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Microscopicimaging of bronchoalveolar fluids of COVID-19 positive intubated patients reveals the different level of SARS-CoV-2 infection on oral squamosal epithelial cells

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COVID-19 pandemic has been a global health emergency due to its association with severe pneumonia and high rate of mortality. In the current study, we reported the direct evidence for the variable level of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) virus infection on several types of oral epithelial cells isolated from the aspirated oropharyngeal and bronchoalveolar secretions of severely infected and intubated patients using cytology, confocal based immunofluorescence imaging (IF), scanning electron microscope (SEM) and transmission electron microscopic (TEM) studies. Cytological analysis showed the presence of keratinised and non-keratinised oral epithelial cells with viral inclusion bodies in stratum granulosum and intermedium cells. IF imaging using SARS-CoV-2 spike protein specific antibody confirmed the presence of virus inside the stratum spinosum/granulosum (keratinised) and stratum intermedium/superficial cells (non-keratinised). No SARS-CoV-2 viruses were seen in stratum corneum cells. SEM analysis also confirms the absence of virus like structure on stratum corneum surface while viruses like structure were seen on the stratum spinosum/granulosum/stratum/intermedium and stratum superficial cells. This advanced microscopic study confirms directly that the virus tends to infect and multiply in the metabolically active or sub-basal cells of oral epithelium and absent in the inactive keratinised stratum corneum from shedding zones of oral mucosa.

Keywords: COVID-19 pandemic, Immunofluorescence, Scanning electron microscope (SEM), Stratum granulosum, Stratum intermedium, Stratum spinosum, Transmission electron microscopic (TEM)

The ongoing 2020 pandemic COVID-19 is caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). It has been a global health emergency due to its high level of infectivity and its association with severe pneumonia and high mortality. SARS-CoV-2 virus may also replicated in the epithelial cells of oropharyngeal region[1]. Primarily, oral symptoms such as taste loss, dry mouth and mucosal blistering occur in the majority of COVID-19 patients. Surprisingly, very little is reported about the infectivity of different cells of oral mucosa by SARS-CoV-2 virus[2]. This virus enters in the cells when its spike protein interacts with the angiotensin-converting enzyme 2 (ACE-2) receptors on epithelial cells[3]. It was already confirmed by *in situ* hybridization, western blotting, immunohisto-chemistry and RT-PCR that the oral and nasal cavity are the main entry point for this pathogen due to overexpression of ACE-2 receptors on epithelial cells[4]. Many reports have confirmed the presence of ACE-2 (a host receptor of SARS-CoV-2 binding), TMPRSS2 and furin (host proteases to cleave the spike protein to release the spike fusion peptide that facilitates host cell entry) on the oral mucosal epithelium that elicits the internalization of SARS-CoV-2 virus to cause the infection[5, 6]. However, the expression levels of these receptors and protease are entirely variable in the different oral anatomical regions as well as the different cellular layers of the same regions. Zhong *et al*., reported that there is a significantly higher expression level of ACE-2 protein in the lip, tongue and oral mucosa, especially towards the basal and sub-basal layer of epithelial cells[7]. The

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corresponding gingiva and palate epithelial cells showed weak positive ACE-2 staining[7]. All these reports speculated indirectly that the ACE-2, TMPRSS-2 and furin positive oral epithelium may provide the favourable condition of SARS-CoV-2 infection[8]. However, there are negligible reports to provide the direct proof of level of infection of SARS-CoV-2 in the different keratinized or non-keratinised oral mucosal cells. In this study, we aimed to provide the direct proof for the presence and level of infection of SARS-CoV-2 virus in the different cells of oral cavity collected from the oropharyngeal and bronchoalveolar secretion of severely infected and intubated COVID-19 patient. These were reported through pap staining (cytology), confocal based immunofluorescence imaging, scanning electron microscope and transmission electron microscope.

Material and Methods

Material

Karnovsky`s fixative (0.5% Glutaraldehyde + 2.0 % Paraformaldehyde), hematoxylin, eosin, orange G., Scott's water, xylene, DPX, PBS, Poly-L-lysin, epoxy embedding kit and DAPI were purchased from Sigma chemical company USA. BSA, ethanol was from Himedia and Triton X-100 was purchased from Fisher Scientific. Osmium tetroxide was procured from Ted Pella, and uranyl acetate from Taab, and lead citrate from Ladd. Polyclonal anti-SARS-CoV-2 specific primary antibody (Abcam; Cat no. ab275759), Alexa fluor-594 conjugated anti-rabbit secondary antibody (Abcam; Cat no. ab150080), 40 nm gold nanoparticles conjugated goat anti- rabbit antibody (Taab) were procures and used mentioned along with them.

Methods

Ethics statement

Experimental procedures for collecting the bronchoalveolar secretion from the severe SARS-CoV-2 infected (COVID-19 positive) and intubated patients from Intensive Care Unit (ICU) were performed after written informed consent from all participants or patients' representatives and after ethical approval by the Institutional Ethics Committee (IEC) of the All India Institute of Medical Sciences New Delhi, India (Ref. No. IEC-307/27.04.2020, RP-10/202).

Sample collection and primary fixation

The aspirated oropharyngeal and bronchoalveolar secretions containing unstimulated suctioned saliva were collected from intubated SARS-CoV-2 positive patients at COVID-19 Intensive Care Unit (ICU), Trauma Centre, All India Institute of Medical Sciences, New Delhi between 4th to 6th days of admission. These samples were collected in freshly prepared Karnovsky`s solution (final 0.5% Glutaraldehyde +2.0 % Formaldehyde). The oropharyngeal and bronchoalveolar secretions (15-20 mL) from the intubated patient were collected in a 50 mL falcon containing 20 mL of 2X fixative (1% Glutaraldehyde and 4% Formaldehyde in 0.2M PB). The collected sample vials were surface sterilized and stored in a specific refrigerator at 4°C after two hours of incubation at room temperature. Available medical records were reviewed before the collection of the samples, for information regarding demographics, symptom history, underlying conditions, infectious disease testing, imaging study findings, treatment and advanced supportive care received. This investigation was reviewed in accordance with the institute ethics for subjects' review procedures.

