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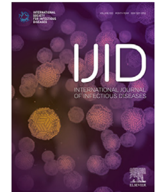

Analytical and clinical evaluation of a heat shock SARS-CoV-2 detection method without RNA extraction for N and E genes RT-qPCR

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Background: The COVID-19 pandemic has caused significant supply shortages worldwide for SARS-CoV-2 molecular diagnosis, like RNA extraction kits.

Objective: The aim of our study was to evaluate the clinical performance and analytical sensitivity of a simple SARS-CoV-2 diagnosis protocol based on heat shock without RNA extraction using both "CDC" (N gene) and "Charite" (E gene) RT-qPCR protocols.

Results: 1,036 nasopharyngeal samples, 543 of them SARS-CoV-2 positive, were analyzed. The heat shock method correctly identified 68.8% (232/337) and 89.4% (202/226) of SARS-CoV-2 positive samples for N gene and E gene, respectively. Analytical sensitivity was assessed for heat shock method using the CDC RT-qPCR protocol, obtaining sensitivity values of 98.6%, 93.3% and 84.8% for limit of detection of 100.000, 50.000 and 20.000 viral RNA copies/mL of sample.

Conclusions: Our findings show that a simple heat shock SARS-CoV-2 RT-qPCR diagnosis method without RNA extraction is a reliable alternative for potentially infectious SARS-CoV-2 positive patients at the time of testing. This affordable protocol can help overcome the cost and supply shortages for SARS-CoV-2 diagnosis, especially in developing countries. In Ecuador, it has been used already by laboratories in the public health system for more than 100.000 specimens.

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Introduction

Humanity is facing the biggest public health crisis since the "Spanish flu" in 1918. The Coronavirus Disease 2019 (COVID-19) pandemic, caused by an infection with Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2), has challenged public health systems worldwide since the initial outbreak in the Chinese city of Wuhan in December 2019. By January 31st 2021, SARS-CoV-2 had caused more than 100 million infections and 2.2 million deaths worldwide (https://coronavirus.jhu.edu/map.html).

The COVID-19 pandemic has challenged public health systems worldwide, not only for patient care and surveillance, but also to guarantee the quality and availability of SARS-CoV-2 related diagnosis tools. The COVID-19 pandemic continues to be a worldwide public health concern and the diagnostic improvements for successful case detection, contact tracing and control of the spread of SARS-CoV-2 infection are still a challenge after more than 10 months of the outbreak (CDC, 2020; Freire Paspuel et al. 2020; Freire Paspuel et al. 2020b). Supply shortage of SARS-CoV-2 testing materials has forced a narrow testing strategy focused on the care of hospitalized patients, hampering efforts to identify and prevent community transmission of SARS-CoV-2, not only in developing countries like Ecuador but even in the USA (Kavanagh et al. 2020; Schneider et al. 2020). Although there are several SARS-CoV-2 di-

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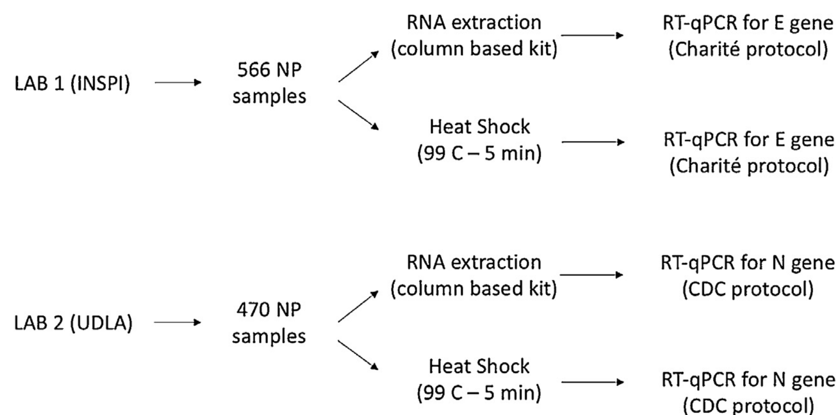

