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Title:

Liquid-based cytological and immunohistochemical study of nasopharyngeal swab from persons under investigation for SARS-CoV-2 infection

Short running title:

Cytological and immunohistochemical study in SARS-CoV-2 infection

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Abstract

Introduction: We describe cytologic and immunohistologic findings in virus transport medium on cases under investigation of SARS-CoV-2 infection.

Methods: Cytologic findings in cases under investigation of SARS-CoV-2 infection from one hundred consecutive nasopharyngeal swab were reviewed. Immunohistochemistry and SARSCoV-2 RT-PCR determination were performed to detect virus.

Results: No viral inclusions were noted in squamous cells obtained from virus transport medium. Immunohistochemistry with monoclonal antibody against SARS-CoV-2 viral nucleoprotein was present in squamous cells. No positivity was present in others cellular components.

Conclusions: SARS-CoV-2 predominantly localizes squamous cells in cytology samples of patients with RT-PCR positive determination of SARSCoV-2. The results of the current study support the notion that the nasopharyngeal region is the anatomical station that SARS-CoV-2 infects first, and the infection can lead to the migration of the virus into the lower airways.

Key words: COVID-19, SARS-CoV-2, liquid-based, RT-PCR, cytology, swab, nasopharynx, immunohistochemical, viral transport medium.

INTRODUCTION

In December 2020, a new type of coronavirus was identified as the causative factor in a series of cases of severe pneumonia in the city of Wuhan, province of Hubei, in the People's Republic of China (1). The World Health Organization gave the official name "COVID-19" for this coronavirus disease, as well as the term "severe acute respiratory syndrome coronavirus 2" (SARS-CoV-2) for the virus (2). This virus is currently the cause of a global pandemic, producing hundreds of thousands of hospital admissions and deaths, with enormous effects on the health and life of the population and serious economic consequences for society.

The tests currently used for the direct identification of SARS-CoV-2 are mainly aimed at detecting viral RNA through nucleic acid amplification, generally using the polymerase chain reaction. The nasopharynx and/or oropharynx are the most tested sampling sites using swabs, with a slightly greater sensitivity shown by the nasopharyngeal swab (3). This test uses virus transport medium to carry it out, however to the best of our knowledge no cytological studies of the virus transport medium have been carried out.

In this article, we report the cytological findings of the universal transport medium used to investigate cases of SARS-CoV-2 infection, as to cell types present and whether there is a viral cytopathic effect in the infected cells. In addition, we included the immunohistochemical study for the SARS-CoV-2 viral nucleoprotein and viral RNA.

MATERIALS AND METHODS

STUDY DESIGN AND PATIENT COHORT

This is a prospective and descriptive cohort study conducted on consecutive cases under investigation (CUI) of SARS-CoV-2 infection in Hospital Universitari de Sant Joan, in Reus, Spain, between 1st April 2020 and 30th July 2020. The study protocol followed the principles of the Declaration of Helsinki 1964 and its subsequent modifications. We included 100 samples of nasopharyngeal swab in universal transport medium (UTM) for SARS-CoV-2 infection submitted to molecular pathology unit of our pathology department. SARS-CoV-2 infection detection was performed by reverse transcription-polymerase chain reaction (RT-PCR) using swab samples from the upper respiratory tract (nasopharyngeal exudate). Tests were carried out with the VIASURE SARS-CoV-2 Real Time PCR Detection kit that detects ORF1ab and N genes (CerTest Biotec, Zaragoza, Spain). RNA was extracted in a QIAcube apparatus with RNeasy reagents (Qiagen N.V., Hilden, Germany) according to the manufacturer's instructions, and analyses were carried out in a 7500 Fast RT-PCR System (Applied Biosystems, Foster City, CA, USA). Patient clinical, and laboratory data were extracted from medical records. The cycle threshold (Ct) values were reported by the cobas SARSCoV-2 test as either "positive" (N and ORF1a genes detected, Ct values <38), "presumptive positive" (N gene or ORF1a gene detected, Ct values <38), or "negative" (N and ORF1a genes not detected, Ct value > 38).

LIQUID-BASED CYTOLOGY

After SARS-CoV-2 RT-PCR determination, the UTM was processed to liquid-based cytology with the Thin Prep 5000™ LBC method (Hologic Co., Marlborough, MA, USA). All the UTM material was fixed with the hemolytic and preservative solution Cytolyt™. The UTM material was spun at 3000 rpm per 5 min.; the sediment was then transferred to 20 ml Preservcyt™ solution, keeping for 15 minutes at room temperature, to be processed with a T5000 automated processor according to the manufacturer's recommendations. Two slides for each sample were obtained and were fixed in 95% ethanol. The second slide was stained with Papanicolaou, whilst the first slide was processed to immunocytochemistry. This was done to try to preserve as much material as possible for the immunohistochemical study. Cell types were assessed and quantitative analysis was performed, taking 10 consecutive fields at 10x.