Sample processing for the cellular enrichment

The collected bronchoalveolar secretion in Karnovsky`s solution was diluted 10 times with 0.1 M NaCl solution and strained through 100 µM pore size nylon mesh cell strainer. The filtrate was centrifuged at 2500 rpm for 3 min in a swinging bucket rotor. The cellular pellets were washed 2-3 times for 10 min with PBS solution to remove the excess mucus. The cellular constituents were enriched by centrifugation at 1200 g for 3 min and resuspended again in the primary fixative A (0.5% Glutaraldehyde 2.0% paraformaldehyde in 0.1M PB buffer) which were further used for Pap staining, immunofluorescence, SEM, and TEM imaging.

Cytological examination/Pap staining of the samples

Pre-treated poly L-lysine coated glass slides were cyto-spun (700 rpm for 5 min) with 200 µL of enriched squamous cells sample to form a smear on the slide. This smear was fixed with 90% ethanol. After the fixation, the slides were washed under running tap water for 1 min. Haematoxylin staining was done for 2-4 min and washed (for 1 min each) sequentially by running tap water, Scott's water and again with tap water. This smear was dehydrated gradually with 70%, 95%, 100% ethanol for 1 min each. Alcohol dehydration with 100% ethanol was repeated twice. The smear on the slides were treated with Orange G for 4 min and again dehydrated with

ethanol series up to 95%. Slides were dip in Eosin stain for 10 min and again treated with 95% ethanol and 100% acetone twice. The stained slides were treated with xylene three times (5 min each) to clean the additional stain and mounted using DPX.

Immunofluorescence using SARS-CoV-2 spike protein specific antibody

Enriched squamous epithelial cells from the bronchoalveolar secretion were rinsed three times with 0.1 M phosphate buffer. Samples (10 μL) were smeared on poly-L-lysine coated glass slides, and air dried at 37°C overnight to fix it. Slide smear was permeabilized using PBST (0.1% Triton X-100 in 1X phosphate buffer saline) and blocked with 2% BSA in PBS for 30 min. Primary antibody (Abcam ab275759, polyclonal against S1 spike protein) at 1:500 dilution was spread on the slides at room temperature for 4 h in a humid chamber and washed with PBS. Fluorophore conjugated secondary antibody (Alexa fluor-594 conjugated anti-rabbit secondary antibody in 1:500 dilution; Abcam, Cat No.- 150080) were incubated for 1 h at RT in dark and washed thoroughly by phosphate buffer. For nuclear recognition, DAPI staining (1 μg/mL) was done for 5 min and washed with PBS to remove the excess amount. Coverslips were mounted in slides using 90% glycerol as mounting medium. Fluorescence imaging was analysed on a laser confocal microscope (Leica SP8 Germany) using different objectives.

Scanning Electron Microscopy

For scanning electron microscopy (SEM), the enriched and primary fixed cellular component of bronchoalveolar secretion were osmicated, dehydrated with ethanol, critical point dried (K850, EMS) and mounted on double- sided tape on aluminium stub. These stubs were sputter coated with a gold-based sputter coater (Agar sputter coater) for 180 seconds. Electron micrographs were obtained on EVO18 (Zeiss, Germany) SEM operated at 20 kV accelerating voltage, between 8-10 mm average working distance with SE detector, and magnifications ranging from ×5000 to ×30000.

Transmission Electron Microscopy

To prepare the specimens for TEM imaging, enriched cellular constituents of bronchoalveolar secretion collected from the ICU wards of AIIMS, New Delhi were further primary fixed if required using primary fixative (2.5% Glutaraldehyde+ 2.0% paraformaldehyde in 0.1M PB buffer). The primary fixed cellular pellets were washed with 0.1 M phosphate buffer (pH 7.4) and post-fixed with 1% osmium tetroxide in 0.1 M phosphate buffer (pH 7.4) for 1 h (secondary fixation) at 4°C. Pellets were again washed with distilled water and en-bloc staining was done with 2% uranyl acetate for 2 h. These samples were again washed with distilled water and dehydrated in an ethanol series (30%, 50%, 70%, 80%, 90%, and 100%). Dehydrated pellets were infiltrated with toluene/resin and finally embedded in epoxy resin. The blocks were polymerised by incubating at 65°C for 48 h. Resin blocks were trimmed, polished and 70 nm thin sections were sectioned using UC7 ultramicrotome (Leica), and mounted on TEM grids. These ultrathin sections were stained with 5% uranyl acetate and 5% lead citrate. Sections were imaged using Transmission Electron Microscopy (Thermo Fisher Scientific Talos F200) using a FEG filament operated at 200 kV.

Results

Sample Collection

The oropharyn geal and bronchoalveolar secretion (total 35 samples) from the collection tube of intubated severe COVID-19 patients were collected between 4^{th} to 6^{th} days of their ICU admission at AIIMS New Delhi in Karnovsky`s solution. The collection tubes were surface sterilized using the 70% alcohols for the decontamination and also to be used in the BSL-2 condition. The collected secretion contains the oral mucosal cells along with the bronchoalveolar cells. The oral mucosal cells were enriched by straining with the help of appropriate size cell strainer (nylon mesh filter). These enriched oral epithelial cells were used for various microscopic studies to explore the type of cells and the level of the SARS-CoV-2 infection.

Papanicolaou (Pap) imaging of bronchoalveolar secretion from COVID-19 infected intubated patients showed viral specific inclusion bodiesin oral epithelium

The oropharyngeal and bronchoalveolar secretion contains numerous oral epithelial cells that were stained either by eosin to produce reddish colour (light to deep red) or as basophilic stain hematoxylin to stain blue colour (from light blue to deep blue) (Fig. 1). This staining clearly differentiates the presence of keratinised and nonkeratinized epithelium of oral origin in the enriched samples[14]. The eosinophilic cells with reddish colour were either from the keratinized origin such as stratum corneum or non-keratinized stratum superficial cells. Very

few stratum corneum cells were present. These cells were confirmed by characteristics keratinization, and absence of nucleus and were also devoid of cytoplasmic inclusion bodies (Fig. 1). However, numerous stratum superficial cells of non-keratinised origin were observed with pyknotic nuclei. These cells were also devoid of any SARS-CoV-2 infection characteristics cytoplasmic inclusion body (Fig. 1). Another keratinised origin stratum granulosum cells with large nucleus along with characteristics oval shaped keratohyalin granules were observed with non-identifiable (SARS-CoV-2 specific or other type) inclusion bodies. Apart from these, there were many metabolically active basophilic (blue) oral epithelial cells such as stratum spinosum, and stratum intermedium in the sample. These cells showed the presence of SARS-CoV-2 specific inclusion body in the cytoplasm (black arrow) and also in the nucleus (white arrowhead) (Fig. 1). On careful imaging at 1000X magnification, the viral infection-based characteristics inclusion body were also seen in stratum superficial, stratum intermediate and striatum spinosum cells (Fig. 1).

