique [12-14]. The combination of RT with Bst polymerase possessing a DNA strand displacement activity allows amplification of target genes at a constant temperature in less than one hour. Large-scale LAMP tests are performed in a similar manner. Reference RT-PCR was performed using pharyngeal washes and samples from respiratory secretions, including sputum, endotracheal secretions, and bronchoalveolar lavage.

2. Objectives

There are several studies that demonstrated satisfying sensitivity and specificity of RT-LAMP for SARS-CoV-2 detection but little is known about its performance of testing clinical samples directly without RNA extraction [11-14]. In this study we evaluated the newly introduced CE-labeled variplex™ SARS-CoV-2 LAMP assay and compared the clinical performance with commercial RT-PCR tests. Testing was performed using pharyngeal washes and samples from respiratory secretions, including sputum, endotracheal secretions, and bronchoalveolar lavage.

3. Study design

3.1. Pharyngeal swabs, RNA extraction, and envelope (E) gene screening RT-PCR

Pharyngeal specimens were collected using eSwab™ transport systems (Copan, Brescia, Italy). Total viral RNA was extracted from 200 μl of the sample medium using the QIAamp Virus/Pathogen Mini Kit (Qiagen, Hilden, Germany). Extraction was performed on the automated Qiasympohmy SP instrument (Qiagen). Purified RNA was eluted in 60 μl AVE buffer and divided into two parts for testing. To rule out cross-reactivity with human coronaviruses 229E and OC43 external quality assessment samples (INSTAND e.V., Düsseldorf, Germany) were processed in a similar manner. Reference RT-PCR was performed using the LightMix® Modular SARS-CoV E-gene primers (TIB Molbiol, Berlin, Germany) and the LightCycler® Multiplex RNA Virus Master (Roche, Penzburg, Germany) [15]. RT-PCR was run on a LightCycler 480 (Roche, Penzburg, Germany).

3.2. Variplex™SARS-CoV-2 RT-LAMP assay using extracted RNA

The variplex™ SARS-CoV-2 is a qualitative molecular assay using a mix of 6 oligonucleotide primers targeting a 282-bp sequence of the membrane protein (M) gene. For a single test 15 μl of RT master mix and 8 μl of eluted RNA were pipetted into two wells of a Genie® test strip (Amplex Diagnostics). 2 μl of the primer mixes for SARS-CoV-2 or the inhibition control were added to one each well. Tests were run at 65 °C for 40 min using a Genie II Mk2A device (Amplex Diagnostics). Amplification was measured by real-time fluorescence detection using a DNA intercalating dye. Data interpretation and calculations were automatically performed by the integrated eazyReport™ software (Amplex Diagnostics).

3.3. Direct testing of respiratory samples by variplex™SARS-CoV-2 RT-LAMP and comparison with the VIASURE and Allplex™ RT-PCR assays

Respiratory samples, including sputum, endotracheal secretions, bronchoalveolar lavages, and pharyngeal washes, collected from COVID-19 patients with an initially positive E gene screening RT-PCR were used for direct RT-LAMP without RNA purification. Specimens from patients with multiple negative E gene RT-PCR results served as negative controls. In a first step all samples were mixed in a ratio of ≤1:1 with Copan sputum liquefying (SL) solution containing dithiothreitol (1 mL ready-to-use tubes, Copan). 75 μl of the suspension was pipetted into 500 μl of LPTV lysis buffer (Amplex Diagnostics) and gently mixed. From this mixture 8 μl were pipetted into two wells of a Genie test strip. 15 μl of RT master mix and 2 μl of primer mixes for SARS-CoV-2 or the inhibition control were added. An additional well consisted of 10 μl sample/LPTV buffer and 15 μl of RT master mix only and served as a lysis control to exclude the occurrence of unspecific fluorescence during amplification.

For comparative RT-PCR analysis the suspensions were processed using the Allplex™2019-nCoV assay which includes the E, N and RdRp genes as viral targets, and the VIASURE SARS-CoV-2 (S gene)-BD MAX™ system. For the Allplex™ assay RNA was isolated from 200 μl of the sample using the QIAamp DSP Virus/Pathogen Mini Kit. 8 μl of RNA was added to 17 μl of the master mix as described in the manufacturer’s instructions. RT-PCR was run on a CFX96 Real-Time PCR Detection System (Bio-Rad, Feldkirchen, Germany). For the VIASURE assay 200 μl of the sample was used for RNA extraction. VIASURE rehydration buffer and gene reaction tubes containing a ready-to-use master mix were loaded onto BD MAX™ ExK™ TNA-3 reagent strips. Nucleic acid extraction and real time RT-PCR were performed on the automated BD MAX™ system (BD).