Figure 1. Sample flow charts for the two SARS-CoV-2 detection protocols followed by the two laboratories involved in the study (NP sample: nasopharyngeal sample).

agnostic tools available on the market, such as RT-LAMP, RNA extraction free systems like Cepheid Xpert Xpress SARS-CoV-2 assay or rapid antigen tests, standard RT-qPCR assays with a previous RNA extraction step remain the gold standard after more than one year of the COVID-19 pandemic. Moreover, the Centers for Disease Control and Prevention (CDC) and the World Health Organization (WHO) both still recommend strict RT-qPCR SARS-CoV-2 molecular diagnostic protocols with specifications for sample collection and RNA extraction (CDC 2020b; WHO 2020).

Under this scenario, RNA extraction kits are among the most highly demanded supplies for SARS-CoV-2 diagnosis. Few reports have already described a potential solution to overcome RNA extraction kits dependency by using a simple heat inactivation and extraction step as an alternative to automated RNA extraction kit-based systems, which are also more expensive and time and labor demanding (Barza et al. 2020; Fomsgaard et al. 2020; Hasan et al. 2020; Lübke et al. 2020; Wing-Ho Chu et al. 2020). The heat shock principle is based on the disruption of the physical integrity of viruses at high temperatures, allowing the release of viral RNA for RT-PCR detection. However, those reports differ on the sensitivity associated to the heat shock protocol, and the comparison is mostly made with an automatized magnetic beads RNA extraction kit (Barza et al. 2020; Fomsgaard et al. 2020; Hasan et al. 2020; Lübke et al. 2020; Wing-Ho Chu et al. 2020). Moreover, those studies were done with a limited number of SARS-CoV-2 positive samples and the analytical sensitivity was not addressed (Barza et al. 2020; Fomsgaard et al. 2020; Hasan et al. 2020; Lübke et al. 2020; Wing-Ho Chu et al. 2020).

The present study evaluated a heat shock method for SARS-CoV-2 detection without RNA extraction using the CDC (N gene) and Charite (E gene) RT-PCR protocols (Corman et at. 2020; Lu et al. 2020), with a significant sample size. We compared the clinical performance and analytical sensitivity of the heat shock method for detection of SARS-CoV-2 to results obtained using a column based manual RNA extraction kit protocol.

Materials and methods

Study design

A total number of 1,036 nasopharyngeal swabs collected on 0.5mL TE pH 8 buffer were included in this study, coming from two different laboratories in the cities of Guayaquil ("Instituto Nacional de Salud Pública e Investigación Leopoldo Izquieta Pérez": "INSPI") and Quito ("Universidad de Las Américas": "UDLA"), as detailed in Figure 1. Also, negative controls (TE pH 8 buffer) were included as control for carryover contamination, one for each set of RNA extractions.

Samples were collected for two different experimental approaches. First, 311 preselected SARS-CoV-2 positive samples were tested for the heat shock-based method (150 at INSPI laboratory and 161 at UDLA laboratory; Supplementary Tables 1 and 2). Second, following reviewers request, 725 samples were included in a blinded study where all the samples were tested for SARS-CoV-2 for the first time in parallel for RNA extraction and heat shock method (Figure 1): 416 at INSPI laboratory and 309 at UDLA laboratory (Supplementary Tables 3 and 4).

RNA Extraction and RT-qPCR for SARS-CoV-2 detection using 2019-nCoV CDC kit (IDT, USA)

161 preselected SARS-CoV-2 positive samples and 309 nasopharyngeal samples with unknown status for SARS-CoV-2 infection were tested following an adapted version of the CDC protocol (Figure 1) by using PureLink Viral RNA/DNA Mini Kit (Invitrogen, USA) as an alternate RNA extraction method, and using a CFX96 BioRad instrument (CDC 2020b; Freire Paspuel et al. 2020c; Freire Paspuel et al. 2020d; Freire Paspuel et al. 2020e). Briefly, The CDC-designed FDA EUA 2019-nCoV CDC kit is based on N1 and N2 amplicons from N gene to detect SARS-CoV-2 and RNase P as an RNA extraction quality control (Lu et al. 2020). These samples were processed at the laboratory of "UDLA" located in Quito. We refer to this SARS-CoV-2 RT-PCR protocol as the "CDC protocol".