Immuno-fluorescence imaging confirms the presence of SARS-CoV-2 virus on the various oral epithelium cells

The bronchoalveolar secretion of intubated COVID-19 patient were analysed to identify the level of SARS-CoV-2 infection in different type of oral epithelium cells by confocal based immunofluorescence imaging using virus spike protein (S1) specific primary antibodies. It was found that the outermost layer of oral epithelium such as stratum corneum or stratum superficial were least infected or not at all infected by the SARS-CoV-2 virus (Fig. 2). However, the stratum intermedium and stratum spinosum cells were heavily infected in the severely infected COVID-19 patient (Fig. 2). We have observed stratum corneum, granulosum, intermedium and superficial cells in one frame with differential

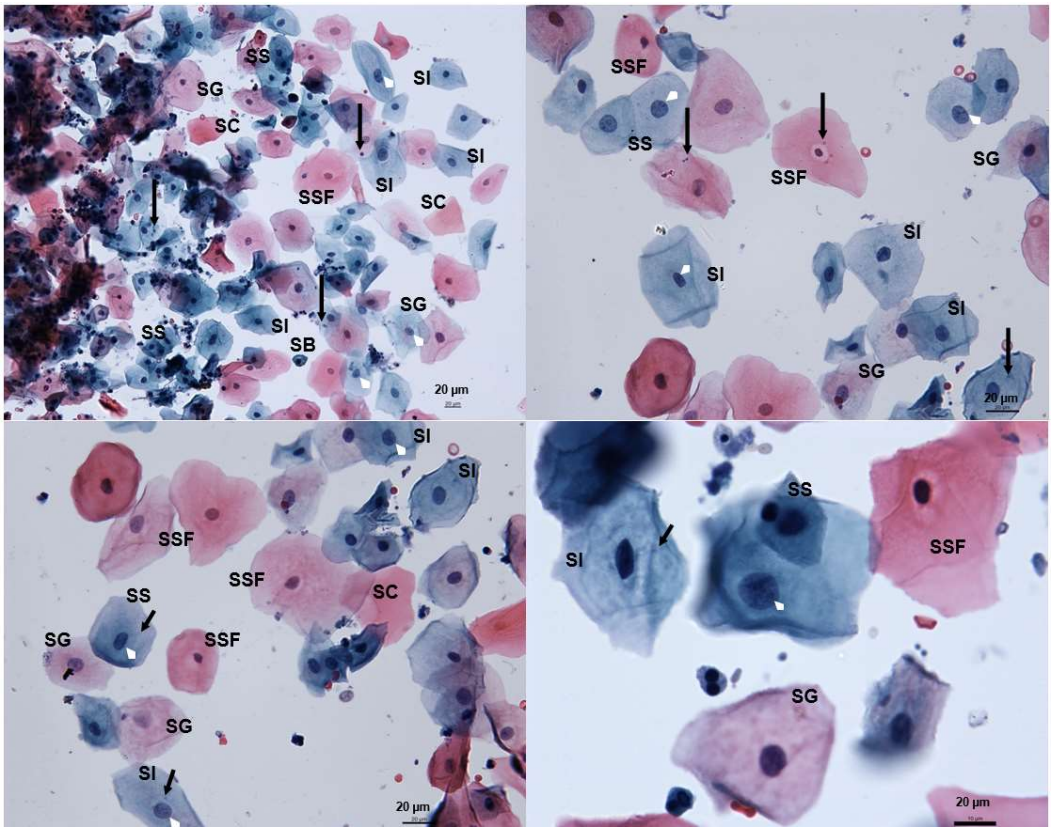
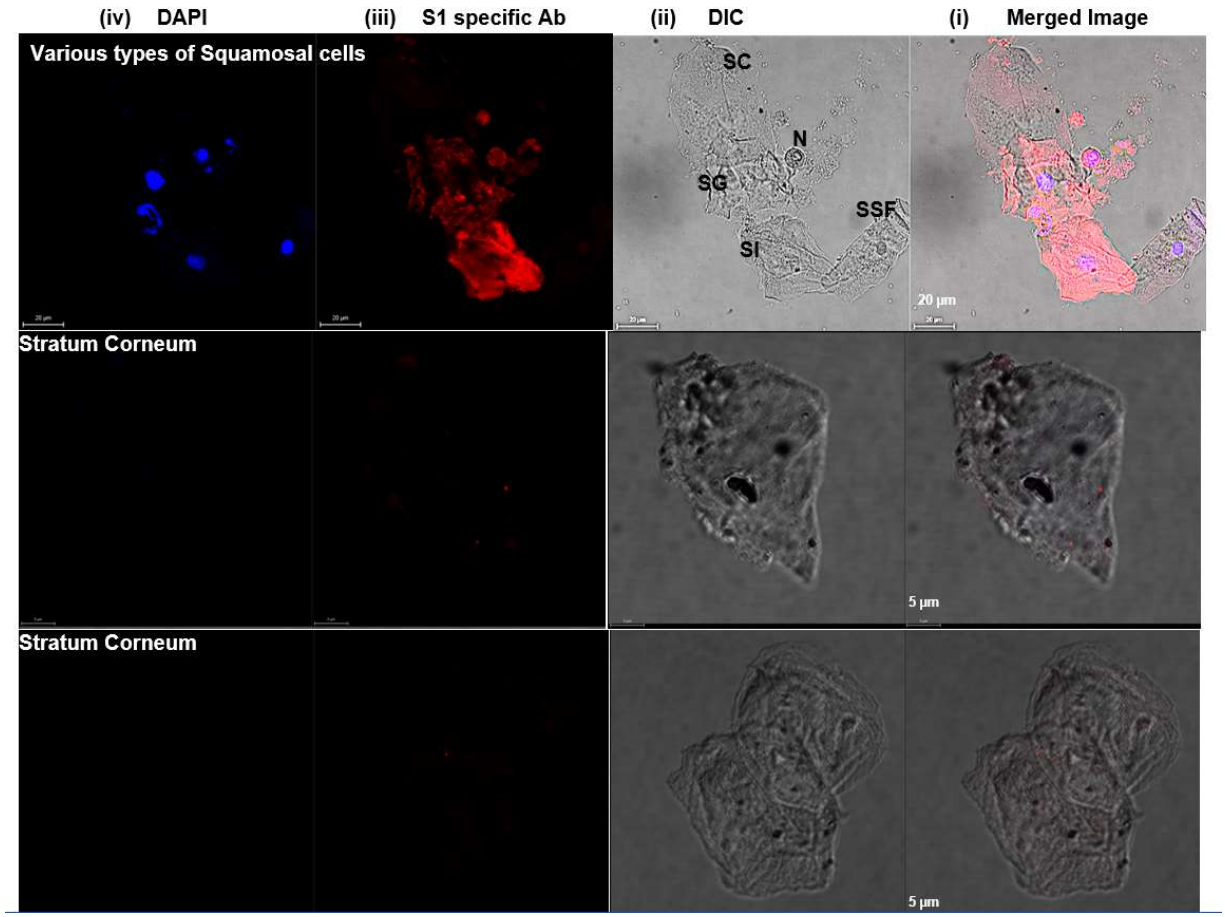


