Medical Molecular Morphology (2022) 55:316–322
https://doi.org/10.1007/s00795-022-00326-9

CASE REPORT

Sputum characteristics of patients with severe COVID-19: report of two cases with immunocytochemical detection of SARS-CoV-2 spike protein

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Received: 27 January 2022 / Accepted: 31 May 2022 / Published online: 18 June 2022

Abstract

Patients with SARS-CoV-2 infection and with severe COVID-19 often have multiple coinfections, and their treatment is challenging. Here, we performed cytology analysis on sputum samples from two patients with severe COVID-19. The specimens were prepared using the rubbing method and stained with Papanicolaou stain. In both cases, several cells with frosted nuclei were observed, and the cytological findings per 100 cells were evaluated. The infected cells were mononuclear to multinuclear, showing chromatin aggregation at the nuclear margins, intranuclear inclusion bodies, eosinophilic cytoplasmic inclusion bodies, and mutual pressure exclusion of the nuclei. Immunocytochemical staining revealed that the cells were positive for AE1/AE3 and negative for CD68 expression, indicating their epithelial origin. Furthermore, infected cells with frosted nuclei were positive for surfactant protein A (SP-A) in Case 2, suggesting infection of type II alveolar pneumocytes or Clara cells. Moreover, in Case 2, the infected cells were positive for herpes simplex virus (HSV) I + II and SARS-CoV-2 spike protein, confirming double infection in these cells. In conclusion, sputum cytology is an important tool for determining the diversity of viral infection, and additional immunocytochemistry can be used for definitive diagnosis.

Keywords Sputum · SARS-CoV-2 · COVID-19 · Herpes simplex virus · SARS-CoV-2 spike protein · Immunocytochemistry

Introduction

Owing to the spread of COVID-19, an increasing number of patients are being hospitalized and treated for worsening respiratory symptoms. Patients with severe COVID-19 often experience multiple coinfections, and their treatment is difficult. Particularly, for patients with underlying diseases, sputum cytology can help to differentiate SARS-CoV-2 infection from other infections such as herpes simplex virus (HSV) or fungal infections, including that caused by *Aspergillus* or *Pneumocystis,* that cause similar symptoms. However, as no treatment method has been established to date, active cytology is not recommended for the confirming the presence of infection. In addition, in laboratories, all respiratory specimens from COVID-19 patients are regarded high-risk, and testing is performed under strict conditions [1]. The pathological characteristics of SARS-CoV-2-infected cells have been reported in some comprehensive reviews based on the analysis of autopsy cases [2, 3]; however, reports on cytological characteristics of clinical specimens are scarce. In addition, morphological methods are not included in the laboratory methods used to identify the causative virus [4].

In this study, we investigated the cytological characteristics related to SARS-CoV-2 using sputum samples from two patients with COVID-19. Both patients had COVID-19 pneumonia, and sputum culture did not show any evidence of secondary bacterial infection, but their symptoms

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worsened during the course of their hospitalization, and their SARS-CoV-2 PCR test results remained positive. Sputum cytology was performed to differentiate between reactivation of SARS-CoV-2 infection and secondary infection by fungi or other viruses. The results of the immunocytochemical staining and Papanicolaou staining were useful and are reported here.

Case report

Case 1 was a man in his 70 s with severe diabetes mellitus and renal failure who was diagnosed with severe COVID-19 infection and admitted to the hospital, and mechanical ventilation was started on admission. Sputum cytology was performed on the 30th day after illness onset. Case 2 was a woman in her 70s with a history of hypertension. She was diagnosed with a severe COVID-19 infection and admitted to the hospital. Oxygen was administered without the use of a ventilator. Pneumonia was thought to have become organizing pneumonia, but new consolidation on chest radiography was observed. Sputum cytology was performed on the 24th day after the onset of illness. In both cases, sputum culture did not show any evidence of secondary bacterial infection, but their symptoms worsened during the course of their hospitalization, and their SARS-CoV-2 PCR test results remained positive at the time of sputum cytology.

