Saudi Dental Journal (2021) 33, 972–978

King Saud University

Saudi Dental Journal

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ORIGINAL ARTICLE

# Interleukin-6 gene polymorphism in Saudi population with recurrent aphthous stomatitis

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Received 24 July 2020; revised 23 June 2021; accepted 4 July 2021
Available online 23 July 2021

**KEYWORD**

Recurrent aphthous ulcers;
Interleukin-6;
Gene polymorphisms

**Abstract** *Introduction:* Recurrent aphthous ulcers are common but poorly understood mucosal disorder. Local and systemic conditions, genetic, immunological, and microbial factors may play a role in the pathogenesis of recurrent aphthous ulceration (RAS). Different aetiologies and mechanisms might be involved in the aetiopathogenesis of aphthous ulceration. Cytokines are thought to play an important role and high levels of interleukin (IL)-6, a pro-inflammatory cytokine, have been detected in the circulation of ulcer tissue. The purpose of the present study was to investigate if polymorphisms of IL-6 gene are associated with RAS in a cohort of specific population.

*Methodology:* A total of 37 RAS patients and 18 healthy controls were included in the study. The genotypes of IL-6 gene −174G\C polymorphisms were determined using polymerase chain reaction and sequencing.

*Results:* Four SNPs were analyzed, one known mutation which been evaluated as a risk factor for RAS, and three new mutations were investigated. The genotype frequencies of −174G\C polymorphism showed no statistically significant differences between RAS patients and controls (p\ 0.629). Polymorphisms of Rs1800795 heterozygous genotype were found in 21.62% of cases, and 33.33% of controls. Homozygous mutant genotype was found in 5.41% of cases and no homozygous mutant genotype was found in control group. The normal alleles were found in 72.97% of cases and 66.67% of control.

*Conclusion:* Thus, according to our study, IL-6 gene polymorphism is not involved in RAS pathogenesis. Further studies should be done on large sample size to detect any association with pathogenesis. However, an alternative reasoning could point out to a complex interactive effect on IL-6 expression that might exist between any of the detected polymorphisms.


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Peer review under responsibility of King Saud University.

Production and hosting by Elsevier

https://doi.org/10.1016/j.sdentj.2021.07.007

## 1. Introduction

Recurrent aphthous stomatitis (RAS), also known as recurrent aphthous ulcers or canker sores are one of the most common chronic oral mucosal inflammatory diseases. They are present as small, ovoid, or round, recurrent multiple ulcers with circumscribed margins, having yellow or gray floors and are surrounded by erythematous haloes. They are diagnosed based on patient's history and clinical presentation (Edgar et al., 2017; Scully and Porter, 2008). The prevalence of RAS is between 5 and 25%. This significant difference is reported due to different origin of examined groups and populations as well as on study design and methodology (Chavan et al., 2012). It has different clinical variants i.e., minor (MiRAS), major (MaRAS) and herpetiform (HU) ulcers. Various etiologic mechanisms or triggering factors includes salivary composition, mechanical injury, tobacco cessation, food allergies, vitamin and microelements deficiencies, hypersensitivity to gluten, trauma, allergy to sodium lauryl sulfate in tooth paste and bacterial or viral factors. Other factors include medical conditions (including Behçet's syndrome, MAGIC syndrome, Sweet's syndrome, PFAPA syndrome, cyclic neutropenia and Crohn's disease), immunologic factor, psychological stress, genetic predisposition, hormonal factors, and drugs (Akintoye et al., 2014; Chavan et al., 2012).

Role of genetic predisposition in RAS was suggested by the author Ship in 1965. They assumed that autosomal recessive or multigene mode of inheritance with modulating influence of environment plays a major role (Ślebioda et al., 2014). Role of genetic factors in etiopathogenesis of recurrent aphthae was further confirmed by studies with positive family history of disease, reported in 24–46% of cases (Ślebioda et al., 2013). Studies on identical twins have also demonstrated the hereditary nature of this disorder (Chavan et al., 2012; Ślebioda et al., 2014). Genetic risk factors which may determine the individual susceptibility to RAS include various DNA polymorphisms distributed in human genome. A special attention was paid to alterations in the metabolism of cytokines including interleukins (IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-10, IL-12), interferon γ (IFN-γ) and tumor necrosis factor-α (TNF-α) (Ślebioda et al., 2013). Few researchers have reported conflicting results when they investigated the relationship between IL-6 promoter polymorphisms and RAS.

