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Screening for SARS-CoV-2 variants in Egypt using multiplex PCR

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ABSTRACT

Background: Since the beginning of SARS-CoV-2 pandemic, numerous variants have emerged as a result of mutations occurring in earlier strains. A number of these variants have been linked with waves of widespread infections. The importance of the Variants of Concern (VOCs) lies in increased transmissibility, morbidity, and mortality, as well as escaping detection by conventional methods and resistance to treatment and vaccines. **Aim of this study:** Our aim is to screen for the presence of six SARS-CoV-2 variants in Egypt, for better understanding of its epidemiology during the epidemic. **Methods:** In this study, 2650 SARS-Cov-2 RNA positive nasopharyngeal specimens were collected at the Reference Laboratory of Egyptian University Hospitals (RLEUH) during the period from December 2020 through October 2021. All the samples were subjected to AllPlex SARS-CoV-2 Master assay, Variant I, II and Novaplex Variant IV Assays. Six VOCs could be detected by this method, which are alpha, beta, gamma, delta, delta plus and epsilon. **Results:** According to our data, the most detected variant was delta variant comprising 1308 (49%) of the cases. This was followed by alpha variant 121 (5%), delta plus 57 (2%) and gamma 8 (0.3%). 1156 (44%) of the cases were designated as other variants. Beta and epsilon variants were not detected in our study. **Conclusions:** Our data show that multiplex PCR assays are helpful in the characterization of the genomic epidemiology of SARS-COV-2. Although the new generation sequencing remains the gold standard for surveillance of variants, AllPlex™ Assays can provide a rapid and affordable option.

Introduction

Since all existing viruses mutate, spreading of multiple strains of the same virus are commonly found, the same applies for the COVID-19 pandemic. SARS-CoV-2 variants are grouped and

defined as those strains containing a common set of genetic mutations [1]. Emerging SARS-CoV-2 variants represent a major health concern as they might result in genetic changes that make the virus more transmissible, virulent, resistant to therapy,

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able to overcome vaccines, or able to escape various diagnostic tests with increased overall morbidity and mortality [2].

Starting September 2020, newly emerging variants of concern (VOC) of SARS-CoV-2 have been detected in Europe [3]. The first to be detected was the United Kingdom alpha variant 20I/501Y.V1 (lineage B.1.1.7) [4], followed by, the South-African beta variant 20H/501Y.V2 (lineage B.1.351) [5] and the Brazilian gamma variant 20J/501Y.V3 (lineage P.1) [6]. Additionally, the eta variant 21D/484K.V3 (lineage B.1.525) has been detected in different countries. [7] Indeed, since the outbreak of the pandemic at least 1250 distinct variants have been identified [8]. The delta (India) variant 21A/478K.V1 (lineage B.1.617.2) as well as the delta plus variant (lineage AY.1/AY.2) have been also detected [7].

The new lineages AY.1/AY.2 have previously been assigned to B.1.617.2 and they are subclades within B.1.617.2. These lineages remain the delta variant, just as B.1.617.2 does, and their designation doesn't imply any functional biological difference from B.1.617.2. The delta variant has become widespread over the last months and B.1.617.2 has been the predominant lineage in many countries worldwide. Classifying viral sequences as B.1.617.2 is useful to chart the rise of B.1.617.2 in a country, but once this lineage is dominant, it doesn't allow researchers to easily do more fine-scale tracking. The AY lineages break up B.1.617.2 into smaller related clusters that can be tracked separately. Each of these clusters is usually associated with a significant epidemiological event [8].

The most significant variants identified in these strains are those located in the S gene, encoding for the Spike protein. Interestingly, one specific mutation, named N501Y, arose independently in all three variants. Since the Spike protein is the key to allow virion entry into host cells, this mutation might augment the Spike protein's ability to bind to its specific cell receptor angiotensin-converting enzyme-2 (ACE-2), increasing the virus ability to infect host cells [9].