IMMUNOHISTOCHEMISTRY

Immunocytochemical staining protocol was previously validated with anti-SARS-CoV-2 NP antibody, Clone # 6F10 (BioVision Incorporated®, CA, USA) in samples obtained from UTM material processed by liquid-based cytology, with 10 positive and 10 negative samples by RT-PCR. Immunocytochemical staining was carried out of all the samples processed for liquid cytology which were placed in a VENTANA® Benchmark ULTRA/LT automatic immunohistochemistry processor (Ventana Medical Systems, Oro Valley, AZ, USA), using the previously standardized protocol for SARS-CoV-2 detection that included recovery solution pH9, 40 min. at 100 °C and the Optiview® DAB Immunohistochemistry Detection Kit (VENTANA®). For incubation with the primary anti-SARS-CoV-2 NP antibody, Clone # 6F10 (BioVision Inc®, Milpitas, CA, USA), after reconstitution with 100 ml of distilled water, a 1:1000 dilution was used, this incubation was carried out for 32 min. at 36 °C. Finally, slides were treated with diaminobenzidine, contrasted with Meyer's hematoxylin, dehydrated with alcohols at increasing concentrations and rinsed with xylol, to finally be examined under an Olympus BX41 light microscope at 3.5x and 60x. The presence of SARS-CoV-2 positivity was considered when cytoplasmic labeling was obtained in cells of liquid-based cytology. The intensity of the reaction was cataloged as negative: no staining; 1+: weak cytoplasmic staining; 2+: moderate cytoplasmic staining, and 3+: intense cytoplasmic staining. Positive cells of SARS-CoV-2 were counted in ten fields consecutive at 20x increase, representing a total area of 1.25 mm². RT-PCR

SARS-CoV-2 test results were unknown at the time of evaluating both the cytological and the immunohistochemical studies.

STATISTICAL ANALYSIS

The results of the cellular analysis are shown as means+SD and percentage. The differences in the results of cell count and SARS-CoV-2 test were examined by the non-parametric Mann-Whitney test for two independent groups. $p < 0.05$ was considered statistically significant. All of the analyses were performed using IBM.SPSS version 23.

RESULTS

Clinical findings:

Our analysis included 54 male and 46 female patients with a median age of 59 years (range, 27–93 years). Sixty-four (64%) cases were asymptomatic. Thirty-six cases (36%) showed symptoms related to SARS-CoV-2 infection, such as fever, dry cough, shortness of breath, fatigue, rhinorrhea and respiratory distress. Four (4%) patients died from COVID-19. Forty (40%) cases were negative to RT-PCR SARS-CoV-2 test. Thirty-three (33%) cases showed N and ORF1a genes positivity (positive test). Gene N or ORF1a positivity was seen in twenty-seven (27%) cases (presumptive positive test) (Gene N positive: 24; Gene ORF1a positive: 3). Table 1 and 2, summarized the results.

Cytological findings:

The cytomorphological characteristics were similar for all groups. Papanicolaou smears showed squamous cells with nucleus/cytoplasm ratio preserved and there was no viral cytopathic effect, such as multinucleation, nuclear hyperchromatism, or eosinophilic nucleolus prominent, etc. Another epithelial component was ciliated respiratory-type epithelial cells, with no evidence of viral cytopathic effect. In three cytologies, fungal structures compatible with *Candida* sp were present. (Figure 1). In 40 cytological smears from PCR-RT-SARS-CoV test negative group the number of squamous cells was 45.08 ± 19.34 , and 67 ± 20 ciliated respiratory-type epithelial cells. In 33 PCR-RT-SARS-CoV test positive group there were 43.74 ± 19.81 squamous cells, and 38.01 ± 18.48 ciliated respiratory-type epithelial cells. In 27 PCR-RT-SARS-CoV test presumptive positive group there were 44.93 ± 22.69 squamous cells, and 51.84 ± 21.01 ciliated respiratory-type epithelial cells. There were no differences between the groups analyzed ($p = 0.97$) (Figure 2). Other cellular component present in the cytological smears were isolated lymphocytes, neutrophils and macrophages. In seven cases the material present

was scarce, with cellular count between 3 to 10 squamous cells and 2 to 12 ciliated respiratory-type epithelial cells.

Immunohistological findings:

Immunohistological study from 37 SARSCoV-2 RT-PCR negative cases, did not show immunoreactivity for anti-SARS-CoV-2 nucleoprotein antibody. Granular cytoplasmatic positivity was observed in 3 SARSCoV-2 RT-PCR negative cases. In 33 SARSCoV-2 RT-PCR positive cases showed granular cytoplasmatic positivity in squamous cells, and the positive cell numbers was 50,52+19.89. Immunoreaction was not observed in ciliated respiratory-type cells. Similarly, macrophages and neutrophils did not show immunoreactivity (Figure 3). Immunohistological study, in 27 SARSCoV-2 RT-PCR presumptive positive cases, showed also granular cytoplasmatic reactivity, and the positive cell numbers was 47.26+18.82. No difference between SARSCoV-2 RT-PCR positive and SARSCoV-2 RT-PCR presumptive positive cases was established (Figure 4). The intensity of cells in SARSCoV-2 RT-PCR presumptive positive cases were lower compared to the SARSCoV-2 RT-PCR positive cases (2+ vs 3+). Table 3.