## 2. Materials and method

### 2.1. Study sample

A total of 57 consecutive samples were recruited for the study including 37 patients with minor and major RAS and 18 control participants. Patients were recruited with same topographical region and had indistinguishable financial status from King Abdulaziz University Hospital, Faculty of Dentistry (KAUFD) and private dental clinics in Mecca and Jeddah, Saudi Arabia. RAS was diagnosed based on approved clinical criteria (Ship et al., 2000). Control group volunteers had no history of RAS or systemic maladies. Avoidance criteria for both study sample and control group were the vicinity, aside from dental caries. Albeit periodontal ailment was not a prohibition measure, none of the people in both study sample and control group introduced periodontitis. The inclusion criteria were Saudi nationality, age, clinically diagnosed with RAS. Inclusion criteria for controls included no family history of recurrent oral ulceration or suspicion of RAS, no oral mucosal lesions, Saudi nationality, and age. The exclusion criteria were systemic diseases (Behçet's disease, systemic lupus erythematous, Celiac disease, Sweet's syndrome, inflammatory bowel disease, Reiter syndrome, PFAPA syndrome and HIV infection), exposure to radiation and drug consumption, tumors of oral cavity and presence of pregnancy. The study was approved by University Ethics Committee and consent was obtained from all the patients or from parents/guardian in case of minors.

### 2.2. Molecular analysis

#### 2.2.1. Genomic DNA extraction

About 2 ml of peripheral blood was collected in an EDTA-containing tube using sterile syringe by a nurse either in the dental clinic or in laboratory of Prince Al-Jawharh Center of Excellence in Research of Hereditary Disorder. Samples were transported in an ice container to the laboratory for further DNA isolation or stored at −20 °C till further use. DNA was isolated from blood using QIAamp® DNA blood kit using instructions provided in the kit. The isolated DNA were quantified using NanoDrop 2000 spectrophotometer (Thermo Scientific) to assess its concentration and purity. The yield was calculated using the formula:

$$\text{Yield } (\mu g) = \text{DNA concentration } (ng/\mu L) \times \text{samplevolume}(\mu L)/1000\ ng/\mu g$$

Also, A260/A280 and A260/A230 ratios were recorded. Agarose gel electrophoresis was done to assess DNA intactness and average size for each sample.

#### 2.2.2. Single nucleotide polymorphism (SNP) genotyping

SNP used in the study was selected based on previous genomic wide association studies (GWAS) and *meta*-analysis studies associated with RAS. Genotyping for selected SNPs were carried out using TaqMan SNP Genotyping assays. Polymerase chain reaction (PCR) primers and TaqMan probes were designed, and reactions were performed in 96 well microplates with Applied Biosystems 7500 sequencing system. SNP ID number and assay number used in RAS genotyping are shown in Table 1.

Mutant allele – C (175 bp *)

#### 2.2.3. Polymerase chain reaction (PCR) for genotyping

PCR was carried out in a total volume of 25 µl which contains 2 µl DNA (50 ng), buffer (50 mM KCl, 10 mM Tris–HCl pH 8.4, 0.1% Triton X-100), 1.5 mM MgCl2, 200 µM dNTPs, Taq DNA polymerase (Promega, USA) and primers (10 pmol/µl). Amplification conditions were 94 °C for 3 min (Initial denaturation) followed by 35 cycles at 94 °C for 30 sec (Denaturation), 57 °C for 30 sec (Annealing) and 72 °C for 30 sec (Extension). The run was terminated by final extension at 72 °C for 5 min. Final product was resolved using 2% agarose gel with stain SYBR® Safe DNA Gel Stain (Invitro-

gen, USA). A total of 5 µl PCR product, positive control, negative control and 100 bp ladder were loaded with 3 µl loading dye (Bromophenol blue). Bands were visualized on the Gel Doc XR + system (Bio-Rad, USA).