Fig. 1 — Cytology of enriched oral mucosal cells from the oropharyngeal and bronchoalveolar secretion using Pap staining. Pap staining was performed in the cellular enriched (by cell strainer) bronchoalveolar secretion of ICU admitted and intubated COVID-19 patients to identify different type of oral epithelium and possibility of presence of SARS-CoV-2 inclusion body. It was found that the enriched secretion contains stratum corneum (SC) stratum granulosum (SG), stratum superficial (SSF), stratum intermedium (SI), stratum spinosum (SS) and very few cells of stratum basale (SB) cells. The probable SARS-CoV-2 inclusion body in cytoplasm (black arrow) and inside the nucleus (white arrow heads) were indicated. The images were taken on Olympus microscope (Olympus BX53) using 63X/100X objectives. The scale bar was given in each image


(iv) DAPI
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Various types of Squamosal cells
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N
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Stratum Granulosum
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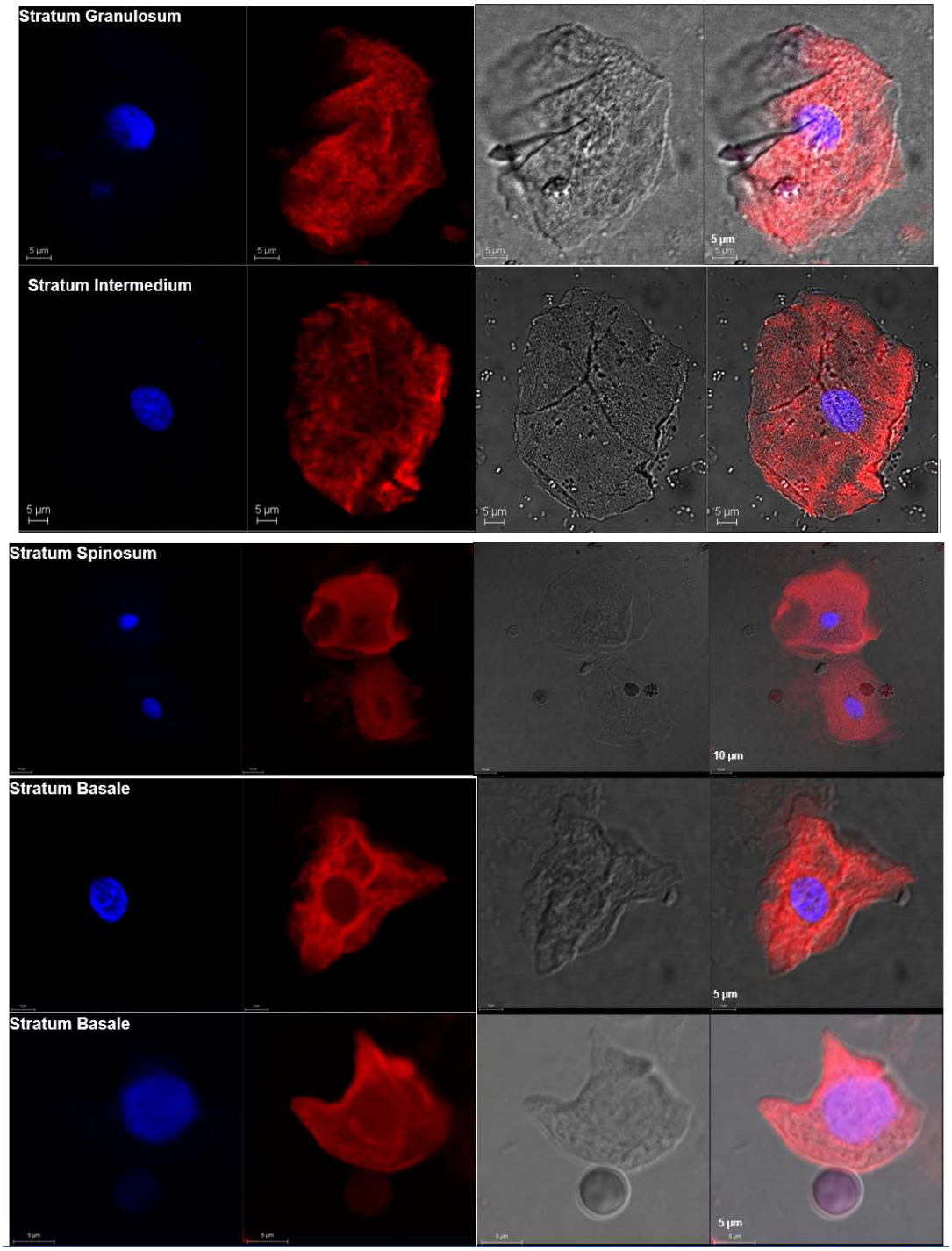