DISCUSSION

We performed a cytological and immunohistochemical study of the universal transport medium for RT-PCR determination of SARSCoV-2 in one hundred patients admitted to our center, regardless of the diagnosis. Reverse transcription-polymerase chain reaction (RT-PCR) has emerged as the primary mode of diagnosis of acute infection with SARS-CoV-2 (4), but there is currently no gold standard for the diagnosis of COVID-19; therefore, sensitivity and specificity could not be calculated (5). The diagnostic strategy recommended by the CDC to identify patients with COVID-19 is to test samples taken from the respiratory tract to assess for the presence of one or several nucleic acid targets specific to SARS-CoV-2 (6). A nasopharyngeal specimen is the preferred choice for SARS-CoV-2 testing (7). In the present study of the viral transport medium on cases under investigation (CUI) of SARS-CoV-2 infection, variable cellular composition was evident, with predominance of squamous cells. Only in seven samples material was limited for cytological evaluation. However, in cases with limited cytological evaluation, adequate results were obtained for analysis RT-PCR determination of SARSCoV-2. These findings confirm that the nasopharyngeal swab is an adequate source of material for the diagnosis of COVID-19.

A finding of our study was the presence of 3 cases with a negative SARS-CoV-2 RT-PCR test, which showed immunohistochemical positivity for viral nucleoprotein. Currently, RT-PCR is the valid diagnostic method for the diagnosis of COVID-19, however different studies have shown a high rate of false negatives of RT-PCR technique (8-10). This may be caused by in-sufficient viral material in the specimen, laboratory error during sampling, or restrictions on sample transportation (8-10). The difference with the three cases found in our series could be explained by a lack of sampling, low amount of RNA template of the sample near or below the detection limit of the reactions, mutations in the nucleic acid sequences targeted, slightly different amplification yield of the targets regions, or other factors related to the RT-PCR test.

Another point of the present study is the diagnostic category of presumptive positive RT-PCR test, when one of the studied genes was positive. The possibility that these are false positive results, arising either from spurious amplification or from detection of a closely related virus, cannot be excluded (5). Another possibility is that these samples contain low levels of virus, near the limit of detection for the cobas SARS-CoV-2 tests but below the threshold of detection. However, in the 33 presumptive positive cases, cytoplasmic reactivity was demonstrated for the viral nucleoprotein SARS-CoV-2. This finding could indicate that cases classified as presumptive positives should be classified as positive.

In the present study, one of our initial approaches was to demonstrate whether there were characteristic viral cytopathic changes related to SARS-CoV-2 infection in cells analyzed from nasopharyngeal swab in universal transport medium, and whether these changes could serve as an indicator of infection. The cytological study showed that no changes were observed associated with the SARS-CoV-2 infection. This could imply that in order to demonstrate the presence of SARS-CoV-2 infection, more specific tests are required to demonstrate genetic targets using molecular biology, RT-PCR, or the demonstration of viral products by immunohistochemistry, such as nucleoproteins.

Histopathological studies on SARS-CoV-2 infection have shown involvement of different organs, being the most relevant findings at pulmonary level (11-15). Non-specific findings are described in early stages, and included oedema, pneumocyte hyperplasia, focal inflammation and multinucleated giant cell formation (11). In advanced stages, histopathological characteristics included diffuse alveolar damage with exudates (10-15; 7-11). However, there is no characteristic viral cytopathic effect of SARS-CoV-2 in the

different organs studied in patients with this infection (11-15). In our study, no definitive viral inclusions were noted in squamous cells correlated with the immunohistochemical study.

The immunohistochemical analysis of the present study showed granular cytoplasmic positivity in squamous cells, without other cellular components showing this reactivity. This finding seems to indicate that SARS-CoV-2 would have the ability to infect squamous cells and, therefore, produce viral replication in these cells, and ACE2 receptors in the nasopharyngeal and oral mucosa could support the infectious capacity of squamous cells (16-18). The ACE2 receptor is necessary for the virus to enter the cell, through endocytosis, to release its RNA, and it uses cellular machinery to replicate and assemble more virions (17). However, other studies have not demonstrated the presence of the ACE-2 receptor in the nasal region (19). However, SARS-CoV-2 infection remarkably induces the expression of ACE2, as an interferon-stimulated gene, in human airway epithelial cells (20,21). In addition, squamous cells in the nasopharyngeal region could serve as reservoir for SARS-CoV-2, since it has been shown that after 4 weeks, 26.3% of the samples by RT-PCR tests remain positive for the SARS-CoV-2 (8). These findings suggest that SARS-CoV-2 viral replication has a relatively long period in infected patients (8) and it is likely that nasopharyngeal squamous cells play a role in viral maintenance. Further studies are required to establish the role of nasopharyngeal squamous cells, ACE2 receptor, and their interaction in SARS-CoV-2 infection.

In conclusion, we demonstrate that SARS-CoV-2 predominantly localizes squamous cells in patients with RT-PCR positive determination of SARSCoV-2. SARS-CoV-2 infects the nasopharyngeal region, likely due to the presence of ACE2 receptors, which facilitates the viral replication and that facilitates the contagious state of the disease. This region is the first anatomical station infected that can allow the migration of the virus into the lower airways.

Author contributions:

David Parada D, Karla Peña: conception, design, primary acquisition and analysis of data (citology and SARS-CoV ICC), drafting and critical revision of manuscript.

Josep Gumà: design, drafting and critical revision of manuscript.

Carmen Guilarte: primary acquisition and analysis of data (citology and ICC), contributed PCR-RT SARS-CoV-2 results.

Francesc Riu: integration and synthesis of relevant clinical information, revisions of manuscript.

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Table 1. Clinical characteristics from persons under investigation for SARS-CoV-2 infection (N=100).