#### 2.2.4. DNA purification and cycle sequencing

Purification of DNA was performed using QIAamp DNA mini kit (Qiagen) according to the manufacturer's instructions. Cycle sequence PCR was performed according to the manufacturer's instructions of commercial kit (Big Dye direct cycle sequencing kit). For obtaining optimal results, high-quality DNA was used with no degradation or contaminants. DNA with an A260/A280 ratio between 1.7 and 1.9 was used for further steps. Cycle sequencing was done using Big Dye direct cycle sequencing kit (Thermo Fischer, USA) according to manufacturer's instructions. For each 10 µl forward or reverse reaction, 1 µl DNA (4 ng\µL), 1.5 µl M13-tailed PCR primer mix (0.8 µM each primer), 5.0 µl BigDye direct PCR master mix 2.5 µl and deionized water was added. The cycle sequencing reaction stages is shown in Table 2.

Further, another step of cycle sequencing was performed. The minimum quantity of PCR product used for sequencing was 20 ng which was confirmed by agarose gel electrophoresis. For each 3 µl total reaction volume, 2 µl Big Dye direct sequencing master mix and 1 µl Big Dye direct M13 forward or reverse primer was used. Reaction mixture was prepared on ice and 3 µl of sequencing reaction mix was added to appropriate well in respective forward or reverse reaction plate. Reaction plate was sealed with adhesive film or caps and spinned briefly. The reactions were run in a thermal cycler as mentioned in Table 3.

Final sequencing product was purified using the Big Dye X Terminator purification kit according to manufacturer's instructions. Final reaction plate was loaded into 96-standard well in sequencer (Applied Biosystems 3500/3500xL-3500/3500xL, Genetic Analyzer, HITACHI, Holland). Final PCR amplicons and sequencing results was sent to the Korea laboratory, Macrogen. The obtained sequences were subjected to BLAST search for identifying similar genes in GenBank.

### 2.3. Statistical analysis

Data analyses were done using statistical package for the social science (SPSS) and SNP tools for Microsoft Excel. The influence and differences in genotype frequencies of SNPs on risk of RAS between patient and healthy groups were tested by chi-square and *t*-test method with confidence interval (95% CI).

**Table 1** SNP ID number used in RAS genotyping.

| **SNP ID** | **rs1800795** |
|---|---|
| Location | Chr.7:22766645 on NCBI Build 37 |
| Gene | IL6 LOC541472 |
| Gene Name | interleukin 6 (interferon, beta 2) |
| SNP Type | Intron, Intragenic |
| Primer sequence | F25AATGTGACGTCCTTTAGCATC3′<br>R 5TCGTGCATGACTTCAGCTTTA3′ |
| Polymorphism | C/G, Transversion Substitution |

**Table 2** Condition for cycle sequencing PCR reaction.

| Stage | Veriti® thermal cyclers | |
|---|---|---|
| | Temperature | Time |
| Hold | 95 °C | 10 min |
| Cycle (35 cycle) | 96 °C | 3 sec |
| | 62 °C | 15 sec |
| | 68 °C | 30 sec |
| Hold | 72 °C | 2 min |
| Hold | 4 °C | ∞ |

## 3. Results

A total of 55 participants were randomly recruited for the present study between ages of 12–75 years. Genomic DNA purified from participant's blood samples were used to amplify target amplicon with specific primers using PCR techniques. Amplicons were resolved in 2% agarose gel which showed a specific band of size 304 bp (Fig. 1).

Amplicons were cut and purified for further use. For further conformation direct DNA sequencing was done of these PCR products. Sequenced fragments were submitted to GenBank database (http://blast.ncbi.nlm.nih.gov) and analyzed in BLAST search program. The sequence showed three possible mutations in promoter region of IL-6 gene. The first mutation was found at position −174G\C. Allelic and genotypic distributions of IL-6 gene −174G\C polymorphism are shown in supplementary Figs. S1-S5. Also, other mutation was found at the end of sequence of IL-6 at position 62. This mutation is unknown and was not listed in GenBank. Further two different mutations at same location were observed, the first was T\- CT (Fig. 2 and Supplementary Fig. S6) and the second was AC\CT (Fig. 3 and Supplementary Fig. S7). All study samples were variants. There was no normal sample.