A mutation of a particular concern is E484K, which reduces the antibody binding to the receptor binding domain (RBD), reducing neutralization efficacy in some SARS-CoV-2 of convalescent human sera by > 10-fold. K417 has been found to be 60–100% buried in class 1

antibody paratopes and K417N has also been shown to significantly reduce sera neutralization. The combination of nearby RBD mutations E484K, N501Y and K417N are found in B.1.351 whereas the mutations E484K, N501Y and K417T are found in P.1 [10].

Testing to identify which viral variant is best accomplished using a sequencing method, commonly called next-generation or metagenomic sequencing or NGS [2]. NGS is a laboratory method that can identify SARS-COV-2 variants, which are reported to public health authorities for the purposes of strain surveillance and epidemiology. In addition to NGS, detection methods, such as reverse transcriptase polymerase chain reaction (RT-PCR) can identify known mutations using rapid, accessible, and high-throughput methods [11].

In Egypt, after a dramatic increase in COVID-19 positive cases during the first half of 2020, a reduction in the number of cases was observed during the second half of the same year. This was followed by a second increase by the end of 2020. The third wave of COVID-19 in Egypt occurred during the first quarter of 2021 [2] which coincided with the appearance of new variants. The aim of this study is to screen for the presence of SARS-CoV-2 variants in Egypt for better understanding of the genomic epidemiology of SARS-CoV-2 in Egypt.

Materials and methods

Specimens collection, transport and study population

This retrospective cross-sectional study included a total of 226,116 nasopharyngeal swabs were collected from adults only attending the Reference Laboratory of Egyptian University hospitals starting December 2020 till October 2021. Samples were placed in viral transport media and an accession number was given as codes for all samples collected. The study participants required laboratory testing either because of presenting with signs and symptoms of Covid-19 infection, suspicion of SARS-CoV-2 due to contact with positive cases, or for regular checkup.

A written informed consent was previously obtained from all patients before sample collection.

Data collection and sample size calculation

To identify the subtypes of SARS-CoV-2 viruses that circulated in Egypt after the emergence of several new variants, all positive samples (14762

samples) archived at the Reference Laboratory of Egyptian University hospitals between December 2020 to October 2021 were obtained. A sampling frame was developed by numbering the 14,762 specimens archived during this period. To get better insight of the SARS-CoV2 subtypes, approximately 20% of the archived specimens were selected randomly for screening of different known variants. Random selection was performed using Epi-info7 software. The number of specimens selected in each month was calculated in proportion to the percent of each month to total positives.

Specimens processing

They were processed in accordance with the manufacturer's instructions. RNA extraction was done using STARMag Universal extraction system (Seegene, Seoul, Korea). Amplification and detection were performed on CFX96™ real-time PCR detection system.

All samples were subjected to Real-Time Reverse-Transcription Polymerase Chain Reaction using AllPlex™ SARS-CoV-2 Master Assay (Seegene, Seoul, Korea).

1. SARS-CoV-2 positive, 5-mutations positive samples were subjected to AllPlex™ SARS-CoV-2 Variant I Assay (Seegene, Seoul, Korea). The results were either positive for alpha (UK) N501 & HV69/70 del, or Beta (SA)/Gamma (Brazil) N501 & E484K.
2. Samples with positive Beta (SA)/Gamma (Brazil) N501Y & E484K were subjected to AllPlex™ SARS-CoV-2 Variant II Assay (Seegene, Seoul, Korea). The results of this assay were either positive for Epsilon (CAL) L452R, W152C, Gamma (Brazil) K417T, Beta (SA) K417N, Delta (India) L452R /Delta Plus L452R. Negative results were presumed to be other variant Like combination of L452R & K417T.
3. Delta/Delta plus samples which were positive for L452R or L452R, P681R and K417N were further subjected to Novaplex™ SARS-CoV-2 Variants IV Assay (Seegene, Seoul, Korea) for presumed Delta (India) and Delta Plus. Their results were either Delta (Confirmed India) P681R, L452R, Delta Plus P681R, L452R, K417N or other variants.
4. Samples that were SARS-CoV-2 positive but 5 mutations negative were subjected to AllPlex™

SARS-CoV-2 Variant II Assay for presumed Epsilon (CAL), Gamma (Brazil), Beta (SA), Delta (India)/Delta Plus. They were also subjected to Novaplex™ SARS-CoV-2 Variants IV Assay for presumed Delta (India) and Delta Plus. Negative results were regarded as other variants (**Figure 1**).