	N	%
Male	54	54
Female	46	46
Symptoms		
Absent	64	64
Present	36	36

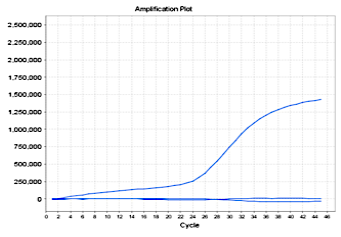
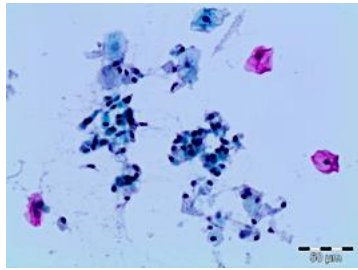
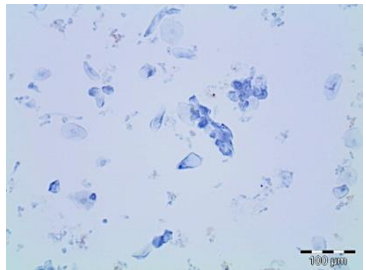
Table 2. RT-PCR SARS CoV-2 test results, immunohistochemical study, clinical symptoms and evolution from persons under investigation for SARS-CoV-2 infection (N=100).

	RT-PCR SARS-CoV-2 test negative (40)	RT-PCR SARS-CoV-2 test positive (33)	RT-PCR SARS-CoV-2 test presumptive positive (27)
ICC positive	3	33	27

ICC: Immunocytochemistry study

ICC Negative	37	0	0
Asymptomatic	28	15	21
Symptomatic	12	18	6
Alive	40	29	27

Table 3. Cases under investigation for SARS-CoV-2 infection from nasopharyngeal swabs. Relation between RT-PCR, cytology and immunohistochemistry study. (Papanicolau staining and DAB, 20x and 10x).

	RT-PCR	Cytology	Immunohistochemistry
<p>SARSCoV-2 negative (case 10)</p>			

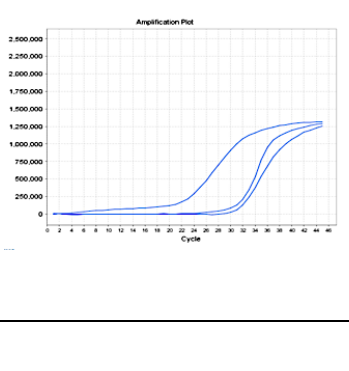
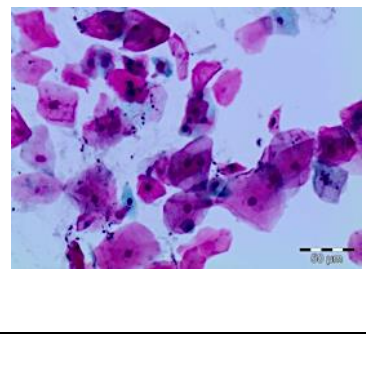

<p>SARSCoV-2 positive (case 50)</p>			

Figure 1. Cytological findings in cases under investigation for SARS-CoV-2 infection from nasopharyngeal swabs. (A) Characteristic finding of squamous cells. No viral cytopathic effect was present. (B) Ciliated respiratory-type epithelial cells, with no evidence of any viral cytopathic effect. (C) Isolated squamous cell metaplasia is present. (D) *Candida* sp pseudohyphal, and hyphal forms are seen. (Papanicolau staining, DA 20x).

