クルーズ船でアウトブレイクしたCOVID-19症例の浜松医療センターでの受け入れ、COVID-19症例の自然経過とウイルス排泄に関する記述と当時世界が認めていなかった唾液検体を用いたPCR検査の従来法との性能評価とその可能性についての報告論文

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【研究内容】

【目的】2020 年 2 月において COVID-19 の診断には、検体採取時にスワブと採取者のＰＰＥを必要とし、医療従事者へのウイルス曝露も生じるに鼻咽頭ぬぐい液検体が必要とされ、診断まで 24 時間必要となるＰＣＲ検査がゴールデンスタンダードであった。スワブやＰＰＥが枯渇していくことは予測され、今後診断が難しくなることが予測された。この欠点を補う目的にて、2020 年 2 月世界がその有用性に注目していなかった唾液検体を用いた、短時間で実施可能なＲＴ-ＰＣＲ検査の開発とその有用性を記述することを目的とした。

【方法】2020 年 2 月 12 日当院へ COVID-19 の診断にてクルーズ船から受け入れを実施した 71 歳男性において、書面にて同意書得、退院基準のための咽頭もしくは鼻咽頭検体にて検査を実施する同日に唾液検体を採取した。なお唾液検体の適切な採取時間を決定するために、入院初期は日中に唾液を採取し、後期には早朝に唾液を採取し、唾液検体の採取時間による違いを比較検討した。基準結果となる咽頭もしくは鼻咽頭スワブは、衛生研究所へ提出し、その PCR 結果を確認した。これとは別に唾液検体は、検体抽出に複雑な機器を使用せず、1 検体あたり 5 分未満でＲＮＡ抽出が可能である Sugar chain-immobilized magnetic gold Nanoparticle 法（以下 SMGNP 法）にて、ＲＮＡ抽出を実施した。抽出した RNA は、鹿児島大学へ冷凍もしくは冷蔵にて輸送し、衛生研究所の結果は未告知にて検査を実施し、その結果を送ってもらい確認した。

【結果】患者は、発症 11 日後には無症状となったが、鼻咽頭検体を用いた PCR 法では、発症から 42 日まで陽性が確認され、唾液から SMGNP 法で抽出した検体では、発症から 37 日まで陽性が確認された。鼻咽頭検体を用いた PCR 方法を標準結果とした場合、日中唾液検体を用いた SMGNP 法での感度は 25%（2/8）、特異度は 100%（1/1）であった。一方、早朝唾液検体を用いた SMGNP 法での場合の感度は 66.7%（4/6）、特異度 100%（4/4）であった。以上から、無症状となった後であっても COVID-19 患者では、PCR 検査が陽性となること、スワブを使用せず患者にとって非侵襲的で医療従事者も検体採取時にウイルス曝露がない唾液検体を用いた簡易で短時間に抽出可能な SMGNP 法での RT-PCR 法にて COVID-19 診断ができる可能性が示唆された。

【考察】唾液検体の基準検体との一致率が日中と早朝で異なった理由には以下のことが考えられる。第一の要因は、唾液検体中のウイルス量の違いである。睡眠中は、唾液流出の停止により口腔内のウイルスクリアランスが低下し、その結果、早朝の唾液検体中のウイルス量が増加する可能性がある。さらに、早朝の唾液は喀痰で汚染されやすくなり、その結果、唾液検体中ウイルス量が増加する可能性がある。第二の要因は唾液検体の量である。ウイルスが唾液中に一様に存在する場合、唾液の量が多くなると、濃縮された検体中のウイルス量が増加することになる。しかし、SMGNP 濃度と RNA 抽出には 600mL の唾液検体を日中、早朝検体とも同容量使用したため、第二の要因は無視できる。第三の要因は、異なる採取時期の唾液検体における RT-PCR 反応に対する阻害剤の存在である。PCR 阻害物質としては、有機化合物、ヘモグロビン、タンパク質、IgG、食品、カルシウムなどが知られている。これらの物質が採取した唾液検体中に存在する場合、検出感度が低下する。例えば、ヘモグロビンは歯磨き後の唾液中に検出されることが知られており、歯磨き後の唾液検体では PCR 阻害が生じえる。また一般的に食品中には、様々な PCR 阻害物質が存在する。よって食後の唾液検体では、食品由来の阻害物質が存在する可能性があり、検出率が低下しえる。以上のことから、朝食前や歯磨き前に採取された早朝唾液は、日中唾液検体よりも多くのウイルスを含み、阻害剤が少ない可能性がある。本原稿の知見は、先行研究で示唆されているように、早朝唾液を使用することの妥当性を裏付ける可能性がある。

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Case Report

A case report of SARS-CoV-2 confirmed in saliva specimens up to 37 days after onset: Proposal of saliva specimens for COVID-19 diagnosis and virus monitoring*

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We present the case of a 71-year-old man who, despite becoming asymptomatic after having some mild symptoms of COVID-19, had SARS-CoV-2 RNA detected for 37 days after onset, from his concentrated and purified saliva specimens using sugar chain-immobilized gold nanoparticles. It was suggested that the early morning saliva specimens were more likely to show positive results than those obtained later in the day.

