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Identification of Viruses in Patients With Postviral Olfactory Dysfunction by Multiplex Reverse-Transcription Polymerase Chain Reaction

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Objectives/Hypothesis: To investigate causative viruses in patients with postviral olfactory disorders (PVOD).

Study Design: Case-control study.

Methods: One hundred fifty-one consecutive patients diagnosed with PVOD were enrolled, and samples from 38 patients who visited the doctor within 3 months of symptom onset were collected and analyzed. Thirty-two individuals who underwent surgery for nasal septal deviation during the same time period were collected as the control group. The Sniffin' Sticks psychophysical olfactory test was used to evaluate olfactory function. Olfactory cleft specimens were collected using nasopharyngeal flocked swabs (COPAN FLOQSwabs). Eighteen viruses were tested for with the Luminex xTAG RVP FAST v2 Assay Kit.

Results: Out of the 38 patients with PVOD, rhinoviruses were detected in 13 patients, and coronavirus OC43 was detected in one patient. The frequency of positive virus detection in the patients with anosmia was higher than in those with hyposmia (58.8% vs. 19.0%, P = 0.018). In control group, rhinovirus was identified in one patient (3.1%). Nasal obstruction was the most common symptom and was experienced by 71.0% of patients.

Conclusions: Rhinovirus and coronavirus are more commonly identified in PVOD. Our methods represent an approach to screen for viruses that may be involved in PVOD.

Key Words: Olfaction disorders, multiplex polymerase chain reaction, respiratory tract infections, virus..

Level of Evidence: 4

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INTRODUCTION

A viral upper respiratory tract infection (URTI) is one of the most commonly identified causes of persistent olfactory disorders. These disorders are also referred to as postviral olfactory disorders (PVODs).[1] Data from several smell and taste centers have demonstrated that approximately 11% to 40% of olfactory disorders treated at hospitals are caused by URTI.[2–5] Recently, Vaira et al.[6] reported that olfactory dysfunction is common in COVID-19 patients. In some patients, this minor and nonspecific symptomatology can represent the only manifestation, which suggests that identification of viruses of PVOD is crucial not only to understanding the etiological factors of PVODs, but also to diagnosis of unusual viral infections.

It is speculated that some particular viruses might contribute to olfactory dysfunction, because there is a strong temporal connection between the onset of the URTI and the development of olfactory disorders. Potential responsible viruses include influenza viruses, parainfluenza viruses, respiratory syncytial virus, coxsackievirus, adenoviruses, poliovirus, enteroviruses (EVs), and herpesviruses, all of which give rise to the common cold and/or neural symptoms.[7] Unfortunately, due to the absence of direct and indirect evidence, causative viruses have not yet been definitively identified. More than a decade ago, several studies have tried to find responsible viruses for PVODs based on epidemiological data or virus detection.[8–11] It remain controversial if influenza viruses and coronaviruses are the causes of PVOD based on epidemiological data.[10,11] As an example, parainfluenza virus type 3 is most likely to be a causative virus via detecting the virus in nasal discharge[8] or turbinate epithelial cells of patients with PVOD using polymerase chain reaction (PCR).[9] Because most patients suffering a post-URTI olfactory loss present long after the insult, and because the methods used to detect viruses in the nasal cavity are complex, the causative viruses of PVOD remain unclear.

In this study, for the first time we simultaneously investigated multiple respiratory viruses in the nasal

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cavity and olfactory cleft in patients with PVOD using the xTAG respiratory viral panel (RVP) (Luminex, Austin, TX), a commercial virus detection platform.

MATERIALS AND METHODS

Participants

This study was performed in outpatient clinics in Beijing Anzhen Hospital of Capital Medical University after approval was obtained from the institutional ethics committee. All subjects provided informed consent for participation.

Between August 2012 and July 2015, 151 consecutive patients diagnosed with postviral olfactory dysfunction were enrolled in this study. Samples from 38 patients who met the inclusion and exclusion criteria were collected and analyzed. The inclusion criteria were as follows: chief complaint of olfactory dysfunction onset after URTI, olfactory dysfunction confirmed by a professional using a subjective olfactory test, no obstruction of the olfactory cleft on nasal endoscopy or radiograph, visited the outpatient clinic within 3 months after the onset of olfactory dysfunction, and >18 years of age. The exclusion criteria were as follows: allergic rhinitis or chronic rhinosinusitis, platelet or bleeding tendency, contracting another cold within 3 months, pregnant or lactating, obvious central nervous system disease, and refusal to sign complementary informed consent.

