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Comparison between Saliva and Nasopharyngeal Swab Specimens for Detection of Respiratory Viruses by Multiplex Reverse Transcription-PCR

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ABSTRACT Nasopharyngeal swabs (NPSs) are being widely used as specimens for multiplex real-time reverse transcription (RT)-PCR for respiratory virus detection. However, it remains unclear whether NPS specimens are optimal for all viruses targeted by multiplex RT-PCR. In addition, the procedure to obtain NPS specimens causes coughing in most patients, which possibly increases the risk of nosocomial spread of viruses. In this study, paired NPS and saliva specimens were collected from 236 adult male patients with suspected acute respiratory illnesses. Specimens were tested for 16 respiratory viruses by multiplex real-time RT-PCR. Among the specimens collected from the 236 patients, at least 1 respiratory virus was detected in 183 NPS specimens (77.5%) and 180 saliva specimens (76.3%). The rates of detection of respiratory viruses were comparable for NPS and saliva specimens ($P = 0.766$). Nine virus species and 349 viruses were isolated, 256 from NPS specimens and 273 from saliva specimens ($P = 0.1574$). Adenovirus was detected more frequently in saliva samples ($P < 0.0001$), whereas influenza virus type A and human rhinovirus were detected more frequently in NPS specimens ($P = 0.0001$ and $P = 0.0289$, respectively). The possibility of false-positive adenovirus detection from saliva samples was excluded by direct sequencing. In conclusion, neither of the sampling methods was consistently more sensitive than the other. We suggest that these cost-effective methods for detecting respiratory viruses in mixed NPS-saliva specimens might be valuable for future studies.

KEYWORDS saliva, respiratory virus, RT-PCR, nasopharyngeal swab

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Detection of viral pathogens in respiratory illnesses can provide valuable information to direct the proper management of patients and to prevent nosocomial transmission. Although various traditional diagnostic methods, such as direct antigen assays and viral cultures, are used for respiratory virus (RV) detection, nucleic acid amplification tests (NAATs) are thought to be superior in many respects, including sensitivity, specificity, time to virus identification, and range of pathogens detected (1–3).

It is generally thought that nasopharyngeal specimens are optimal for detecting RVs, particularly when conventional methods are used (4). Currently, for adult patients, multiplex real-time reverse transcription (RT)-PCR assays using nasopharyngeal swabs (NPSs) are widely applied. However, acquiring NPSs is not as easy as obtaining other types of specimens, such as saliva specimens; this may result in suboptimal specimens, particularly if specimens are obtained by inexperienced personnel. Moreover, the

TABLE 1 Demographic features and clinical presentations of the participants ($n = 236$)

Characteristic	Finding
Age (yr)	
Median	22
Range	20–26
Male (no. [%])	236 (100)
Symptoms (no. [%])	
Cough	178 (75.4)
Sputum	168 (71.2)
Tonsil enlargement	44 (18.6)
Rhinorrhea	166 (70.3)
Sore throat	73 (30.9)
Headache	16 (6.8)
Fever	3 (1.3)

procedure for obtaining NPS specimens causes coughing in most patients. Coughing can cause the production of airborne particles containing infectious virus and thus may increase the risk of nosocomial spread of RVs such as influenza virus (INF) (5).

Unlike direct antigen assays, NAATs do not require the presence of infected cells or high concentrations of viruses, which suggests that other types of specimens, such as saliva specimens, can yield comparable results. NPSs and other types of specimens, such as oropharyngeal swab (OPS), sputum, and saliva specimens, as samples for RT-PCR have been compared in a number of studies (6–9). The purpose of this study was to assess the comparability of saliva samples (the easiest samples to obtain) and NPSs as specimens for multiplex RT-PCR for RV detection.

RESULTS

Demographic characteristics and clinical presentations of the enrolled 236 patients are shown in Table 1. The patients were all male recruits 20 to 26 years of age, and most had a history of cough (75.4%), sputum (71.2%), and rhinorrhea (70.3%).

