Original Article

Nasal and oral swab Expressions of ACE2 and IL6 in Nigerian COVID-19 Patients with Varying Disease Severity

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Abstract

Background: ACE2 receptor is crucial for SARS-CoV 2 entry into host cells, and pro-inflammatory cytokine storm causes mortality in COVID-19 patients. Blood levels of both ACE2 inhibitor and IL6 antagonists were considered for therapeutic management strategies during COVID-19. The study determined the molecular expressions of ACE2 and IL6 in nasal and oral swabs of COVID-19 Nigerian patients of different severities.
Materials and Method: Forty-five COVID-19 patients (mild, moderate and severe) were recruited from the Biorepository Clinical Virology Laboratory, University of Ibadan, Nigeria. Ten (10) healthy individuals served as control. Expression levels of ACE2 and IL6 were determined using q-RT-PCR. The threshold (Ct) values of the expressions were determined and fold changes ($2^{-\Delta\Delta CT}$) values calculated. $P < 0.05$ was taken as significant.
Results: The mean swab Ct value of ACE2 expression was significantly reduced in combined COVID-19 cases compared with control, while mean swab IL6 Ct values were similar in cases and control. The mean swab ACE2 Ct values were significantly reduced in severe- and moderate-COVID-19 cases compared with control, while mean swab IL6 Ct value was significantly increased in mild COVID-19 cases compared with control. Sixty (60) %, 93% and 100% of mild, moderate and severe COVID-19 patients respectively had low swab ACE2 expression while 93%, 100% and 93% of mild, moderate and severe COVID-19 patients respectively had low swab IL-6 expression. Also, 67% and 50% of male and female COVID-19 patients respectively had low Ct values of swab IL6 expression. Thirty-three percent of females or ≥40yrs old COVID-19 patients had low Ct values of swab ACE2 expression while 33% and 100% of <40yrs and ≥40yrs old COVID-19 patients respectively had low Ct values of swab IL-6 expression.
Conclusion/Recommendations: ACE2 expression in swab might be used as an indicator of SARS-CoV 2 severity. Thus, the development of ACE2-based therapeutic measure against COVID-19 is supported by this study.
Keywords: ACE2 receptor, Cytokine storm, COVID-19, Nasal and oral swab.

Introduction The advent of the novel coronavirus caused by SARS-CoV-2 in late 2019 caused worldwide catastrophy[1,2] Thus, the study of SARS CoV-2 immuno-biology[1-4] and pathophysiology[5-7] to identify novel biomarkers, prognostic tools and repurposed therapeutic options were carried out.[1-7]The mode of infection of SARS- CoV-2 was via its spike S-glycoprotein binding with ACE2 on the host epithelial surface.[8]

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This process was supported by transmembrane protease serine 2 (TMPRSS2)[7] leading to robust immune response including increased levels of inflammation mediators.[9]Due to the pivotal role of ACE2 in SARS-CoV-2 entry into host cell and its involvement in multi-organ pathology,[8, 10] the development of ACE inhibitors in the management of COVID-19 patients was attempted.[11]

Following SARS- CoV2 infection via ACE2 receptor binding, IL6 is produced rapidly by a variety of cells stimulating acute phase reactions, haematopoiesis, and immune responses.[12] IL6 cytokine's mRNA expression was observed to be high in COVID-19 patients' peripheral blood mononuclear cells and was associated with the severity of sickness.[13]The treatment of COVID-19 patients with IL6 receptor antagonist demonstrated improved outcomes or no effect in some cases.[14,15]

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Existing data on IL-6 level[16,17] and ACE2 expression[18] in COVID-19 patients were determined in patients' blood, with limited studies on nasopharyngeal. The choice of nasal and oral swabs in this study is because SARS-CoV-2 primarily enters through its spike protein attachment to the host cell ACE2 receptor of nasal and oral epithelia. The semi-invasive nature of mucosal sample collection and established efficacy in qRT-PCR-based COVID-19 testing[19] added credence to the sampling and analytical method used. The present study assessed the levels of expression of ACE2 and IL6 by nasal and oral cells in relation to COVID-19 severities, age and sex of COVID-19 patients in Nigeria.