1. Introduction

COVID-19 was reported in Wuhan, China on December 31, 2019, and spread around the world quickly. On March 11, 2020, the WHO declared it to be a pandemic [1]. The current definitive diagnosis of COVID-19 is mainly performed using real-time reverse transcription polymerase chain reaction (RT-PCR) from lower respiratory tract specimens or nasopharyngeal specimens [2]. Virus monitoring is performed on confirmed cases, and hospital discharge requires two days' consecutive negative confirmation of nasopharyngeal specimens [3]. Despite this monitoring standard being widely used globally, it has the following disadvantages: healthcare workers being exposed to the virus during specimen collection, thus creating a need for personal protective equipment (PPE) despite the current shortage of medical resources, and the performance of uncomfortable or invasive procedures on patients. To overcome these disadvantages, we came up with an RT-PCR test using saliva specimens, which were pre-treated with sugar chain-immobilized magnetic gold nanoparticles (SMGNP) to concentrate and purify virus particles at a rate of 5 min for one specimen. In evaluating RT-PCR using saliva specimens, we found the appropriate timing to collect saliva specimens, and present a case in which viral RNA was detected in saliva specimens for 37 days after onset. Our report contributes to knowledge of virus shedding and alternative testing methods.

2. Case report

On February 12, 2020, a Japanese man aged 71 years with only a history of allergic rhinitis was transported to our hospital from a cruise ship with an outbreak of COVID-19, anchored in Yokohama for quarantine. He had been on the cruise ship since January 20,

* Yasuhisa Tajima designed the study, and drafted the work. Yasuo Suda developed RT-PCR for SARS-CoV-2 using saliva specimens and did all RT-PCR using saliva specimens. Yasuo Suda and Kunio Yano revised the work critically for important intellectual content. All authors contributed to the acquisition, analysis, or interpretation of data for the work. All authors have given the final approval of the version to be published and agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy of any part of the work are appropriately investigated and resolved.

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2020. He complained of body aches on February 5. On February 7, his temperature reached 37.5 °C. RT-PCR was performed, and on February 9, COVID-19 was confirmed. When he came to our hospital, his vital signs were within normal range and his laboratory results were quite normal. He had a dry cough and nasal discharge but his functions were otherwise normal. He was hospitalized for follow-up and confirmation of a RT-PCR negative result for SARS-CoV-2. On February 13, we received his written informed consent to participate in a study to establish an alternative and rapid diagnostic method using saliva specimens. This study was approved by the Institutional Review Board of Hamamatsu Medical Center (2019-122) based on the Ethical Guidelines for Medical Research Targeting Humans, provided by the Japanese Ministry of Health, Labor and Welfare. Saliva specimens were collected on the same day as the oropharyngeal or nasopharyngeal specimens submitted to the National Institute of Infectious Diseases (NIID) for viral monitoring. To determine the best time for obtaining the saliva specimens, daytime saliva specimens (DSS) were collected until March 1 and early morning saliva specimens (EMSS) were collected from March 3. We gave him a collection container marked with a 600-mL line the day before his submitting saliva specimens. Saliva specimen collections were carried out by him alone, spitting saliva up to the marked line, which was confirmed by the nurse especially in the case of EMSS. We concentrated and purified virus particles from 600mL of his saliva specimens using SMGNP, and extracted the RNA. SMGNP is composed of iron and gold of about 5 nm size, immobilized with sugar chain (sulfated oligosaccharide), to which the virus binds. The following procedure was used according to the previously established method with modification [4e 6]. When SMGNPs are added to the viral solution, SMGNPs adsorbs on the surface of the viral particles via the sugar chain to capture the viruses. A secondary solution of magnetic microparticles (MMPs, size: about 1 mm) composed of iron were added to the solution to collect the SMGNP-captured viruses. Then, magnetic separation was carried out to obtain the SMGNP-virus-MMP complex, in which viral particles were separated from the viral solution. Finally by adding a detergent (0.1% sodium lauryl sulfate aqueous solution) to the complex, the viral RNA was eluted. Since the viral particles were purified during the separation step, it was possible to directly apply the RT-PCR without further purification. The extracted RNA was cryopreserved (from February 13 to March 1, 2020) or refrigerated (from March 3 to 20), and sent to Kagoshima University to perform RT-PCR (intercalation method) using Centers for Disease Control and Prevention (CDC)-proposed primer sets with a slight modification (Forward primer: GACCC-CAAAATCAGCGAAATG, Reverse primer: ATGTTGAGTGA-GAGCGGTG) [7]. The assays at Kagoshima University (KU) were all done without any information about the NIID RT-PCR results.