Patient distribution according to the number of isolated viruses is shown in Table 2. Among the 236 patients, 204 (86.4%) were identified, by either of the sampling methods, as being infected with at least 1 RV. The detection rate with NPSs was 77.5% (183 of 236 patients), and that with saliva samples was 76.3% (180 of 236 patients). The difference between the two detection rates was not statistically significant ($P = 0.766$, by McNemar's test). Coinfection with multiple viruses was identified in NPSs for 54 patients, in saliva samples for 73 patients, and in either NPS or saliva specimens for 98 patients. Of the coinfected samples, 2, 3, and 4 viruses were detected for 37, 16, and 1 patients, respectively, by testing NPS samples and for 55, 16, and 2 patients by testing saliva samples. Combining both NPS and saliva results showed that 60, 30, and 7 patients had 2, 3, and 4 viruses, respectively. The maximum number of viruses detected was 5 in 1 patient, using a combination of the two sampling methods.

The results of comparisons based on each virus detected are shown in Table 3. A

TABLE 2 Distribution of the numbers of respiratory viruses isolated from the collected samples ($n = 236$)

	No. (%) of positive samples		
No. of viruses isolated	NPS	Saliva	NPS or saliva
0	53 (22.5)	56 (23.7)	32 (13.6)
1	129 (54.7)	107 (45.3)	106 (44.9)
2	37 (15.7)	55 (23.3)	60 (25.4)
3	16 (6.8)	16 (6.8)	30 (12.7)
4	1 (0.4)	2 (0.8)	7 (3.0)
5	0 (0.0)	0 (0.0)	1 (0.4)
Total	236 (100.0)	236 (100.0)	236 (100.0)

TABLE 3 Comparison between NPS and saliva samples according to the virus type

Virus	No. (%) of positive samples			P[a]
	NPS and saliva	NPS	Saliva	
ADV	3	3 (1.2)	43 (15.8)	<0.0001
INF type A	4	23 (9.0)	8 (2.9)	0.0001
RSV type B	0	1 (0.4)	0 (0.0)	NA[b]
PIV-2	0	0 (0.0)	1 (0.4)	NA[b]
PIV-4	2	3 (1.2)	3 (1.1)	1.0000
CoV-229E	6	14 (5.5)	8 (2.9)	0.0565
CoV-OC43	32	41 (16.0)	46 (16.8)	0.3483
EV	70	83 (32.4)	89 (32.6)	0.3357
HRV	63	88 (34.4)	75 (27.5)	0.0289
Total	179	256 (100.0)	273 (100.0)	0.1574

[a]Pearson's chi-square test with Yates's continuity correction.
[b]NA, not applicable. The statistics were not estimated due to zero cells.

total of 349 viruses were identified in 204 patients by either sampling method, and 179 viruses were identified by both sampling methods. A total of 256 viruses (73.4%) were identified from NPSs and 273 (78.2%) from saliva samples; the difference between these proportions was not statistically significant ($P = 0.1574$). Among the 16 viruses that can be detected with the Anyplex II RV16 assay, 9 were identified in this study, namely, adenovirus (ADV), INF type A, respiratory syncytial virus (RSV) type B, parainfluenza virus (PIV) types 2 and 4, coronavirus 229E (CoV-229E) and CoV-OC43, enterovirus (EV), and human rhinovirus (HRV). Enterovirus was the most frequently detected virus, followed by HRV, CoV-OC43, CoV-229E, ADV or INF type A, and PIV type 4, for both sampling methods. ADVs were detected more often in saliva samples ($P < 0.0001$), whereas INF type A and HRV were detected more often in NPS specimens ($P = 0.0001$ and $P = 0.0289$, respectively). The differences in the rates of detection of other viruses were not statistically significant or could not be statistically evaluated because of the limited number of infection-positive cases.

The results of agreement analyses are shown in Table 4. The values for total, positive, and negative agreement of the two sampling methods were 95.5%, 68.1%, and 97.6%, respectively. The overall kappa value was 0.66, which is categorized as indicating substantial agreement. The low positive agreement values for RSV type B, PIV type 2, PIV type 4, and CoV-229E can be explained by the low prevalence (limited number of positive cases). The low positive agreement values for ADV and INF type A can be attributed to both relatively low prevalence and disagreement between the two sampling methods. In general, low prevalence results in decreased kappa values, and prevalence-adjusted bias-adjusted kappa (PABAK) values can be used to alleviate the effect of prevalence on kappa values (10). In our study, the overall PABAK value was 0.91, indicating nearly perfect agreement between the two sampling methods.