Thirty-two individuals who underwent surgery for nasal septal deviation during the same time period were collected (23 men, nine women), ranging in age from 18 to 48years (mean = 29.4 years). The exclusion criteria were as same as above.

Olfactory Testing and Subjective Assessments of Nasal Symptoms

The Sniffin' Sticks test battery was used to evaluate patients' subjective olfactory sense. This battery consists of a perceptual threshold test (T), an odor discrimination test (D), and an odor identification test (I).[12–14] The TDI score, which corresponds to the sum of the results obtained for the threshold, discrimination, and identification measures, was calculated and used to represent olfactory function. The cutoff level of hyposmia is 24.9 in subjects younger than 15 years, 30.3 for those aged 16 to 35 years, 27.3 for those aged 36 to 55 years, and 19.6 for subjects older than 55 years. A TDI score of <15 indicates anosmia.[15]

To collect the patients' cold symptoms, related medical history, and other demographic data, the patients enrolled were sent questionnaires that included questions about age, gender, family history, symptoms, medication history, time of disease onset, and hospitalization time. The symptom categories and items in the questionnaires included systemic, nasal, pharyngeal, and lower respiratory tract symptoms.[11]

Specimen Collection and Preparation

During the first visit, nasal discharge and exfoliated cells were collected from one nasal cavity and olfactory cleft (the relatively larger one) of chosen patients using COPAN's patented nasopharyngeal flocked swabs (FLOQSwabs; COPAN, Murrieta, CA) under nasal endoscope without local anesthesia. Unlike traditional fiber wound swabs, FLOQSwabs have no internal absorbent core to disperse and entrap the specimen, and the entire sample stays close to the surface for fast and complete elution. The perpendicular nylon fibers serve as a soft brush and enable improved collection of cell samples. A disposable clean plastic tube was placed in the anterior nares to avoid contamination. After the samples were collected, the swabs were immediately put into UTM Viral Transport Media (COPAN), which is a viral transport medium for the collection, transport, maintenance, and long-term frozen storage of viruses that is stable at room temperature. The samples were stored at −80°C for up to 6 months or until viral testing. Samples from the control group were obtained in the same way technically as the clinic patients after general anesthesia before starting the surgery.

Detection of Respiratory Viruses With the xTAG RVP

The xTAG Respiratory Viral Panel Fast v2 (RVP FAST v2; Luminex) was used to detect nucleic acids from multiple respiratory viruses in swabs collected from clinical patients. The first-generation version was the first panel for the molecular detection of respiratory viruses that received Food and Drug Administration (FDA) approval. This multiplex PCR kit can simultaneously detect 18 common respiratory viruses and subtypes in a single working shift. The following virus types and subtypes are identified: influenza virus (IVs) A subtype H1; influenza A subtype H3; influenza A subtype 2009 H1N1; influenza B; respiratory syncytial virus (RSVs); coronavirus (CoV) subtype 229E; OC43; NL63; HKU1; parainfluenza virus (PIVs) 1, 2, and 3; human metapneumovirus (hMPV); enterovirus/rhinovirus (EVs/RVs); adenovirus (AdVs); and human bocavirus (HBoV). The limits of detection for each virus at 95% confidence range is available in Supporting Appendix S1.

First, viral nucleic acids were extracted using the QIAamp MinElute Virus Spin Kit (QIAGEN, Hilden, Germany). RVP FAST v2 testing was performed in accordance with the package insert. The testing was conducted by a single operator at each of the clinical sites. The data generated by the Luminex LX 200 were analyzed by the xTAG data analysis software to provide a report summarizing the viruses present in the sample.

Due to the design of the primers and the similarity between RVs and EVs, which are all members of the picornavirus family and consist of a small, non-enveloped particle containing an RNA genome, the RVP FAST v2 cannot directly distinguish between these two types of viruses. Therefore, additional differentiation of RV/EV-positive specimens was performed based on an in-house reverse-transcription PCR and VP4-based phylogenetic analysis.[8,16,17] Briefly, EVP4 (nt 541–560, 5′-CTACTTTGGGTGTCCG TGTT-3) and OL68 (nt 1178–1197, 5′-GGTAA[C/T]TTCCACCAC CA[A/T/G/C]CC-3′) were used as forward and reverse primers, respectively. The PCR products were separated by denaturing agarose gel electrophoresis, and then the purified amplicons were identified by sequence analysis.

