VariplexTM test system fails to reliably detect SARS-CoV-2 directly from respiratory samples without RNA extraction

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Abstract

Diagnosis of COVID is performed by PCR methods, but their capacitiy is limited by the requirement of high-level facilities and instruments. The loop-mediated isothermal amplification (LAMP) method has been utilized for the detection of isolated virus-specific RNA. Preliminary data suggest the possibility of isothermal amplification directly from respiratory samples without RNA extraction.

All patients admitted to our hospital were screened for SARS-CoV-2 by routine. Respiratory samples were tested by variplex system based on LAMP method directly without RNA extraction and by PCR. Confirmation or exclusion of clinical COVID diagnosis was based on history, examination and findings. Primary endpoint was the false negative rate of variplex test compared to PCR.

A total of 153 patients were included in the study. Median age was 79 years and male/female ratio was 45%/55%. Prevalence of clinical COVID diagnosis was 61.4%. Variplex test and PCR assay was positive in 15.0% and 45.5%, respectively. In 109 patients variplex test and PCR assay were performed simultaneously. False negative rate of variplex test compared to PCR was 83.0%.

The potential of LAMP-technology using isolated RNA has been demonstrated impressively by others and excellent sensitivity and specivity of detecting SARS-CoV-2 has been reported. However, without RNA extraction the variplex test system failed to reliable detect SARS-CoV-2 directly in respiratory samples.

Introduction

Since December 2019, an emerging infectious disease (COVID-19), caused by the novel coronavirus SARS-CoV-2, has emerged in Wuhan, China [1, 2]. As of 1st April 2020, it has caused 876,898 infections in 203 countries, including 43,477 deaths demonstrating the strong human-to-human transmission capacity of SARS-Cov-2. Initially screening focused on patients with foreign travel or contacts with known cases. Both of these foci no longer reflects the current status of the pandemic [3]. The majority of cases have mild or asymptomatic course [4] and symptoms of the COVID-19 infection are highly nonspecific, including respiratory symptoms, fever, cough, dyspnea, and viral pneumonia [5]. Thus, diagnostic tests specific to this infection are urgently required to confirm suspected cases, screen patients, and conduct virus surveillance. In this scenario, a point-of-care device, i.e., a rapid, robust, and cost-efficient device is crucial and urgently needed for the detection of COVID-19 [6, 7].

At present, the identification of SARS-CoV-2 requires routine and confirmatory diagnosis through real-time polymerase chain reaction (RT-PCR). In recent years, the loop-mediated isothermal amplification (LAMP) method that includes an exponential amplification of specific nucleic acid sequences at a constant temperature, has been widely utilized for the rapid detection of virus-specific genes [8]. The specificity and sensitivity of this method is generally comparable to those of the conventional RT-PCR [9]. The LAMP assays merged with reverse transcription steps have been developed for the detection of RNA viruses, including SARS-CoV-2 [10].

In March 2020, some patients were admitted with suspicion of COVID-19 to our small general hospital in Bavaria, Germany. Patients were treated at one normal care unit of the medical department as well as our interdisciplinary intensive care unit under strict hygiene standards. At that time, delay of RT-PCR, performed by an external institute, for SARS-COV-2 test results was up to 9 days. When a nosocomial SARS-COV-2 outbreak at a normal care unit of the department of trauma surgery and geriatrics was detected, there was urgent need for simple, rapid and reliable detection of SARS-Cov-2 in our own laboratory.

We decided to use the fast and cheap variplex SARS-CoV-2 test system, which is a ready to use isothermal amplification system for both DNA and RNA using LAMP-technology.