TABLE 4 Analysis of agreement between the two sampling methods

Virus	Agreement (% [95% CI])[a]			Kappa (95% CI)	PABAK (95% CI)
	Total	Positive	Negative		
ADV	83.1 (78.3–87.8)	13.0 (0.0–26.3)	90.6 (87.7–93.5)	0.11 (0.01–0.22)	0.66 (0.55–0.75)
INF type A	90.3 (86.5–94.0)	25.8 (5.4–46.1)	94.8 (92.6–96.9)	0.22 (0.02–0.42)	0.81 (0.71–0.87)
RSV type B	99.6 (98.7–100.0)	0.0 (0.0–0.0)	99.8 (99.3–100)	0 (0.00–0.00)	0.99 (0.95–1.00)
PIV-2	99.6 (98.7–100.0)	0.0 (0.0–0.0)	99.8 (99.3–100)	0 (0.00–0.00)	0.99 (0.95–1.00)
PIV-4	99.2 (98.0–100.0)	66.7 (23.1–100.0)	99.6 (98.9–100)	0.66 (0.22–1.00)	0.98 (0.94–1.00)
CoV-229E	95.8 (93.2–98.3)	54.5 (29.4–79.6)	97.8 (96.4–99.1)	0.52 (0.27–0.78)	0.92 (0.84–0.96)
CoV-OC43	90.3 (86.5–94.0)	73.6 (63.1–83.9)	94.0 (91.5–96.4)	0.68 (0.55–0.80)	0.81 (0.71–0.87)
EV	86.4 (82.1–90.8)	81.4 (75.0–87.7)	89.3 (85.6–93.0)	0.71 (0.61–0.80)	0.73 (0.62–0.81)
HRV	84.3 (79.7–89.0)	77.3 (70.1–84.4)	88.0 (84.1–91.8)	0.65 (0.55–0.76)	0.69 (0.58–0.77)
Total	95.5 (94.9–96.2)	68.1 (63.4–72.6)	97.6 (97.2–97.9)	0.66 (0.61–0.71)	0.91 (0.90–0.92)

[a]CI, confidence interval.

As shown in Table 3, among the 43 ADV-positive samples, only 3 showed infection positivity in both saliva and NPS specimens. Direct sequencing of ADV-positive saliva specimens was performed to exclude any false-positive results. Two samples were excluded because of PCR failure. Therefore, 41 ADV-positive samples were eligible for direct sequencing, and all showed amplification of ADV. ADV type 2 was identified in 44% of samples, followed by type 1 (27%), type 5 (27%), and type 6 (2%). Three positive NPS specimens were found to have different ADV serotypes, i.e., ADV types 1, 2, and 5.

DISCUSSION

It is generally thought that the optimal specimen type for detecting RVs is nasopharyngeal aspirate (NPA) specimens, although this may not be true for all RVs and detection techniques (4). Given the complexity and invasive nature of the procedure used to acquire NPA specimens, NPSs have emerged as an alternative specimen type. NPSs were shown to be superior or equivalent to NPA specimens for RT-PCR in many studies (8, 11, 12). However, the collection of NPS specimens is somewhat invasive and causes coughing in most patients, which may increase the risk of nosocomial spread of respiratory viruses, such as influenza virus, through the production of airborne particles containing infectious virus (5, 9). In addition, it remains unclear whether nasopharyngeal specimens are optimal for detection of most RVs by multiplex RT-PCR.

In a previous study comparing NPS and sputum specimens for RV detection by multiplex RT-PCR, sputum samples yielded significantly higher detection rates for most types of viruses (6). The superiority of sputum samples to NPSs was more evident in the detection of INF type A and RSV type A, which is known to be associated with lower respiratory tract infections. However, the authors stated that the use of sputum samples has some limitations. First, because of its high viscosity, sputum requires an additional pretreatment procedure for nucleic acid extraction, which has not been standardized and thus may lead to invalid RT-PCR results if applied improperly. Second, some patients, such as young children and elderly patients, cannot produce sputum.