3.4. Virus stock dilutions

To assess the analytical sensitivity of the assays the SARS-CoV-2 isolate Jena/2020/5159 propagated and titrated on Vero-76 cells was used. 10-fold serial dilutions of a virus stock of 10^7 TCID50/mL in a pharyngeal wash were mixed with Copan SL solution and processed for the different assays as described above.

3.5. Statistical analysis

The qualitative performance of the assays was assessed by calculating the specificity, sensitivity, negative and positive prospective values, and accuracy. For reference a sample was defined as true-positive when at least two different tests gave a positive result. Concordance of two diagnostic tests was examined by Cohen’s κ coefficient analysis. Correlation between RT-PCR Ct values and RT-LAMP threshold time was estimated using Pearson coefficient analysis.

4. Results

First, we analyzed a panel of pharyngeal swabs sent to the laboratory for routine SARS-CoV-2 diagnostics performed by LightMix® E gene RT-PCR. An aliquot of the RNA eluate was applied to the variplex™ RT-LAMP M gene assay. 96 PCR-positive and 41 negative RNA aliquots were tested. Samples with divergent results between LightMix® RT-PCR and RT-LAMP were verified by VIASURE and Allplex™ assays in order to identify false-positively tested specimen. 10 out of 96 RNA eluates that were LightMix® E-positive could not be confirmed by a second test and were defined as false-positive. Their median Ct value was 36.6 (IQR 36.1-37.6). In contrast, no false-positive results were observed using the variplex™ RT-LAMP. However, the sensitivity of RT-LAMP was only 75 % (Table 1). When only samples with an E gene RT-PCR Ct value <35 as indicative for potentially infectious patients were considered for analysis, sensitivity of RT-LAMP reached 86.4 % (Table 1). The median threshold time of positive signals was 15.75 min (IQR 13.25-24).

To verify the sensitivity of RT-LAMP extracted RNA from a log-dilution series of a virus stock was tested. The variplex™ assay achieved a reliable detection at 1 TCID50/mL, corresponding to 0.03 TCID50/reaction. In comparison LightMix® RT-PCR showed 100 % detection down to 0.1 TCID50/mL. This concentration was positive by RT-LAMP in 33 % of the samples (Table 2).

Next, we investigated a panel of clinical samples that were tested by RT-LAMP without RNA extraction. Only samples from respiratory secretions and pharyngeal washes were used because in preliminary
Table 1
Diagnostic performance of the variplex™ RT-LAMP assay using isolated RNA from pharyngeal swabs.

| Virus concentration | Variplex™ M | LightMix® E |
|---------------------|------------|-------------|
| TCID<sub>50</sub> | Positive replicates M gene, mean threshold time [min] (SD) | Positive replicates E gene, mean Ct (SD) |
| 100 | 6/6, 8.5 (1.25) | 6/6, 25.4 (0.5) |
| 10 | 6/6, 11.5 (1.75) | 6/6, 28.8 (0.5) |
| 1 | 6/6, 22.5 (6.25) | 6/6, 33.3 (1) |
| 0.1 | 2/6, 36 | 6/6, 36.1 (1.5) |
| 0.01 | 0.00003 | 2/6, 37.1 |
| 0.001 | 0.00003 | – |

Table 2
Limits of detection of the variplex™ RT-LAMP and LightMix® RT-PCR conducted on RNA eluates from SARS-CoV-2 virus stock dilutions.

| TCID<sub>50</sub> | Positive replicates M gene, mean threshold time [min] (SD) |
|-------------------|-------------------------------------------------------------|
| 0.01 TCID<sub>50</sub>/mL | 2/6, 35 (0.5) |
| 0.05 TCID<sub>50</sub>/mL | 6/6, 22.5 (6.25) |
| 0.1 TCID<sub>50</sub>/mL | 6/6, 36.1 (1.5) |
| 0.001 TCID<sub>50</sub>/mL | 2/6, 37.1 |
| 0.0001 TCID<sub>50</sub>/mL | – |

Table 3
Comparison of RT-LAMP and RT-PCR assays conducted on respiratory samples.