As of 1st April 2020 the variplex test system has not received in vitro diagnostic (IVD) certificate. In the meantime it received IVD certificate restricted to the use of isolated RNA. RNA extraction is the first step of any RNA virus testing, such as RT-PCR and isothermal amplifications systems. Commercially available extraction kits make this step more easy, however, RNA extraction may be the limiting step in smaller laboratories in general and may not be available due to the scarcity during pandemic in particular. Therefore, we performed a few variplex tests without RNA extraction directly from respiratory samples. Among this few cases were true positive as well as true negative results. Based on this preliminary promising results and driven by the pressure of the pandemic and a nosocomial SARS-CoV-2 outbreak in our small general hospital, we decided to skip RNA extraction by routine. Here we report on the retrospective analysis of the variplex test system without RNA extraction compared to conventional RT-PCR in all patients admitted to our general hospital in April 2020.

Materials And Methods

This retrospective chart review study involving human participants was in accordance with the ethical standards of the institutional and national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. The Ethics Committee of the Ludwig-Maximilians-University, Munich, Germany approved this study (approval number 20-432).

During SARS-CoV-2 pandemic patients admitted to our hospital were screened for SARS-CoV-2 by routine. Two separate respiratory samples (oropharyngeal or nasopharyngeal swabs) were taken by flexible standard swabs with rayon flocking (MASTASWAB, Mast Group Ltd., Reinfeld, Germany).

COVID-19 RT-PCR assays were performed by an external institute, namely Medizinisches Labor Rosenheim MVZ GbR, Rosenheim, Germany, member of Limbach Gruppe SE, Heidelberg, Germany.

LAMP Reaction

The variplex SARS-CoV-2 test system (Amplex Diagnostics, Gars-Bahnhof, Germany) was used along with Genie II Mk2 instrument (OptiGene Limited, Horsham, UK). This ready to use test system is based on the

loop-mediated isothermal amplification (LAMP) method. Swabs (without medium) were dipped, swirled and squeezed in sputum liquifying solution (SLSolution, Copan Italia, Brescia, Italy). Variplex test was performed immediately without RNA extraction according to standard procedure provided by Amplex Diagnostics.

75 µL (100 µL since 15th April) of the sample in SLSolution was suspended in 500 µL HYPLEX LPTV buffer. A 25 µL reaction mixture for the sample (15 µL Master Mix, 2 µL primer “SARS-CoV-2”, 8 µL LPTV suspension), inhibition control (15 µL Master Mix, 2 µL primer “inhibition control”, 8 µL LPTV suspension) and lysis control (15 µL Master Mix, 10 µL LPTV suspension) was mixed homogeneously. LAMP and fluorescence signal measurement were performed using Genie II thermocycler at 66°C for 35 minutes.

Inclusion and exclusion criteria

Records of all patients admitted to our general hospital from 1st April to 30th April 2020 were analyzed for SARS-CoV-2 routine screening results, as well as for history, examination, and findings necessary for confirmation or exclusion of clinical COVID-19 diagnosis. Patients were excluded only, if data were incomplete and did not allow confirmation or exclusion of clinical COVID-19 diagnosis.

COVID-19 Diagnosis

COVID-19 was diagnosed clinically based on history, examination, and findings in particular laboratory values and chest CT. Previuos findings such as SARS-CoV-2 PCR results were recorded and used for clinical COVID-19 diagnosis only. According to CO-RADS, which provides a standardized assessment scheme with a five-point scale of suspicion for pulmonary involvement of COVID-19 on chest CT [11], we developed the **clin**ical **CO**VID-19 **R**ating **A**nd **D**iagnosis **S**ystem (clinCO-RADS) with a five-point scale for level of suspicion from “very low“ and “low” to “high” and “very high“. In between we defined “intermediate“ for cases otherwise classified “very low“ or “low“, if any PCR result for COVID-19 was positive. “High“ and “very high“ was classified clinically only, i.e. independent of PCR results. Variplex test results were not used for clinical COVID-19 diagnosis and classification.