RT-qPCR for SARS-CoV-2 detection using LightMix SarbecoV E-gene plus EAV control kit (TIB MOLBIOL, Germany)

150 preselected SARS-CoV-2 positive samples and 416 nasopharyngeal samples with unknown status for SARS-CoV-2 infection were tested following the manufacturer's manual (Figure 1). PureLink Viral RNA/DNA Mini Kit (Invitrogen, USA) and a LightCycler 480 II system Roche instrument were used for RNA extraction and thermal cycling. Briefly, the "Charité University- Berlin Institute of Virology" (Berlin, Germany) designed LightMix SarbecoV E-gene plus EAV control kit is based on E gene detection probe for SARS-CoV-2 and EAV as an RNA extraction quality control (Corman et al. 2020). These samples were processed at the laboratory of "INSPI" located in Guayaquil. We referred along the text to this SARS-CoV-2 RT-PCR protocol as de "Charite protocol".

Table 1
Clinical performance of the heat shock method for SARS-CoV-2 detection without RNA extraction for CDC (N gene) and Charite (E gene) RT-qPCR protocol using preselected SARS-COV-2 positive samples (% values of true positive is the sensitivity)

RT-PCR protocol	true positive samples	false negative samples	total positive samples
N gene	109 (67.7%)	52 (32.3%)	161
E gene	133 (88.7%)	17 (11.3%)	150

RT-qPCR for SARS-CoV-2 detection using heat shock method without RNA extraction

All the samples processed by the "CDC" and "Charite" protocols using RNA extraction kits were also processed using our heat shock method without RNA extraction. The remaining volume (approximately 300 uL) of transport medium after RNA extraction (200 uL) was used for the heat shock method. The samples were centrifuged for 1 min up to 14,000 rpm, supernatant was carefully removed and 50uL of RNase free water was added, followed by vortexing for 30 seconds. Samples were then placed in 0.2mL tubes on a thermal cycler. A heat shock of 99°C for 5 minutes was applied, followed by cooling at 4°C for 5 minutes. To avoid RNA degradation, samples were processed for RNA extraction and heat shock method within the same day.

Analytical Sensitivity

Limit of detection (LoD) thresholds for sensitivity calculations were addressed by calculating viral loads of the samples processed by the "CDC" protocol. The 2019-nCoV N positive control (IDT, USA), provided at 200.000 genome equivalents/mL was used for calibration curves to obtain the viral loads of the samples. Viral loads can be expressed as copies/uL of RNA extraction or copies/mL of sample; the conversion factor is 200, as 0.2mL of sample is used for RNA extraction and 40uL is used as final elution volume of RNA extraction.

Statistics

Student t-test was performed to compare Ct values.

Ethics statement

All samples have been submitted for routine patient care and diagnostics. Ethics approval was not sought because the study involves laboratory validation of test methods and the secondary use of anonymous pathological specimens that falls under the category 'exempted' by "Instituto Nacional de Salud Pública e Investigación Leopoldo Izquieta Pérez" review board and "Comité de Etica para Investigación en Seres Humanos" from "Universidad de Las Américas".

Results

Clinical performance for heat shock SARS-CoV-2 RT-PCR detection method without RNA extraction for preselected SARS-CoV-2 positive samples

150 SARS-CoV-2 positive samples with RNA extraction were tested following the "Charite protocol" for the heat shock method. 133 out of 150 SARS-CoV-2 positive samples were also positive for the heat shock method, resulting in a sensitivity of 88.7% (Table 1). All the SARS-CoV-2 positive samples that tested negative for the heat shock method had Ct values larger than 31.52 (see Supplementary Table 1). The average value for the E gene Ct was significantly different ($p<0.001$) for the heat shock method (Ct = 31.6) compared to the RNA extraction method (Ct = 24.4).