| Compared assays | P/P | P/N | N/P | N/N | % agreement | Cohen’s κ (CI) | Scale |
|-----------------|-----|-----|-----|-----|-------------|----------------|-------|
| Variplex™ M/Allplex™ E | 21 | 8 | 9 | 35 | 76.7 | 0.52 (0.32–0.72) | Moderate agreement |
| Variplex™ M/Allplex™ RdRP | 22 | 7 | 9 | 35 | 78.1 | 0.55 (0.35–0.74) | Moderate agreement |
| Variplex™ M/Allplex™ N | 23 | 7 | 9 | 35 | 79.4 | 0.53 (0.59–0.77) | Moderate agreement |
| Variplex™ M/VIASURE S | 28 | 9 | 6 | 38 | 79.4 | 0.56 (0.37–0.77) | Moderate agreement |
| Allplex™ RdRP/Allplex™ N | 30 | 1 | 1 | 41 | 97.3 | 0.94 (0.87–1) | Almost perfect agreement |
| Allplex™ RdRP/Allplex™ E | 29 | 1 | 2 | 41 | 95.5 | 0.92 (0.82–1) | Almost perfect agreement |
| a | CI, 95% confidence interval. |
Another reason was that the supply of swabs with technology [9, 13]. For direct testing we focused on samples from respiratory secretions and pharyngeal washes instead of swabs because RT-LAMP would be highly attractive as an alternative easy-to-use technology [9, 13]. The virus stock was diluted in a pharyngeal wash mixed with Copan SL solution.

# Table 4

Diagnostic performance of the variplex™ LAMP assay directly conducted on respiratory samples in comparison with RT-PCR.

|                | True-positive | True-negative | False-positive | False-negative | Sensitivity, % (Ct) | Specificity, % (Ct) | PPV\(^a\), % (Ct) | NPV\(^b\), % (Ct) | Accuracy, % (Ct) |
|----------------|---------------|---------------|----------------|----------------|---------------------|---------------------|-------------------|------------------|-------------------|
| Variplex™ M    | 29            | 35            | 0              | 9              | 76.3 (59.8–88.6)    | 100 (90–100)        | 100               | 79.6 (68.7–87.3) | 87.7 (77.9–94.2) |
| VIASURE S      | 26            | 35            | 0              | 12             | 68.4 (31.4–82.5)    | 100 (90–100)        | 74.5              | 83.6             | 92.1              |
| Allplex™ E     | 30            | 35            | 0              | 8              | 80.0 (62.7–90.4)    | 100 (90–100)        | 81.4              | 89 (79.5–95.2)   | 90.4              |
| Allplex™ RdRP  | 32            | 35            | 0              | 6              | 84.2 (68.8–94)      | 100 (90–100)        | 85.4              | 91.8             | 91.8              |
| Allplex™ N     | 31            | 35            | 0              | 7              | 81.6 (65.7–92.3)    | 100 (90–100)        | 83.3              | 90.4             | 92.4              |
| Allplex™ E + RdRP + N | 32       | 35            | 0              | 6              | 84.2 (68.8–94)      | 100 (90–100)        | 85.4              | 91.8             | 92.4              |
| Allplex™ RdRP + VIASURE S | 33    | 35            | 0              | 5              | 86.8 (71.9–95.6)    | 100 (90–100)        | 87.5              | 93.2             | 93.2              |
| Variplex™ M + VIASURE S | 35      | 35            | 0              | 3              | 92.1 (78.6–98.3)    | 100 (90–100)        | 92.1              | 95.9             | 95.9              |
| Variplex™ M + Allplex™ RdRP | 38     | 35            | 0              | 0              | 100 (90.8–100)      | 100 (90–100)        | 100               | 100 (95.1–100)   | 100 (95.1–100)   |

\(^a\) CI, 95 % confidence interval.

\(^b\) PPV, positive predictive value; NPV, negative predictive value.

# Table 5

Fluctuating RT-LAMP and RT-PCR results of SARS-CoV-2 in an ICU patient.

| Day | Material             | Variplex™ M (threshold time, min) | Allplex™ E (Ct) | Allplex™ RdRP (Ct) | Allplex™ N (Ct) | VIASURE S (Ct) |
|-----|----------------------|----------------------------------|-----------------|--------------------|-----------------|---------------|
| 0   | Bronchoalveolar lavage | Negative                         | 28              | 30.7              | 30.9            | 34.5          |
| 5   | Endotracheal secretion | 39.75                             | 31.2            | 32.8              | 33.9            | 35.9          |
| 12  | Endotracheal secretion | 30.25                             | Negative        | 38.2              | Negative        | Negative      |
| 19  | Endotracheal secretion | 33.4                              | Negative        | Negative           | Negative        | Negative      |
| 26  | Bronchoalveolar lavage | Negative                          | (71.9–95.6)     | 92.1              | (78.6–98.3)     | 95.9          |
| 30  | Endotracheal secretion | 39.75                             | Negative        | Negative           | Negative        | Negative      |

\(^a\) Confirmed by LightMix® E-gene RT-PCR.

