Comparison of in-house SARS-CoV-2 genome extraction procedures. A need for COVID-19 pandemic

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Comparison of in-house SARS-CoV-2 genome extraction procedures. A need for COVID-19 pandemic

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Abstract

Purpose: Among the methods used to diagnose COVID-19, those based on genomic detection by q(RT)-PCR are the most sensitive. To perform these assays, a previous genome extraction of the sample is required. The dramatic increase in the number of SARS-CoV-2 detection assays has increased the demand for extraction reagents hindering the supply of commercial reagents. Homemade reagents and procedures could be an alternative.

Methods: Nasopharyngeal samples were extracted by seven different methods as well as the automatic method MagNaPure96, to detect SARS-CoV-2.

Results: All protocols show sensitivity higher than 87%, in comparison with reference method, for detecting SARS-CoV-2 as well as human β- globin.

Conclusions: Our results support that these procedures, using common and cheap reagents, are effective to extract RNA (from SARS-CoV-2) or DNA (from human β-globin) genome from nasopharyngeal swabs. Furthermore, these procedures could be easily adopted by routine diagnostic laboratories to implement detection methods to help to fight against COVID-19 pandemic.

Keywords

SARS-CoV-2; COVID-19; genome extraction; In-house protocol; Heat extraction.

Introduction

In December 2019, Chinese health authorities identified the new betacoronavirus SARS-CoV-2 as the cause of the respiratory illness COVID-19, which was declared pandemic by World Health Organization in January 2020 (1, 2). High sensitivity diagnostic methods to detect and contain potential outbreaks are required to fight against this pandemic.

Among these methods, those based on genomic detection by quantitative retrotranscriptase (RT)-PCR have been proved to be the best for a quick and sensitive detection of COVID-19 infected patients. To perform these assays, a previous genome extraction of the sample is required. This step is essential since both quantity and quality of the genome obtained could affect the further amplification process (3). Because of that, some studies comparing different commercial and/or manual procedures for genome extraction from different type of samples, such as fecal, blood or respiratory specimens have been reported (4-6).

The current global health emergency due to SARS-CoV-2 pandemic has caused a dramatic increase in the number of detection assays performed by diagnostic laboratories and, therefore, a huge demand for extraction reagents making difficult the supply of commercial reagents. Homemade reagents and manual procedures are an alternative. Our team has conducted a comparative study between seven manual procedures with commercial and homemade reagents.

The aim was to evaluate alternative protocols to commercial genome extraction procedures to be used for SARS-CoV-2 detection by routine diagnostic laboratories.

Material and methods

Samples: A total of 58 nasopharyngeal samples from patients with suspicious of SARS-CoV-2 infection were collected. The original volume of each sample (200 µL) was diluted 8 times to a final volume of 1.6 mL to get enough volume to perform all extraction procedures.

Genome extraction procedures: The automatic extraction method MagNa Pure 96 System (Roche, Ginebra, Switzerland), which is usually developed in the laboratory, was taken as reference. Seven different procedures were carried out manually. MagNA Pure 96 System and MagNA Pure 32 System (Roche) were performed using commercial reagents following manufacturer´s instructions. Other four protocols named as One-Step Method A (OSM-A), One-Step Method B (OSM-B), Two-Step Method (TSM) and "Bikop" method, were performed using common homemade reagents. The last protocol, which is based on heat treatment was performed in a SureCycler 8800 (Agilent Technologies, Santa Clara, CA) and no reagents were necessary. Protocols and reagents of the different genome extraction procedures were tested one time for each sample and are shown in Table 1.

qRT-PCR: all extracted samples were tested with a multiple qRT-PCR directed to two regions of the SARS-CoV 2 genome (Orf1ab and N gene), as well as the human β- globin gen. Briefly, 5 µl of sample, previously extracted by any of the tested methods, were added to 10 µl of TaqMan Fast 1-Step Master Mix (Life technologies, Carlsbad, CA) supplemented with a mixture of primers (Thermo Fisher Scientific, Walthman, MA) and taqman MGB probes (Applied Biosystems, Foster City, CA) (Table 2). Amplification and subsequent analysis were carried out using the Applied Biosystems 7500 Real-time PCR

System (Applied Biosystems). The cycling protocol was as follows: (50ºC, 20 min; 95ºC, 5 min; 45 cycles of 95ºC, 10 sec; 55ºC, 15 sec and 60ºC, 30 sec).