Endpoints

Primary endpoint was the false negative rate (1-sensitivity) of variplex test compared to PCR assay as gold standard. Secondary endpoints were prevalence of clinical COVID-19 diagnosis, positive rates of variplex test and RT-PCR assay, sensitivity, specifity, positive and negative predictive value, and accuracy of variplex test compared to RT-PCR assay. Previous PCR results were used for clinical COVID-19 diagnosis (clinCO-RADS “intermediate”) only, but not for other primary and secondary endpoints.

Statistics

According to the retrospective nature of this study sample size calculation was not performed but will be discussed in the discussion section. Results are given in numbers and percent, median and interquartile range (IQR). Exact 95% confidence interval was calculated by the method of Clopper and Pearson.

Results

From 1st April to 30th April 2020 173 patients were admitted to our hospital. In 153 cases clinical diagnosis concerning COVID-19 was made or could be excluded based on routine data records. Patient characteristics and routine laboratory values on admission were listed in table 1 and table 2, respectively.

Table 1 Patient characteristics (n=153). Data are median (IQR) or number (%)

Age, years	79 (64 – 84)
Male/female	57/70 (45%/55%)
Duration of hospital stay, days	4 (2 – 7)
Survivors/non-survivors	129/24 (84.3%/15.7%)
Department	
Internal Medicine	122 (79.9%)
Surgery	25 (16.3%)
Otorhinolarynology, Head and Neck Surgery	6 (3.9%)
Chest CT scan	107 (69.9%)

Table 2 Routine laboratory values on admission.

Laboratory value	normal value	median	IQR
White-cell count, /nL	4.3 – 10.0	7.2	5.2 – 10.2
Lymphocyte count, /nL	1.30 – 3.4	0.94	0.67 – 1.40
Platelet count, /nL	150 – 350	210	159 – 266
LDH, U/L	< 248	264	186 – 351
CRP, mg/dL	< 0.30	3.82	0.86 – 9.64
High-sensitivity cardiac troponin I, pg/mL	< 60.4	20.5	9.1 – 40.5
D-dimer, mg/L	< 0.50	1.18	0.60 – 2.53

LDH = Lactate dehydrogenase, CRP = C-reactive protein, IQR = interquartile range

In 127 patients variplex test and/or RT-PCR assay was performed. PCR test results as previous findings were recorded in 37 out of 153 patients, thereof 34 positive for SARS-CoV-2. Prevalence of clinical COVID-19 diagnosis was 61.4% (94/153, 95% CI 53.2% – 69.2%) with 71 out of 94 (75.5%) patients surviving. Number of patients according to level of suspicion of clinical COVID-19 diagnosis for survivors and non-survivors are shown in figure 1.

Variplex test and RT-PCR assay was performed in 115 and 123 patients, respectively. In two patients variplex test results were invalide. Variplex test and RT-PCR assay was positive in 15.0% (17/113, 9.0% – 23.0%) and 45.5% (56/123, 36.5% – 54.8%), respectively. In 109 patients variplex test and RT-PCR assay were performed simultaneously. False negative rate of variplex test compared to RT-PCR was 83.0% (39/47, 69.2% – 92.4%). Sensitivity, specifity, positive and negative predictive value, and accuracy of variplex test compared to RT-PCR as gold standard are listed in table 3.

Table 3 Results of variplex test and RT-PCR assay performed simultaneously in 109 patients (95% CI)

Prevalence	43.1% (33.7% – 53.0%)
Sensitivity	17.0% (7.6% – 30.8%)
Specifity	88.7% (78.1% – 95.3%)
PPV	53.3% (26.6% – 78.7%)
NPV	58.5% (47.9% – 68.6%)
Accuracy	57.8% (48.0% – 67.2%)

Sensitivity of variplex test and RT-PCR assay detecting clinical COVID-19 diagnosis was 15.3% (7.2% – 27.0%) and 78.9% (67.6% – 87.7%), respectively. Results of variplex test and RT-PCR assay for different levels of suspicion of clinical COVID-19 diagnosis are shown in figure 2 and 3.