Statistical Analysis

Each group was described with the median and quartiles, and differences were determined by analysis of variance using SPSS version 17.0 (IBM, Armonk, NY). Between-group differences in detection rate were determined by Fisher exact test. A P value <.05 was considered significant.

RESULTS

The Demographic and Clinical Characteristics of PVOD

Over nearly 3 years, 151 patients (48 males, 103 females) visiting the outpatient clinic with a major complaint of olfactory dysfunction after a URTI were diagnosed with PVOD. One hundred thirteen patients were excluded from nasal sample collection based on the

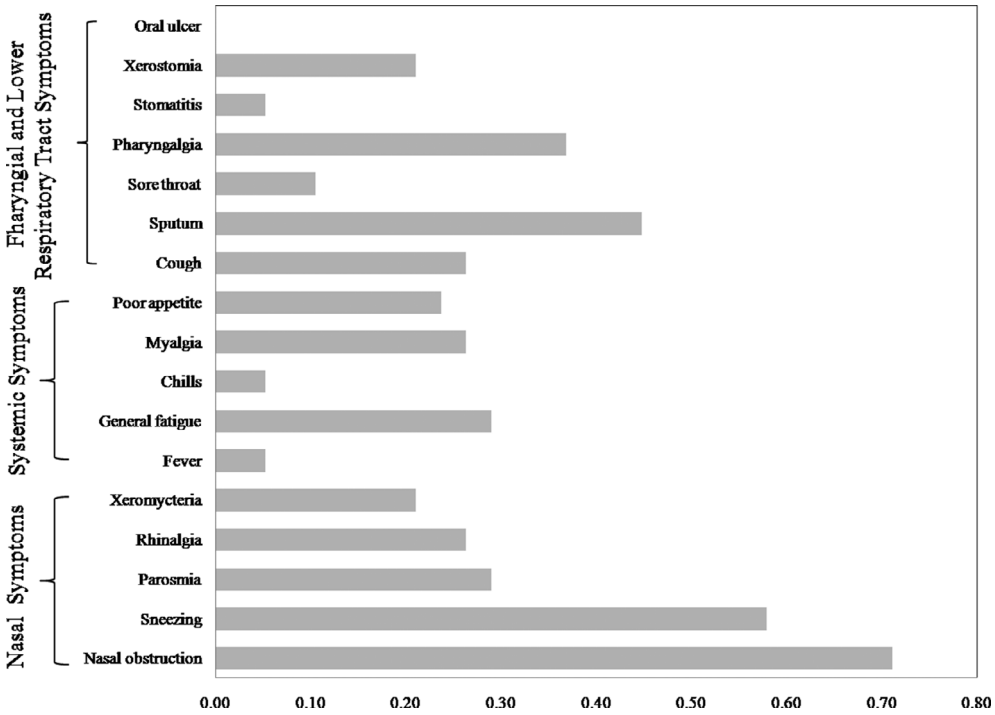

Fig. 1. Percentage of the patients with postviral olfactory dysfunction reporting each symptom.

strict exclusion and inclusion criteria: 64 because their visit took place more than 3 months after the onset of olfactory dysfunction, 12 because of allergic rhinitis, nine because of chronic rhinosinusitis, 26 due to refusal to provide informed consent, and two due to the contraction of another cold within 3 months. Thus, 38 patients (14 males, 24 females) were enrolled in the study. The male-to-female ratio for patients was 1:1.71. The patients' median age was 50.1 years (range = 27–77 years), with no sex-related differences (female patients: mean age = 48.5 years, male patients: mean age = 52.7 years, $P = 0.432$).