Statistical studies: A T-Student test, whose null hypothesis was that in-house protocols works in the same way that the reference method, with a p-value of 0.05, was performed.

Results

Nasopharyngeal samples were extracted by seven different manual methods, as well as by the automatic extraction method MagNA Pure 96 used as reference, and amplified by qRT-PCR. According to the reference automatic method, β-globin gene was detected in all samples, being 30 of them also positive to SARS-CoV-2 genome. The cycle threshold (Ct) of each amplification and mean, rank and 95% CI of each method are shown in tables 3 and 4.

The sensitivity for SARS-CoV-2 detection of the manual methods was calculated by comparison to automatic method. For OSM-A sensitivity was 93.3% while for MP32 achieved 100%. The limits of 95% CI were 30.16 and 32.7 in automatic method MP96. By mean difference, in MP32 were 32.34 and 33.66 and in OSM-A were 33.36 and 34.84.

The sensitivity for β-globin detection of the manual methods was also calculated by comparison to automatic method. For “Bikop” extraction sensitivity was 87.9% while for OSM-A reached 100%. The limits of 95% CI were 30.16 and 32.38 in automatic method MP96. By mean difference, in “Bikop” extraction were 34.53 and 36.25 and in MP32 were 35.96 and 37.58.

Discussion

The expansion of the outbreak of the new coronavirus SARS-CoV-2 and the global pandemic situation caused by its spread has provoked the need of a quick and efficient tool for diagnosis of viral disease. Genome amplification based on PCR has been raised as the best method and as important as the amplification process is the previous genome extraction step. In this process, the viral genome as well as cellular genome is purified. Both genomes are used to amplify and quantify the viral genome and the human gene β-globin, respectively. The ratio between the two values allows calculating a normalized viral load and determining the sample quality (8, 9).

Considering that the use of PCR commercial kits is very usual in most clinical laboratories, the supply of reagents during pandemic peak was a common problem. Trying to obtain a solution for future similar situations, several extraction genome procedures were tested with the idea of obtaining a method to be included in manual or even automatic diagnostic procedures used routinely in clinical laboratories. Similar methods were also developed by other authors (10-12). In our study, the sensitivity for SARS-CoV-2 of the tested procedures was higher than 93%. These results were also observed in β-globin gene amplification, were all methods bowed a sensitivity higher than 87%. According to these data, any method has enough sensitivity and can be used routinely in a clinical laboratory.

Data were a little bit worse when Cts of SARS-CoV-2 were compared with reference methods, Also, higher Cts were observed in β-globin detection. Because sensitivity was decreased in both targets (SARS-CoV-2 and β-globin), viral load was minimally underestimated, no more than 0.5 log considering that each 3 Cts means a difference in the viral load of 1 log. A possible explanation to these results is that manual processing

92 is less precise and more prone to error than automatic methods using a robot.
93 Automatization of these procedures can solve this situation.
94 One limitation of the tested procedures can be that they are not quick enough. A
95 possible alternative is cut time at least in half for washing steps. In preliminary studies
96 in our laboratory changes in results were not observed. The main advantage is the use
97 of common reagents making possible these procedures can be easily adapted by any
98 other laboratory directly or changing the reagents for other with similar properties.
99 Furthermore, these procedures could also be used for the extraction of RNA or DNA
100 genome of other viruses.
101 Heat extraction method, whose sensitivities for SARS-CoV-2 and β-globin were 96.7%
102 and 87.9%, respectively, can be used for a quick screening of infected patients with
103 suspicious of a high viral load. Recent reports using a similar method support the viability
104 of this technology to SARS-CoV-2 genome extraction (13, 14). Considering that this is the
105 quickest (only 15 minutes), cheapest and easiest (only need a thermoblock) protocol of
106 all the tested procedures, it appears as a clear alternative to be implemented in small
107 diagnostic laboratories, favoring a decentralization of SARS-CoV-2 diagnostic and
108 desaturating central hospitals. In this method, Cts were similar than in others, and good
109 enough for using when samples level is high.
110 In summary, in-house procedures evaluated can substitute commercial techniques
111 performing a successful SARS-CoV-2 genome extraction using common and cheap
112 reagents. On the other hand, human genome can also be successfully extracted allowing
113 the use of β-globin gene as sample quality control and for normalized viral load calculus.
114 These procedures could be easily adopted by clinical laboratories and be used to extract
115 human samples with suspect of any viral presence. It is worth to note that Heat