Table 2
Values of the sensitivity for several limits of detection or viral load thresholds for the heat shock SARS-CoV-2 RT-qPCR detection method without RNA extraction for the N gene

Viral load	Heat shock vs total positives samples	sensitivity
100.000 copies / mL	72/73	98.6%
50.000 copies / mL	84/90	93.3%
30.000 copies / mL	91/101	90.1%
20.000 copies / mL	95/112	84.8%

161 SARS-CoV-2 positive samples with RNA extraction were tested following the "CDC protocol" for the heat shock method. 109 out of 161 SARS-CoV-2 positive samples were also positive for the heat shock method, resulting in a sensitivity of 67.7% (Table 1). All the SARS-CoV-2 positive samples that tested negative for the heat shock method had Ct values larger than 29.1 (see Supplementary Table 2). The average value for the N1 amplicon Ct was significantly different ($p<0.001$) for the heat shock method (Ct = 29.8) compared to the RNA extraction method (Ct = 26.5). The average value for the N2 amplicon Ct was not significantly different ($p = 0.19$) for the heat shock method (Ct = 31.4) compared to the RNA extraction method (Ct = 30.1). The average value for the RnaseP Ct was significantly different ($p<0.001$) for the heat shock method (Ct = 30.4) compared to the RNA extraction method (Ct = 25.1).

Analytical sensitivity: calculation of the sensitivity dependent on limit of detection (LoD) for the heat shock SARS-CoV-2 RT-PCR diagnosis method without RNA extraction

The viral loads detailed in Supplementary Table 2 were calculated running a calibration curve with 2019-nCoV N positive control. The LoD for the "CDC protocol" was set at 1000 viral RNA copies per mL of sample on previous studies (15-17). As detailed in Table 2 the sensitivity of the heat shock SARS-CoV-2 RT-PCR detection method without RNA extraction using the "CDC protocol" was calculated for different LoDs. For viral loads above 100.000 copies/mL, 72 out of 73 samples were positive for the heat shock method, resulting in a sensitivity of 98.6% compared to RNA extraction method; for viral loads larger than 50.000 copies/mL, 84 out of 90 samples were positive for the heat shock method, resulting in a sensitivity of 93.3% compared to the RNA extraction method; for viral loads larger than 20.000 copies/mL, 95 out of 112 samples were positive for the heat shock method, resulting in a sensitivity of 84.8% compared to RNA extraction method.

Clinical performance for heat shock SARS-CoV-2 RT-PCR detection method without RNA extraction on a blind study

416 samples of unknown status for SARS-CoV-2 infection were tested following the "Charite protocol" for both RNA extraction and the heat shock method. 69 out of 76 SARS-CoV-2 positive samples for RNA extraction were also positive for the heat shock method, resulting in a sensitivity of 90.8% (Table 3; Supplementary Table 3).

Table 3
Blind assay for the clinical performance of the heat shock method for SARS-CoV-2 detection without RNA extraction for CDC (N gene) and Charite (E gene) RT-qPCR protocol (% values of true positives is the sensitivity)

RT-PCR protocol	true positive samples	false negative samples	total positive samples
N gene	123 (69.9%)	53 (30.1%)	176
E gene	69 (90.8%)	7 (9.2%)	76

Table 4
Comparison of the clinical performance for SARS-CoV-2 detection without RNA extraction among several studies published

sample treatment	sample size (SARS-CoV-2 +)	sensitivity (%)	reference
65°C/20min	86	94.0	8
98°C/5min - no treatment	40	58.0 - 56.0	9
98°C/5min	39	97.4	10
99°C/5min	91	81.3	11
65°C/10min	19	95.0	12
95°C/10min - no treatment	150	92.0 - 84.0	19
99°C/5min	543	77.5	our study

