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Evaluation of simple nucleic acid extraction methods for the detection of SARS-CoV-2 in nasopharyngeal and saliva specimens during global shortage of extraction kits

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ABSTRACT

Background: The severe shortage of nucleic acid extraction kits during the current COVID-19 pandemic represents a key limiting factor in testing capacity.
Objectives: This study compared the results of SARS-CoV-2 RT-PCR using different simple nucleic acid extraction methods on nasopharyngeal and saliva specimens.
Study design: Fifty nasopharyngeal swab and saliva specimens previously tested positive for SARS-CoV-2 were retrieved. Three different methods of nucleic acid extraction were compared. The first method involves incubating the specimen with proteinase K, and then heat treatment at 98 °C for 5 min (PKH); the second method involves heat treatment at 98 °C for 5 min without proteinase K pre-incubation (heat only); the third method involves no pre-processing steps (direct). The products from all 3 methods were tested by SARS-CoV-2 RT-PCR.
Results: PKH had significantly higher positive rate in SARS-CoV-2 RT-PCR (80 %) than those of heat only (58 %; P = 0.001) or direct (56 %; P = 0.002). The median Ct value was significantly earlier for PKH (median Ct: 37.0, IQR 31.7–40) than that of heat only (median Ct: 40, IQR 36.2–41; P < 0.0001) and direct (median Ct, 37.5; IQR 33.9–41.0; P = 0.0049). Subgroup analysis showed that PKH had higher detection rate, lower limit of detection and earlier Ct values than the other two groups for both NPS and saliva specimens.
Conclusions: PKH pre-processing resulted in the highest detection rate of SARS-CoV-2 by RT-PCR, and represents an alternative method for nucleic acid extraction when commercial extraction kits are not available.

1. Background

The rapid spread of SARS-CoV-2 has overwhelmed the healthcare system [1]. Early laboratory diagnosis allows prompt isolation of COVID-19 patients and quarantine of their close contacts to break the transmission chain [2,3]. Furthermore, early diagnosis and initiation of antiviral treatment can result in better patient outcome [4].

Most clinical laboratories use commercial kits to extract nucleic acid for reverse transcription-polymerase chain reaction (RT-PCR). However, during the COVID-19 pandemic, there is a severe shortage of nucleic acid extraction kits due to the sudden surge in demand, the reduced production capacity, and delays in shipments. Hence, alternative methods for nucleic acid extraction is urgently needed.

Fomsgaard et al. has recently shown that heating at 95 °C or 98 °C could achieve a sensitivity of about 95 % in the detection of SARS-CoV-2 for oropharyngeal swabs [5]. Sung et al. showed that proteinase K could enhance the detection of Middle East respiratory syndrome coronavirus (MERS-CoV) in sputum specimens that were spiked with inactivated virus [6].

2. Objectives

We assessed whether the combination of proteinase K incubation and heat treatment can enhance the detection of SARS-CoV-2 by RT-PCR.

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3. Study Design

3.1. Clinical specimens

This study included 50 specimens, including 25 nasopharyngeal swab (NPS) and 25 posterior oropharyngeal saliva specimens. NPS and saliva specimens were collected in viral transport medium as described previously [7,8]. For the initial testing, these specimens were extracted using NucliSENS easyMAG extraction system (BioMerieux, Marcy-l'Étoile, France) as described previously [9]. The study was approved by the Institutional Review Board of The University of Hong Kong/Hospital Authority Hong Kong West Cluster (UW 20-286).

3.2. Pre-RT-PCR specimen processing and real-time RT-PCR

This study evaluated 3 different protocols in specimen processing before RT-PCR. The first method involves the pre-treatment of specimen with proteinase K and heat (PKH). Proteinase K solution (Qiagen, Hilden, Germany) was added to NPS or saliva in 1:5 ratio, incubated for 15 min at 56 °C, followed by 5 min at 98 °C, and then cooled for 2 min at 4 °C. The second method involves only heat processing (heat only). Respiratory specimen was heated for 5 min at 98 °C, and then cooled for 2 min at 4 °C. For the third method, there was no pre-processing step before RT-PCR (direct). For all methods, 5 μL of the processed specimens were used for subsequent real time RT-PCR targeting SARS-CoV-2 RdRp-Hel gene as described previously [7,10]. To avoid potential confounding factors, each specimen was extracted with the three different methods at the same time, and real-time RT-PCR of all 3 processed specimens was performed in the same run.

3.3. Statistical analysis

Statistical analysis was performed using PRISM 6.0. The positive rates of each method were compared using McNemar's test. The Ct values were compared using ANOVA Friedman test with Dunn's multiple comparisons test. A Ct value of 41 was assigned to specimens that tested negative in the real-time RT-PCR assay. A P value of < 0.05 was considered statistically significant.

