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Evaluation of saliva as diagnostic materials for influenza virus infection by PCR-based assays

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ABSTRACT

Background: Immunochromatographic antigen tests have been widely used for detection of influenza virus; however its low sensitivity restricts the use of clinical materials other than nasopharyngeal swabs. Saliva is obtained non-invasively and has utility for diagnosis of influenza. Polymerase chain reaction (PCR) is not typically used for rapid testing because it is time consuming. We evaluated the utility of saliva as diagnostic materials for influenza virus infection by PCR-based assays.
Methods: Nasopharyngeal swabs and saliva were simultaneously collected from 144 patients and investigated by reverse transcription-quantitative PCR (RT-qPCR) and droplet-RT-PCR.
Results: Overall concordance of results from nasopharyngeal swabs and saliva were 95.8%. Influenza gene was detectable in less than 12 min in saliva by the droplet-RT-PCR. Saliva as well as nasopharyngeal swabs contained more than 1×10^2 copies/μl of the influenza gene. About half of the patients provided positive results in nasopharyngeal swabs and saliva within 24 h from the onset of the symptoms.
Conclusion: The study demonstrates that saliva can be used as an alternative specimen source to nasopharyngeal swabs. When rapid PCR assay including RNA extraction to be full-automation in a miniaturized machine, point-of-care test based on PCR may be realized using saliva without restriction of materials.
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1. Introduction

Influenza virus causes acute febrile respiratory infection with severe illness and life-threatening complications, especially in young children, elderly adults, and immunocompromised patients [1,2]. Even outside these vulnerable populations, the extent of the infection during epidemic outbreaks leads to increased workplace absenteeism, thereby leading to a dramatic impact on economies [3]. The ability to rapidly diagnose influenza infections is critical for early clinical treatment and isolation of patients.

Immunochromatographic antigen (IC) tests are widely used in clinical laboratories to detect the influenza viral nucleoprotein; however, the low sensitivity of the IC test is a major problem for influenza diagnosis in the early stages of infection [4]. On the other hand, detection of genomic RNA by polymerase chain reaction (PCR) analyses is the gold standard for identifying and classifying influenza virus [5,6].

Most influenza viruses infect the respiratory tract and replicate productively in the airway epithelial cells, including the nasopharynx [7,8]. Nasopharyngeal specimens are generally used for isolation of influenza virus [9–11], though saliva can be sampled more easily than nasopharyngeal swabs. Such a non-invasive test, particularly for children, would provide potentially valuable materials for detection of the influenza virus by reverse transcription-quantitative PCR (RT-PCR) [12–14].

RT-PCR is one of the most sensitive methods for detecting the presence of RNA, and various samples, including saliva, can be subjected to RT-PCR analysis. This makes RT-PCR a valuable tool for the diagnosis of influenza virus infections if the turnaround time of the PCR-based assay is improved. We previously reported the sensitivity of the droplet-RT-PCR for influenza virus detection was similar to the conventional RT- quantitative PCR (RT-qPCR) [15]. RT-qPCR is as sensitive for influenza detection as viral culture isolation is [16,17], making droplet-RT-PCR potentially one of the most reliable methods for the detection of influenza virus. PCR performed in a small volume can achieve efficient amplification while retaining specificity, as exemplified by emulsion PCR, in which the reaction mixtures are compartmentalized [18].

In this study, we evaluated the utility of saliva as diagnostic materials for influenza virus infection using the conventional RT-qPCR and the high-speed droplet-RT-PCR.

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2. Materials and methods

2.1. Sample collection

Nasopharyngeal swabs and saliva were obtained simultaneously from 144 patients who had provided informed consent. The saliva was collected via dropper. The study population included 64 female (mean age: 39.5 years old, range 24–62) and 80 male (mean age: 41.6 years old, range 27–63) individuals. Patients enrolled in this study were selected based-on the following influenza-like symptoms; fever, cough, headache, sore throat, myalgia, congestion, malaise, and chills and were subjected to the immunochromatographic antigen (IC) tests (ESPLINE Influenza A & B-N, Fujirebio Inc., Tokyo, Japan) of nasopharyngeal swabs in Shinshu University Hospital from January 2012 through March 2014. The IC tests using the nasopharyngeal swabs diagnosed 24 patients as having influenza A or B virus. Five control samples were obtained from normal individuals without influenza-like symptoms. This study was approved by the Institutional Review Board of Shinshu University (no. 1785).

2.2. RNA extraction

RNA was extracted from the nasopharyngeal swabs suspended into sterile PBS (70 μL) and saliva (140 μL) according to the collection volume of each sample using the QIAamp viral RNA Mini kit (Qiagen, Valencia, CA) according to the manufacturer's instructions.