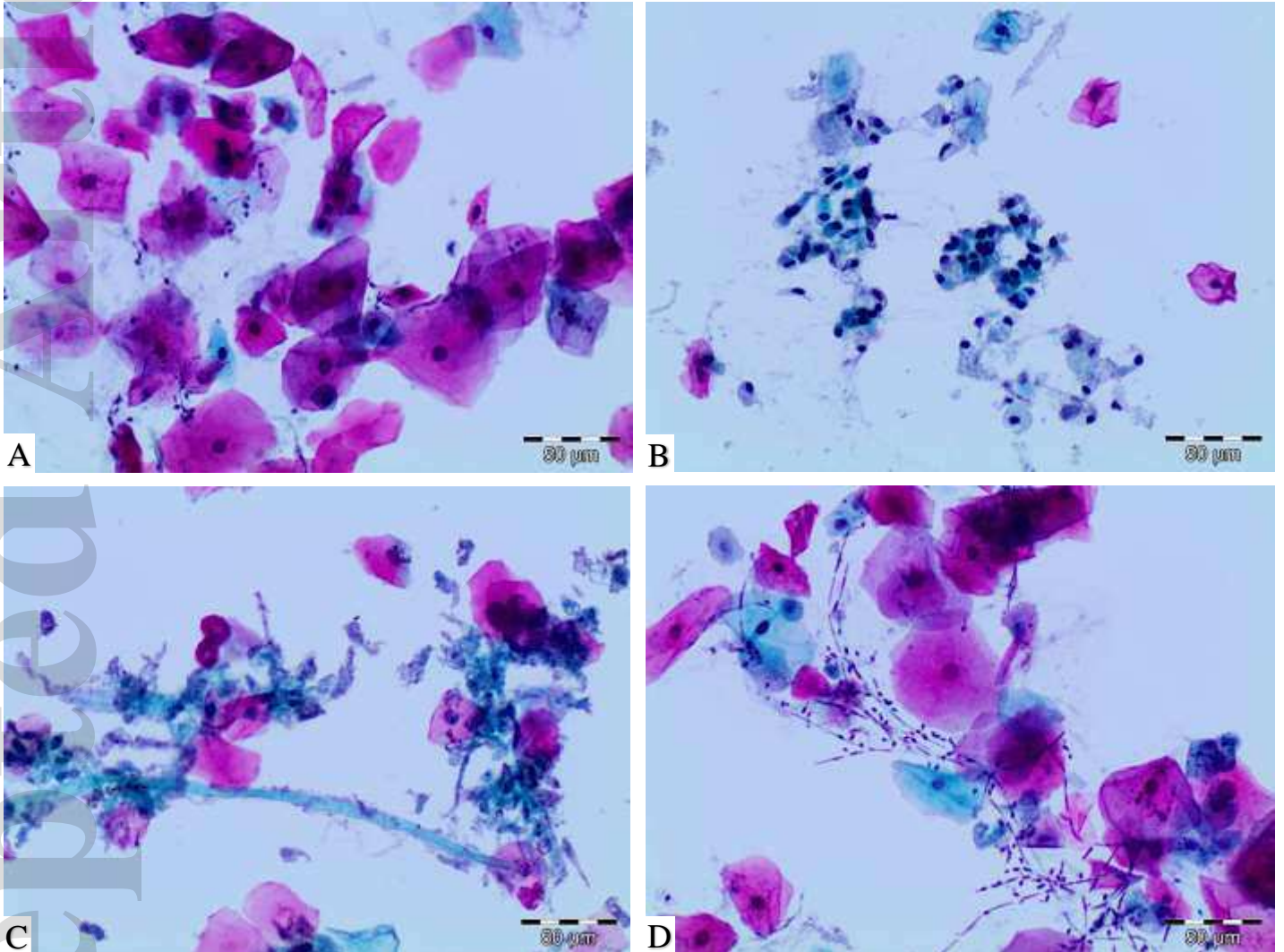


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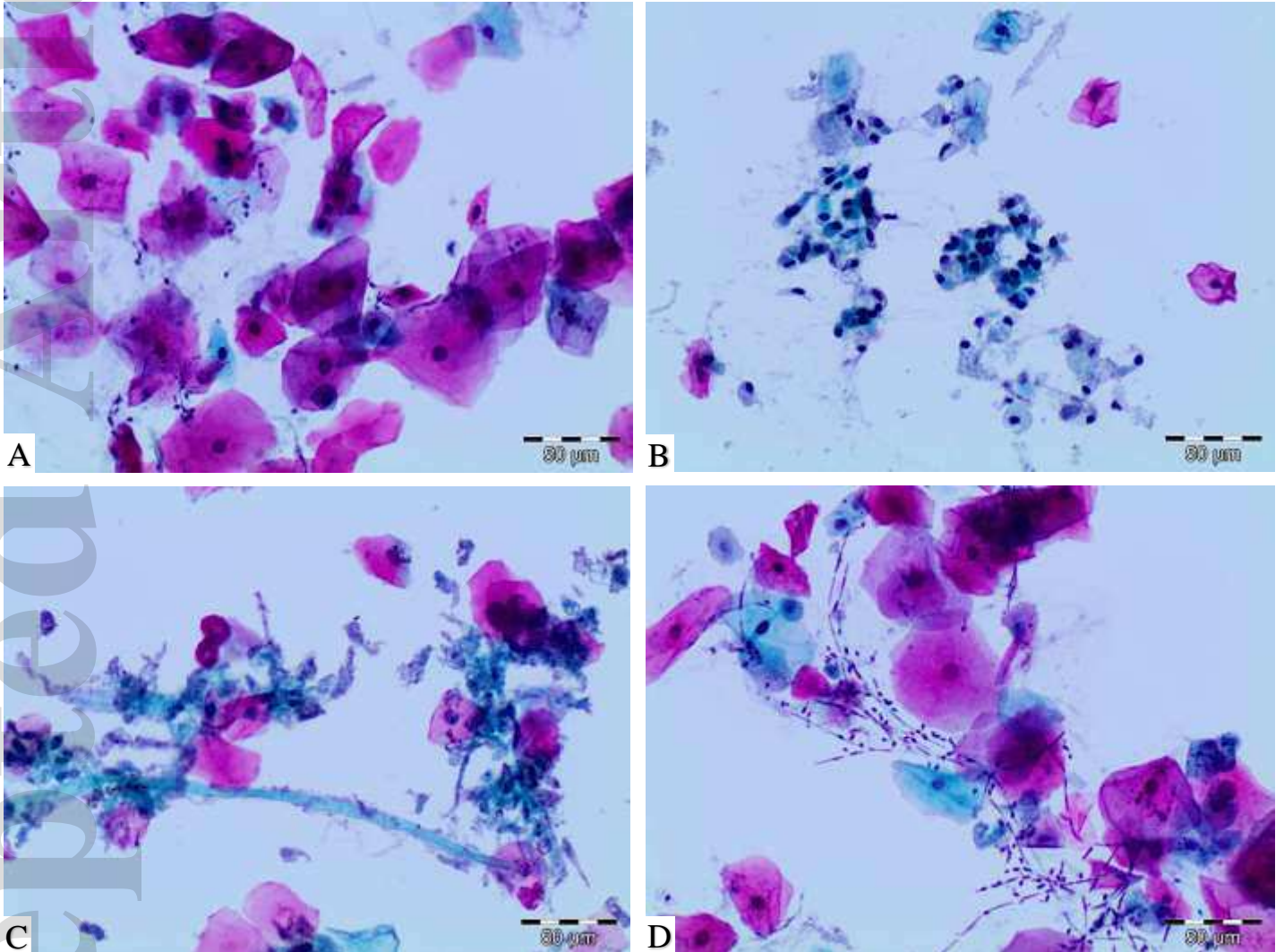
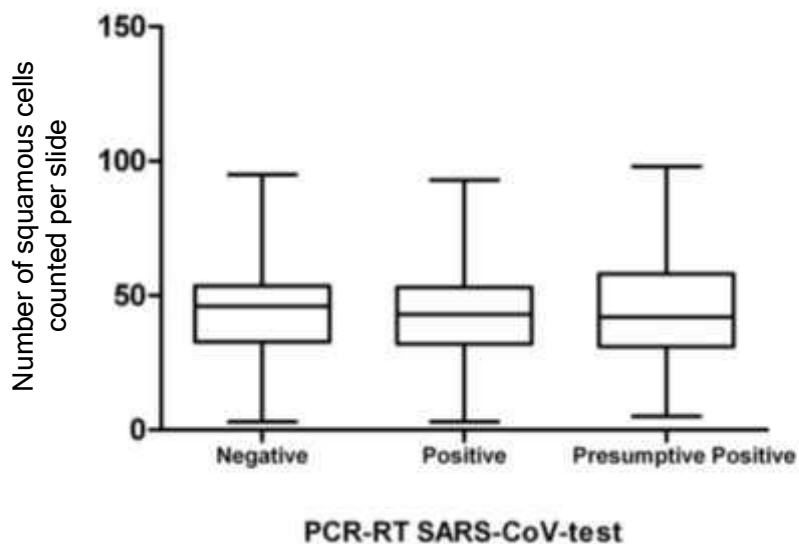
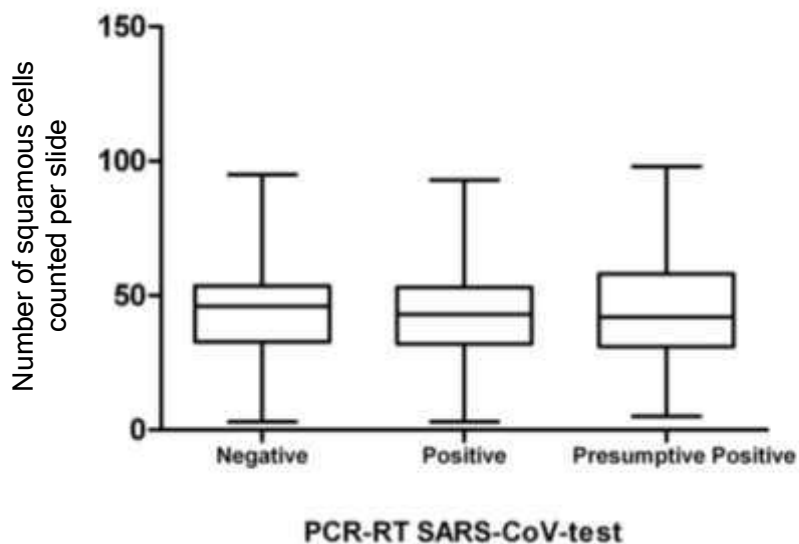