Discussion

Here, we report on the performance evaluation of a new and faster in vitro diagnostic method in patients of a general hospital following a nosocomial SARS-Cov-2 outbreak. This outbreak was detected in March 2020 at a normal care unit of the department of trauma surgery and geriatrics. COVID-19 was confirmed by RT-PCR assays performed by an external institute and latency of results was up to 9 days at that time. For efficient SARS-CoV-2 screening of every person in our hospital, i.e. all patients and the whole staff, as

well as for realtime results we decided to use a point-of-cate device based on LAMP-technology as recommended by others [6, 12].

The potential of LAMP-technology has been demonstrated impressively. Synthesized RNA of SARS-CoV-2 could be amplified to detectable levels in dilutions as low as 2 – 100 copies per reaction [12, 13]. Sensitivity of LAMP in detecting intact viral RNA, that was extracted from cell culture supernatants of isolates from COVID-19 patients, has been reported to be ten-fold lower than that of qRT-PCR (10^{-7} versus 10^{-8} dilutions), while specifity was high against all viruses tested [10]. In clinical specimens sensitivity and specifity of LAMP after RNA extraction was 100% and 98.7% – 100%, respectively [10, 14]. In general, nucleic acid-based methods are thought to be sensitive but prone to false positive [15].

After introduction of the variplex test system end of March 2020 in our hospital we rarely observed discrepant results compared to RT-PCR suggestive of false positive. However, negative variplex test results in patients with CT-scans typical for COVID-19 as well as with positive RT-PCR results were noticed. Therefore, since 15th of April we increased sample volume (SLSolution) from 75 µL to 100 µL according to the manufacturers advice to increase sensitivity. When observations of false negative variplex test results persisted, we performed an interim analysis. Endpoint of the interim analysis was the false negative rate of variplex test system compared to RT-PCR in all patients admitted in April 2020.

Assuming a sensitivity of 60% poor and one of 75% good, corresponding false negative rates would be 40% and 25%, respectively. The false negative rate of the variplex SARS-CoV-2 test system compared to RT-PCR in all patients admitted to our general hospital in April 2020, in whom simultaneous swabs could be obtained (n = 109), was 83% and sensitivity was 8/47 = 17% (95%CI 7.6% – 30.8%). As a consequence, we discontinued variplex testing without RNA extraction by routine and initiated further analysis of all data recorded. In addition to the false negative rate compared to RT-PCR as primary endpoint, we were interested in a comparison to a clinically based COVID-19 diagnosis as secondary endpoint and some retrospective sample size calculations for self control.

Sample size calculations were based on the optimal two stage designs by R. Simon [16]. In addition to the assumptions mentioned above (sensitivity of 60% being poor, of 75% good), a type I error of 10% and a power of 90% would result in a first stage sample size of 43 RT-PCR positive patients (MinMax Design). Further assuming a sensitivity of RT-PCR of 70% in throat swabs in COVID-19 patients and a prevalence of 60% of COVID-19 in all patients admitted to our hospital, a total of 102 patients had to be screened simultaneously for SARS-CoV-2. Upper limit for first stage rejection of variplex test in this setting is 25, hence much more than the 8 positive variplex tests out of 47 RT-PCR positive patients observed. Ignoring the first stage stopping rule due to the significant delay of RT-PCR results and in regard to modification of our throat swab sample volume from 75µL to 100 µL, a sample size of 64 PCR positive patients, corresponding a total of 152 patients admitted to our hospital had to be screened. This is in line with the total of 153 patients screened, thereof 109 patients with simultaneous swabs for variplex test and RT-PCR assay, reported in this study. Upper limit for 2nd stage rejection in this setting is 43 variplex test positive out of 64 RT-PCR positive patients.

SARS-CoV-2 viral load in upper respiratory specimens of infected patients decreases in the course of the disease [1]. This temporal dynamics in viral shedding [17] could be the reason for negative results of variplex test as well as of RT-PCR assay and raises the need for a clinical suspected diagnosis independent of viral detection. Clinical diagnosis of COVID-19 pneumonia is based on CT scan predominantly. A strong correlation of increasing levels of suspicion of pulmonary involvement with the positive rate of PCR assays has been demonstrated recently and ranged from 6% for CO-RADS 1 up to 93% for CO-RADS 5 [11].