OPS specimens are easier to obtain than NPS specimens but more difficult to obtain than saliva samples. In addition, some previous studies showed that OPSs are inferior to NPSs as specimens for RV detection by RT-PCR (8, 13).

A limited number of previous studies have utilized saliva samples as specimens for NAATs for RV detection. One study compared throat swab and saliva specimens with NPA samples for the detection of four RVs (RSV, INF types A and B, and PIV) in pediatric patients with presumed lower respiratory tract infections (9). When a NPA specimen was positive for a RV, testing of the patient's corresponding saliva and throat swab specimens for the same virus was performed. Detection rates could not be directly compared between sampling methods because NPA-negative but saliva-positive cases could not be counted. In another study, all patients diagnosed as H1N1 positive by RT-PCR using NPS specimens were also positive for H1N1 using their saliva specimens (14).

The results of this study showed that the overall detection rate from saliva samples was comparable to that from NPS specimens when multiplex RT-PCR was used for RV detection. Variability in detection rates according to the species of virus was observed; notably, the rate of adenovirus detection from saliva samples was significantly higher than that from NPS samples. Lower rates of adenovirus detection from NPS specimens, compared to other types of specimens, have been reported in several previous studies (7, 11). This difference may reflect the fact that the major site of initial replication of adenovirus is the nonciliated respiratory epithelium of the oropharynx (7). Saliva samples may be considered reasonable replacements for NPS specimens during adenovirus outbreaks. The specific relationships between the genotypes of adenoviruses and their detection in NPS specimens were not determined in our study.

In this study, the combined detection rate with NPS and saliva samples was 86.4% (204 of 236 patients), which was higher than the rates of 77.5% (183 of 236 patients) with NPS specimens and 76.3% (180 of 236 patients) with saliva samples alone (Table 2). We found relatively high rates of RV detection, compared to those found in another

military study (15). That study showed that 49.1% of cases tested were positive for at least one virus between 2009 and 2013 in the respiratory disease sentinel surveillance system in the Singapore military. In addition, several studies found a wide prevalence range (38 to 64.7%) for respiratory viruses in the Republic of Korea, using the same RV assay (16–18). There are several reasons why differences in virus prevalence were observed. First, the differences may be related to the subjects examined in the study. We enrolled subjects who were placed in specific circumstances, compared to those in other studies. All subjects were the new military recruits trained as medics who had started their military careers ≤6 weeks earlier. This suggests that they were vulnerable to respiratory infections because the recruit training program is physically and psychologically demanding, which might have depressed the immune system (19–21). In addition, floor-based accommodations were close and crowded, which can increase the prevalence of respiratory viruses. Several studies of U.S. military recruits have demonstrated that respiratory disease rates are directly associated with increasing levels of crowding (22–25). Second, evidence of high overall rates of positivity in this study was from the South Korean Army Training Center Influenza and Respiratory Virus Surveillance System, which found that the prevalence of RVs ranged from approximately 50% per week to 80% per week during the study period (data not published and only reported to the superior authority). Specimens containing at least two viruses (coinfection) were detected at rates of 21.1% (54/236 patients) and 30.9% (73/236 patients) among NPS and saliva specimens, respectively. The coinfection rate increased to 41.5% (98/236 patients) with combined NPS and saliva specimens. Other studies reported similar coinfection rates of approximately 10 to 30% with NPS specimens (1). HRV was the most commonly detected virus in coinfected NPS samples, followed by EV, CoV-OC43, INF type A, and CoV-229E. HRV was also the most frequently detected virus in saliva samples, followed by EV, ADV, CoV-OC43, CoV-229E, and INF type A. The clinical severity of viral coinfection is controversial (26, 27). At the time of the initial study design, we did not consider factors related to the severity of illnesses because this study was part of the Army Training Center Influenza and Respiratory Virus Surveillance System, which focuses on the patterns of outbreaks of respiratory illnesses in the army.