Although the patient looked healthy and had only body aches, a one-day-fever, and dry cough lasting several days, he had positive RT-PCR from NIID until day 42 after onset. His EMSS was positive up to day 37, and changed to negative on day 39. On day 45, he received 2 days' consecutive negative RT-PCR NIID results based on his nasopharyngeal specimens, and was discharged in good health (Fig. 1).

3. Discussion

The WHO has claimed that virus shedding patterns are not yet well understood and further investigations are needed to better understand the timing, compartmentalization and quantity of viral shedding to inform optimal specimen collection [2]. No mention was made of saliva specimens. The use of saliva specimens for diagnosis or virus monitoring has several advantages: reducing virus exposure to healthcare workers, saving PPE and collection time, and being non-invasive.

The detection of SARS-CoV-2 in saliva has been reported [8,9]. The study also reported using saliva collected in the early morning, but it was not stated whether these samples were compared with specimens collected later in the day [9]. In our case, saliva specimens collected during the day had a lower rate of positive concordance when compared to NIID results. For DSS, the sensitivity was 25.0%(2/8) and the specificity was 100%(1/1) based on NIID results. In contrast, when the EMSS were used, the results came close to matching the NIID results of RT-PCR performed on the nasopharyngeal specimens (Fig. 1). The number of the EMSS was small, but the sensitivity based on NIID results was 66.7%(4/6) and the specificity was 100%(4/4).

There may be several factors affecting the different detection rates relating to the time of saliva specimens collection. The first factor is the difference in the amount of virus in the saliva specimens. During sleep, the cessation of salivary outflow can result in a decrease in oral viral clearance, resulting in an increase in viral load in salivary specimens in early morning saliva [10]. In addition, early morning saliva may be more likely to be contaminated with sputum, resulting in an increased viral load in the saliva specimens [9]. The second factor is the volume of the saliva specimens. If the viruses are uniformly present in the saliva, a higher volume of saliva would increase the amount of virus in the concentrated specimen. However, since we uniformly used 600mL saliva specimens for the SMGNP concentration and RNA extraction, the second factor can be ignored. The third factor is the variation of inhibitory agents on RT-PCR reaction in saliva specimens at different collection times. Known inhibitors for PCR include organic compounds, hemoglobin, protein, IgG, food, and calcium [11]. Detection sensitivity would be reduced if these substances are present in the collected saliva specimens. For example, hemoglobin is known to be detected in saliva after tooth brushing [12], and PCR inhibition can occur in saliva specimens after tooth brushing. In general, a variety of PCR inhibitors are found in foods [11]. In post-meal saliva specimens, food-derived inhibitors may be present, resulting in a reduced detection rate. Based on the above considerations, EMSS collected

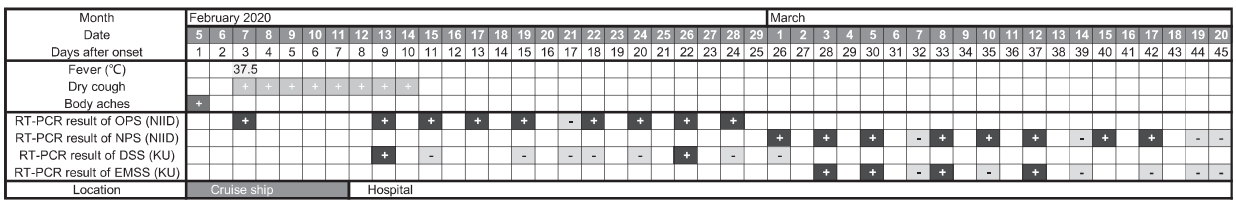

Fig. 1. Symptoms and RT-PCR results of the patient with COVID-19 from onset to discharge. Two saliva specimens on day13 (February 17) and day 40 (March 15) had not been submitted. Abbreviations; OPS: oropharyngeal specimens; NPS: nasopharyngeal specimens; DSS: daytime saliva specimens; EMSS: early morning saliva specimens; NIID: The National Institute of Infectious Diseases; KU: Kagoshima University.

before breakfast and tooth brushing have the potential to contain more virus and fewer inhibitory agents than DSS. The finding of this manuscript may support the validity of using EMSS, as suggested in another study [9].