### 3.1. Statistical analysis

Statistical analysis was performed using a SNP tool which is a general add-in tool for Microsoft Excel program for data conversion and basic analysis for SNP data (http://www.bioinformatics.org/snp-tools-excel/). The influence and differences in

**Table 3** Condition for cycle sequencing reaction.

| Stage | Veriti thermal cyclers | |
|---|---|---|
| | Temperature | Time |
| Hold | 37 °C | 15 min |
| Hold | 80 °C | 2 min |
| Hold | 96 °C | 1 min |
| Cycle (25 cycle) | 96 °C | 10 sec |
| | 50 °C | 5 sec |
| | 60 °C | 75 sec |
| | 4 °C | ∞ |

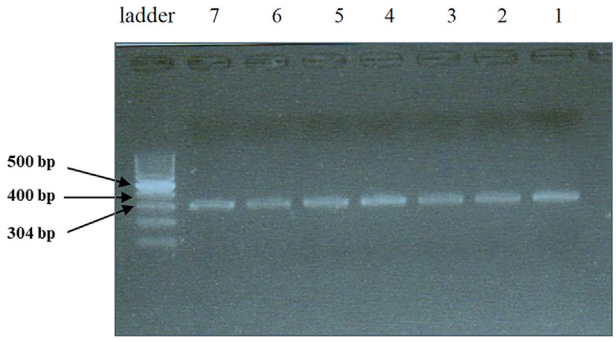


**Fig. 1** Agarose gel photograph showing amplicons of 304 bp.

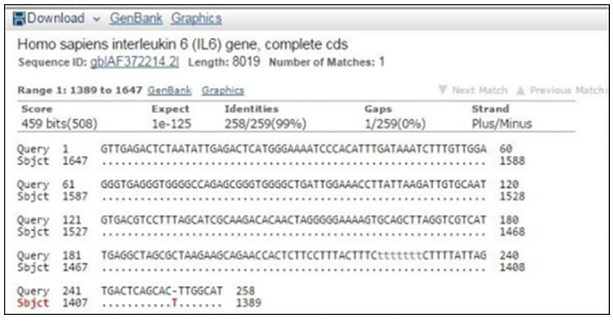


**Fig. 2** Photograph showing T\- mutation in BLAST search.

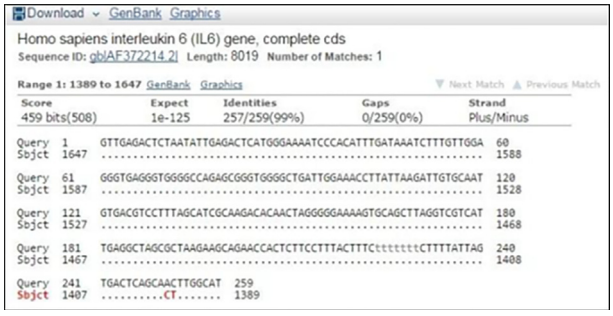


**Fig. 3** Photograph showing AC\CT mutation in BLAST search.

genotype frequencies of SNPs on the risk of RAS between patients and healthy controls were tested by chi-square and *t*-test with confidence interval (CI) 95%.

In total 4 SNPs were analyzed out of which three was new mutation and one mutation was previously known (Rs1800795) and has been evaluated as a risk factor for RAS (Bazrafshani et al., 2002). Chi square test was done for the SNP Rs1800795 with p value 0.629, odd ratio (OR) 0.741% and CI = 0.219–2.507. Polymorphisms of Rs1800795 heterozygous genotype were found in 21.62% of cases and 33.33% of controls. Homozygous mutant genotype was found in 5.41% of cases and only one was found in control percentage. Normal allele was found in 72.97% of cases and 66.67% of control. Similarly, allele polymorphisms of other new mutation or SNPs and the rest of genotype result using Chi square test and OR of five selected SNPs are shown in tables below (Table 4a–4e).