2.3. One-step high speed droplet-RT-PCR

The primers and TaqMan probes for universal influenza A (H1N1) and B virus were used according to the Centers for Disease Control and Prevention (CDC) protocol [5,6] as follows: influenza A: forward primer, 5′-GACCRATCCTGTCACCTCTGAC-3′, reverse primer, 5'-AGGGCATTYTGGACAAAKCGTCTA-3′, and probe, 5′-FAM-TGCAGTCCTC GCTCACTG GGCACG-BHQ1-3′; influenza B: forward primer, 5′-TCCTCA ACTCACTCTTCGAGCG-3′, reverse primer, 5′-CGGTGCTCTTGACCAAAT TGG-3′, and probe, 5′-FAM-CCAATTCGAGCAGCTGAAACTGCGGTG-BHQ1-3′. One-step real-time RT-PCR was performed using the droplet-PCR machine (Seiko Epson, Matsumoto, Japan) [16]. The RT-PCR mixture contained template RNA, SuperScript III/Platinum Taq Mix (Life Technologies, Grand Island, NY), 800 nmol/L of each primer, designed as above, 200 nmol/L TaqMan probe, and reaction buffer composed of Tris–HCl, pH 9.0, KCl, and $MgCl_2$, in a total volume of 5 μL. One microliter of the reaction mixtures was used for the one-step droplet-RT-PCR assay. The reaction conditions used for Influenza A were: RT at 50 °C for 1 min, RT inactivation at 98 °C for 10 s, and 50 cycles of 98 °C for 3 s and 58 °C for 6 s. The reaction conditions used for Influenza B were: RT at 50 °C for 1 min, RT inactivation at 98 °C for 10 s, and 50 cycles of 98 °C for 5 s and 60 °C for 8 s. All samples were analyzed in duplicate. We determined that the influenza gene was present when the amplification exceeded 10^1 copies per reaction at 40 cycles.

2.4. Conventional RT-qPCR

The conventional RT-qPCR was performed with QuantStudio 12 K flex systems (Life Technologies, Carlsbad, CA) using the Superscript III One-Step qRT-PCR kit (Life Technologies, Grand Island, NY). The RT-qPCR reaction mixture (25 μL) comprised 5 μL template RNA, 0.5 μL SuperScript III/Platinum Taq Mix, 12.5 μL 2× Reaction Mix with ROX, 800 nmol/L of each primer, and 200 nmol/L of TaqMan probe. The reaction conditions were as follows: initial denaturation at 94 °C for 5 min, followed by RT at 50 °C for 30 min, 95 °C for 2 s for inactivation of RT, and 50 cycles of 95 °C for 15 s and 55 °C for 30 s.

The inputs for the standards were known concentrations (copies/μL) of plasmids (10^1–10^6) carrying the relevant 100 bp-regions of the influenza A (H1N1) and B genome for PCR amplification. The standard curves were calculated as equivalent numbers of influenza A or B amplicons. A standard curve was generated for each assay to validate the reaction conditions and calculate the number of amplicoms. All samples were analyzed in triplicate.

3. Results

3.1. Influenza gene in the nasopharyngeal swabs and saliva by the PCR-based assays

The conventional RT-qPCR and droplet-RT-PCR provided the completely-consistent results from the nasopharyngeal swabs and saliva (Table 1). Among the 144 patients, 28 and 110 were positive or negative in both samples, while 4 and 2 patients were only positive nasopharyngeal swabs or saliva, respectively (Table 1). The overall concordance of the results from both samples was 95.8% (Table 1). In both nasopharyngeal swabs and saliva from patients, the droplet-RT-PCR method was able to detect influenza A within 8.6 min at 40 cycles and influenza B within 11.3 min at 40 cycles (Fig. 1). On the other hand, no amplification was detected in 5 control samples obtained from normal individuals without influenza-like symptoms.

3.2. Quantitative evaluation of influenza gene in the nasopharyngeal swabs and saliva

The quantity of the influenza gene in the nasopharyngeal swabs and saliva was evaluated by the conventional RT-qPCR. Nasopharyngeal swabs or saliva positive for influenza virus infection contained more than 1×10^2 copies/μl of the influenza A or B gene (Fig. 2). The 2 patients negative for the virus in nasopharyngeal swabs had 6×10^3 and 8×10^3 copies/μl in their saliva, respectively, whereas the 4 patients with saliva negative for the virus had 2×10^3–8×10^4 copies/μl in their nasopharyngeal swabs (Fig. 2).

3.3. Early detection of influenza a virus in the nasopharyngeal swabs and saliva

We determined the positive rate of influenza A detection in nasopharyngeal swabs and saliva from 23 patients available for information on the time from the onset of clinical symptoms. Within 24 h, 50% of the nasopharyngeal swabs and 55% of saliva were found positive for influenza gene, which were confirmed by either the RT-qPCR or droplet-RT-PCR. (Fig. 3).

4. Discussion

In this study, we showed that saliva could be used for the diagnosis of influenza virus infection. High degree of result concordance was obtained from the nasopharyngeal swabs and saliva. The droplet-RT-PCR assay could amplify influenza A or B virus in the saliva as well as in nasopharyngeal swabs in less than 12 min. Nasopharyngeal swabs or throat swabs samples have been commonly used for IC tests because they have a higher concentration of influenza virus than is found in other samples [9–11]. The previous study suggested that the saliva was potentially valuable for diagnosis of the respiratory virus infection disease because the viruses exist in saliva [13,19,20]. Our study

Table 1
Detection of Influenza A or B virus in nasopharyngeal swabs and saliva using conventional RT-qPCR and droplet-RT-PCR.

PCR-based methods	Nasopharyngeal swabs (+)		Nasopharyngeal swabs (−)	
	Saliva (+)	Saliva (−)	Saliva (+)	Saliva (−)
Conventional RT-qPCR	28	4	2	110
Droplet-RT-PCR	28	4	2	110

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