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ORIGINAL ARTICLE

Comparison of Throat and Nasopharyngeal Swabs for the Molecular Detection of Enterovirus in Pediatric Patients

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SUMMARY

Background: **Enterovirus infections frequently occur in children worldwide. Molecular assays are widely used to detect enterovirus. Nasopharyngeal swabs (NPS) and throat swabs (TS) are common specimen types used in clinical practice. Here, the reliability of TS for detecting enterovirus in pediatric patients was compared with that of NPS using real-time reverse transcription polymerase chain reaction (RT-rPCR).**
Methods: **Results obtained using the Allplex Respiratory Panel 2 (Seegene, Korea) for NPS (NPS-RP) and AccuPower EV Real-time RT-PCR (Bioneer, Korea) for TS (TS-EV), which were performed simultaneously between September 2017 to March 2020, were initially compared. Cross examination (Allplex Respiratory Panel 2 assay using TS and AccuPower EV assay with NPS) was performed for specimens collected between July 2019 to March 2020 to evaluate the performance of the enterovirus assays based on each specimen type.**
Results: **Among the 742 case results of initial tests, 597 cases (80.5%) tested negative in both assays, and 91 cases (12.6%) tested positive in both assays. Fifty-four discrepant results were observed: 39 cases (5.3%) tested positive in TS-EV and negative in NPS-RP, and 15 cases (2.0%) tested positive in NPS-RP and negative in TS-EV. The overall percent agreement was 92.7%. In the 99 cases cross examined, overall percent agreements were 98.0%, 94.9%, 92.9%, and 89.9% for TS-EV vs. TS-RP, NPS-RP vs. NPS-EV, TS-EV vs. NPS-EV, and NPS-RP vs. TS-RP, respectively.**
Conclusions: **TS yields a high agreement rate with NPS in detecting enterovirus, regardless of single-plex or multiplex RT-rPCR assays. Thus, TS could be a good alternative specimen in pediatric patients who are reluctant to NPS sampling.**
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KEYWORDS

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INTRODUCTION

Enteroviral infections occur frequently in children worldwide [1]. Generally, enteroviruses can cause mild respiratory or febrile illness in infants and young children; however, they can also cause severe diseases, including encephalitis, myocarditis, myelitis, paralysis, and sepsis [2]. Hand-foot-mouth disease (HFMD) is common in young children and HFMD outbreaks caused by enteroviruses were reported in Asia and Eu-

rope [3,4]. In Korea, the incidence of HFMD in 2011 - 2017 was reported as 8.4 out of 100 persons under the age of six [5]. Typically, the diagnosis of enteroviral infection is mainly based on clinical manifestations; recently, laboratory tests have become important to support their diagnosis. Real-time reverse transcription-polymerase chain reaction (RT-rPCR) is widely used to detect enteroviral infections in many countries including Korea. Clinical samples, such as cerebrospinal fluid (CSF), blood, stool, and respiratory specimens, are recommended for diagnostic testing depending on clinical manifestations. Among the sample types, respiratory specimens can be readily obtained from patients and retain detectable enteroviral load for a longer period than other sample types [2,6].

In Korea, two types of respiratory specimens are frequently used for enterovirus detection: throat swab (TS) and nasopharyngeal swab (NPS). NPS is usually recommended when the clinical manifestation is associated with upper respiratory infection [7], and it can be used for multiplex testing of other respiratory viruses (RV). Meanwhile, TS is preferred by pediatricians as it reduces the possibility of pain and injury in the nasopharynx of young children with HFMD. However, no comparative study that evaluates TS and NPS for the molecular diagnosis of enterovirus infection has been conducted in areas of high prevalence of pediatric patients with HFMD.

In this study, the reliability of using TS over NPS for enterovirus detection was examined using two RT-rPCR reagents: AccuPower EV Real-time RT-PCR (Bioneer, Daejeon, Korea) and Allplex Respiratory Panel 2 (Seegene, Seoul, Korea).