Co-RADS 3 implies equivocal findings for pulmonary involvement of COVID-19 based on CT features that can also be found in other viral pneumonias or non-infectious etiologies and CO-RADS 6, was introduced to indicate proven COVID-19 as signified by a positive RT-PCR test for virus-specific nucleic acid [11]. Based on the experience of our interim analysis we developed a clinically based COVID-19 rating and diagnosis system (clinCO-RADS). We adopted the levels of CO-RADS except for level 3 and level 6. We decided to split CO-RADS 3 by means of the whole clinical information such as history, examination and other findings and classified in a first step to a four point scale (clinCO-RADS 1, 2, 4, 5). In a second step, patients classified clinCO-RADS 1 or 2 were classified clinCO-RADS 3, if SARS-CoV-2 was detected by PCR.

Sensitivity of RT-PCR in detecting clinCO-RADS levels 3 – 5 was almost 80%, hence in the expected range, resulting in a false negative rate of about 20%. We could demonstrate a increasing positive rate of RT-PCR assays with higher clinCO-RADS levels (figure 3) similar to the findings of Prokop et al. [11]. Furthermore, number and rate of non-survivors correlated with clinCO-RADS levels (figure 1), consistent with the association of radiologic findings with mortality of COVID-19 patients [18]. In contrast to the expected results of RT-PCR assays, the sensitivity of variplex test in detecting clinCO-RADS levels 3 – 5 was 15% and the false negative rate was 85% (figure 2).

In view of this disappointing results we analysed the whole process from the technique of throat swabs to the release of variplex test results. Detection of SARS-CoV-2 depends on type of clinical specimen. While positive rates in bronchoalveolar lavage fluid is highest with 93%, positive rates with nasal swabs are 63% and with pharyngeal swabs 32%, respectively. However, the number of specimens analysed were different and partly very low (BAL n = 15, nasal swabs n = 8, pharyngeal swabs n = 398) [19]. In our hospital, simultaneous swabs were taken by well trained nurses, both oropharyngeal or nasopharygeal, with identical swabs. Diagnostic yield depends on sampling and therefore on swabs. Swabs with short fiber strands such as FLOQSwabs (Copan) may be superior compared to standard swabs with rayon flocking. However, identical swabs were used for variplex test as well as for RT-PCR assay. Furthermore, due to pandemic caused scarcity of resources, only standard swabs were available in our hospital.

Conclusion

LAMP-technology may be the answer to the urgently needed rapid, robust, and cost-efficient tests for the detection of COVID-19. Linked to obligate RNA extraction before isothermal amplification, excellent

sensitivity and specifity up to 100% has been reported. Without RNA extraction, the variplex test system failed to reliable detect SARS-CoV-2 directly in respiratory samples.

Declarations

Compliance with Ethical Standards

Funding: none

Conflict of Interest: The authors declare that they have no conflict of interest.

Ethics approval: This retrospective chart review study involving human participants was in accordance with the ethical standards of the institutional and national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. The Ethics Committee of the Ludwig-Maximilians-University, Munich, Germany approved this study (approval number 20-432).