Sputum samples submitted to the pathology laboratory were processed in a Class II biological safety cabinet by technicians wearing full personal protective equipment, according to community-acquired pneumonia guidelines [1]. Specimens were prepared by the rubbing method (gently rubbing two glass slides together). Two sputum samples, from patients without pulmonary infection clinically, and normal cytological findings, were used as clinical controls. One hundred cells with frosted nuclei, which are considered characteristic of virus-infected cells, were examined for the number of nuclei, presence of nuclear inclusion bodies, mutual pressure exclusion images of the nuclei, and presence of eosinophilic granules in the cytoplasm. If mutual pressure exclusion images were present, the nuclei were considered parallel to each other.

To confirm the origin of these cells and the infecting virus, a cell transcription assay was performed. After Papanicolaou staining, the samples were sealed with mounting medium. After immersion in xylene until the cover glass spontaneously peeled off, the cytoplasm was decolorized by passing through a further two baths of new xylene, followed by two baths of anhydrous alcohol (combined, about 30 min), and then rinsed under running water. Next, hematoxylin was decolorized with 1% hydrochloric acid alcohol (hydrochloric acid in 70% ethanol in a ratio of 1%) and then rinsed under running water. After immersion in 95% ethanol and a water-soluble aerosol fixative (Cytokeep II, Alfresa Pharma Corp., Osaka, Japan) for exfoliated cytology, the fixative solution was allowed to dry [5].

Cell immunostaining was performed using the same cells that were decolorized for Papanicolaou staining. Primary antibodies were against cytokeratin (AE1/AE3) (Millipore-Sigma, Burlington, MA, USA, mouse monoclonal, clone MAB3412, dilution 1:200), HSV I + II (Dako, Glostrup, Denmark, rabbit polyclonal, 1:2000), VZV (Millipore-Sigma, mouse monoclonal, clone MAB8612, 1:2000), CD68 (mouse monoclonal, clone M0876, 1:250), and SP-A (mouse monoclonal, clone M4501, 1:300). Each set of reagents was allowed to react at room temperature for 30 min. Secondary antibodies were Leica Bond polymer system (Leica Biosystems, Newcastle Upon Tyne, United Kingdom), IHC refine kit (DS9800), and 3,3′ diaminobenzidine (DAB), a chromogen. In addition, for double staining, a Vina Green™ Chromogen kit (Biocare Medical, Concord, CA, USA,) was used for blue coloration. Positively stained histopathology specimens were used as positive controls. The primary antibody to SARS-CoV-2 spike protein was mouse monoclonal antibody GTX632604 (GeneTex, Irvine, CA, USA) diluted 500 times and reacted 4 times overnight, and the secondary antibody was Histofine MAX-PO (M) (Nicherei Biosciences Inc., Tokyo, Japan), colored with DAB. As a methodological control, we confirmed that SARS-CoV-2 spike protein could be detected by immunohistochemistry after Papanicolaou staining and subsequent decolorization, using the SARS-CoV-2-infected VeroE6/TMPRSS2 cell line [6] cultured in DMEM, obtained from the Japanese Collection of Research Bioresources (JCRB) Cell Bank. VeroE6/TMPRSS2 cell line without SARS-CoV-2-infection was used as a negative control. In addition, as a negative control of clinical samples, we used cytologically normal sputum cells collected from two patients who were clinically free of respiratory infection. These controls were stained as same as described above (Supplemental Fig. 1).

In both cases, Papanicolaou staining revealed several cells with a frosted appearance that appeared to be infected with the virus. These cells were mononuclear or multinuclear with chromatin aggregation at the nuclear margins, eosinophilic intranuclear inclusion bodies, mutual pressure exclusion of nuclei, and eosinophilic granules in the cytoplasm (Fig. 1a–c). In a sample obtained from Case 1, there were 44 mononuclear cells, 56 multinucleated cells, 5 intranuclear inclusion bodies, and 86 acidophilic granules, and 56 multinuclear cells (100%) showed a mutual pressure exclusion image. Fungal cells (*Aspergillus niger*) were also observed. In a sample obtained from Case 2, 68 mononuclear cells, 32 multinuclear cells, 10 intranuclear inclusion bodies, and 86 eosinophilic granules and 31 of 32 multinuclear cells (96%) showed mutual nuclear exclusion (Table 1). Immunocytochemical staining revealed the expression of AE1/