extraction is a quick, cheap, and easy diagnostic method, which could be used in small diagnosis laboratories.

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Declarations

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Conflict of interest statement: the authors declare no potential conflicts of interest.

Availability: of data and material: Not applicable

Code Availability: Not applicable

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Table 1: *Extraction procedures steps*

Steps	MagNa Pure 96[1] (MP96)	MagNa Pure 32[1] (MP32)	One-step method A (OSM-A)	One-step method B (OSM-B)	Two-steps method (TSM)	"Bikop" method	Heat Extraction
1	Lysis buffer MP96 Incubate (10'- RT*)	Lysis buffer MP32 Incubate (10'- RT)	Lysis buffer 1[2] Incubate (10'- RT)	Lysis buffer 1[3] Incubate (10'- RT)	Lysis buffer 1[4] Incubate (10'- RT)	Lysis buffer 1[2] Incubate (10'- RT)	Hot Spot (10' - 95ºC)
2					Lysis Buffer 2[2] Incubate (10' - RT)		Freeze (5' - 4ºC)
3	Binding Buffer MP96 Incubate in shaker (10' – RT) Magnetize* & Remove SN*	Binding Buffer MP32 Incubate in shaker (10' - RT) Magnetize & Remove SN	Binding Buffer[5] Incubate in shaker (10' - RT) Magnetize & Remove SN	Binding Buffer[5] Incubate in shaker (10' - RT) Magnetize & Remove SN	Binding Buffer[5] Incubate in shaker (10' - RT) Magnetize & Remove SN	Binding Buffer[5] Incubate in shaker (10' - RT) Magnetize & Remove SN	
4	Wash Buffer 1 MP96 Incubate (10' - RT) Magnetize & Remove SN	Wash Buffer 1 MP32 Incubate (10' - RT) Magnetize & Remove SN	Wash Buffer 1[6] Incubate (10' - RT) Magnetize & Remove SN	Wash Buffer 1[6] Incubate (10' - RT) Magnetize & Remove SN	Wash Buffer 1[6] Incubate (10' - RT) Magnetize & Remove SN	Wash Buffer 1[6] Incubate (10' - RT) Magnetize & Remove SN	
5	Wash Buffer 2 MP96 Incubate (10' - RT) Magnetize & Remove SN	Wash Buffer 2 MP32 Incubate (10' - RT) Magnetize & Remove SN	Wash Buffer 2[7] Incubate (10' - RT) Magnetize & Remove SN	Wash Buffer 2[7] Incubate (10' - RT) Magnetize & Remove SN	Wash Buffer 2[7] Incubate (10' - RT) Magnetize & Remove SN	Wash Buffer 2[7] Incubate (10' - RT) Magnetize & Remove SN	
6	Wash Buffer 3 MP96 Incubate (10' - RT) Magnetize & Remove SN	Wash Buffer 3 MP32 Incubate (10' - RT) Magnetize & Remove SN	Wash Buffer 3[8] Incubate (10' - RT) Magnetize & Remove SN	Wash Buffer 3[8] Incubate (10' - RT) Magnetize & Remove SN	Wash Buffer 3[8] Incubate (10' - RT) Magnetize & Remove SN		
7	Elution Buffer MP96 Magnetize & Collect SN	Elution Buffer MP32 Magnetize & Collect SN	Elution Buffer[9] Magnetize & Collect SN	Elution Buffer[9] Magnetize & Collect SN	Elution Buffer[9] Magnetize & Collect SN	Elution Buffer [9] Magnetize & Collect SN	
Time (estimate)	**50'**	**50'**	**50'**	**50'**	**60'**	**40'**	**15'**