MATERIALS AND METHODS

Patients and specimens

Patients who were admitted to Hanyang University Guri Hospital on presenting febrile symptoms and were subjected to simultaneous assays for enterovirus and RV detection between September 2017 to March 2020 were enrolled in this study. TS and NPS were collected by the same medical practitioner at the same time for each patient. If examination was not performed within 1 hour, the specimens were stored at 4°C up to 3 days. Initial assays were performed using the Allplex Respiratory Panel 2 for NPS (NPS-RP) and AccuPower EV Real-time RT-PCR for TS (TS-EV) for the entire study period. Cross examination was conducted using the Allplex Respiratory Panel 2 assay for TS (TS-RP) and the AccuPower EV assay for NPS (NPS-EV); specimens were collected between July 2019 to March 2020. Specimens were stored in -70°C after the initial assay for the cross examination. Furthermore, the influence of freeze-thawing was examined using 22 NPS and 14 TS specimens. This study was approved by the Institutional Review Board (IRB) of Hanyang University Guri Hospital (IRB No. 2021-06-002). Informed consent from the participants was waived by the IRB.

Nucleic acid preparation

A nylon flocked swab (FLOQSwabs; Copan, Murrieta, CA, USA) was used to obtain TS, while the ESwab® (Copan) was used to collect NPS. TS was put into a sterile tube with 1 mL of phosphate buffer solution. Nucleic acid extraction was performed using the eMAG instrument (bioMerieux, Marcy l'Etoile, France), and the nucleic acid was eluted in 60 µL of elution buffer, according to the manufacturer's instructions [8].

RT-rPCR for enterovirus

The AccuPower EV Real-time RT-PCR kit is a one-step RT-rPCR assay that detects the enterovirus RNA based on the highly conserved 5`-nontranslated region in the human enterovirus genome [9]. The Exicycler™ 96 real-time quantitative thermocycler (Bioneer) was used for the detection of the amplified product [10]. A cycle threshold (Ct) value ≤ 40 was considered a positive result.

The Allplex Respiratory Panel for RV (RP1, RP2, and RP3) is a RT-rPCR assay that detects 16 respiratory viruses: adenovirus (AdV), bocavirus, coronavirus OC43/NL63/229E, human enterovirus, human metapneumovirus (MPV), influenza virus A/B, parainfluenza virus (PIV) type 1/2/3/4, respiratory syncytial virus (RSV) A/B, human rhinovirus, and three influenza virus A subtypes (H1, H1 pdm09, and H3). Enterovirus is included in the RP2, together with AdV, PIV, and MPV. RT-rPCR was performed using CFX96 real-time PCR detection system (Bio-Rad, Hercules, CA, USA) [11]. Automated analysis of the results was run using the Software Seegene Viewer. The software considers targets that generate an adequate, exponential fluorescence curve, with Ct < 42 cycles as a positive result.

Enterovirus genotyping

Enterovirus genotyping was performed by Bioneer and Macrogen (Daejeon, Korea) using reverse transcription PCR and sequencing according to the WHO guideline [12]. Viral RNA was amplified using specific primer sets for VP1 in the Exicycler™ V4 thermocycler (Bioneer). PCR products were visualized via electrophoresis and sequenced using the ABI BigDye Terminator v3.1 Cycle Sequencing Kits (Applied Biosystems, San Francisco, CA, USA) and ABI 3730XL DNA Analyzer (Applied Biosystems). The genotypes were confirmed using Basic Local Alignment Search Tool (BLAST) on the National Center for Biotechnology Information (NCBI) website.

Statistics

All statistical analyses were performed using MedCalc for Windows, version 20.111. Categorical variables are summarized as absolute counts and percentages, while continuous variables are presented as median and range. The positive percent agreement (PPA), negative percent agreement (NPA), and overall percent agreement (OPA)

were calculated using the 2 x 2 contingency table and presented as value of kappa along with 95% confidence intervals (CI). Continuous variables were analyzed using the Mann-Whitney U test. Differences between the paired values were compared using the Wilcoxon test and McNemar test. All tests were two-sided, and $p < 0.05$ was considered statistically significant.