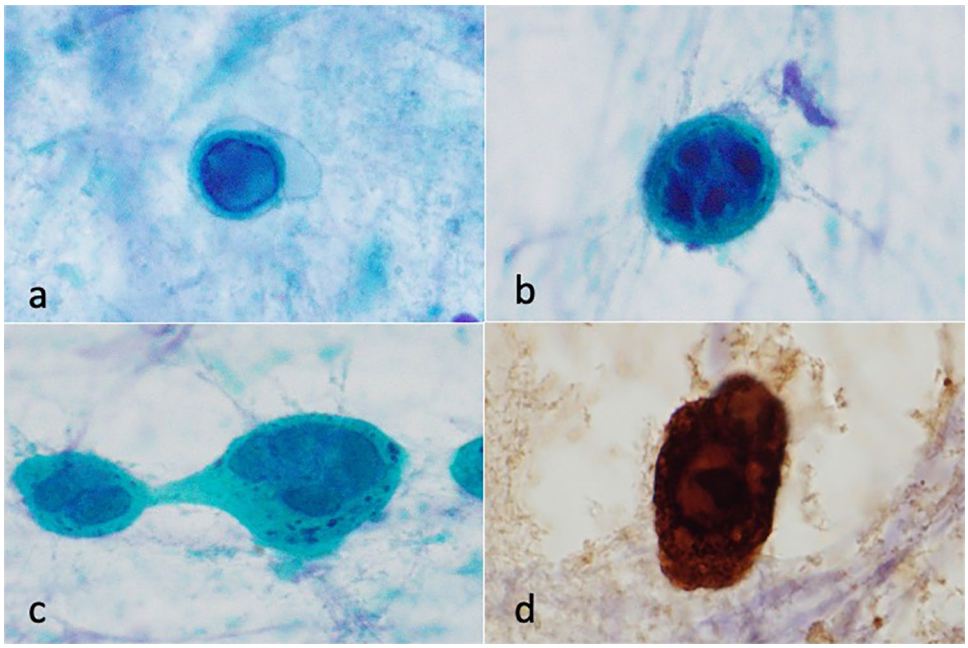

Fig. 1 Virus-infected cells in the sputum of Case 1. **a** Mononuclear cell with frosted nuclei on Papanicolaou staining; **b** multinuclear cell with frosted nuclei on Papanicolaou staining; **c** mononuclear cells with eosinophilic granules in the cytoplasm on Papanicolaou staining; **d** mononuclear cell positive for cytokeratin (AE1/AE3) on immunocytochemistry. Magnification × 1000

Table 1 Characteristics of cells with frosted nuclei (per 100 cells)

	Case 1	Case 2
Mononuclear:multinuclear ratio	44:56	68:32
Intranuclear inclusions	5	10
Eosinophilic granules in the cytoplasm	86	86
Mutual pressure exclusion image	100	96

AE3 (+) (Fig. 1d), HSV I + II (+) (Fig. 2b), varicella-zoster virus (VZV) (−) (Fig. 2d), SARS-CoV -2 spike protein (+) (Fig. 3b), and CD68 (−) cells (Fig. 4). Examination of the Papanicolaou-stained specimens revealed both mononuclear and multinuclear cells among the SARS-CoV-2 spike-protein-positive cells, including cells with large nuclear inclusion bodies. In Case 2, HSV I and II double staining