Materials and Methods

Nasal and oral swab samples were collected from forty-five COVID-19 patients from Oyo State, Nigeria, along with ten SARS-CoV-2 uninfected healthy donors, who acted as controls. The COVID-19 patients were grouped into three categories: mild (with fever, cough and fatigue), moderate (with difficulty in breathing and mild pneumonia) and severe (with severe pneumonia, other organ failure and possible death).[20]

Diagnosis of COVID-19: The COVID-19 tests by qRT-PCR were carried out in the Biorepository Clinical Virology Laboratory, College of Medicine, University of Ibadan. All the compliances regarding the regulations were fulfilled. The procedure was approved by the University of Ibadan/University College Hospital, Ibadan Institutional Ethical Committee (UI/EC/21/0749). The ACE2 and IL6 in the swab were assessed using qRT-PCR in accordance by established gold-standard laboratory benchmarks.[21,22] Samples were collected with disposable sterile swab stick (Luxus Lebenswelt GmbH, Willich, Germany) into a sterile Virus Transport Media (VTM) (RemelMicrotest M4RT dual swab kit, Thermo Fisher Diagnostics, Landsmeer, The Netherlands). Before to the nasal and oral sample collection, the participants were instructed to clear their noses to avoid any kind of blockage. The volunteers' head was kept at approximately 70^0 angle and a sterile nylon swab was inserted into the fossa until a resistance was felt for a few seconds to absorb the nasal and oral fluid, removed with rotation, immediately placed inside the virus transport medium, vortexed and frozen at -80^0C until further application. RNA was extracted from the samples using QiagenQIAamp Viral RNA Mini extractionkit (QIAGEN GmbH, QIAGEN Strasse 1, Hilden, Germany) as per the manufacturer's instructions and frozen at -80^0C.[21] The RNA was further synthesized into complementary DNA (cDNA) with an iScriptcDNAsynthesis kit (Bio-Rad, Hercules, CA, USA) as per the manufacturer's instructions.[22] The cDNA was used as a template for qRT-PCR and iTaq Universal SYBR Green supermix was used from the Bio-Rad for amplification and identification. Calculated cycle threshold (Ct) value depend on Livak equation as follow: ΔCt (Patient sample) = Ct ACE – Ct β-actin ΔCt(Healthy control) = Ct ACE – Ct β-actin $\Delta\Delta Ct = \Delta Ct$ (Patient.) – ΔCt (Control). Folding = $2^{-\Delta\Delta Ct}$ A cycle threshold (Ct) is defined as the number of amplification cycles required to reach a fixed background level of fluorescence at which the diagnostic result of the real-time PCR changes from negative (not detectable) to positive (detectable). A high Ct value often correlates with a low viral load or analyte of interest.[21] In other words, Ct is a semi-quantitative value that can broadly categorise the concentration of viral genetic material in a patient sample following testing by RT PCR as low, medium or high – that is, it tells the approximately how much genetic material is in the sample. A low Ct indicates a high concentration of viral genetic material, which is typically associated with high risk of infectivity. A high Ct indicates a low concentration of viral genetic material which is typically associated with a lower risk of infectivity.[22]

Expression of IL6 and ACE2 in nasopharyngeal swab: cDNA produced from the nasal swab was used for the expression of IL6 and ACE2 using the following primers below.

Primers	Sequences
β-Actin F	CTGTGGCATCCACGAAACTA
R	GTGTTGGCGTACAGGTCTT
IL6 F	GCACCTCAGATTGTTGTTGT
R	ACTGTCCTAACGCTCATACTTT
ACE2 F	TGGGACTCTGCCATTTACTTAC
R	CCCAAGATCTCTCGCTTCATA

Data analysis:

All the data generated from qRT-PCR were sorted using MS Excel (Microsoft Corporation, Washington, USA), and ΔΔCt was calculated. The fold change ($2^{-\Delta\Delta Ct}$) values were plotted usingBar Chart. Fisher's exact test was run for each set of data obtained at 5% level of significance using computer software SPSS version 27. All the relative expression values above 1 in $2^{-\Delta\Delta Ct}$ calculation were considered as higher expression and all the relative expression values below1 were considered as lower expression. All the heatmaps were generated in R using Complex Heatmap package.