*RT: Room Temperature
*Using a 96R Ring Magnet Plate (Alpaqua, Beverly, MA)
*SN: supernatant
[1] Reagents and volumes suggested by manufacturer were used in these protocols.
[2] Lysis Buffer 1: 200µL GIT (GIT 4M + sarcosyl 0,5% + sodium citrate 25mM) and 10µL pK (10µg/µL)
[3]Lysis Buffer 2: 200µL GIT, 10 µL pK and 200 µL SDS (SDS 0,5% + Tris (pH 8) 10mM + EDTA 1mM)
[4]Lysis Buffer 3: 200µL SDS (SDS 0,5% + Tris (pH 8) 10mM + EDTA 1mM)
[5]Binding Buffer: 200µL magnetic beads in Isopropanol (1,5g/mL), (Roche, Ginebra, Switzerland)
[6]Wash Buffer 1: 200µL isopropanol
[7]Wash Buffer 2: 200µL EtOH 80%
[8]Wash Buffer 3: 200µL NaCl 0,5M
[9]Elution Buffer: 100 µL water

Table 2: *Primers and taqman MGB probes used to detect SARS-CoV2 and human β-globin.*

Design	Gen	Function	Name	Sequence (5´-3´)
In-house	ORF1ab	Forward primer	CoV-2-OVI-S	ATCAAGTTAATGGTTACCCTAACATGT
	SARS-CoV2	Reverse primer	CoV-2-OVI-A	AACCTAGCTGTAAAGGTAAATTGGTACC
		MGB FAM probe	CoV-2-OVI-FAM	CCGCGAAGAAGCTA
CDC[1]	N	Forward primer	2019-nCoV-N1-F	GACCCCAAAATCAGCGAAAT
	SARS-CoV2	Reverse primer	2019-nCoV-N1-R	TCTGGTTACTGCCAGTTGAATCTG
		MGB VIC probe	2019-nCoV-N1-P-VIC	CCGCATTACGTTTGGT[2]
In-house	β-globin	Forward primer	Beta-TR-S	ACACAACTGTGTTCACTAGC
	Human	Reverse primer	Beta-TR-A	CCAACTTCATCCACGTTCACC
		MGB Cy5 probe	Beta-Cy5	TGCATCTGACTCCTGAGGA

[1] Sequences published by Center for Disease Control and Prevention (CDC) (7)

Table 3:*Results of amplification of SARS-CoV-2 and human β-globin (Ct SARS-CoV-2/Ct β-globin)*