4. Results

The overall RT-PCR positive rate was significantly higher for PKH (80 % [40/50]) than those of heat only (58 % [29/50]; P = 0.001) and direct (56 % [28/50]; P = 0.002) (Table 1). Subgroup analysis showed that the positive rate for PKH remained to be the highest for either NPS or saliva when compared with other methods. In particular, for saliva specimens, the positive rate for PKH group (76 % [19/25]) was significantly higher than of direct (44 % [11/25]) (P = 0.021); for NPS, the positive rate for PKH group (84 % [21/25]) was significantly higher than of heat only (60 % [15/25]) (P = 0.0125).

Next we compared the Ct values of the 3 methods (Fig. 1). The median Ct value of PKH (37.0) was significantly earlier than heat only (40; P < 0.0001) or direct (37.5; P = 0.0049), but there was no significant difference between direct and heat only (P = 0.1072). For subgroup analysis of the NPS specimens, the median Ct value of PKH (32.1) was significantly earlier than heat only (40; P < 0.0001). For saliva specimens, the median Ct value of PKH (37.7) was significantly earlier than that of heat only (40; P = 0.0486) and of direct only (41; P = 0.040).

The limit of detection (LOD) of all 3 processing methods was evaluated using serially-diluted NPS and saliva specimens of a patient. For NPS, the LOD was 1:1000 for PKH, 1:10 for heat only, and 1:100 for direct (Table 2). For saliva, the LOD was 1:10000 for PKH, 1:100 for heat only and 1:1000 for direct.

Table 1
Comparison of different respiratory specimen processing steps.

	Extraction methods			P values		
	PKH	Heat only	Direct	PKH vs heat only	PKH vs direct	Heat only vs direct
All Samples (n = 50)						
Positive	40 (80)	29 (58)	28 (56)	0.001	0.002	1.000
Negative	10 (20)	21 (42)	22 (44)			
NPS (n = 25)						
Positive	21 (84)	15 (60)	17 (68)	0.031	0.0125	0.500
Negative	4 (16)	10 (40)	8 (32)			
Saliva (n = 25)						
Positive	19 (76)	14 (56)	11 (44)	0.063	0.021	0.453
Negative	6 (24)	11 (44)	14 (56)			

Data are expressed as no. (%).

5. Discussion

In this study, we found that the combination of proteinase K and heat (PKH group) had significantly higher positive rate than the heat only group and the direct group. The Ct values were also significantly earlier for the PKH group than either heat group or direct group, including both NPS and saliva specimens. The LOD was also significantly lower for PKH than either heat or direct.

Unlike previous studies, we evaluated the different extraction methods on posterior oropharyngeal saliva specimens. Saliva is a convenient non-invasive specimen type that is now gaining popularity during the COVID-19 pandemic. We and others have previously shown that saliva can be used for diagnosis and monitoring of viral load for SARS-CoV-2 and influenza viruses [4,7,8,11–13]. Saliva has also been used successfully in massive screening of SARS-CoV-2 in Hong Kong [14].

There are several mechanisms for which proteinase K can improve the extraction of nucleic acid from clinical specimens. First, proteinase K can digest RNase, which prevents the degradation of SAR-CoV-2 RNA in the NPS or saliva specimens [15]. Second, proteinase K can digest proteins in NPS or saliva which may lower the efficiency of RT-PCR reaction. Third, proteinase K can help to homogenize saliva specimens which may have been mixed with the more viscous secretions descending from the nasopharynx or ascending from the airway.

Although the sensitivity of PKH is lower than that of commercial extraction assays, there are several advantages of using PKH. First, proteinase K is widely available and the supply of proteinase K is not severely affected during the current pandemic. Second, extraction with PKH method is much cheaper than that of using commercial kits, an important factor for massive screening projects especially in developing counties. Third, PKH is technically simple. Fourth, the amount of original sample required is only 5 μL, and therefore important when the amount of original sample is limited.

A previous study by Fomsgaard et al. showed that heat treatment improved the detection of SARS-CoV-2 in oropharyngeal swab when compared with no treatment (direct) [5]. However, in our current study, the difference between heat only group and the direct group were not significant. The difference may be related to the RT-PCR assay used, as Fomsgaard et al. has shown that the type of RT-PCR assay can affect the results.

There are several limitations in this study. First, we evaluated NPS and saliva, but not lower respiratory tract specimens. Since the extraction efficiency can be affected by the specimen type, further evaluation on other specimen types should be conducted. Second, we only evaluated adult patients in this study. Third, we evaluated only proteinase K in this study because of the wide availability of this enzyme.