Figure 2. Squamous cells present in samples obtained from universal transport medium, processed by liquid-based cytology. Negative (N=40), positive (N=33) and presumptive positive (N=27). (No significant differences were observed between groups).



1) Negative. Median: 46; Q1: 32.75%; Q3: 53.59%. Minimum: 3. Maximum: 95.
2) Positive. Median: 43; Q1: 32%; Q3: 53%. Minimum: 3. Maximum: 93. 3)
Presumptive positive. Median: 42; Q1: 31%; Q3: 58%; Minimum: 5; Maximum:
98.

Figure 2. Squamous cells present in samples obtained from universal transport medium, processed by liquid-based cytology. Negative (N=40), positive (N=33) and presumptive positive (N=27). (No significant differences were observed between groups).



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Presumptive positive. Median: 42; Q1: 31%; Q3: 58%; Minimum: 5; Maximum:
98.

Figure 3. Immunohistological findings in cases under investigation for SARS-CoV-2 infection from nasopharyngeal swabs. (A,B) Cytoplasmatic immunostaining in squamous cells (RT-PCR, positive). (C) Squamous cells positive, with no reactivity seen in neutrophils, cylindrical cells, or macrophages. (D) RT-PCR presumptive positive showing immunostaining in squamous cells, note that the staining is weaker. (DAB staining. DA 10x and 20x).