Principle of the assay

This approach is based on stepwise qualitative detection of SARS-CoV-2 and its variants with multiplex real-time reverse transcription PCR from nasopharyngeal aspirate, nasopharyngeal swab, bronchoalveolar lavage, oropharyngeal swab, sputum and saliva. Each step involves the simultaneous amplification of a number of genes together with internal control. The assays used are:

1. Allplex™ SARS-CoV-2 Master Assay which detects five notable mutations in S gene (HV69/70 deletion, Y144 deletion, E484K, N501Y, and P681H) in one signal, in addition to four genes of SARS-CoV-2 which are E gene, N gene, RdRP gene and S gene.
2. Allplex™ SARS-CoV-2 Variants I which detects RdRP gene and 3 S gene mutations (E484K, HV69/70 deletion, N501Y).
3. Allplex™ SARS-CoV-2 Variants II Assay which detects 4 S gene mutations (K417N, K417T, L452R, W152C).
4. Novaplex™ SARS-CoV-2 Variants IV Assay which detects 3 S gene mutations found in Delta variants (L452R, P681R, K417N).

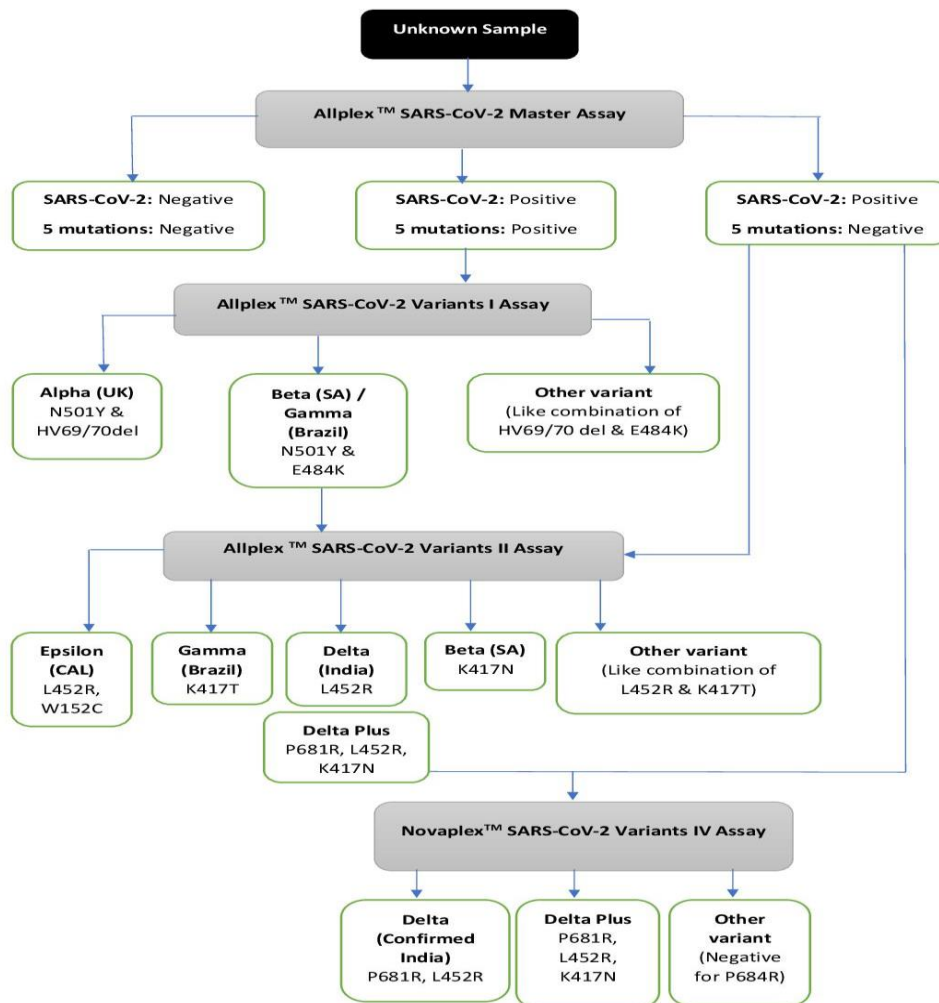
The presence of specific gene sequences in the reaction is reported as a Ct value through Seegene Viewer analysis software.