Samples	MP96 (automated)	MP96 (handmade)	MP32	OSM-A	OSM-B	TSM	"Bikop" method	Heat Extraction
1º	24 / 36	30 / 40	30 / 40	31 / 40	30 / 40	29 / 40	29 / 36	31 / 38
2º	24 / 29	30 / 33	30 / 36	35 / 34	31 / 34	29 / 36	27 / 32	30 / 34
3º	25 / 34	29 / 38	31 / 40	31 / 39	31 / 40	32 / 39	27 / 35	27 / 38
4º	26 / 30	31 / 34	31 / 37	31 / 39	33 / 36	30 / 34	30 / 35	32 / 36
5º	27 / 31	31 / 35	30 / 37	32 / 40	32 / 35	31 / 35	34 / 36	33 / 40
6º	27 / 29	33 / 34	32 / 37	33 / 36	34 / 36	31 / 35	31 / 34	31 / 36
7º	28 / 39	33 / 40	30 / 40	32 / 40	32 / 40	31 / 40	32 / 40	30 / 40
8º	30 / 29	34 / 31	33 / 37	36 / 35	32 / 34	33 / 35	31 / 32	34 / 33
9º	30 / 40	31 / 36	32 / 40	31 / 40	31 / 40	30 / 40	31 / 40	32 / 40
10º	31 / 31	33 / 38	33 / 39	33 / 34	34 / 34	34 / 36	34 / 34	34 / 33
11º	32 / 29	33 / 32	34 / 35	34 / 34	33 / 36	33 / 33	33 / 33	33 / 40
12º	32 / 34	37 / 39	34 / 37	36 / 39	35 / 38	39 / 37	37 / 35	36 / 35
13º	32 / 33	34 / 38	31 / 34	33 / 40	32 / 40	32 / 38	33 / 39	34 / 40
14º	32 / 36	31 / 40	32 / 38	33 / 40	32 / 40	32 / 38	32 / 40	34 / 40
15º	33 / 37	33 / 40	34 / 37	39 / 40	38 / 40	33 / 40	33 / 38	34 / 40
16º	33 / 37	34 / 40	33 / 40	36 / 40	36 / 40	0 / 40	33 / 40	34 / 40
17º	33 / 27	34 / 40	33 / 32	34 / 35	35 / 32	33 / 35	33 / 31	0 / 34
18º	33 / 35	36 / 38	35 / 40	35 / 40	35 / 40	36 / 40	0 / 40	37 / 40
19º	0 (31)* / 33	33 / 40	34 / 40	0 / 38	33 / 37	34 / 36	33 / 36	34 / 35
20º	33 / 36	34 / 40	34 / 40	34 / 37	33 / 40	33 / 40	33 / 40	34 / 40
21º	34 / 34	34 / 36	36 / 40	37 / 39	36 / 40	34 / 38	38 / 38	36 / 40
22º	34 / 32	33 / 35	33 / 40	0 / 35	35 / 37	33 / 36	35 / 35	35 / 34
23º	34 / 23	34 / 40	34 / 39	34 / 40	34 / 40	33 / 37	33 / 40	34 / 40
24º	34 / 28	37 / 31	37 / 33	34 / 32	34 / 32	34 / 31	34 / 30	35 / 28
25º	34 / 40	34 / 40	34 / 40	35 / 40	36 / 40	34 / 40	35 / 40	35 / 40
26º	35 / 40	36 / 40	35 / 40	37 / 40	36 / 39	35 / 40	39 / 40	38 / 40
27º	35 / 35	35 / 40	36 / 40	37 / 40	37 / 40	37 / 40	37 / 39	36 / 40
28º	36 / 38	34 / 40	33 / 40	34 / 40	35 / 40	35 / 40	35 / 40	35 / 40
29º	0 (35)* / 27	34 / 30	33 / 31	35 / 31	35 / 32	33 / 30	0 / 32	38 / 31
30º	0 (36)* / 33	0 / 0	33 / 39	33 / 39	0 / 40	35 / 38	34 / 40	35 / 38
31º	- / 22	- / 26	- / 29	- / 28	- / 28	- / 28	- / 26	- / 25
32º	- / 23	- / 0	- / 35	- / 33	- / 31	- / 33	- / 32	- / 33
33º	- / 24	- / 32	- / 36	- / 33	- / 33	- / 32	- / 34	- / 33
34º	- / 25	- / 33	- / 37	- / 34	- / 34	- / 34	- / 35	- / 33
35º	- / 26	- / 31	- / 32	- / 30	- / 31	- / 30	- / 30	- / 31
36º	- / 27	- / 32	- / 35	- / 31	- / 32	- / 32	- / 34	- / 32
37º	- / 27	- / 40	- / 31	- / 31	- / 30	- / 30	- / 30	- / 38
38º	- / 28	- / 32	- / 40	- / 33	- / 34	- / 33	- / 35	- / 34
39º	- / 28	- / 0	- / 40	- / 35	- / 36	- / 32	- / 33	- / 33
40º	- / 28	- / 0	- / 36	- / 32	- / 33	- / 32	- / 35	- / 33
41º	- / 29	- / 32	- / 32	- / 34	- / 35	- / 0	- / 0	- / 0
42º	- / 29	- / 0	- / 40	- / 33	- / 33	- / 35	- / 34	- / 37
43º	- / 29	- / 31	- / 31	- / 32	- / 33	- / 33	- / 0	- / 0
44º	- / 29	- / 33	- / 34	- / 33	- / 33	- / 34	- / 33	- / 32
45º	- / 30	- / 33	- / 33	- / 35	- / 37	- / 33	- / 0	- / 33
46º	- / 30	- / 36	- / 38	- / 34	- / 33	- / 38	- / 35	- / 0
47º	- / 30	- / 34	- / 34	- / 33	- / 32	- / 33	- / 34	- / 31
48º	- / 31	- / 35	- / 35	- / 39	- / 37	- / 35	- / 0	- / 32
49º	- / 31	- / 34	- / 40	- / 34	- / 34	- / 37	- / 35	- / 40
50º	- / 31	- / 33	- / 33	- / 36	- / 36	- / 35	- / 0	- / 0
51º	- / 31	- / 33	- / 39	- / 36	- / 34	- / 36	- / 0	- / 34
52º	- / 32	- / 37	- / 37	- / 33	- / 34	- / 35	- / 33	- / 32
53º	- / 32	- / 33	- / 33	- / 35	- / 35	- / 31	- / 35	- / 36
54º	- / 32	- / 35	- / 35	- / 40	- / 0	- / 35	- / 35	- / 38
55º	- / 33	- / 36	- / 40	- / 37	- / 35	- / 36	- / 36	- / 0
56º	- / 34	- / 38	- / 38	- / 37	- / 0	- / 0	- / 0	- / 37
57º	- / 34	- / 0	- / 0	- / 39	- / 37	- / 36	- / 36	- / 40
58º	- / 34	- / 0	- / 35	- / 39	- / 37	- / 33	- / 35	- / 0