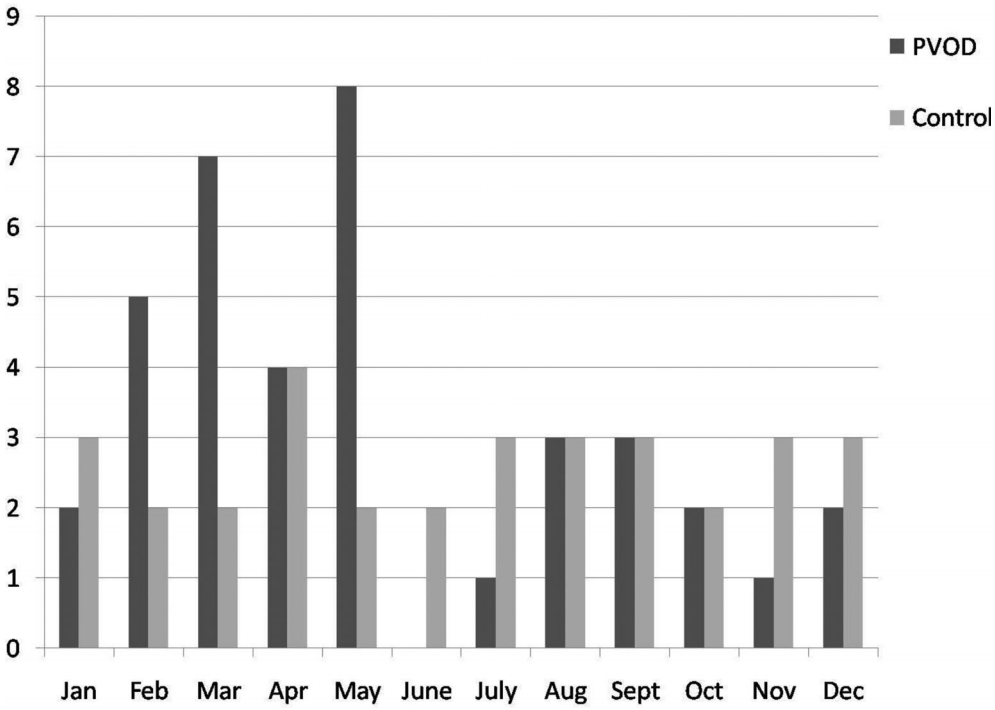

Fig. 2. The monthly distribution of patients with postviral olfactory dysfunction (PVOD) and the control group with septal deviation.

Regarding the severity of olfactory loss, 17 (45.7%) and 21 (54.3%) PVOD patients were identified as anosmic and hyposmic, respectively. Control group subjects were all normal for olfactory function.

According to the questionnaire items regarding cold symptoms, nasal obstruction was the most common symptom and was experienced by 71.0% of patients, whereas nasal cleft obstruction was not obvious through clinical observation. Parosmia appeared in 29.0% of the patients. The observation frequency of each item and symptom category are shown in Fig. 1. The monthly distribution of when the patients from the two groups presented is shown in Fig. 2.

Identification of 18 Viruses

RV was identified in 13 patients (34.2%), and CoV OC43 was found in one patient (2.6%) (Table I). Furthermore, the RVs found in the specimens were further classified as HRV-78 (six patients), HRV-40 (four patients),

TABLE I.
Identification of 18 Respiratory Viruses in the Olfactory Cleft of Patients With Postviral Olfactory Dysfunction by Multiplex Polymer Cain Reaction Detection.

Case No.	Age, yr	Gender	Duration of Smell Loss, mo	T	D	I	TDI	Severity	Virus
1	65	M	1	7	6	4	17	Hyposmia	—
2	56	M	1	6.5	7	7	20.5	Hyposmia	—
3	32	F	1	0	7	11	18	Hyposmia	—
4	36	F	1	5.5	10	10	25.5	Hyposmia	—
5	31	F	1	6	6	7	19	Hyposmia	—
6	44	F	3	5.5	8	11	24.5	Hyposmia	—
7	64	M	3	3.5	6	8	17.5	Hyposmia	Coronavirus
8	72	F	1	3.5	5	9	17.5	Hyposmia	—
9	34	M	3	4.25	8	6	18.25	Hyposmia	—
10	35	M	1	7	9	11	27	Hyposmia	Rhinovirus
11	35	F	1	5.5	8	12	25.5	Hyposmia	—
12	40	F	1	0	3	4	7	Anosmia	—
13	51	F	3	0	3	3	6	Anosmia	Rhinovirus
14	39	M	3	0	5	4	9	Anosmia	—
15	34	M	2	0	2	4	6	Anosmia	Rhinovirus
16	65	M	2	0	2	2	4	Anosmia	Rhinovirus
17	61	F	3	0	3	5	8	Anosmia	Rhinovirus
18	34	F	1	1	1	2	4	Anosmia	Rhinovirus
19	76	F	1	0	3	4	7	Anosmia	Rhinovirus
20	54	F	2	0	4	3	7	Anosmia	—
21	58	M	1	1	5	4	10	Anosmia	Rhinovirus
22	69	M	1	2	9	8	19	Hyposmia	
23	38	F	3	3	14	8	25	Hyposmia	
24	63	F	1	0	4	6	10	Anosmia	
25	50	F	1	1	11	12	24	Hyposmia	
26	34	F	1	4.5	10	12	26.5	Hyposmia	Rhinovirus
27	33	F	1	3.5	11	10	24.5	Hyposmia	
28	50	F	1	0	14	6	20	Hyposmia	Rhinovirus
29	47	M	2	3	5	8	16	Hyposmia	
30	44	F	1	4.5	9	12	24.5	Hyposmia	
31	77	F	1	0	1	1	1	Anosmia	
32	58	M	1	0	0	3	3	Anosmia	Rhinovirus
33	62	F	1	0	0	1	1	Anosmia	
34	41	F	1	0	1	2	3	Anosmia	Rhinovirus
35	64	F	1	0	0	2	2	Anosmia	Rhinovirus
36	27	M	3	1	13	14	28	Hyposmia	
37	54	M	1	1	0	4	5	Anosmia	
38	57	F	1	3	11	4	18	Hyposmia	