Results

In Table 1, the mean swab Ct value of ACE2 was significantly reduced in combined (mild + moderate + severe) COVID-19 cases compared with control ($p<0.05$), while the mean Ct value of IL-6 was non-significantly different in combined COVID-19 cases compared with control ($p>0.05$). The mean swab Ct

values of ACE 2 were significantly reduced in severe- and moderate- COVID-19 cases compared with control, while the mean Ct value of IL-6 was significantly increased in mild COVID-19 cases compared with control ($p<0.05$). The mean swab Ct value of ACE2 was significantly reduced in all COVID-19 cases compared with control, while the mean swab Ct value of IL-6 was non-significantly increased in all COVID-19 cases compared with control.

In Table 2, 60%, 93% and 100% of mild, moderate and severe COVID-19 patients respectively had low Ct values of nasal and oral swab ACE 2 expression. Therefore, a higher proportion of severe COVID-19 patients followed by a moderate proportion of COVID-19 patients expressed high levels of ACE 2 in the nasal and oral swabs. Also in Table 2, 93%, 100% and 93% of mild, moderate and severe COVID-19 patients respectively had low Ct values of nasal and oral swab IL-6 expressions. Therefore, the level of IL-6 in the nasal and oral swab of COVID-19 patients seems not to be dependent on COVID-19 severity. See Table 2.

In Table 3, zero (0)% and 33% of male and female COVID-19 patients respectively had low Ct values of nasal and oral swab ACE 2 expressions. Therefore, a higher proportion of female COVID-19 patients expressed high levels of ACE 2 in the nasal and oral swabs. Also in Table 3, 67% and 50% of male and female COVID-19 patients respectively had low Ct values of nasal and oral swab IL-6 expression. Therefore, higher proportion of male COVID-19

Table 1: The Ct values (mean ± standard deviation) of nasal and oral swab ACE2 and IL6 expressions in COVID-19 patients and control

	ACE2	IL6
Control	33.36 ± 2.64	29.44 ± 6.45
All COVID-19 participants	30.74 ± 2.72	31.04 ± 3.97
Mild COVID-19 participants	32.05 ± 2.19	33.77 ± 1.88
Moderate COVID-19 participants	29.62 ± 3.09	30.58 ± 4.00
Severe COVID-19 participants	30.54 ±2.01	28.77 ± 6.02
t-,p[a]	2.81, 0.01*	0.76, 0.20
t-,p[b]	1.31, 0.20	2.06, 0.05*
t-,p[c]	2.83, 0.01*	0.63, 0.20
t-,p[d]	2.88, 0.01*	0.26, 0.20

*Significantly different from control ($p<0.05$).
a= Control compared with all COVID-19 participants
b= Control compared with mild COVID-19 participants
c= Control compared with moderate COVID-19 participants
d= Control compared with severe COVID-19 participants

Table 2: The prevalence (%) of mild, moderate, or severe COVID -19 patients having low and high Ct values of nasal and oral swab ACE2 and IL6 expressions.

	ACE2		IL6	
	High expression	Low expression	High expression	Low expression
Mild	40	60	07	93
Moderate	7	93	0	100
Severe	0	100	7	93

Table 3: Gender and age prevalence (%) of COVID-19 participants having low and high values of Ct values of nasal and oral swab ACE2 and IL6 expressions.

	ACE 6		IL 6	
	High Expression	Low expression	High expression	Low expression
Males	100	0	33	67
Females	67	33	50	50
≤ 40 yrs. of age	100	0	67	33
≥ 40 yrs. of age	67	33	0	100

patients expressed high level of IL-6 in the nasal and oral swabs.