Statistical analysis

Data was analyzed using the statistical package SPSS (Statistical Package for the Social Sciences) version 25. Frequency (count) and relative frequency (percentage) was used for categorical data. Comparisons between categorical data was done using Chi square (χ^2) test. Exact test was used instead when the expected frequency is less than 5. [12]. *P* value less than or equal 0.05 was considered statistically significant.

Ethical approval

This study was approved by the Ethics Committee at the Supreme Council of University Hospitals on 2/1/2022, number: NO-0316.

Figure 1. Covid-19 variants test algorithm.

Results

This study included 2650 SARS-CoV-2 RNA positive nasopharyngeal specimens from 1534 (58%) males and 1116 (42%) females. The patients' ages ranged from 11 to 92 years (50.5 ± 18.6). Out of the 2650 SARS-CoV-2 samples collected during the whole study duration from December 2020 to October 2021, the majority (1308, 49.4%) were of the delta variant. The other detected variants were as follows: 121 (4.5%) alpha variant, 8 (0.3%) gamma variant and delta plus variant accounted for 57 (2.2%) of the studied specimens. About 43.6% of the studied samples could not be identified by Allplex SARS-CoV-2 assays and were defined as other variants (**table 1**).

In the beginning of this study most of SARS Cov-2 detected in patients was not identified by Allpex SARS-CoV-2 assays and was designated as "other variants". Since January 2021 the delta variant started to appear in our study group and

rapidly increased till it reached 297 cases in May 2021. The delta variant represented the most distinctly predominant one from March through October 2021, and its detection continued till the end of the study. The rate of the delta variant gradually increased till it reached 90% at the end of the study.

The alpha variant was also detected, in much lower rates compared to the delta variant, and reached its peak in March and April then rapidly declined until it no further detected in August 2021. Its rate among other variants ranged from 0.7% to 9.3%.

Very few numbers of the gamma variant were detected only from March through June 2021 (< 1% among other variants).

We noticed the emergence of the delta plus variant in our study since March 2021, however, in considerably lower rates than the delta variant. It approximately reached 15% of all detected variants

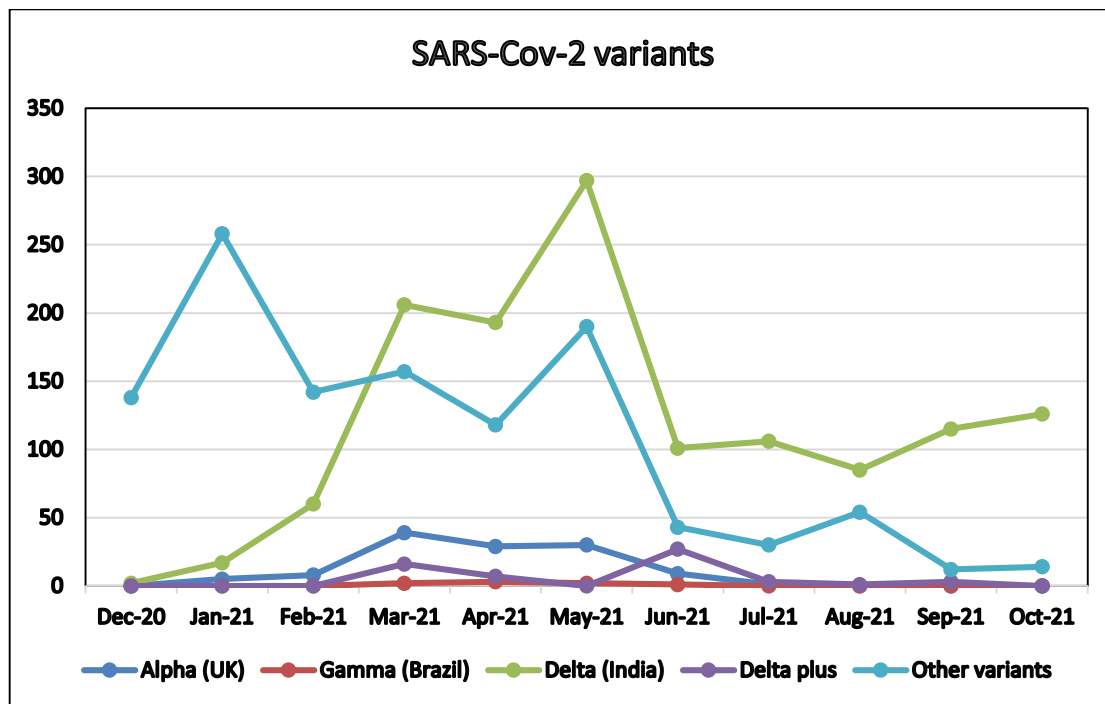
in June 2021 but it rapidly declined to only 0.7-2.3% in later months (**Figure 2**).