F = female; M = male; T = threshold test; D = discrimination test; I = identification test.

HRV-75 (two patients), and HRV-28 (one patient). In the control group, RV was identified in one patient (3.1%). There were no other viruses found in the control group.

The frequency of positive virus detection in the patients with anosmia was higher than in those with hyposmia (58.8% [10/17] vs. 19.0% [4/21], $P = .018$).

DISCUSSION

In this study, we demonstrated the convenience and feasibility of testing samples from the olfactory clefts of PVOD patients using flocked swabs, universal viral transport media, and RVP FAST v2. Among the 38 samples tested, 13 were positive for RV and one was positive for CoV OC43, suggesting that RVs and CoVs are major causative agents of PVOD. We also found that the positive detection rate was higher in the PVOD patients with anosmia than in those with hyposmia, suggesting that the persistence of the virus may be one factor that results in more severe injury to the olfactory system. Viruses were identified in patients who visited a physician nearly 3 months after the onset of symptoms of the common cold. The observation indicates that the virus has not completely disappeared from the olfactory cleft by the time the diagnosis is confirmed, at least at this early time point.

Because most PVOD patients tend to delay visiting a physician, and because the viruses that cause URTIs are thought to be detectable in nasal lavage samples for up to 4 weeks after symptom onset, the isolation and detection of PVOD-causing viruses is difficult. In recent years, some scholars have looked for traces of virus in patients with PVOD. Unfortunately, it is hard to directly compare the results of such investigations due to the use of different inclusion criteria, sample sizes, and virus detection methods, as well as variability in the geographic origin of patients.

Sugiura et al.[11] obtained indirect etiological evidence from epidemiological data by comparing the monthly incidence of PVOD with the monthly frequency of the isolation of various viruses using public infectious disease surveillance data. These results were further examined by testing serum antibody titers for the viruses in 127 PVOD samples. Parainfluenza type 3 was the most likely causative virus. The authors did not consider CoVs and RVs because they were not reported sufficiently frequently in the surveillance data because they are difficult to isolate. Suzuki et al.[8] presented direct etiological evidence by detecting 10 viruses, including RVs, CoVs, IVs, PIVs, RSVs, AdVs, EVs, Epstein-Barr virus (EBVs), herpes simplex virus (HSVs), and varicella-zoster virus (VZVs), in the nasal secretions of 24 patients with PVOD by reverse-transcription PCR. RVs were detected in 10 patients who visited an outpatient clinic within 72 hours after the onset of olfactory dysfunction. CoV and parainfluenza virus were also detected in one patient each. Consistent with our results, the study provided additional powerful evidence that RVs can cause olfactory dysfunction. Wang et al.[9] reported a high detection rate (88.0%) of PIV3 in the turbinate epithelial cells of 22 PVOD patients compared with a low incidence rate in a healthy population. In contrast, we did not find PIV3 in this study. However, it did not mean that PIV3 has been excluded as a candidate causal agent due to the limited sample size. Furthermore, studies have shown that parainfluenza virus type 1[18,19] infects olfactory neurons and persists in nerve tissue of mice for a long time. Direct detection of pathogens in PVOD patients may provide more reliable and direct evidence compared with animal experiments. More clinical research should be done to determine if PIV3 infects olfactory receptor neurons in humans like it does in mice.

The human rhinoviruses (HRVs), members of the family *Picornaviridae* are the most common cause of URTIs and include more than 100 serotypes.[20] Though HRVs preferentially infect nasal epithelial cells and are usually rather harmless, there is increasing evidence that HRVs set the stage for more dangerous conditions, such as severe diseases of the lower respiratory tract, asthmatic exacerbation, and even autoimmunity.[21,22] However, the evidence linking RV infection and olfactory dysfunction is insufficient, because there is no evidence that RVs have the ability to spread from respiratory pathways to the central nervous system through the olfactory tract like the influenza virus.[23] The mechanisms underlying RV-induced olfactory dysfunction may differ from those responsible for influenza-induced olfactory dysfunction.