## 4. Discussion

RAS is one of the most common oral diseases whose etiology is still unknown. Some literature reported that RAS may have immunological, psychological, genetic, and microbiological causes (Akintoye et al., 2014; Edgar et al., 2017). Although studies have tried to identify the role of immune mechanisms in RAS, the immune pathogenesis remains yet to be established (Chavan et al., 2012). According to Karakus et al., RAS is supposed to be formed by a local mucosal damage in an individual genetically predisposed to an abnormal cytokine cascade (Karakus et al., 2014). Although RAS patients did not show increase level of IL-6 in serum, its high level have been shown in ulcer tissues (Pekiner et al., 2012; Yamamoto et al., 1994). The IL-6 gene is located on chromosome 7p21 whose expression is generally regulated at transcriptional level by control elements in promoter region of gene. Several polymorphisms have been found in promoter region of IL-6 gene. The IL-6 gene −174G\C and −572G\C polymorphisms in promoter region are important regulators of transcription. The −174G\C polymorphism is located immediately upstream of a multi-response element at positions −173 to −151. The −572G\C polymorphism is located near a potential glucocorticoid receptor element at position −557 to −552 (Hulkkonen et al., 2001). The IL-6 production is modulated by these polymorphisms and has been associated with susceptibility to several pathological conditions (Cussigh et al., 2011; Song et al., 2013). There are only few reports which studied the relationship between IL-6 promoter polymorphisms and RAS, and their results were inconsistent. While author Bazrafshani et al., reported a positive association between carriage of IL-6 −174GG genotype and RAS, others could not confirm this observation (Bazrafshani et al., 2002; Guimarães et al., 2007; Jing and Zhang, 2015). According to some studies, presence of low producer phenotype of IL-6 gene −174G\C and −572G\C polymorphisms may protect from RAS (Karakus et al., 2013). As no previous work was done among Saudi population on this issue, the purpose of present study was to investigate if IL-6 gene polymorphisms were associated with the development of RAS in a cohort of a Saudi population.

In the present study, four SNPs were analyzed, one known mutation which has been previously evaluated as risk factor for RAS by author Bazrafshani et al., and three new mutations. Polymorphisms of Rs1800795 heterozygous genotype were found in 21.62% of cases, and 33.33% of controls. Homozygous mutant genotype was found in 5.41% of cases and one homozygous mutant genotype in control. Normal allele was found in 72.97% of cases and 66.67% of control. Other mutation was found at the end of sequence of IL-6 at position −62, the mutation is unknown and was not listed in gene bank, two different mutations were observed at same location, the first one is T\- and the second one is AC\CT. The study done by Bazrafshani et al., showed that the greatest risk of RAS was associated with G/G homozygosity (OR = 3.4; 95% CI 1.9–6.2; p = 0.0001). No significant association was noted with IL-6–573 polymorphism. The IL-6–174 and −573 polymorphisms are very close and association with one may result in associating with other. However, G allele at position −573 occurs in over 90% of population and therefore a large sample size is needed to be evaluated for any association at this position. The IL-6–174 promoter polymorphism

**Table 4a** Genotypes and allele frequencies of SNP Rs1800795.

| Rs1800795 | Cases | Percentage (%) | Allele (C) Frequency | Controls | Percentage (%) | Allele (C) Frequency |
|---|---|---|---|---|---|---|
| CC | 2 | 5.41% | 0.232495277 | 1 | 0.00% | 0.183503419 |
| GC | 8 | 21.62% | Allele (G) Frequency | 6 | 33.33% | Allele (G) Frequency |
| GG | 27 | 72.97% | 0.767504723 | 12 | 66.67% | 0.816496581 |
| Total | 37 | 100.00% | 1 | 18 | 100.00% | 1 |

**Table 4b** Genotypes and allele frequencies of new mutation 1.

| New mutation 1 | Cases | Percentage (%) | Allele (del) Frequency | Controls | Percentage (%) | Allele(del) Frequency 2 |
|---|---|---|---|---|---|---|
| T0 | 29 | 80.56% | 0.897527468 | 14 | 77.78% | 0.881917104 |
| TA | 0 | 0.00% | Allele (T) Freq. | 0 | 0.00% | Allele (T) Freq. |
| TT | 7 | 19.44% | 0.102472532 | 4 | 22.22% | 0.118082896 |
| Total | 36 | 100.00% | 1 | 18 | 100.00% | 1 |

**Table 4c** Genotypes and allele frequencies of new mutation 2.

| New mutation 2 | Cases | Percentage (%) | Allele (C) Frequency | Controls | Percentage (%) | Allele (C) Frequency 2 |
|---|---|---|---|---|---|---|
| CC | 7 | 19.44% | 0.440958552 | 4 | 22.22% | 0.471404521 |
| CA | 0 | 0.00% | Allele (G) Frequency | 0 | 0.00% | Allele (G) Freq. |
| AA | 29 | 80.56% | 0.559041448 | 14 | 77.78% | 0.528595479 |
| Total | 36 | 100.00% | 1 | 18 | 100.00% | 1 |