RESULTS

Between September 2017 to March 2020, 684 patients were enrolled in this study, and assay results of 742 pairs of samples were analyzed. The median age of patients was 2 years (range: 0 - 18 years); 43.1% (295/684) of the patients were female, while 56.9% (389/684) were male. A total of 597 cases (80.5%) tested negative in both assays, while 91 cases (12.6%) tested positive in both assays (Table 1). Among the 54 cases that showed discrepant results, 39 (5.3%) tested positive in TS-EV and negative in NPS-RP, and 15 (2.0%) tested positive in NPS-RP and negative in TS-EV. The positive rate of enterovirus in TS-EV (17.5%) was higher than that in NPS-RP (14.2%) ($p < 0.001$). The OPA was 92.7% (kappa: 0.73, 95% CI: 0.66 - 0.80), and the PPA and NPA were 77.1% and 95.7% respectively. Among the 106 NPS-RP-positive cases, the median Ct of 91 cases that were also positive in TS-EV was 31.97 (range: 18.23 - 40.47); meanwhile, that of 15 cases that were negative in TS-EV was 37.56 (range: 27.32 - 40.49) ($p = 0.002$, Figure 1). Among the 130 TS-EV-positive cases, Ct analysis results were available for 109 cases. The median Ct of 75 cases that were positive for both assays was 27.76 (range: 18.37 - 39.02), while that of the 34 cases that were positive only in TS-EV assay was 33.08 (range: 22.95 - 38.29) ($p < 0.001$).

A cross examination was performed using 99 specimen pairs obtained between July 2019 to March 2020, with 10 cases showing discordant results in the initial tests (five cases were positive in NPS-RP and negative in TS-EV, and five cases were positive in TS-EV and negative in NPS-RP). Among the 99 cross examined cases, the positive rates of TS-EV, TS-RP, NPS-RP, and NPS-EV were 26.3% (26/99), 24.2% (24/99), 25.3% (25/99), and 24.2% (24/99), respectively (Table 2). The OPA was highest in TS-EV and TS-RP (98.0%) and lowest in NPS-RP and TS-RP (89.9%). The kappa index for concordance in TS-EV vs. TS-RP, NPS-RP vs. NPS-EV, TS-EV vs. NPS-EV, and NPS-RP vs. TS-RP were 0.95, 0.86, 0.81, and 0.73, respectively. Among the 19 cases that were positive in the four specimen-assay combinations, no significant difference was observed in the Ct values between the two PCR assays using TS. The median Ct value of TS-EV was 27.50 (range: 22.73 - 39.02) and that of TS-RP was 28.71 (range: 19.51 - 38.49) ($p = 0.431$, Figure 2). However, the Ct values of NPS were significantly different according to the PCR assays. The median Ct value of NPS-EV was 25.03 (range: 19.06 - 34.46) and that of NPS-RP was 31.78 (range: 19.85 - 38.35) ($p < 0.001$). No significant differences were noted between the Ct values of two specimen types using each PCR assay. Two specimen pairs that were negative in both initial assays were revealed to be positive in cross examination (Ct: 34.55 at NPS-EV and 40.05 at TS-RP, data not shown). In addition, the influence of freeze-thawing was not significant in both NPS and TS specimens. The median Ct of NPS changed from 31.03 (range: 18.23 - 39.61) to 32.72 (range: 19.89 - 40.18) after freezing for 13 - 187 days. Meanwhile, the median Ct of TS changed from 27.75 (range: 24.68 - 34.17) to 27.63 (range: 24.97 - 34.78) after freezing for 5 - 367 days.