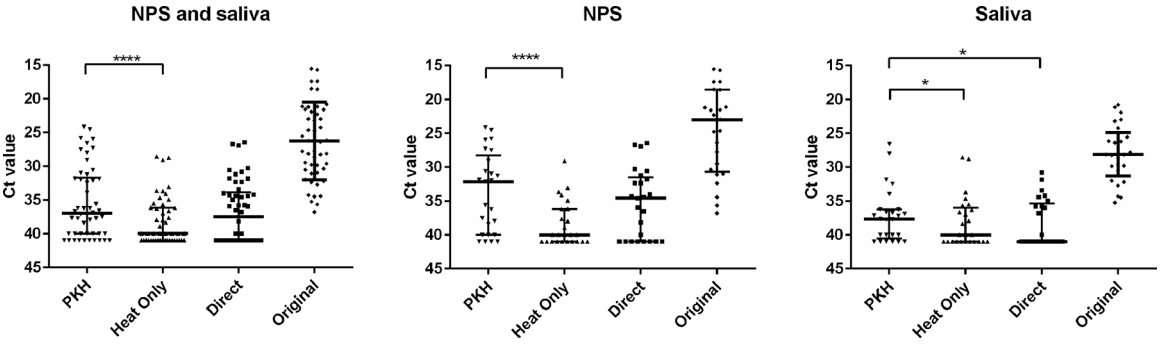

Fig. 1. Comparison of RT-PCR results from different nucleic acid extraction methods. The Ct values were compared between the PKH, heat only and direct groups. PKH: specimens pre-incubated with proteinase K for 15 min at 56 °C, followed by 5 min at 98 °C. Heat only: specimens were heated for 5 min at 98 °C. Direct: no pre-processing step before RT-PCR. Original: Total nucleic acid was extracted using NucliSENS easyMAG extraction system. *, < 0.05; ****, < 0.0001.

Table 2
Evaluation of the limit of detection (LOD) for the three processing methods using serially diluted NPS and saliva specimens from a COVID-19 patient.

NPS Sample									
Sample Dilution	PKH			Heat Only			Direct		
	Test 1	Test 2	Test 3	Test 1	Test 2	Test 3	Test 1	Test 2	Test 3
1:10	35.65	35.37	35.16	36.74	36.65	36.57	38.18	36.42	40.00
1:100	38.98	37.23	38.03	–	–	–	40.00	40.00	38.30
1:1000	37.77	40.00	40.00	–	–	–	–	–	–
1:10000	–	–	–	–	–	–	–	–	–
1:100000	–	–	–	–	–	–	–	–	–
Saliva Sample									
Sample Dilution	PKH			Heat Only			Direct		
	Test 1	Test 2	Test 3	Test 1	Test 2	Test 3	Test 1	Test 2	Test 3
1:10	31.81	32.20	32.27	35.29	35.05	34.88	33.11	32.85	31.88
1:100	36.13	36.91	36.75	38.46	40.00	40.00	37.79	36.09	35.78
1:1000	39.10	38.84	39.59	–	–	–	40.00	38.02	38.30
1:10000	40.00	40.00	40.00	–	–	–	–	–	40.00
1:100000	–	–	–	–	–	–	–	–	–

Data represent Ct values. The Ct values for the total nucleic acid of the undiluted NPS and saliva specimen extracted using NucliSENS easyMAG extraction system is 21.59 and 21.14, respectively.

The evaluation of other proteases and RNA protectors should also be considered.

We have demonstrated that the combination of proteinase K and heat pre-treatment significantly improved the detection of SARS-CoV-2 by RT-PCR when compared with heat only or no pre-treatment for NPS and saliva specimens. PKH is technically simple and require only reagents that are widely available and low cost. Hence, PKH would be a practical substitute for nucleic acid extraction when there is a significant shortage of commercially available extraction assays.

CRediT authorship contribution statement

Allen Wing-Ho Chu: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Data curation, Writing - original draft, Writing - review & editing. **Wan-Mui Chan:** Methodology, Investigation, Writing - review & editing. **Jonathan Daniel Ip:** Investigation, Writing - review & editing. **Cyril Chik-Yan Yip:** Validation, Formal analysis, Writing - review & editing. **Jasper Fuk-Woo Chan:** Writing - review & editing. **Kwok-Yung Yuen:** Resources, Writing - review & editing, Supervision. **Kelvin Kai-Wang To:** Conceptualization, Formal analysis, Data curation, Writing - original draft, Writing - review & editing, Supervision.