In Table 3, 0% and 33% of <40yrs and ≥40yrs of age COVID-19 patients respectively had low Ct values of nasal and oral swab ACE 2 expression. Therefore, a higher proportion of ≥40 yrs old COVID-19 patients expressed high level of ACE 2 in the nasal and oral swabs. Also in Table 3, 33% and 100% of <40yrs and ≥40yrs of age COVID-19 patients respectively had low Ct values of nasal and oral swab IL-6 expression. Therefore, a higher proportion of ≥40 yrs old COVID-19 patients expressed high levels of IL 6 in the nasal and oral swabs. See Table 3.

Discussion

The pandemicity of the COVID-19 outbreak which began in the 2020 led to urgent requests for treatment strategies and better clinical management of patients.[7] SARS-CoV-2 infects the host by binding via its spike protein to host cell epithelial ACE2 receptors, which is largely expressed in the nasal and oropharyngeal epithelium.[8] This is followed by viral activation of the innate and adaptive immune responses.[23] The role of ACE2 as a first step in SARS-CoV 2 infection, suggested its choice as the basis for COVID-19 targeted therapy.[22, 23] This study shows that the mean Ct value of nasal and oral swab ACE2 was significantly reduced in combined COVID-19 cases compared with control. The implication of this is that there was a higher load of ACE2 in COVID-19 patients compared with the control. This was because ACE2, being the cellular receptor of SARS-COV-2 provided more available receptors for viral entry and hence a higher viral load associated with poor prognosis. It is reasonable to hypothesise that increased expression of transmembrane ACE2-SARS-CoV-2 receptor could be harmful, due to the possibility of favouring viral entrance and involvement in inflammation.[24] Upregulation of ACE2 mRNA expression was observed in bronchoalveolar lavage fluid cells[25] and lung cells[26] of COVID-19 patients. Throughout COVID-19 episode, there were no targeted drugs specifically against SARS-CoV-2 infection but some repurposed drugs were found to be effective in the treatment.[5] However, ACE/ARB inhibitors (ClinicalTrials.gov Identifier: NCT

04330300) was repurposed for the management of SARS-CoV-2 infection.[27] The basis for the use of this inhibitor was not stated but could have been suggested to be through the blocking of SARS-CoV-2 associated ACE activity caused by increased ACE expression in COVID-19 patients compared with control as found in this study. Our present study thus provided additional insight into the ACE-based therapeutic strategy of SARS-CoV-2 infection.

The pathogenesis of COVID-19 is complex including excessive production of cytokines and chemokines, profound recruitment of inflammatory cells, insufficient interferon response, and presence of auto-antibodies.[1-6]Chemokines and interleukins(IL) such as IL-1, IL-6, IL-12, IL-8, monocyte chemoattractant protein-1 (MCP-1) and interferon-gamma-inducible protein 10 (IP-10) were significantly elevated in the plasma of COVID-19 patients especially in severe COVID-19 patients.[28] Autopsy studies of SARS patients reported cytokine expression in SARS-CoV-infected ACE2+ cells, but not in tissues without infected ACE2+ cells[29]All these are corroborated by our finding of significantly low mean Ct values of ACE2 in moderate and severe COVID-19 patients (but not in mild COVID-19 patients) compared with control. A low Ct value of ACE 2 indicates high expression of ACE.[21, 22]The similarity of mean Ct nasal and swab IL-6 expression in our combined COVID-19 patients and control indicated that IL-6 was not needed at the attachment or invasion stage of SARS-CoV 2 into the host but at the level of systemic host humoral innate response. This suggestion was further emphasised by the significant reduction of nasal and oral swab IL6 in mild COVID-19 patients as indicated by significantly higher Ct value of IL6 expression in mild COVID-19 patients. An earlier study reported increased IL6 levels in the blood of COVID-19 patients.[8]