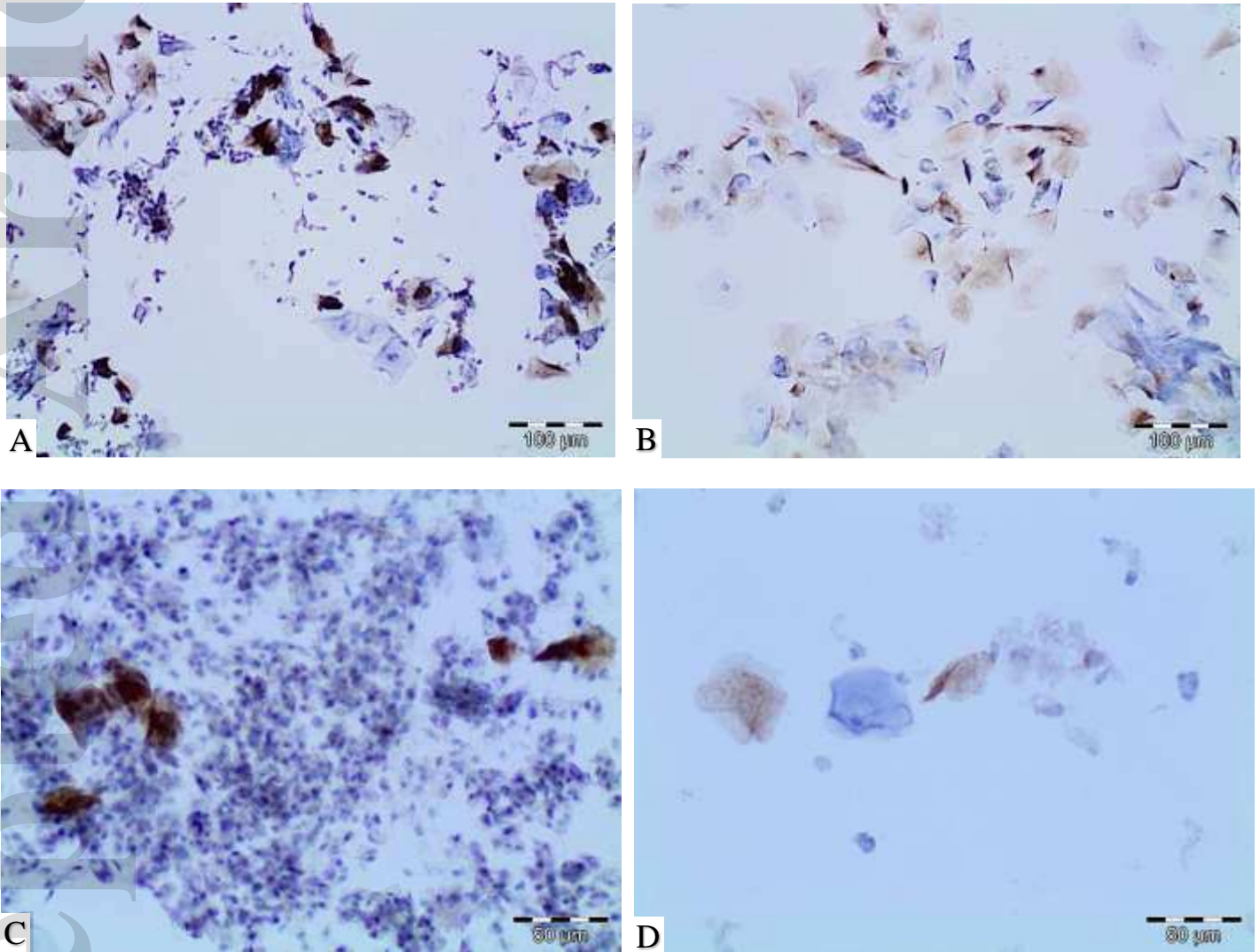


Figure 3. Immunohistological findings in cases under investigation for SARS-CoV-2 infection from nasopharyngeal swabs. (A,B) Cytoplasmatic immunostaining in squamous cells (RT-PCR, positive). (C) Squamous cells positive, with no reactivity seen in neutrophils, cylindrical cells, or macrophages. (D) RT-PCR presumptive positive showing immunostaining in squamous cells, note that the staining is weaker. (DAB staining. DA 10x and 20x).