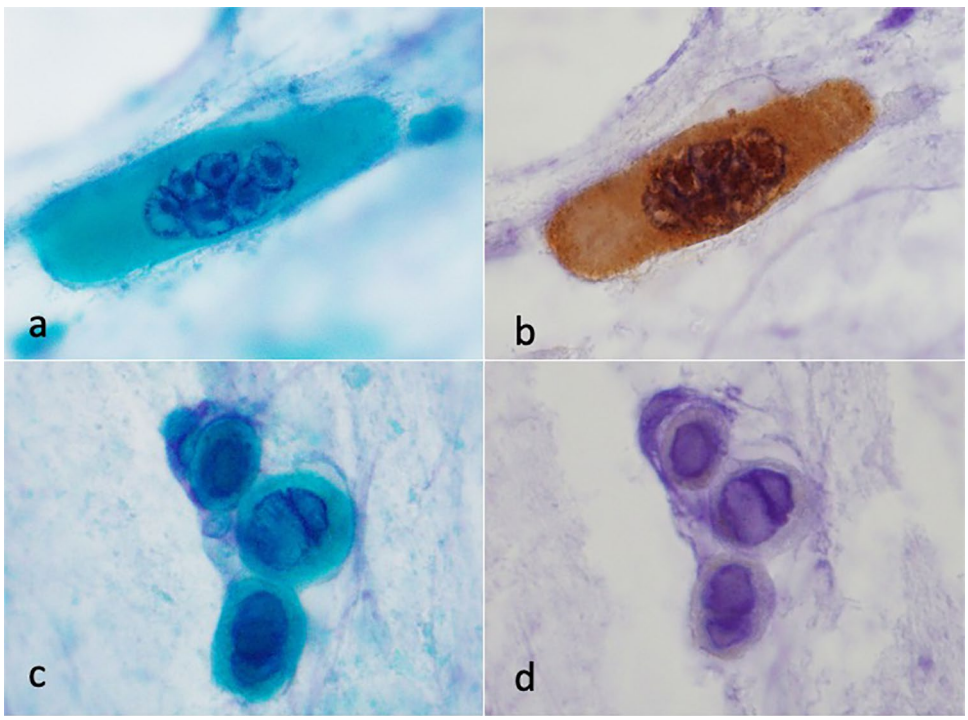

Fig. 2 Identification of herpesvirus-infected cells in the sputum of Case 2. **a** Multinucleated cell with frosted nuclei on Papanicolaou staining; **b** multinucleated cell positive for HSV I and II on immunocytochemistry; **c** mononuclear cells with frosted nuclei on Papanicolaou staining; and **d** mononuclear cells negative for VZV on immunocytochemistry. Magnification × 1000. *HSV* herpes simplex virus, *VZV* varicella-zoster virus