*these samples were negative on the MP96 automated extraction, so the Ct before dilution (in parenthesis) was used

Table 4: ***Statistic data***

SARS CoV2 samples	MP96 (automated)	MP96 (manual)	MP32	OSM-A	OSM-B	TSM	"Bikop"method	Heat Extraction
Positives (sensitivity)	30 (100%)	29 (96.7%)	30 (100%)	28 (93.3%)	29 (96.7%)	29 (96.7%)	28 (93.3%)	29 (96.7%)
Mean ± σ	31.43 ± 3.54	33.27 ± 2	33 ± 1.86	34.1 ± 2.08	33.79 ± 2.01	33.03 ± 2.26	33.07 ± 2.85	33.82 ± 2.44
Range	[24 – 36]	[29 - 37]	[30 - 37]	[31 - 39]	[30 – 38]	[29 - 39]	[27 – 39]	[27 - 38]
95% IC	[30.16- 32.7]	[32.55- 33.99]	[32.34 – 33.66]	[33.36 – 34.84]	[33.07 – 34.51]	[32.22 – 33.84]	[32.05 – 34.09]	[32.95 – 34.69]
Mean difference[1]	-	1.84	1.57	2.67	2.36	1.59	1.64	2.39
p-value	-	0.03	< 0.001	0.04	0.01	0.06	0.27	0.01

β-globin samples	MP96 (automated)	MP96 (manual)	MP32	OSM-A	OSM-B	TSM	"Bikop" method	Heat Extraction
Positives (sensitivity)	58 (100%)	51 (87.9%)	57 (98.3%)	58 (100%)	56 (96.6%)	56 (96,6%)	51 (87.9%)	51 (87.9%)
Mean ± σ	31.27 ± 4.31	35.62 ± 3.59	36.77 ± 3.14	36.12 ± 3.35	35.87 ± 3.29	35.5 ± 3.20	35.39 ± 3.32	35.88 ± 3.77
Range	[22 - 40]	[26 - 40]	[29 - 40]	[28 – 40]	[28 - 40]	[28 - 40]	[26 - 40]	[25 - 40]
95% IC	[30.16 – 32.38]	[34.70 – 36.54]	[35.96 – 37.58]	[35.26 – 36.98]	[35.02 – 36.72]	[34.68 – 36.32]	[34.53 – 36.25]	[34.91 – 36.85]
Mean difference[1]	-	4.35	5.5	4.84	4.6	4.23	4.12	4.61
p-value	-	0.006	< 0.001	< 0.001	< 0.001	< 0.001	0.01	< 0.001

[1] Difference between means of the manual and automatic methods.