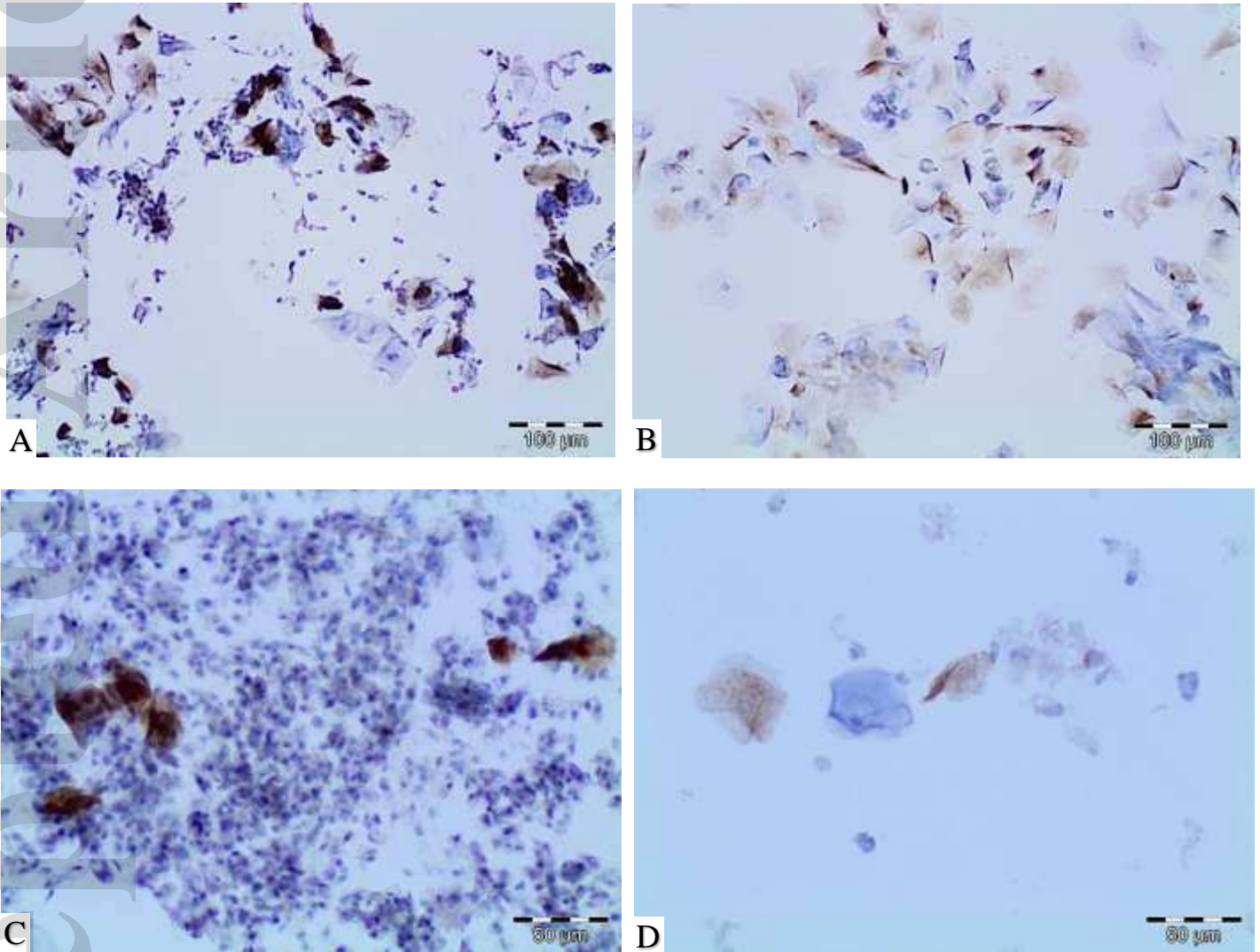
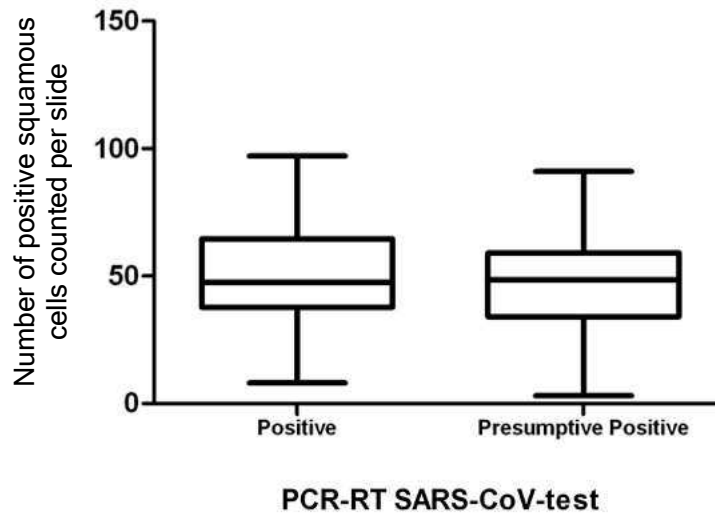
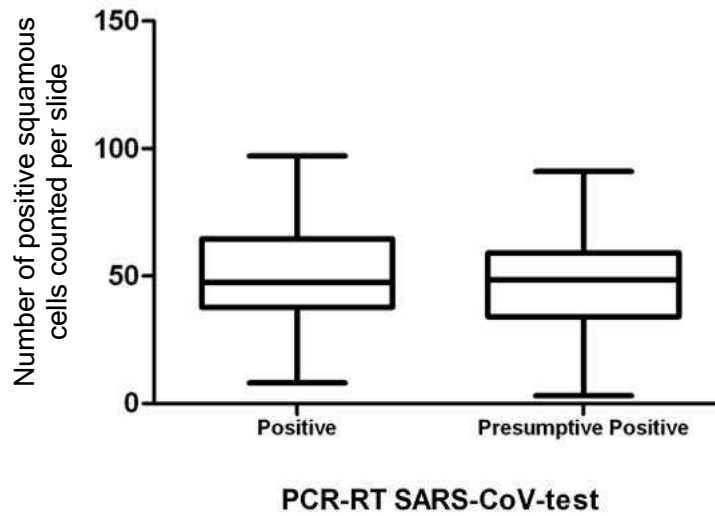


Figure 4. Immunohistological positive squamous cells in cases of RT-PCR SARS-CoV-2 infection positive and presumptive positive. (No significant differences was observed between groups).



1) Positive. Median: 47.50; Q1: 37.75%; Q3: 64.50%. Minimum: 8. Maximum: 97.
2) Presumptive positive. Median: 48.50; Q1: 34%; Q3: 59%; Minimum: 3; Maximum: 91.

Figure 4. Immunohistological positive squamous cells in cases of RT-PCR SARS-CoV-2 infection positive and presumptive positive. (No significant differences was observed between groups).



1) Positive. Median: 47.50; Q1: 37.75%; Q3: 64.50%. Minimum: 8. Maximum: 97.
2) Presumptive positive. Median: 48.50; Q1: 34%; Q3: 59%; Minimum: 3; Maximum: 91.