**Table 4d** Genotypes and allele frequencies of new mutation 3.

| New mutation 3 | Cases | Percentage (%) | Allele (T) Frequency | Controls | Percentage (%) | Allele (C) Frequency.2 |
|---|---|---|---|---|---|---|
| TT | 7 | 19.44% | 0.440958552 | 4 | 22.22% | 0.471404521 |
| TC | 0 | 0.00% | Allele (A) Frequency | 0 | 0.00% | Allele (G) Frequency |
| CC | 29 | 80.56% | 0.559041448 | 14 | 77.78% | 0.528595479 |
| Total | 36 | 100.00% | 1 | 18 | 100.00% | 1 |

**Table 4e** Table showing chi square test and OR of the four SNPs.

| | Mutation cases | Normal cases | Mutation controls | Normal controls | OR | 95% CI | X2 | P value | Power | $OR_{MH}$ | $X^2_{MH}$ | $P_{MH}$ |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Rs1800795 | 10 | 27 | 6 | 12 | 0.741 | 0.219 | 2.507 | 0.629 | 0.070 | 0.741 | 0.229 | 0.632 |
| New Mutation 1 | 29 | 7 | 14 | 4 | 2.071 | 0.297 | 4.725 | 0.811 | 0.043 | 1.184 | 0.056 | 0.813 |
| New Mutation 2 | 7 | 29 | 4 | 14 | 1.750 | 0.212 | 3.372 | 0.811 | 0.043 | 0.845 | 0.056 | 0.813 |
| New Mutation 3 | 7 | 29 | 4 | 14 | 1.750 | 0.212 | 3.372 | 0.811 | 0.043 | 0.845 | 0.056 | 0.813 |

appears to be functional, lying immediately upstream of a multi-response element at positions −173 to −151. This element is important in IL-1 and TNF modulated expression of IL-6 and inheritance of G allele at position −174 has been shown to encode higher levels of IL-6 than the C allele (Bazrafshani and Hajeer, 2003). Thus, our present results are not in accordance with such previous work or conclusions. On the other hand, our results are in line with those of

Guimaraes et al. (2007) and Jing and Zhang (2015), who could not find a single IL-6 gene polymorphism to be involved in RAS pathogenesis. They concluded that polymorphisms on these genes were not associated with RAS and this inconsistency was probably due to population heterogeneity or the study group size (Guimarães et al., 2007; Jing and Zhang et al., 2015). Thus, as the sample size in our study is small, sample heterogeneity variation is low, we also agree with above theory. Therefore, these reasons demand to increase the size of study sample and codifying study sample to get significant and convinced results. The main reason for small study sample is the difficulty in obtaining blood samples from people, particularly in Saudi society and also due to the location of disease i.e., mouth. However, an alternative reason could point out to a complex interactive effect on IL-6 expression that might exist between any of the detected polymorphisms. High production of IL-6 could be an important etiological factor for RAS and could explain elevated IL-6 tissue levels reported in RAS. It is also important that corticosteroids and thalidomide, two of the most effective therapeutic agents in RAS, are inhibitors of IL-6 production. However, the effect of mutations could be due to the coexistence of two or more mutations.

## 5. Conclusion

In conclusion, no single IL-6 gene polymorphism can be related to the etiology of RAS in our present study. A combined effect of more than one gene mutation could interplay in the pathogenesis of RAS lesions. Also, studies on a larger scale are needed to be done to confirm our results to use them as genetic markers for RAS susceptibility among study population.

## Ethical statement

The ethics committee of King Abdulaziz University Hospital has approved the study protocol. The study convention was sanction by the King Abdulaziz University Hospital, Faculty of Dentistry (KAUFD) Ethics Committee and consent was obtained from all patients or from the relations when subjects were less than 18 years.

## CRediT authorship contribution statement

**Samah Jameel Saeed Shabana:** Data curation, Formal analysis, Investigation, Methodology, Project administration, Software, Writing - original draft. **Muhammad Hamid Zaini Mutawakkil:** Conceptualization, Methodology, Funding acquisition, Project administration, Resources, Supervision, Validation, Writing - review & editing. **Hassan Mamdouh Aly El–Ashmaoui:** Formal analysis, Investigation, Methodology, Project administration, Validation, Visualization, Writing - review & editing. **Fathya Mohammed Abdel-Qawi Zahran:** Data curation, Project administration, Validation, Visualization, Writing - review & editing.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Acknowledgement

None.

## Funding

None.

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