A total of 179 viruses were isolated from NPS and saliva samples together, whereas 256 and 273 viruses were isolated from NPS and saliva samples alone, respectively (Table 3). HRV and EV together accounted for 66.8% and 60.15% in NPS and saliva samples, respectively. HRV and EV are generally thought to cause common colds in adults with no underling disease. Although we did not evaluate the exact clinical severity and diagnostic data, most subjects were assumed to have upper respiratory tract infections. A significant number of patients tested had cough and sputum, suggesting lower respiratory tract infections. However, these symptoms may be attributed in part to environmental factors, such as exposure to dust, smoke, and air pollution and lack of adequate ventilation in the accommodations (28). Comparisons of the two sampling methods revealed that ADVs were detected significantly more often in saliva samples ($P < 0.0001$), whereas INF type A and HRV were detected more often in NPS specimens ($P = 0.0001$ and $P = 0.0289$, respectively). The differences in the rates of detection of other viruses were not statistically significant or could not be statistically evaluated because of the limited number of detected cases. The higher detection rate with the combination of multiple specimens can be explained by the variety of target viruses detected by multiplex RT-PCR and possible heterogeneity in their primary sites of replication. Using the two types of samples in parallel might double the cost, but physically mixing the two types of samples and running a single assay only minimally increased cost. Because saliva samples can be obtained with minimal additional effort and cost, we suggest that the detection of RVs in mixed NPS-saliva specimens should be evaluated in future studies.

There were some limitations to the present study. First, this study was limited by the detection ability of two sampling methods without proper clinical diagnosis. Future studies should evaluate upper and lower respiratory tract infections and disease severity. Second, we could not include a control group of asymptomatic individuals to

rule out the possibility of viral shedding (chronic or transient) in the saliva samples. Because it is difficult to differentiate between viral shedding and acute infection with RV NAATs, clinical implementation of the use of saliva samples requires further analysis, including control samples. However, the current study included subjects within 1 week after respiratory symptom onset, to overcome some problems of viral shedding. Finally, this study enrolled only young adult male subjects for a short period. To generalize the results, further studies with subjects of various ages and both genders should be conducted for year-round RV detection.

To our knowledge, this is the first study to assess the comparability of saliva and NPS specimens in multiplex RT-PCR assays for RV detection in adults. The results of this study showed that the overall performance of saliva samples was equivalent to that of NPS specimens in RV detection by multiplex assays. Neither sampling method was consistently more sensitive than the other, based on the types of viruses detected. Mixing NPS and saliva specimens appears to be a promising strategy, considering the different detection patterns of the different types of specimens.

MATERIALS AND METHODS

Patients and specimens. We conducted a prospective study using paired NPS and saliva specimens from adult male patients, within 1 week after respiratory symptom onset, at the Armed Forces Medical School (Daejeon, Republic of Korea). Adult male patients between 20 and 26 years of age, who were recruits training as medics and showed signs and symptoms of acute respiratory illnesses, such as cough, sputum, tonsil enlargement, rhinorrhea, sore throat, headache, and fever, were recruited between 25 November 2015 and 8 January 2016. They had begun their military careers ≤6 weeks earlier and lived close to each other in a floor-based military barracks, with approximately 20 colleagues per room. During the study period, 256 pairs of NPS and saliva specimens were collected; 20 pairs were rejected because of inadequate quantity or quality and 236 pairs were included in the study. The demographic features and clinical presentations of the participants are shown in Table 1. NPS specimens were obtained by experienced military physicians using flocked swabs and were transported in 3 ml universal transport medium (COPAN, Murrieta, CA, USA). For collection of saliva, the participants received brief explanations regarding the difference between saliva and sputum. They were instructed to spit their saliva into sterile sputum containers. The minimum amount of saliva required was 2 ml. The color and viscosity of each saliva specimen were evaluated by visual inspection, and 20 samples suspected to include sputum were excluded from the study. Collected specimens were immediately stored at 4°C and were tested by multiplex RT-PCR within 48 h. All subjects provided informed consent, and the study was approved by the ethics committee of the Armed Forces Medical Command (protocol AFMC-15090-IRB-15-081).