Informed consent: not applicable (retrospective chart review)

Patient consent: The need for consent was waived by the approving ethics committee due to the retrospective nature of this study

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Figures

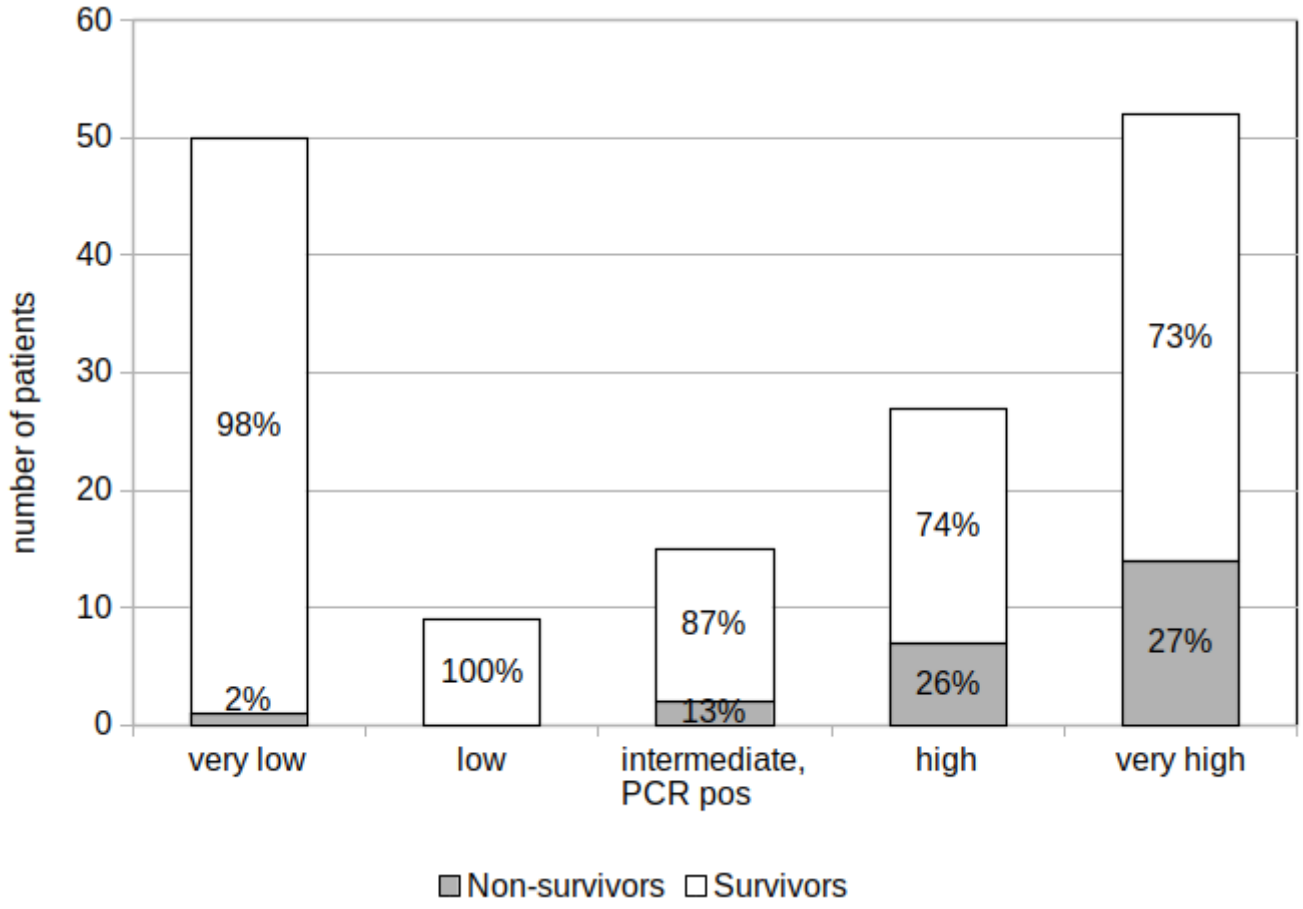

Figure 1

shows the level of suspicion of clinical COVID-19 diagnosis (clinCO-RADS) for all patients (n = 153), survivors and non-survivors