Genotyping was performed using the 10 cases showing discordant results in the initial assays (Table 3). Among the five NPS-RP positive specimens (case 1 - 5), three NPS (cases 2, 3, 4) were positive in the EV assay, and their genotypes were identified as Coxsackievirus A6, B5, and A5, respectively. Two NPS specimens (cases 1 and 5) were negative in both EV assay and genotyping. Meanwhile, the TS specimens of the five patients (case 1 - 5) were negative in the RP and EV assays except for case 2 that tested positive in the RP assay (Ct = 41.4). The TS specimens of cases 6 - 10 were positive in both EV and RP assays, whereas those of cases 7 - 10 were positive in genotyping. Cases 7 - 10 were identified as Echovirus E9, Coxsackievirus A6, Coxsackievirus B1, and Echovirus E30, respectively. In addition, among cases 6 - 10, two NPS specimens were positive in the EV assay (case 8, 10), wherein case 8 was identified as Enterovirus 71 via genotyping.

DISCUSSION

Enterovirus infections are prevalent worldwide and cause self-limiting febrile illness to severe neurologic symptoms in infants and young children [13]. For the proper and early diagnosis of enterovirus infection, RT-PCR targeting 5′ noncoding regions is recommended recently [2]. The type of specimen used for RT-PCR varies according to the time from the symptom onset and clinical manifestations. Among the recommended sample types, respiratory specimens are frequently used for enterovirus detection as the specimen can be easily obtained and yields reliable results using molecular assays [2,6]. Nasopharyngeal aspirate or nasal wash (NW) specimens have been the specimen of choice for detecting respiratory pathogens in traditional diagnostic methods, such as antigen detection or viral culture [14]. Since the use of molecular methods for detecting RV has increased, NPS has been considered as a specimen of choice for its simplicity and improved standardization [15,16]. However, TS has also been used frequently as an alternative sample in areas with prevalent HFMD, including Korea, to make sample collection from young children painless and less invasive, both of which are crucial factors for ensuring patient compliance and preventing potential injury [17,18].

Table 1. Detection of enterovirus using NPS-RP and TS-EV assays.

TS-EV	NPS-RP			Kappa	OPA (%)	PPA (%)	NPA (%)
	Positive	Negative	Total				
Positive	91	39	130	0.73	92.7	77.1	95.7
Negative	15	597	612				
Total	106	636	742				

NPS-RP - Allplex Respiratory Panel 2 assay using nasopharyngeal swab, TS-EV - AccuPower EV assay using throat swab, OPA - overall percent agreement, PPA - positive percent agreement, NPV - negative percent agreement.

Table 2. Comparison of cross examination results from 99 patients.

TS-RP	TS-EV			NPS-EV	NPS-RP			NPS-EV	TS-EV			TS-RP	NPS-RP		
	Pos	Neg	Total		Pos	Neg	Total		Pos	Neg	Total		Pos	Neg	Total
Pos	24	2	26		22	3	25		21	4	25		20	6	26
Neg	0	73	73		2	72	74		3	71	74		4	69	73
Total	24	75	99		24	75	99		24	75	99		24	75	99
OPA (%)	98.0				94.9				92.9				89.9		
PPA (%)	96.0				89.8				91.3				80.0		
NPA (%)	98.7				96.6				92.8				93.2		
Kappa (95% CI)	0.95 (0.87 - 1.00)				0.86 (0.75 - 0.98)				0.81 (0.67 - 0.95)				0.73 (0.58 - 0.89)		

TS - throat swab, NPS - nasopharyngeal swab, RP - Allplex respiratory panel 2, EV - AccuPower EV assay, Pos - positive, Neg - negative, OPA - overall percent agreement, PPA - positive percent agreement, NPA - negative percent agreement, CI - confidence interval.

Table 3. Genotypes of 10 specimen pairs showing discordant results in NPS-RP and TS-EV assays.