Sixty (60)%, 93% and 100% of mild, moderate and severe COVID-19 patients respectively had low Ct values of nasal and oral swab ACE2 expression. Therefore, a higher proportion of severe COVID-19 patients followed by a moderate proportion of COVID-19 patients expressed high levels of ACE2 in the nasopharynx. This supports an earlier report that advanced and severe forms of COVID-19 are associated with cytokine storm syndrome,[29] since ACE2 and the inflammation process was found to be linked through tumour necrosis factor-alpha converting enzyme (TACE), also known as ADAM17 (a disintegrin and metalloproteinase 17) which activates inflammatory cytokines such as TNF-α and IL6.[30]None (0%) and 33% of male and female COVID-19 patients respectively had low Ct values of nasopharyngeal swab ACE2 expression. Therefore, a higher proportion of female COVID-19 patients expressed high levels of ACE2 in the nasopharynx. Also, 0% and 33% of <40yrs and ≥40yrs of age COVID-19 patients respectively had low Ct values of nasal and oral swab ACE2 expression. Thus, a higher proportion of ≥40 yrs old COVID-19 patients expressed high levels of ACE 2 in the nasopharynx. Male rats had reduced ACE2 level compared with

female, also oestrogen increases the expression of ACE2.[31] In addition, ACE2 is said to be located on the X chromosome and its protein expression is expected to be higher in females than in males in murine models,[32] which is also consistent with our hypothesis on a higher level of ACE2 in female gender expression as presented in this study.

Several theories have been proposed to explain why COVID-19 was mild at younger age.[33-37] Cristiani et al., (2020) proposed higher strength of the innate immunity of young persons, which enhances their trained immunity.[34] Cyranoski(2020) proposed the uncompromised state of the endothelial system of children which protects them from the severe complications of COVID-19 that originate from endothelial inflammation and dysfunction.[35] However, the theory that supports our present finding is the one based on the variation of the viral receptor ACE2 with age. It was observed that ACE2 mRNA and serum protein is lower in younger age than in older ages,[36] and it was proposed that lower receptor levels protect young ones from severe SARS-CoV 2 infection.[37] Our present finding of increased expression level of swab ACE2 in>40yrs old COVID-19 patients compared with <40 yrs old patients supports the earlier report of higher prevalence of COVID-19 in adults and females.[38]

In the present study 93%, 100% and 93% of mild, moderate and severe COVID-19 patients respectively had low Ct values of nasopharyngeal IL-6 expression. Therefore, the level of IL6 in the nasopharynx of COVID-19 patients seems not to be dependent on COVID-19 severity. In contrast, a previous study showed that an increase in IL6 expression in lung tissue was associated with severity of COVID-19 infection.[39] Another prospective cohort study revealed that elevated blood IL-6 levels were related to the prolonged hospital stay of COVID-19 patients.[40] Sixty-seven (67)% and 50% of male and female COVID-19 patients respectively had low Ct values of nasopharyngeal swab IL-6 expression. Therefore, a relatively higher proportion of male COVID-19 patients expressed high levels of IL-6 in the nasopharynx. A study showed that persistently elevated IL6 levels in males are associated with a higher rate of multiple organ failure.[41] Thirty-three (33)% and 100% of <40yrs and ≥40yrs of age COVID-19 patients respectively had low Ct values of nasopharyngeal IL-6 expression. Therefore, higher proportion of ≥40 yrs old COVID-19 patients expressed high level of IL-6 in the nasal and oral swabs. Thus, in the context of natural aging, a study reported that the natural aging process caused increased levels of IL-6.[42]

Conclusions:

This study concluded that nasal and oral swab ACE2 is fundamental to age- and gender-based severity of COVID-19. Thus, the development of receptor-based targeted treatment against COVID-19 is supported by this study.

Limitations and further study

The study is constrained by a relatively small sample size, which may impact the generalisability of the findings to a broader population. Thus, multicenter and longitudinal studies are encouraged particularly on the stored swab samples since COVID-19 is no longer pandemic. Further study is recommended on different age strata, ethnic groups and races with or without co-morbidities,

Author Contributions: GOA conceived the concept of the manuscript. OKA carried out the laboratory procedures. AF was the consultant physician to the study. All authors carried out the statistical analysis, contributed to the writing of the manuscript and approved its publication.

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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