Laboratory testing. Multiplex RT-PCR was performed using the Anyplex II RV16 detection kit (Seegene, Seoul, South Korea), which has been certified for *in vitro* diagnostic product (IVD) use by Conformité Européenne (CE) marking and the South Korean Food and Drug Administration (FDA), as described previously (16). Briefly, nucleic acid extraction was performed using the Microlab STARlet system (Hamilton, Reno, NV, USA), with the STARMag 96 virus kit (Seegene), from 500 μl of universal transport medium or 500 μl of saliva. Bacteriophage MS2, which was used as an internal control, was added to the specimens before nucleic acid extraction and was incorporated into the products as an exogenous whole-process control, for monitoring of all steps from nucleic acid extraction to result analysis. cDNA synthesis was performed using cDNA Synthesis Premix (Seegene). Reverse transcription was conducted in a final volume of 20 μl, containing 8 μl of RT buffer, 2 μl of random hexamers, 2 μl of RT enzyme, and 8 μl of nucleic acids from the samples, under the following conditions: 5 min at 25°C, 60 min at 37°C, and 2 min at 95°C. Next, the 16 target viruses were subjected to multiplexed PCR in two tubes, using RV detection kits A and B. Multiplex real-time PCR was conducted in a 20-μl final volume, containing 8 μl of cDNA, 5 μl of 4× TOCE Oligo Mix primer, 5 μl of 4× PCR master mix, and 2 μl of RNase-free water, with the CFX96 real-time PCR detection system (Bio-Rad, Hercules, CA, USA). Thermal cycling was performed under the following condition: 50°C for 4 min and denaturation at 95°C for 15 min, followed by 50 cycles of 95°C for 30 s, 60°C for 1 min, and 72°C for 30 s. During cycles 30, 40, and 50 of the real-time reaction, catcher melting temperature analysis (CMTA) was performed by cooling the reaction mixture to 55°C, maintaining it at 55°C for 30 s, and heating it from 55°C to 85°C. Data analyzed by the Seegene viewer software were automatically presented for 16 RVs, i.e., bocavirus, EV, INF types A and B, PIV types 1, 2, 3, and 4, RSV types A and B, ADV, metapneumovirus, CoV-OC43, CoV-229E, CoV-NL63, and HRV. The software assigns designations as follows: +++, viruses detected from the first CMTA (after 30 cycles); ++, viruses detected from the second CMTA (after 40 cycles); +, viruses detected from the third CMTA (after 50 cycles). Samples containing large viral loads are generally detected during early-cycle analysis; however, the manufacturer does not provide clear data regarding viral load ranges according to the testing results. In our study, all results from + to +++ were regarded equally as positive results.

Direct sequencing was performed with the adenovirus-positive saliva samples to verify the results, as most NPS samples from the same patients were negative for adenovirus. Nested PCR, targeting approximately 900 bp of the partial hexon gene containing hypervariable regions 1 to 6, and sequencing were performed using the primer pairs and conditions used in a previous study (29). To determine the

genotypes of the ADV strains detected in our study, ADV sequences were analyzed using nucleotide BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and MEGA software (version 5.1). The nucleotide sequences of reference strains for each adenovirus type were obtained from the GenBank nucleotide database and a previous study (30).

Data analyses. McNemar's test was used to compare the detection rates for the two sampling methods in terms of the numbers of patients. Pearson's chi-square test was used to compare the sampling methods in terms of the numbers of viruses detected. Methods for analysis of interrate agreement statistics, including percent agreement and kappa statistics, were used to determine the comparability of the two sampling methods. Underestimation of kappa values results from a low prevalence of each virus; therefore, PABAK values were also calculated. Kappa statistics were interpreted as follows: 0, poor; 0 to 0.2, slight; 0.21 to 0.4, fair; 0.41 to 0.6, moderate; 0.61 to 0.8, substantial; 0.81 to 1.0, almost perfect (31). Statistical analyses were performed using SPSS (version 19.0; SPSS Inc., Chicago, IL, USA) and R (version 3.3.1) software.

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