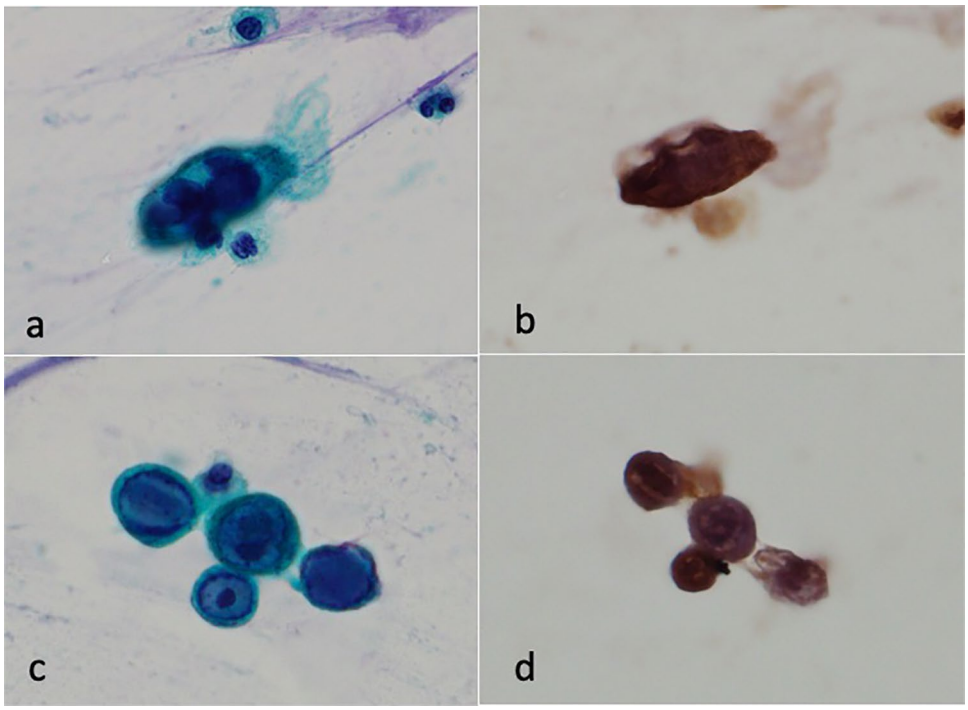

Fig. 3 Identification of SARS-CoV-2-infected cells in the sputum of Case 2. **a** Multinuclear cell with frosted nuclei on Papanicolaou staining; **b** multinuclear cell positive for SARS-CoV-2 on immunocytochemistry; **c** Mononuclear cells with frosted nuclei on Papanicolaou staining; and **d** mononuclear cells positive for SARS-CoV-2 on immunocytochemistry. Magnification × 1000. Both the mononuclear and the multinuclear infected cells were positive for SARS-CoV-2 spike protein

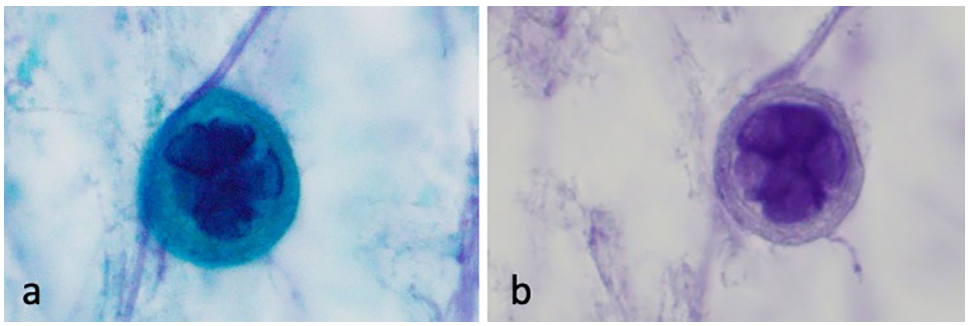

Fig. 4 Immunostaining for CD68 in Case 1. **a** Multinuclear cell with frosted nuclei on Papanicolaou staining; and **b** multinuclear cell negative for CD68 on immunocytochemistry. Magnification × 1000. The cytoplasm was decolored after Papanicolaou staining

of SARS-CoV-2 spike protein-positive cells was positive, suggesting that a single epithelial cell was heavily infected (Fig. 5a–c). The distribution of positive and negative epithelial cells on double staining is shown in Table 2. Of the 76 epithelial cells considered, 5 (7%) were double positive. Both mono- and multinucleated cells were included. Furthermore, infected cells with frosted nuclei in Case 2 were positive for SP-A, suggesting a type II alveolar pneumocyte or Clara cell (Fig. 5d).

Discussion

COVID-19 was diagnosed using PCR or immunochromatography performed on nasopharyngeal swabs or saliva samples. Cytology and histopathology specimens are not used to diagnose COVID-19 because the follow-up and treatment of respiratory tract infections are based on PCR testing. However, it is important to identify the pathologies to clarify the disease progression and the mechanism of lung damage. Examination of autopsy cases has shown that in addition to diffuse alveolar damage (acute respiratory distress syndrome), COVID-19 can also cause vascular damage and secondary pneumonia due to various coinfections [2, 3].

Sputum cytology of samples obtained from patients with severe COVID-19 reveals macrophages with foamy cytoplasm and multinucleation [7]. In addition, squamous metaplasia and repair cells, multinuclear cells, and type II pneumocytes with nuclear enlargement have been reported. These are considered non-specific patterns that reflect acute lung injury. From the perspective of biosafety, performing routine sputum cytology for SARS-CoV-2 is not recommended; however, the primary purpose of cytological examination is to identify coinfections. Therefore, it is necessary to clarify the characteristics of SARS-CoV-2-infected cells.