Case	Gender	Age	NPS-RP /TS-EV	NPS			TS		
				RP, Ct	EV, Ct	Genotype	RP, Ct	EV, Ct	Genotype
1	F	4	+/-	38.68	-	-	-	-	-
2	F	2	+/-	27.32	25.5	CV-A6	41.1	-	-
3	M	0	+/-	28.05	23.3	CV-B5	-	-	-
4	M	3	+/-	35.17	30.17	CV-A5	-	-	-
5	F	4	+/-	37.56	-	-	-	-	-
6	F	1	-/+	-	-	-	36.06	38.18	-
7	F	0	-/+	-	-	-	34.82	34.33	E9
8	M	2	-/+	-	29.88	EV71	35.22	26.37	CV-A6
9	M	1	-/+	-	-	-	22.84	25.04	CV-B1
10	M	14	-/+	-	31.37	-	27.36	27.31	E30

TS - throat swab, NPS - nasopharyngeal swab, RP - Allplex respiratory panel 2, EV - AccuPower EV assay, Ct - cycle threshold, CV - Coxsackievirus, E - Echovirus, EV71 - Enterovirus 71.

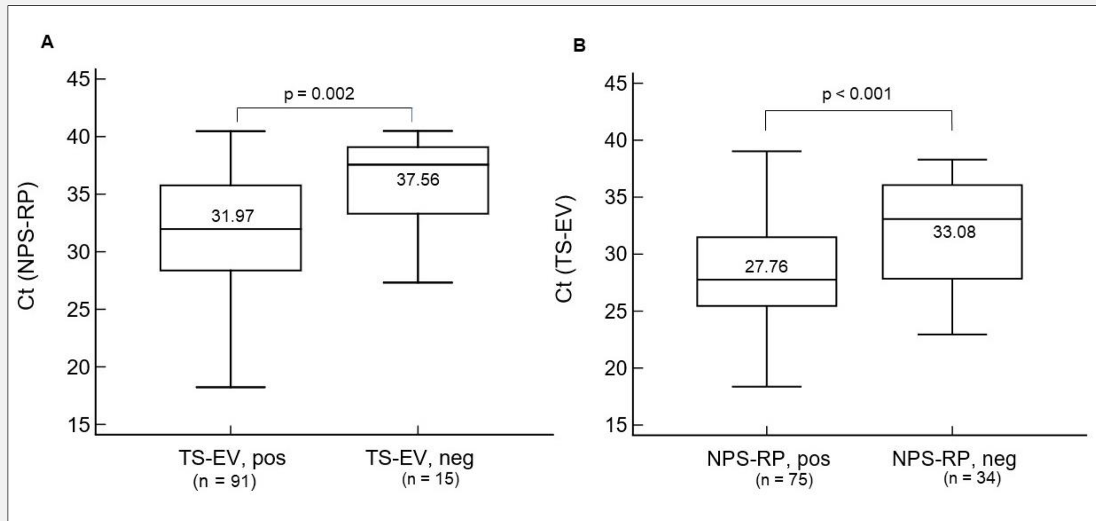

Figure 1. Comparison of the Ct values of NPS-RP between TS-EV-positive and -negative cases (A), and Ct values of TS-EV between NPS-RP-positive and -negative cases (B).

Ct - cycle threshold, NPS-RP - Allplex Respiratory Panel 2 assay using nasopharyngeal swab, TS-EV - AccuPower EV assay using throat swab, pos - positive, neg - negative.