CoVs, which belong to the *Coronaviridae* family, cause 30% of upper and lower respiratory tract infections in humans.[24] Human coronavirus 229E (HCoV-229E), OC43 (HCoV-OC43), HKU1 (HCoV-HKU1), and NL63 (HCoV-NL63) are detected globally and evoke only mild symptoms in most patients,[25] whereas severe acute respiratory syndrome coronavirus (SARS-CoV) and Middle East respiratory syndrome coronavirus can elicit severe symptoms and even death.[26] Akerlund et al. reported that the olfactory threshold increased after nasal inoculation with CoV 229E.[27] In addition to the lungs, the brain is also infected in some patients with SARS-CoV; such an infection results in long-term neurological sequelae. Studies of transgenic mice that express the SARS-CoV receptor (human angiotensin-converting enzyme 2) have shown that the virus enters the brain primarily via the olfactory bulb, and that infection results in rapid transneuronal spread to connected areas of the brain.[28] The primary cell targets of the mouse hepatitis virus strain JHM, a murine CoV, are also olfactory neurons; infection of mitral neurons in the olfactory bulb is followed by spread to the brain along olfactory and limbic pathways.[29–31] In this study, CoV was identified in one patient based on clinical evidence, suggesting that CoV might be one of the causes of PVOD.

Recently, the identification of respiratory viruses has become more important because of the unexpected emergence of several terrible viruses, such as SARS-CoV and the influenza A viruses H5N1 and H1N1.[32] However, given the number of etiological agents that have been implicated in URTIs, several conventional monoplex PCR assays have to be used in parallel, which is tedious, time consuming, expensive, unstable, and requires large amounts of biological samples.[33,34] Compared with laboratory-developed real-time reverse-transcription PCR

assays, a number of commercial multiplex PCR assays for the detection of respiratory viral infections have been proposed that enable the detection of 12 to 33 different pathogens at the same time.[35] The RVP FAST v2 is the first kit for multiplex detection of respiratory virus nucleic acids to obtain FDA approval. The performance of this kit in testing nasopharyngeal samples has been validated in a clinical laboratory setting. For RSV, hMPV, EV, and RV, it demonstrated an improved overall detection rate over specialized viral reverse-transcription PCR assays.[36] Despite the controversy surrounding assay sensitivity, due to the specificity and cost-effectiveness of commercial multiplex respiratory virus detection assays compared with monoplex real-time reverse-transcription PCR, and in-house–developed multiplex reverse-transcription PCR,[37–40] a commercially available assay for rapid diagnosis would be a very useful tool for routine screening of respiratory specimens in clinical settings and hospital-based laboratories. Consistent with previous studies,[32,34,41] our study has proven it to be a rapid, simple, reliable, and cost-effective platform to identify respiratory viruses in one analysis. Based on this pilot study, specialized multiplex molecular tests should be designed to determine the causative agent of PVOD.

Compared to the disposable mucus extractor used by Suzuki et al.[8] or to other special sample collection instruments such as suction, clysis, or brush, the fine nasopharyngeal flocked swabs used in this study are commercially available and provide an optimal specimen for the diagnosis of respiratory infections by reverse transcription PCR.[42] Furthermore, collecting samples via flocked swabs is easier to perform in an outpatient setting without causing more patient discomfort, even when doing so from the narrow olfactory cleft.

There are several limitations in this work. First, a better control population would have been patients up to 3 months after an URTI without smell loss and with a similar monthly distribution, which might provide additional evidence to causative virus identification. Wang et al.[9] proposed that the control group of patients with nasal septal deviation would be an appropriate comparator. The phenotype of PVOD might be determined by many factors, such as gender, age, and immune state. Due to the limited number of positive samples, our data cannot answer the question of whether higher viral yields are present earlier in the course of PVOD. The disadvantage of the qualitative test panel is that it lacks quantitation; we also did not perform replicate assays. These issues can be addressed in future studies.

In routine practice, tests for viral URTI are not always available, and currently, there are often no treatments available for particular viral infections. Nevertheless, investigating the causative virus is the first step in understanding the pathogenic mechanism of PVOD.

CONCLUSION

RVs and CoVs may be causative viruses in PVOD. More work needs to be done to clarify how the pathogenic viruses identified through screening affect olfactory function.

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