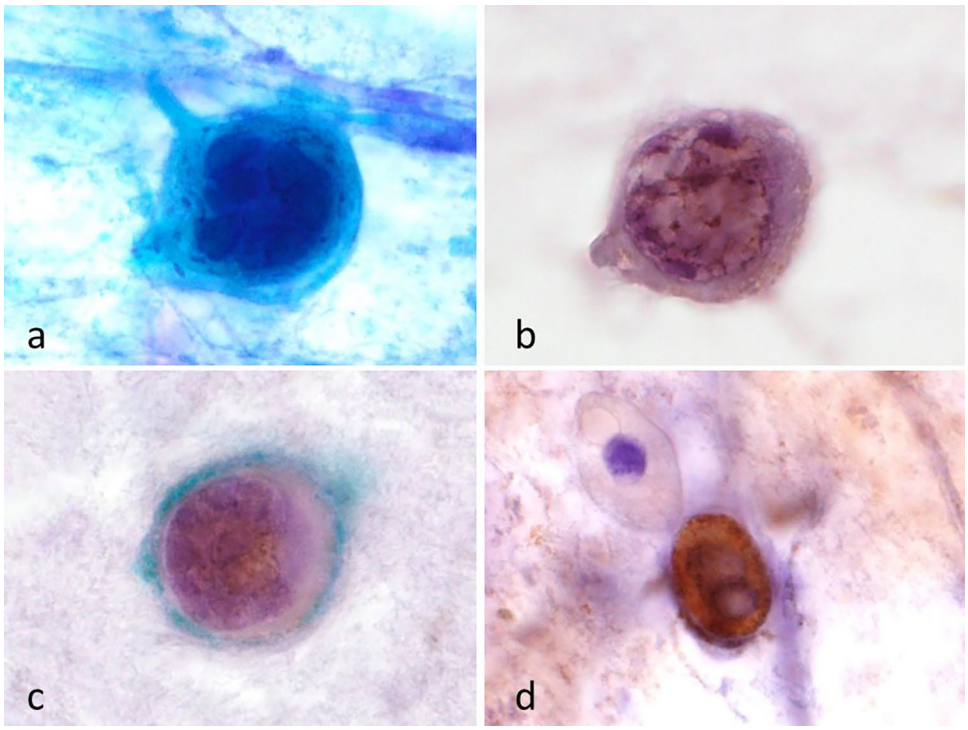

Fig. 5 Immunostaining of infected cells in Case 2. **a** Mononuclear cell with frosted nuclei on Papanicolaou staining; **b** mononuclear cell positive for SARS-CoV-2 on immunocytochemistry; **c** mononuclear cells with nuclear positivity for SARS-CoV-2 (brown color) and cytoplasmic positivity for HSV I and II (blue color) on immunocytochemistry; and **d** mononuclear cells positive for SP-A on immunocytochemistry. Magnification × 1000. *SP-A* surfactant protein A

Table 2 Distribution of positive and negative epithelial cells on double staining for SARS-CoV-2 and herpes simplex virus in Case 2

		HSV I + II		
		Positive	Negative	
SARS-CoV-2	Positive	5 (7%)	30 (39%)	35 (46%)
	Negative	19 (25%)	22 (29%)	41 (54%)
		24 (32%)	52 (68%)	76 (100%)

HSV herpes simplex virus, *SARS-CoV-2* severe acute respiratory syndrome coronavirus 2

Frosted nuclei and inclusion bodies are generally observed in cytological examination of samples obtained from patients with viral infections, and the number of nuclei can be used to identify the viral species. For example, measles virus (an RNA virus) infection is characterized by multinucleated giant cells with inclusion bodies in the cytoplasm and nucleus [8], whereas HSV (a DNA virus) infection is characterized by frosted nuclei, multinucleated cells, mutual pressure exclusion of nuclei, and inclusion bodies in the nucleus [9]; however, the cellular findings change over time after infection. Cytomegalovirus (a DNA virus) infection is characterized by cells with smudgy nuclei, large intranuclear inclusion bodies with characteristic halos, and cytoplasmic inclusion bodies [10]. The nuclei are either mononuclear or binuclear.