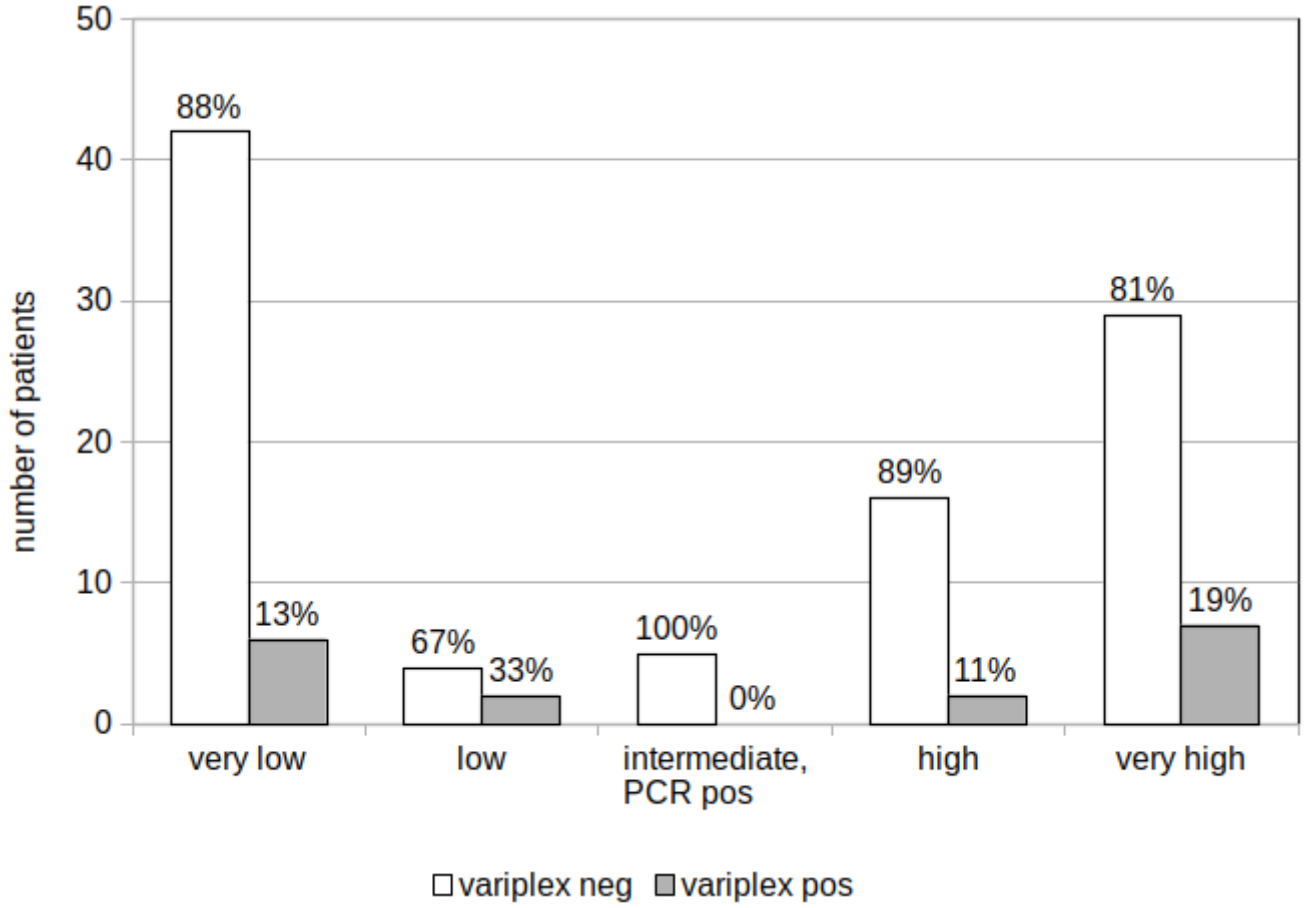

Figure 2

Results of variplex test according to level of suspicion of clinical COVID-19 diagnosis (clinCO-RADS), n=113