Fig. 2 — Confocal immunofluorescence microscopy of enriched oral epithelium from bronchoalveolar secretion of ICU admitted and intubated COVID-19 patients showing differential internalization of SARS-CoV-2 virus inside the different cells of oral epithelium. Immunofluorescence was performed using the virus spike protein (S1) specific antibody as primary antibody and Alexa-flour 594 conjugated goat anti rabbit secondary antibody. SARS-CoV-2 virus showed prominent internalization in the stratum spinosum (SS) of keratinized origin and stratum intermedium of non-keratinised origin cells. The presence of virus was gradually decreased in stratum granulosum, stratum superficial cells and least or negligible in stratum corneum. It is a novel finding that virus was found mostly towards the sub-basal lamina and least in the primarily exposed shedding surface of epithelium. Column (i) for nuclear stain by DAPI (blue) (ii) red fluorescence indicating the presence of SARS-CoV2 virus as Alexa-flour 594 (iii) DIC image and (iv) merged image of all three. The scale bar are given with each figure. The imaging was done either using 63X or 100X objective lens having DIC. SC: Stratum corneum; SG: stratum granulosum; SSF: stratum superficial cells; SI: stratum intermedium; and N:Neutrophil

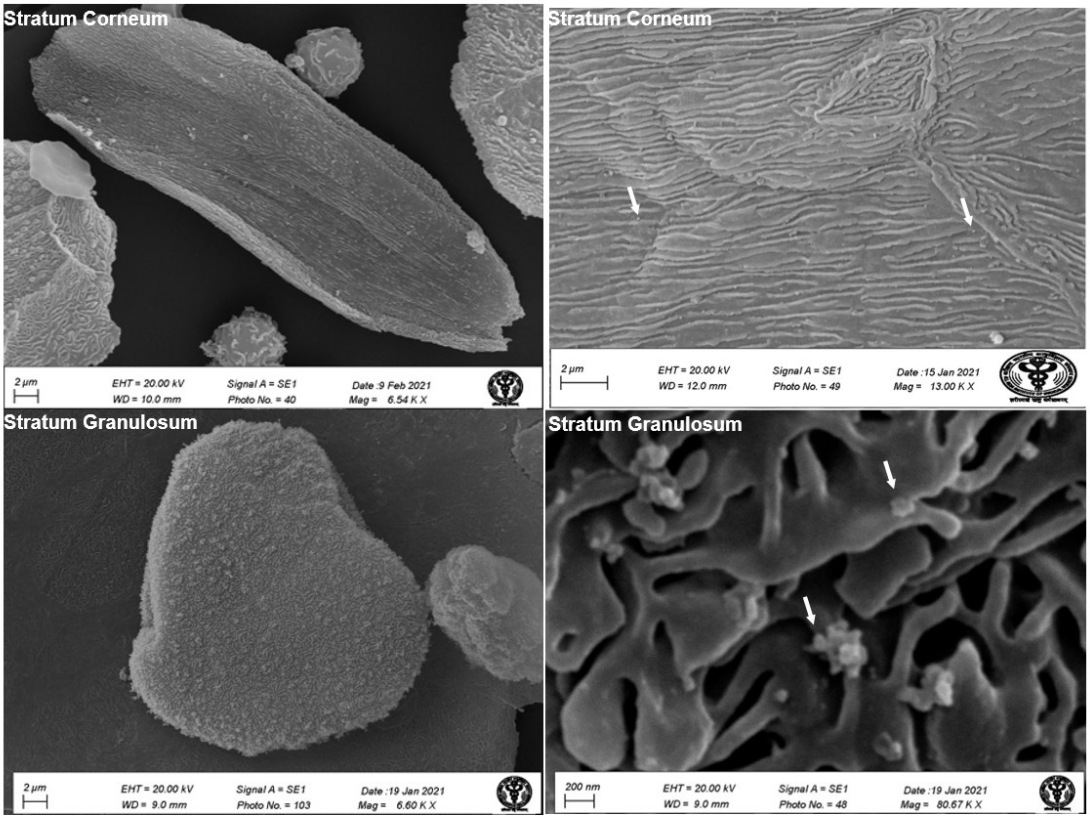
level of infection of SARS-CoV-2 virus (Fig. 2, first image). Interestingly, the levels of infection were maximum in stratum spinosum and intermediate cells followed by stratum granulosum and stratum basale cells (Fig. 2).

Scanning electron microscopic imaging reveals the presence of viruses on the surface of different oral epithelium cells

The oral epithelium cells of bronchoalveolar secretion were imaged by scanning electron microscopes to identify the presence as well as level of SARS-CoV-2 virus infection on the surface of different types of oral epithelium cells. Very few stratum corneum cells were present with characteristic flat and keratinised structure. We did not find any alteration on the surface structure in comparison to literature reported in the stratum corneum cells as well as could not identify the presence of SARS-CoV-2 on the surface of these cells. However, small vesicles like structure, as a possible SARS-CoV-2 virus were seen in the stratum granulosum (small dotted shaped) and relatively less amount on the stratum superficial cells (Fig. 3). The relatively highest number of small dotted structures (could have possibility of SARS-CoV-2 virus) were seen in the stratum intermedium and stratum spinosum cells (Fig. 3). It was found that the least or negligible viruses were present on the stratum corneum as well as on stratum superficial cells. The stratum intermedium cells have a relatively high number of viral presences on the surface (Fig. 3).