In lung tissue autopsies of patients with COVID-19, mononuclear and multinuclear cells with features such as smudge nuclei and chromatin aggregation at the nuclear margins are observed, and the cells are positive for cytokeratin and CD68 expression [11]. In our two patients, Papanicolaou staining revealed mononuclear and multinuclear cells with frosted nuclei, chromatin aggregation at the nuclear margins, and intranuclear inclusion bodies. In the multinuclear cells, mutual pressure exclusion of nuclei was also observed. In addition, eosinophilic granules of different sizes were observed in the cytoplasm (Table 3). Some cells possessed cilia, suggesting an airway epithelial origin.

Bronchial epithelium, type II alveolar epithelium, and histiocytes test positive for SARS-CoV-2 RNA using in situ hybridization [12]. However, in our study, SARS-CoV-2 spike protein-positive cells expressed cytokeratin and were of epithelial origin, whereas in Case 2, the cells were also SP-A-positive, indicating that the infected cells were type II alveolar pneumocytes or Clara cells.

The morphology of the cells infected with SARS-CoV-2 that test positive for SARS-CoV-2 spike protein closely resembled the morphology of HSV-infected cells. However, these cells also tested positive for HSV, and double staining results showed that multiple viruses had infected the same cells. To our knowledge, no previous study has reported direct morphological evidence of multiple infections of the same cells, and this may be the first report of double infection in clinical cytology. However, the limited number of cells was obtained in each case, we were unable to immunocytologically observe a single cell with a large number of double stains, such as SARS-CoV-2

Table 3 Morphological characteristics of virus-infected cells, including cells from the sputum of the two current cases

	Case 1	Case 2
Nuclear chromatin	Frosted	Frosted
Number of nuclei	Mononuclear and polynuclear, mutual pressure exclusion image	Mononuclear and polynuclear, mutual pressure exclusion image
Intranuclear inclusion	+	+
Intracytoplasmic inclusion	+ Granular	+ Granular

spike protein/AE1/AE3, SARS-CoV-2 spike protein/SP-A, SARS-CoV-2 spike protein/CD68. So, the type of lung epithelium that was positive for double staining has not been determined. In addition, since only Case 2 out of the two cases was confirmed, it is necessary to investigate further cases to determine the number of severe SARS-CoV-2 infection cases that cause this phenomenon. Another limitation of our study is that we did not morphologically analyze the sputum cells from non-COVID-19 control cases precisely. They were considered as control, because of clinically no pulmonary disease and cytologically no abnormality by screening, but no comparative study of cell morphology or other immunocytochemistry had been performed.

Cells of the upper respiratory tract origin that are infected with SARS-CoV-2 do not show morphological changes such as multinucleation that are characteristic of viral infection [13]. Although the sites of specimen collection in the previous study differed from those of the two patients in this study, the time course after infection may also play a role. In our patients, 30 and 24 days had passed since the onset of the disease, and the relationship between disease progression and changes in cell morphology should be considered. As the samples from both patients were handled in isolation and the technicians used protective equipment, it is unlikely that any new infection was introduced from other sources. We believe that HSV is an opportunistic infection after SARS-CoV-2 infection becomes severe. Many patients who become severely ill are older adults or have underlying diseases, and the possibility of prior HSV infection before SARS-CoV-2 infection cannot be ruled out. In other words, it is possible that SARS-CoV-2 infection reactivates HSV infection or that SARS-CoV-2 infection enhances susceptibility to HSV infection [14]. We hope that similar reports in the future will clarify COVID-19 pathogenesis. Nevertheless, a severe infection in the lower respiratory tract, as in the present case, may indicate severe disease, and cytological findings may serve as a warning of the possibility of disease progression.

In patients with severe COVID-19, sputum cytology is a useful test for detecting pathogens such as fungi and viruses. If cells suggestive of viral infection are identified, immunocytochemical staining for SARS-CoV-2 spike protein should be used to identify SARS-CoV-2-infected cells, and the presence of frosted nuclei or multinucleated cells should be considered a sign of HSV infection and severe disease.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00795-022-00326-9.

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