309 samples of unknown status for SARS-CoV-2 infection were tested following the "CDC protocol" for both RNA extraction and the heat shock method. 123 out of 176 SARS-CoV-2 positive samples for RNA extraction were also positive for the heat shock method, resulting in a sensitivity of 69.9% (Table 3; Supplementary Table 4). Among those 123 positive samples for the heat shock method, 29 samples were only positive for N1 gene target (these samples are called presumptive positive at Supplementary Table 4, but we considered them positive for further analysis).

Overall clinical performance for heat shock SARS-CoV-2 RT-PCR detection method without RNA extraction

The total number of 1,036 samples was analyzed for both standard RNA extraction and heat shock method. 543 samples were positive for RNA extraction-RT-qPCR, and 434 of them were also positive for heat shock-RT-PCR, yielding an overall sensitivity for the heat shock method of 77.1% (434/563; see Table 4).

For the Charité protocol, a total number of 566 samples were analyzed for both standard RNA extraction and heat shock method. 226 samples were positive for RNA extraction-RT-qPCR, and 202 of them were also positive for heat shock-RT-PCR, yielding an overall sensitivity for the heat shock method followed by RT-PCR for E gene of 89.4% (202/226).

For the CDC protocol, a total number of 470 samples were analyzed for both standard RNA extraction and heat shock method. 337 samples were positive for RNA extraction-RT-qPCR, and 232 of them were also positive for heat shock-RT-PCR, yielding an overall sensitivity for the heat shock method followed by RT-PCR for N gene of 68.8% (232/337).

Discussion

Our results support that the direct detection of SARS-CoV-2 using a heat shock method based on a 99°C heat inactivation and release step for 5 minutes without RNA extraction is a reliable alternative to the use of column based manual RNA extraction kits, although low viral loads samples would not be detected. Thermal treatment is important as it inactivates virus, causes exposure of the viral genome and denatures inhibitors of the PCR that may be present on the sample. It has been suggested that heating at high temperatures above 95°C for direct RT-qPCR without RNA extraction often results in lower RT-qPCR sensitivity compared to moderate temperatures of 65°C (Barza et al. 2020; Bhattacharya et al. 2004). However, our results and other reports show that it is not the case for SARS-CoV-2 diagnosis and high temperature heat shock may yield a sensitivity above 90% (Fomsgaard et al. 2020). Moreover, although a recent report describes a highly sensitive direct detection of SARS-CoV-2 RNA by RT-qPCR even with neither RNA extraction nor heat shock, the authors suggested that the heat shock method would improve diagnostic sensitivity for low viral loads samples (Bruce et al. 2020).

We observed a significant switch toward higher Ct values for E, N1 and RNaseP amplicons using the heat shock method. We performed the RT-qPCR with the same amount of sample regardless whether heat shock of RNA extraction was performed. Also, similar volume was used for heat shock (remaining volume of 300uL) or RNA extraction (200 uL). Therefore, the lack of sensitivity observed is probably due to the less efficient RNA concentration by centrifugation and elution on a smaller volume, compared to the use of ionic binding columns like the ones included on RNA extraction kits. Additionally, the sensitivity for the heat shock method was better for E gene based RT-qPCR (89.4%) than for N gene (68.8%), but because each of the RT-qPCR protocols were developed at a different laboratory, we cannot completely rule out that those differences may be not associated to the gene targets but to experimental variability.