# Table 6

Limits of detection of the variplex™ RT-LAMP directly conducted on SARS-CoV-2 stock dilutions without RNA isolation and comparison to RT-PCR assays.

| Virus concentration (TCID\(_{50}\)/mL) | Variplex™ M | VIASURE S | Allplex™ | | | |
|----------------------------------------|-------------|-----------|----------|---|---|
| Positive replicates M gene, mean threshold time (Ct (SD)) | Positive replicates S gene, mean Ct (SD) | Positive replicates E gene, mean Ct (SD) | Positive replicates RdRP gene, mean Ct (SD) | Positive replicates N gene, mean Ct (SD) |
| 300                                    | 0.4         | 5/5, 10.5 (0.75) | 3         | 5/5, 28.3 (2.1) | 8     | 5/5, 27.1 (0.8) | 5/5, 28 (1.1) | 5/5, 27.6 (2.1) |
| 30                                     | 0.04        | 5/5, 17 (5) | 0.3      | 5/5, 31.5 (1.8) | 0.8   | 5/5, 30.5 (0.8) | 5/5, 30.8 (0.7) | 5/5, 31.2 (1.5) |
| 3                                      | 0.004       | 5/5, 30 (3) | 0.03     | 5/5, 34.4 (1.1) | 0.08  | 5/5, 33.2 (0.9) | 5/5, 33.9 (1)  | 5/5, 34.2 (1.7) |
| 0.3                                    | 0.00004     | –         | 0.003    | 2/5, 37.8      | 0.008 | 5/5, 35.4 (0.8) | 5/5, 36.6 (0.6) | 5/5, 37.1 (1.9) |
| 0.03                                   | 0.00004     | –         | 0.00003  | –              | 0.0008 | 2/5, 37      | 1/5, 37.3       | 1/5, 35.8 |

\(^a\) The virus stock was diluted in a pharyngeal wash mixed with Copan SL solution.

Positive RT-PCR tests that were associated with a high Ct value [18].

A major advantage of RT-LAMP is that it allows a simple testing of specimens when unprocessed samples are used, bypassing the bottleneck of RNA extraction [9,19]. Against the background of irregularities regarding the delivery of RNA isolation kits by many manufacturers, RT-LAMP would be highly attractive as an alternative easy-to-use technology [9,13]. For direct testing we focused on samples from respiratory secretions and pharyngeal washes instead of swabs because several transport media can inhibit or reduce RT activity, as reported in recent studies [10,11]. Another reason was that the supply of swabs with fluid transport media was running into a critical shortage in a phase of significantly increased demand for testing.

The direct comparison of the variplex™ RT-LAMP with commercial RT-PCR assays showed that no method was able to detect all positive samples and fluctuating results during the course of the disease were observed in several patients. It soon became obvious during this study that there is only a moderate agreement between RT-LAMP and RT-PCR results in COVID-19 patients. Combining both techniques led to a sensitivity of 92–100 %. The complementation of the methods may be due to the difference in sample preparation. RNA extraction has the advantage that viral RNA is concentrated in a RT-PCR compatible buffer [8]. However, column-based extraction as used in many commercial tests can also result in a loss of RNA [8]. By using RT-LAMP to test unprocessed samples this problem is avoided but RT activity may be
inhibited by carbohydrates and salts depending on the sample composition [10]. In this context suitable specimen types have to be carefully evaluated. Saliva which has been described to contain high virus copy numbers may also represent a potential specimen type for direct RT-LAMP testing [13,20].

In conclusion this study shows that the variplex™ SARS-CoV-2 RT-LAMP assay may serve as an easy-to perform rapid molecular test to be combined with RT-PCR in order to ensure an efficient workflow of timely and accurate diagnosis even at times of high work load and increased testing requests. The major limitation of this work was the relatively small sample size due to low numbers of COVID-19 patients in our hospital. Future studies are needed to examine the utility of RT-LAMP under routine conditions with high sample throughput.

Ethical statement

The study protocol for the evaluation of the variplex™ SARS-CoV-2 assays for clinical samples was reviewed and approved by the ethics committee of the Jena University Hospital (2019–1549.1-MV).

CRediT authorship contribution statement

Jürgen Rödel: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing - original draft, Writing - review & editing, Project administration. Renate Egerer: Investigation, Writing - review & editing. Ayrun Suleyman: Investigation, Formal analysis, Writing - review & editing. Beatrice Sommer-Schmid: Investigation, Writing - review & editing. Andreas Henke: Methodology, Investigation. Birgit Edel: Methodology, Writing - review & editing. Bettina Löfler: Writing - review & editing, Project administration.

Declaration of Competing Interest

The authors report no declarations of interest.

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