TEM provided the direct evidence of presence of SARS-CoV-2 virus on oral epithelium

Para-keratinized and non-keratinised oral epithelial cells were clearly identified by the presence of conspicuous tono-fibrils and dispersed tonofilaments, respectively (Fig. 4). The transmission electron microscopic imaging of enriched oral epithelium from the oropharyngeal and bronchoalveolar secretion of intubated COVID-19 positive patients showed SARS-CoV-2 virus with prominent surface projections (spikes). The SARS-CoV-2 virus was not identified in the stratum corneum cells but some of the viruses were seen in the stratum superficial cells (Fig. 4). Most of the stratum granulosum cells with bunch of



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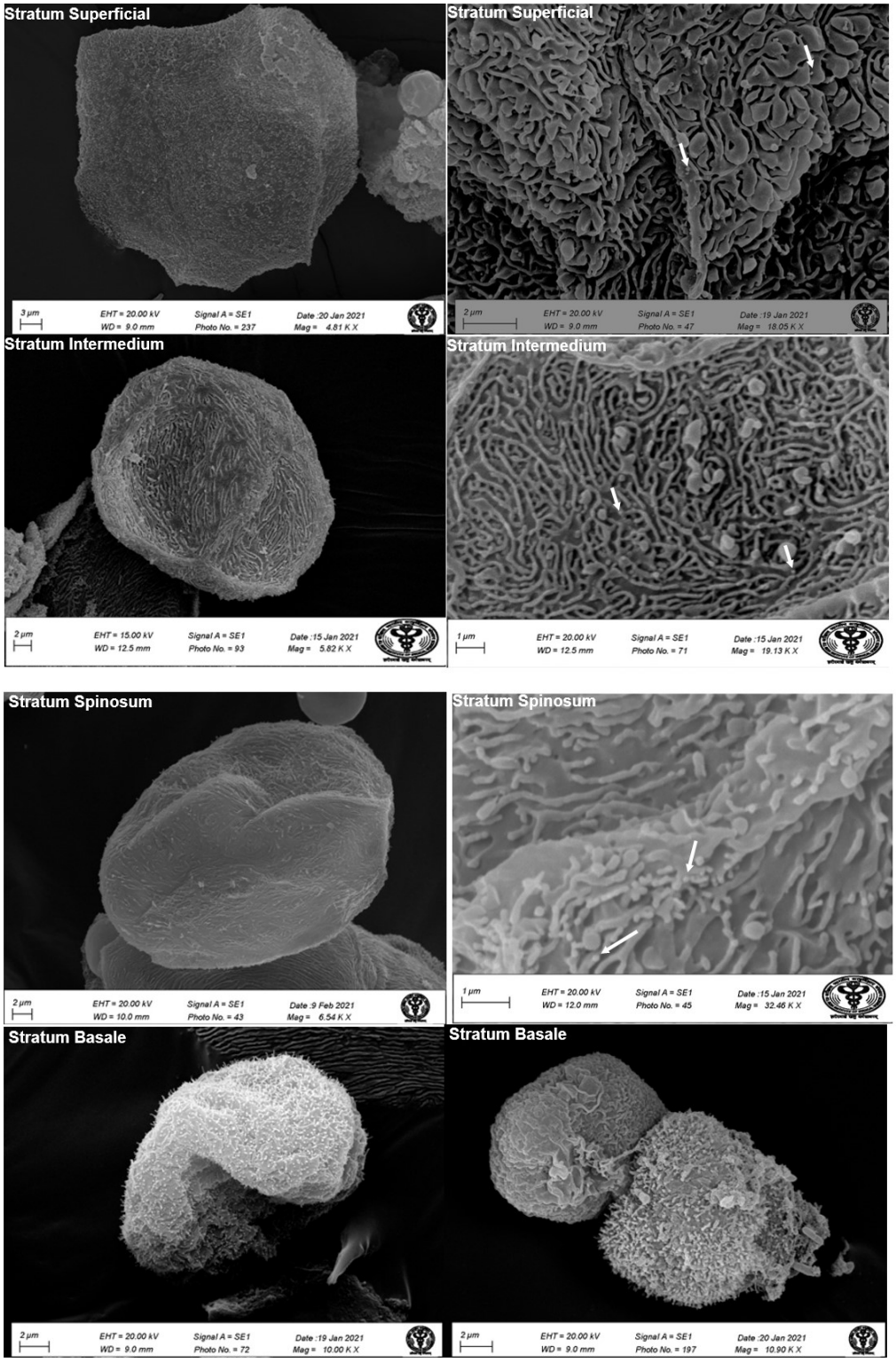

Fig. 3 — Scanning electron micrograph of oral epithelium from the oropharyngeal and bronchoalveolar secretion of COVID-19 positive intubated patients. Scanning electron microscopy were performed on the enriched oral epithelium cells from secretion after the critical point drying and sputter coating. Different oral epithelium cells from keratinised and non-keratinized origin were identified based on the size, shape and surface morphology. The magnified image of each type of epithelium cells with SARS-CoV-2 (white arrow) on surface were reported using many patient samples. The SARS-CoV-2 virus were identified/denoted based on the size and morphology only