Several reports have already shown heat shock methods for SARS-CoV-2 RT-qPCR diagnosis without RNA extraction (Barza et al. 2020; Bruce et al. 2020; Fomsgaard et al. 2020; Hasan et al. 2020; Lübke et al. 2020; Wing-Ho Chu et al. 2020).However, none of those studies used a column based manual RNA extraction kit, but automated magnetic beads based RNA extraction systems (Barza et al. 2020; Bruce et al. 2020; Fomsgaard et al. 2020; Hasan et al. 2020; Lübke et al. 2020; Wing-Ho Chu et al. 2020). Moreover, with a sample size of 543 SARS-CoV-2 positive samples, our study is to our knowledge the most statistically significant, as the previous ones were carried out with substantially smaller SARS-CoV-2 positive sample sizes of 86 (Barza et al. 2020), 40 (Wing-Ho Chu et al. 2020), 39 (Fomsgaard et al. 2020), 91 (Lübke et al. 2020), 19 (Hasan et al. 2020) and 150 (Bruce et al. 2020). Also, our study is the only one using two of the most-used worldwide RT-qPCR protocols for SARS-CoV-2 diagnosis, the CDC and Charite protocols (CDC 2020b; Corman et al. 2020; Lu et al. 2020). Nevertheless, we found an overall sensitivity for our heat shock method of 77.5%; similar values of sensitivity ranging from 58% to 97.4% have been reported on previous studies (Barza et al. 2020; Bruce et al. 2020; Fomsgaard et al. 2020; Hasan et al. 2020; Lübke et al. 2020; Wing-Ho Chu et al. 2020), as summarized in Table 4.

The previous publications addressing heat shock methods without RNA extraction for SARS-CoV-2 detection clearly indicated that failure to detect SARS-CoV-2 positive samples happened for high Ct values in the range of 32 to 40 (Barza et al. 2020; Bruce et al. 2020; Fomsgaard et al. 2020; Hasan et al. 2020; Lübke et al. 2020; Wing-Ho Chu et al. 2020). However, only one of those reports address the sensitivity of the heat shock methods in terms of viral load, reporting a sensitivity of 95% for samples above 66.000 viral copies/mL (Hasan et al. 2020). Although the main limitation of that study is the reduced sample size, only 18 SARS-CoV-2 positive samples (Hasan et al. 2020), those results are in agreement with the sensitivity of 93.3% that we obtained for viral loads larger than 50.000 copies/mL.

It is important to note that our heat shock SARS-CoV-2 RT-qPCR method has a sensitivity up to 98.6% for viral loads larger than 100.000 copies/mL. Considering the viral load frequency distribution for SARS-CoV-2, this high LoD would potentially exclude more than 30% of true positive cases (Kleiboeker et al. 2020; Lavezzo et al. 2020). It has also been recently reported that only patients with viral loads over 1 million copies/mL would be infectious (Wölfel et al. 2020), and potentially all of them would be detected with our heat shock SARS-CoV-2 RT-qPCR method. However, the viral load threshold for SARS-CoV-2 transmission is still a matter of discussion (Avanzato el al. 2020; Freire-Paspuel et al. 2021; Rodríguez-Grande et al. 2021), so reduced sensitivity for low viral loads is still a limitation of RNA extraction free SARS-CoV-2 detection protocols.

Considering the worldwide high demand for reagents for SARS-CoV RT-qPCR detection , supply shortage is a fact, hampering extensive testing in developing countries like Ecuador. Under this scenario, SARS-CoV-2 detection methods alleviating cost and dependency on supplies are crucial to increase SARS-CoV-2 detection capacity and even to avoid COVID-19 diagnosis disruption. This was the case with the SARS-CoV-2 National Reference Laboratory at "Instituto Nacional de Salud Pública Leopoldo Izquieta Pérez" in Ecuador, where the method described in this study has been used for more than 100.000 samples at a time that RNA extraction kits were not available in the country.

Declaration of Competing Interest

All authors have no conflict of interest to declare.

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Authorship contribution statement

All authors contributed to study conceptualization, experimental procedures and revision and approval of final version of the manuscript.

Alfredo Bruno, Byron Freire-Paspuel, Domenica de Mora performed most of the experiments and analyzed the data. Miguel Angel Garcia Bereguiain wrote the manuscript.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ijid.2021.06.038.

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