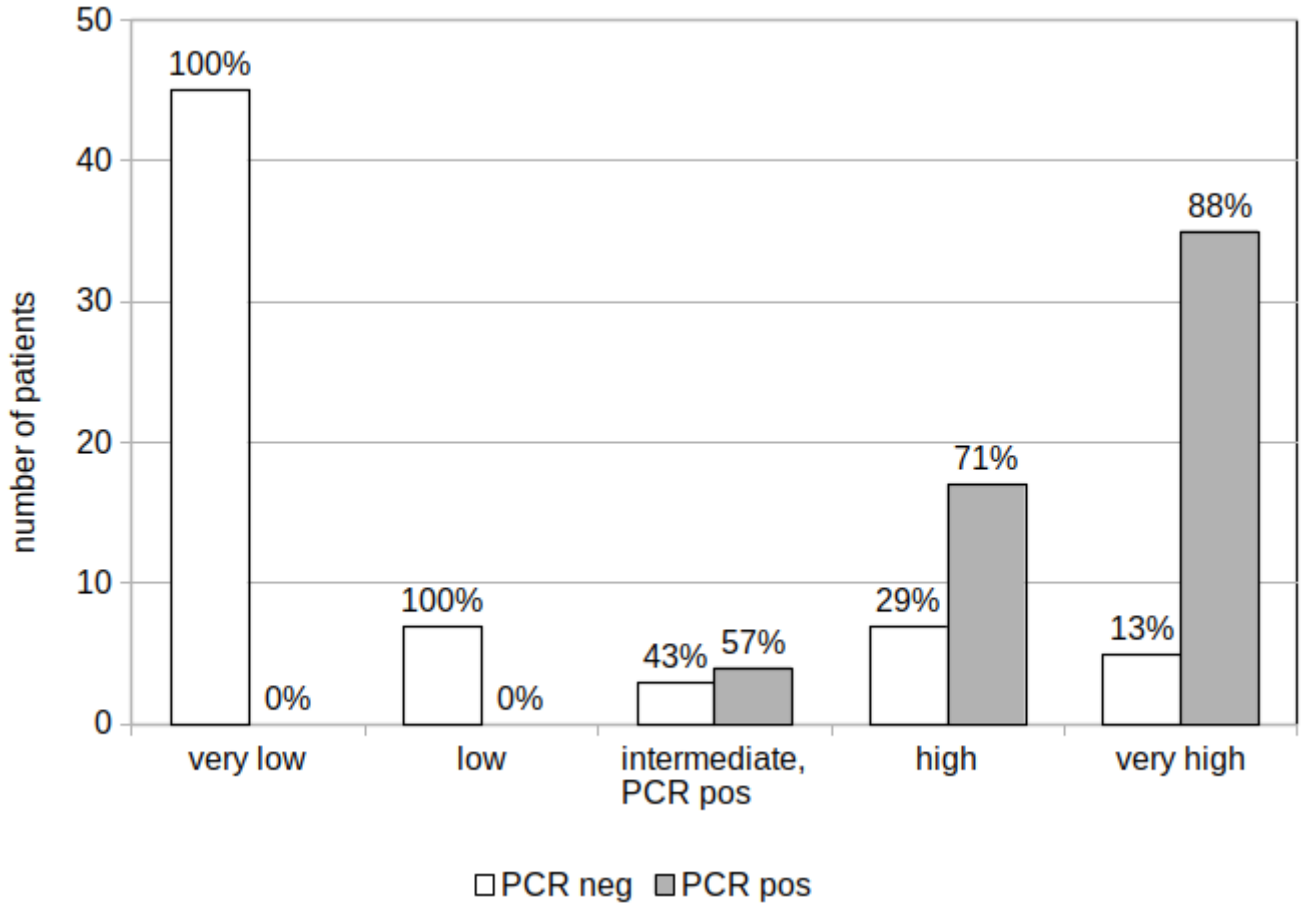

Figure 3

Results of RT-PCR assay according to level of suspicion of clinical COVID-19 diagnosis (clinCO-RADS), n=123