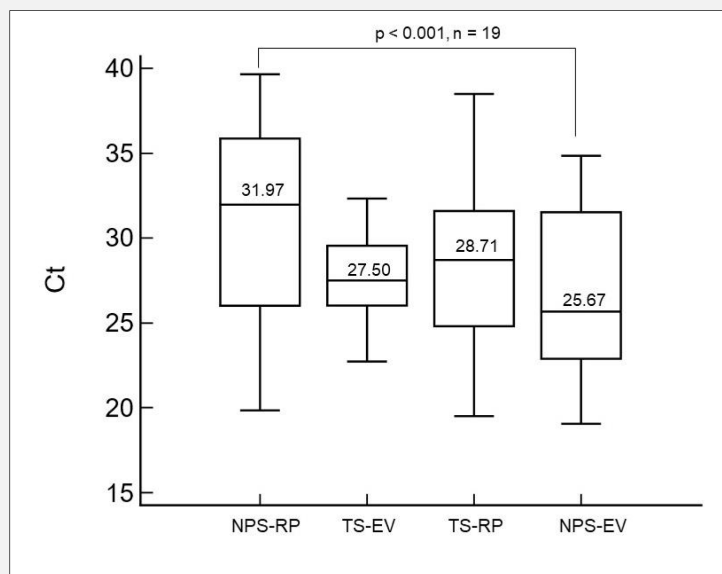

Figure 2. Ct values of the 19 enterovirus-positive cases in the four specimen-assay combinations.

Ct - cycle threshold, NPS - nasopharyngeal swab, TS - throat swab, RP - Allplex Respiratory Panel 2 assay, EV - AccuPower EV assay.

The scavenger receptor class B, member 2 (SCARB2) has been identified to be associated with enterovirus [19]. SCARB2, a receptor for enterovirus 71 and coxsackievirus A16, has been reported to be present in the tonsillar crypt squamous epithelium, which supports active viral replication and represents an important source of viral shedding [6,19]. This observation supports TS as an appropriate specimen. A previous study compared oropharyngeal swab (OPS) with NPS or NW for detecting respiratory pathogens and showed that NPS yielded the highest sensitivity for detecting rhinovirus and adenovirus in adults with acute pharyngitis [20]. However, for enterovirus, the difference among NPS, NW, and OPS was not statistically significant. Furthermore, a study evaluating TS and NPS for enterovirus detection in pediatric patients is not yet available. In the present study, relatively high overall agreement between TS-EV and NPS-RP (92.7%) was observed, and the positive rate of enterovirus covering three seasonal outbreaks was higher in TS-EV (17.5%) than NPS-RP (14.2%) ($p < 0.001$), although it was not an analytical comparison between respective specimen types or assays. In the direct evaluation of 99 specimens, comparison test using TS showed the highest overall agreement (98%). These results indicate that TS could be an alternative sample type that can provide robust and reliable results for the detection of enterovirus in pediatric patients. However, further direct evaluation with larger cohorts is needed to confirm its analytical performance.

In temperate regions including Korea, enterovirus infections are prevalent in the summer and early autumn, and predominant enterovirus genotypes change slightly every year [17,21]. EV-A and EV-B species presented different epidemiologic trends in the national enterovirus surveillance program from 2012 to 2019 in Korea [17]. They reported that EV-A was detected year-round, affecting children aged 1 - 3 years old and was associated with herpangina and HFMD, whereas EV-B was detected predominantly in the summer and early fall and associated with neurologic manifestations. In this national surveillance, the five most common genotypes in 2019 were EV71, E30, CV-A6, E9, and CV-A16, which were consistent with genotyping results of the present study.

This study has some limitations. Because cross examination was performed in the latter part of the study, an extensive direct evaluation of the performance of the assays according to specimen types or reagents is needed in further studies. Genotyping for enterovirus was successful in only specimens with a low Ct ($Ct \leq 35$ in RP and ≤ 34 in EV). In addition, genotyping was not performed for two specimen pairs, which were initially negative but were found to be positive during the cross examination. Serotype coverage was slightly different between two RT-rPCR reagents used, wherein one reagent covers more enterovirus serotypes.

In conclusion, TS specimens yielded similar performance to NPS in detecting enterovirus in pediatric patients. As TS has benefits over NPS, it can be used as an alternative specimen type for young children who are suspected of having enterovirus infections.

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Declaration of Interest:
The authors declare no competing financial interests.

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