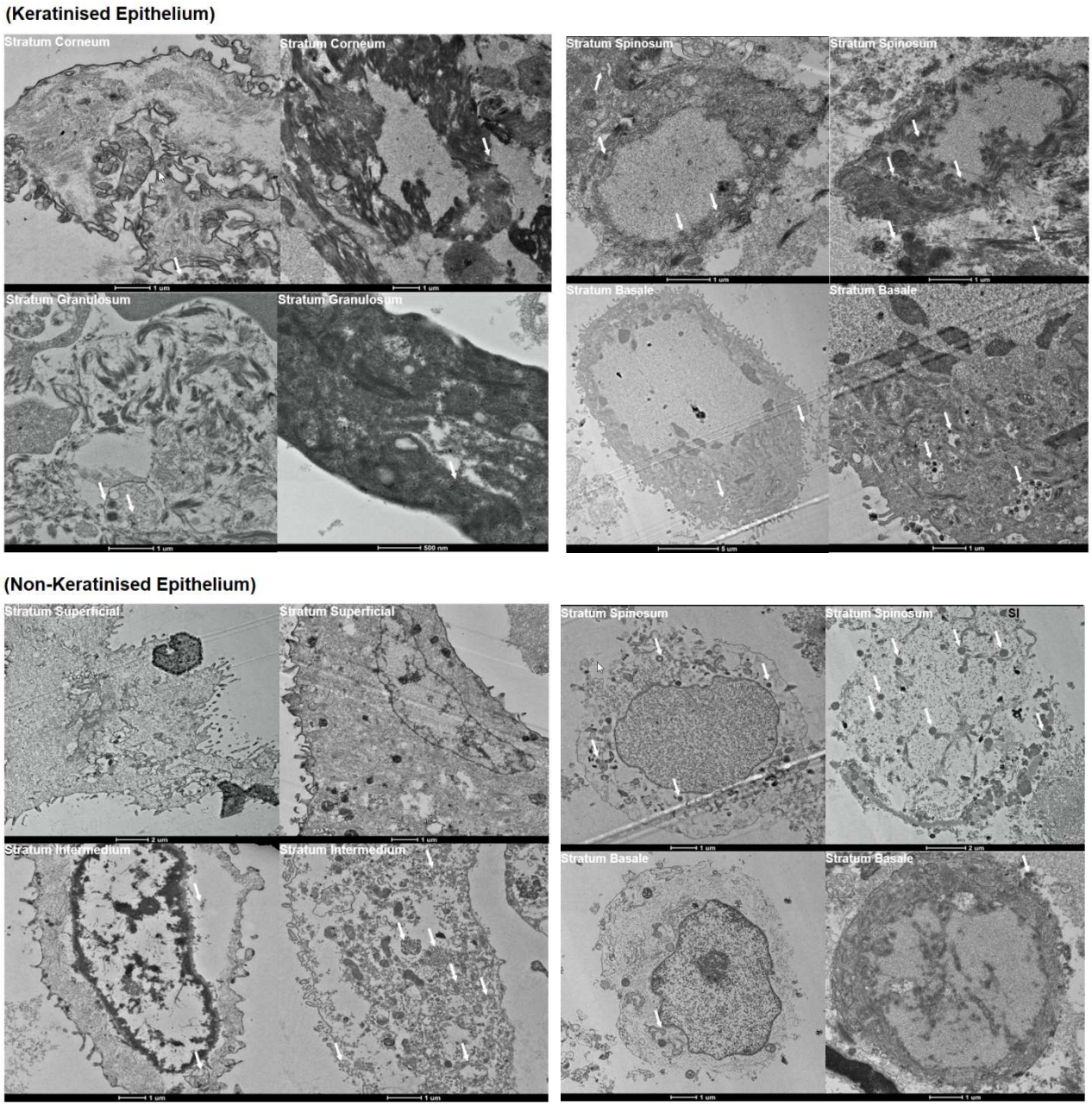

Fig. 4 — Transmission electron microscopy of oral epithelium from oropharyngeal and bronchoalveolar secretion of COVID-19 positive intubated patients.The enriched squamous epithelium cells were embedded in epoxy resin, ultra-sectioned and imaged on Talos TEM after staining with uranyl acetate and lead citrate. The keratinised and nonkeratinized cells were identified by presence of conspicuous tonofilament (keratinised origin) and dispersed filament (non-keratinized origin). The types of epitheliums in both origin (keratinised and non-keratinized origin) were identified by shape, size, granules, presence of cell organelles, nucleus size and shape. The presence of SARS-CoV-2 in these cells are denoted through the white arrow. It was found that most the SARS-CoV-2 virus were present in the stratum spinosum and stratum intermedium cells

tono-fibrils (keratinised) showed the SARS-CoV-2 virus of size 110-205 nm in the vesicular body. However, these viruses, present near the nuclear membrane were relatively large in number and bigger size. Rounded shaped contrast dots in stratum spinosum, and stratum basale of keratinised origin were seen. The characteristics crown shape of the SARS-CoV-2 were prominently visible in the non-keratinised origin epithelium especially in the stratum intermedium and stratum spinosum (Fig. 4). The highest number of these viruses with well shapes crowned structure were observed in non-keratinised

stratum spinosum cells. The stratum intermedium and stratum spinosum cells were heavily infected by the SARS-CoV-2 virus. Very few SARS-CoV-2 viruses were present in the stratum basale cells (Fig. 4).

Discussion

The novel severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is an enveloped single stranded positive-sense RNA virus of size 50-200 nm (*in vivo*) with specific crown appearance due to presence of spikes protein. This virus is a causal agent of pandemic COVID-19 that primarily infected and internalized by interacting with angiotensin-converting enzyme 2 (ACE-2) receptors of host cells through its spike protein (S protein) as a targeted cell internalization[5]. The host cell proteases TMPRSS2 (transmembrane protease serine 2) cleaved the viral spike protein and released the spike fusion peptide that facilitates the virus internalization to the host cell[9,10]. The ACE-2, transmembrane protease serine 2 (TMPRSS-2), and furin are the key components that assists the viral internalization and establishment of infection into the host cells[5,9-11]. ACE-2, TMPRSS-2, and furin mRNA expression was observed in taste bud-derived cultured cells, which was further confirmed by immunofluorescence[5]. ACE-2 and TMPRSS-2 protein expression were reported in various human tissues such as epithelial cells of the lung, intestine, kidney, oral mucosa and colon[5,12-15]. Among these tissues, the expression of these receptors on oral mucosa cells was higher in the metabolically active epithelial cells such as stratum spinosum or basale cells[5,16]. ACE-2 receptor was reported in the tongue tissues, oral mucosa, exfoliated epithelial cells in the saliva[5]. Single-cell RNA sequencing data of severely infected patients also reported to confirm the presence of SARS-CoV-2 in oral epithelium especially in supra-basal cells of the mucosae[2]. The probability of infection and replication of SARS-CoV-2 virus on oral mucosa is critical to understand the underappreciated role in transmitting this virus.

The oral cavity are lined by two types of specialized stratified squamous mucosa such as keratinized (attached gingiva and hard palate) and the non-keratinized (oral, labial, ventral tongue, oropharyngeal, and unattached gingiva) mucosae[17]. Due to presence of different kinds of epithelial cells in oropharyngeal secretions, it is challenging to understand the role of these oral cavity specific cells in infectivity, transmission and multiplication of COVID-19 virus using the molecular techniques. Though the role of ACE-2 and TMPRSS-2 are reported, there is no direct evidence of the level of infectivity and presence of these viruses in different kinds of oral mucosal cells. For the comprehensive descriptions of viral infection and to predict the vulnerabilities across the oral cavity, we have performed a series of microscopic imaging using imaging (for identification of cell types and inclusion bodies formed due to virus infection), immunofluorescence imaging (for direct identification of SARS-CoV-2 infection), scanning electron microscope (SEM) (for identifying the viruses on the surface of epithelial cells), and transmission electron microscope after ultra-sectioning of resin block for direct visualization of SARS-CoV-2 virus and its level of infectivity).