The results of the last four saliva specimens point towards an appropriate collection method. It can be suggested that virus monitoring after definitive diagnosis should be performed with EMSS concentrated and purified using SMGNP, and then performed with a nasopharyngeal specimen after the EMSS produces negative results.

For the virus shedding period, viral RNA was detected up to 25 days after symptom onset in previous reports [9]. For specimens other than saliva, it has been reported that virus RNA was detected up to 37 days after onset [13]. In our case, viral RNA was confirmed for a longer period than these reports. Kelvin Kai-Wang To et al. reported that the higher the initial viral load, the longer the detection period, and that older individuals tended to have higher peak viral loads [9]. Alraddadi BM et al. reported that in MERS-CoV person with allergic rhinitis had a relative risk of 2.21 for infection [14]. Our case was elderly and had allergic rhinitis, which may be the reason why the viral RNA was confirmed over a longer period of time. In COVID-19 cases with allergic rhinitis, it is necessary to verify whether the virus excretion period is prolonged.

One of the prospects for saliva as a diagnostic specimen is its application to rapid antigen testing. Previous studies have estimated that coronavirus levels in saliva specimens are as low as 1 in 10e1000 compared to nasopharyngeal or lower respiratory tract specimens [9,15]. The detection sensitivity of rapid antigen testing has been reported to be between 10^5 and 10^7 copies/mL for influenza diagnosis, which is 10^2 to 10^4 times less sensitive than PCR [16]. Therefore, rapid antigen testing using saliva specimens is considered impractical due to its low sensitivity.

Our case presented: First, when using saliva specimens for virus monitoring, early morning specimens should be used. Second, EMSS concentrated and purified using SMGNP may be an alternative method for virus monitoring, when followed up with nasopharyngeal specimens. Third, it is possible that the virus can be detected in saliva for 37 days after onset, even after the patient becomes asymptomatic.

There are some limitations to our case. Firstly, the comparison standards for DSS and EMSS are different. In order to compare the sensitivity and specificity, the same standard test must be compared. However, the specimens submitted to NIID were changed in the middle of the process, and most of the DSS specimens were compared to oropharyngeal specimens (8/9), and all of the EMSS specimens were compared to nasopharyngeal specimens (10/10). When comparing nasopharyngeal specimens and oropharyngeal specimens, it has been reported that nasopharyngeal specimens may have a higher viral load [17]. It has also been reported that the viral load tends to decrease in excretion over time from onset [9]. In other words, EMSS was compared to a more sensitive standard test with a lower viral load than DSS was. Therefore, we can state that the efficacy of EMSS is not overestimated in comparison to DSS. Second, 4e6 days elapsed between virus extraction and RT-PCR of the saliva specimens, which may have resulted in RNA disruption and reduced sensitivity compared to NIID results obtained by RT-PCR on the day of specimen collection or the next day.

Ethics approval and consent to participate

This study was approved by the Institutional Review Board of Hamamatsu Medical Center (2019-122) based on the Ethical Guidelines for Medical Research Targeting Humans provided by the Japanese Ministry of Health, Labor and Welfare.

Consent for publication

We received the patient's written informed consent. A copy of the written consent is available for review by the Editor of this journal.

Availability of data and materials

The datasets used for the current study are available from the corresponding author on reasonable request.

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Declaration of Competing interest

The authors: No reported conflict of interest except Dr. Suda. Dr. Suda reports other from SUDx-Biotec Corporation, during the conduct of the study; grants from Japan Ministry of Agriculture, Forestry and Fisheries, grants from Japan Agency for Medical Research and Development, outside the submitted work; In addition, Dr. Suda has a US patent #9464281 licensed and Dr. Suda is also a president of SUDx-Biotec Corporation. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest.

The Individuals included in acknowledgements: Bo reported as an employee of Kagoshima University with a remit to support the internationalization of the university, she received no payment or services for her contribution to this paper, which was limited to proofreading for grammatical errors and providing stylistic suggestions. Arima reported she is an employee of SUDx-Biotec Corporation. Regarding this paper, she helped the PCR assay, however, did not receive any additional salary. All individuals included in acknowledgements have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest.

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