Declaration of Competing Interest

None.

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References

[1] J.F. Chan, S. Yuan, K.H. Kok, K.K. To, H. Chu, J. Yang, et al., A familial cluster of pneumonia associated with the 2019 novel coronavirus indicating person-to-person transmission: a study of a family cluster, Lancet (2020), https://doi.org/10.1016/

S0140-6736(20)30154-30159.
[2] V.C.C. Cheng, S.C. Wong, K.K.W. To, P.L. Ho, K.Y. Yuen, Preparedness and proactive infection control measures against the emerging novel coronavirus in China, J. Hosp. Infect. 104 (2020) 254–255.
[3] J. Peto, Covid-19 mass testing facilities could end the epidemic rapidly, BMJ 368 (2020) m1163.
[4] I.F. Hung, K.C. Lung, E.Y. Tso, R. Liu, T.W. Chung, M.Y. Chu, et al., Triple combination of interferon beta-1b, lopinavir-ritonavir, and ribavirin in the treatment of patients admitted to hospital with COVID-19: an open-label, randomised, phase 2 trial, Lancet (2020), https://doi.org/10.1016/S0140-6736(20)31042-31044.
[5] A.S. Fomsgaard, M.W. Rosenstierne, An Alternative Workflow for Molecular Detection of SARS-CoV-2 - Escape From the NA Extraction Kit-shortage, Copenhagen, Denmark, March 2020. Euro Surveill (2020), p. 25.
[6] H. Sung, D. Yong, C.S. Ki, J.S. Kim, M.W. Seong, H. Lee, et al., Comparative evaluation of three homogenization methods for isolating middle east respiratory syndrome coronavirus nucleic acids from sputum samples for real-time reverse transcription PCR, Ann. Lab. Med. 36 (2016) 457–462.
[7] K.K. To, O.T. Tsang, W.S. Leung, A.R. Tam, T.C. Wu, D.C. Lung, et al., Temporal profiles of viral load in posterior oropharyngeal saliva samples and serum antibody responses during infection by SARS-CoV-2: an observational cohort study, Lancet Infect. Dis. (2020), https://doi.org/10.1016/S1473-3099(20)30196-1.
[8] K.K. To, O.T. Tsang, C. Chik-Yan Yip, K.H. Chan, T.C. Wu, J.M.C. Chan, et al., Consistent detection of 2019 novel coronavirus in saliva, Clin. Infect. Dis. (2020), https://doi.org/10.1093/cid/ciaa149.
[9] C.C. Yip, C.C. Ho, J.F. Chan, K.K. To, H.S. Chan, S.C. Wong, et al., Development of a novel, genome subtraction-derived, SARS-CoV-2-Specific COVID-19-nsp2 real-time RT-PCR assay and its evaluation using clinical specimens, Int. J. Mol. Sci. (2020) 21.
[10] J.F. Chan, C.C. Yip, K.K. To, T.H. Tang, S.C. Wong, K.H. Leung, et al., Improved molecular diagnosis of COVID-19 by the novel, highly sensitive and specific COVID-19-RdRp/Hel real-time reverse Transcription-PCR assay validated in vitro and with clinical specimens, J. Clin. Microbiol. (2020) 58.
[11] K.K.W. To, C.C.Y. Yip, C.Y.W. Lai, C.K.H. Wong, D.T.Y. Ho, P.K.P. Pang, et al., Saliva as a diagnostic specimen for testing respiratory virus by a point-of-care molecular assay: a diagnostic validity study, Clin. Microbiol. Infect. 25 (2019) 372–378.
[12] E. Williams, K. Bond, B. Zhang, M. Putland, D.A. Williamson, Saliva as a non-invasive specimen for detection of SARS-CoV-2, J. Clin. Microbiol. (2020), https://doi.org/10.1128/JCM.00776-20.
[13] E. Pasomsub, S.P. Watcharananan, K. Boonyawat, W. Suksuwan, S. Sungkanuparph, A. Phuphuakrat, Saliva sample as a non-invasive specimen for the diagnosis of coronavirus disease-2019 (COVID-19): a cross-sectional study, Clin. Microbiol. Infect. (2020), https://doi.org/10.1016/j.cmi.2020.05.001.
[14] The Hong Kong SAR Government, Home Quarantine for Inbound Travellers - Frequently Asked Questions, (2020) Available at https://www.coronavirus.gov.hk/eng/inbound-travel-faq.html Accessed on May 16, 2020.
[15] M.F. Carey, C.L. Peterson, S.T. Smale, The RNase protection assay, Cold Spring Harb. Protoc. 2013 (2013) pdb.prot071910.