Light microscopy cannot be directly used for viral identification purposes, but the presence of viral infection can be identified by different morphological features observed on the specific infected cells. Pap staining of cells were reported to reveal the viral presence by identifying some morphological features such as intracytoplasmic and intranuclear cytopathic inclusion[18], distinct vacuolisation in the cytoplasm and multinucleation[19]. We have also found the presence of intracytoplasmic and intranuclear inclusion bodies in the cytoplasm and nucleus of different oral squamous epithelial cells (Fig. 1). This presence of inclusion body might be due to the viral infection, although we cannot differentiate between various viral infections (adenovirus, influenza, para influenza, herpes simplex virus, respiratory syncytial virus, cytomegalovirus, and measles). Hence, already confirmed SARS-CoV-2 positive patient's cells containing inclusion bodies can be a sign of infection in those cells (Fig. 2).

It was already shown by immunohistochemical analyses that ACE-2 was differentially expressed in the different oral epithelium from basal part to sub basal and shedding outermost area[8]. Immunohistochemistry based analysis of keratinised squamous epithelium confirms the presence of ACE-2 expression in the nuclei and cytoplasm of the spinosum and basal cell layers. However, in the horny layer, the signal was absent in the nucleus as well as cytoplasm and the cell membrane[20]. These reports proved that ACE-2 and TMPRSS-2 were highly expressed in the sub basal region in comparison to basal and superficial shedding region of oral

epithelium by *in situ* hybridisation (ISH)[20]. However, the presence of differential SARS-CoV-2 virus in stratum granulosum, stratum intermedium and stratum spinosum and stratum corneum were not explored. These oral epithelia are also one of the first cells that are encountered by SARS-CoV-2 virus. It is already known that the supra-basal cells of oral mucosa were continuously shed into saliva multiples times per day, so the expression patterning of supra-basal mucosal cells raised the possibility that sloughed cells may be infected and serve as a viral carrier to other parts of the body or to other humans[2]. Surprisingly, in our finding, based on immunofluorescence imaging, the infection level of SARS-CoV-2 was highest in sub basal epithelial cells and these findings correlated well with the higher expression level of ACE2 and TMPRSS-2 (Figs. 1 & 2). Viral antigens were detected by immunofluorescence in different kinds of oral epithelial cells in the severely infected patients (Figs. 1 & 2). The high viral loads in the non-keratinized oral epithelium of intubated patients support the potential for persons infected with SARS-CoV-2 to readily transmit the virus, with prolonged and continued viral shedding in severe cases.

The identification of presence of SARS-CoV-2 on the surface of the different oral mucosal cells are important to understand the infectivity and spread of the SARS-CoV-2 virus. It is obvious that these viruses would be present on the surface of oral mucosal cells from oropharyngeal and bronchoalveolar secretion of severally infected ICU admitted patients with ARDS (acute respiratory distress syndrome). These viruses were generated in large amounts in the alveolar sac of severally infected patients and can be adsorbed on the surface (simple adsorption) as well as internalise or multiply in the cytoplasm (infection and multiplication) of oral mucosal cells. There are many reports of SARS-CoV-2 infected cultured cells and in the nasopharyngeal swab for the internalization and multiplication[21,22]. However, the presence of SARS-CoV-2 virus in the seriously infected patients with respect to various oral epithelium was not reported. In our SEM study, we have shown the presence of SARS-CoV-2 virus on the surface of different oral squamous epithelium. Very few SARS-CoV-2 viruses were present on the surface of the stratum corneum. These viruses may be adhered to the cell membrane by simple adsorption (because of high multiplication in critical condition) and were washed during the multiple washing steps involved in the sample preparation in immunofluorescence. This may be the reason that these viruses could not be detected in the immunofluorescence imaging on stratum corneum but detected in very low number in SEM (Fig. 2 & 3).The presence of SARS-CoV-2 viruses are least in stratum corneum, and gradually increased from stratum superficial, stratum basale, stratum granulosum to stratum intermedium cells and highest in the stratum spinosum cells (Fig. 3). This finding confirms the differential infection, multiplication and release of viruses through oral epithelium. The differential presence of ACE-2 receptors on these cells correlated with these finding[2,5].

TEM examination of oral epithelium cells from bronchoalveolar secretions showed virions with prominent surface projections (spikes) characteristic of the family *Coronaviridae*. Transmission electron microscopy finding also corroborated with the abundance of ACE-2 markers detected on the different oral mucosal cells[2, 5] as well as confirmed by our study of immunofluorescence imaging (Figs. 2, 3 & 4). SARS-CoV-2 virion particles are spherical in shape having coronal fibrils on the outer surface[23] and electron dense material inside the particle[24]. Virion were visible in the membrane bound vesicles inside the cytoplasm and also freely in the cytoplasm (Fig. 4). The viruses were not detected in the stratum corneum cells of keratinoid origin as these cells are devoid of cellular machinery that helps in the internalisation of SARS-CoV-2 virus[2,5]. However, the outermost shedding area cells of non-keratinized origin with characteristic dispersed tono-filaments showed the presence of SARS-CoV-2 viruses on the cytoplasm.These viruses may be carried over from the pre-infected SARS-CoV-2 virus on sub basal cells of non-keratinised origin during shedding process. This is because the sub basal cells such as stratum intermedium or stratum spinosum were heavily infected from the crown like SARS-CoV-2 virus. This higher infection in these cells corroborates with the overexpression of ACE-2 and TMPRSS-2receptors.

Conclusion

This report describes the specific cellular and extracellular localization of SARS-CoV-2 in different oral epithelium cells, with Pap, immune-fluorescence, SEM, and TEM imaging. Together, these findings provide insight into SARS-CoV-2 pathogenesis, infectivity, and multiplication in the oral epithelium.

The ultra-structural observations are consistent with molecular biology for SARS-CoV-2 infection.

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Conflict of interest

All authors declare no conflict of interest.

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