Other variants found by the Allplex SARS-Cov-2 assays such as the beta and the epsilon variants were not observed in the present study.

Table 1. SARS-Cov-2 variants detected in the study.

Variant	Number (%)
Alpha (UK)	121 (4.5%)
Gamma (Brazil)	8 (0.3%)
delta (India)	1308 (49.4%)
Delta plus	57 (2.2%)
Other variants	1156 (43.6%)
Total	2650 (100%)

Figure 2. Distribution of SARS-Cov-2 variants among the study duration.



Discussion

The emergence of new variants is a considered a global threat. This is attributed to the altered properties of these variants which might include their ability to spread, the severity of infection, their resistance to vaccines or current therapies. Continuous monitoring and assessment of the evolution of SARS-COV-2 by centers and institutes is considered crucial worldwide. The characterization of specific Variants of Interest (VOIs) and Variants of Concern (VOCs) is of great value, in order to take the proper response to the variant and prevent its spread.

In our study, we aimed at characterizing the genomic epidemiology of SARS-COV-2 in Egypt. For this purpose, we screened a representative sample of all positive specimens archived at the Reference Laboratory of Egyptian University hospitals between December 2020 to July 2021, for six variants of concern (VOC); alpha (UK), beta (south Africa), gamma (Brazil), delta (India), delta plus and epsilon (US-California) using Allplex SARS-Cov-2 assays.

Out of 2650 SARS-Cov-2 RNA positive nasopharyngeal specimens, the alpha variant was first identified in September 2020 in the UK [2]. Afterwards, it has spread across Europe to become

the most dominant lineage, outweighing the antecedent strains. Within two months, and starting from near 0%, its rate has accelerated to 50%. Soon later it peaked to over 98% of all sequenced samples in England. This could be explained by a 50% to 80% increased transmissibility, owing to the various mutations occurring in the spike protein receptor-binding domain [13].

In our study the alpha variant first detected in January 2021. It reached its peak in March and April. Then the rate rapidly declined until it no further detected in August 2021. It represented 121 (4.5%) of all positive samples.

The rapid drop of the rates of Alpha strain could be attributed to several factors. The first is the antagonism by newer and more contagious strains. The second is the unequal effect of vaccination on the different strains [14]. According to **Bian et al.**, the response of the Alpha lineage to vaccines did not deteriorate, unlike other lineages. [15] Another explanation is the increasing rates of false negative results due to failure of S- gene detection by genomic sequencing owing to the 69-70 deletion in Alpha lineage. This target failure has risen from near 0% in October 2020 to 98.8% in March 2021. However, this problem has not been encountered in PCR-tested samples. [13]

The delta variant started to appear in our study group in January 2021. Its rate rapidly increased till it reached 297 cases in May 2021. The delta variant represented the most distinctly predominant strain from March through October 2021. Its detection continued till the end of the study. The total number of detected delta strain was 1308, accounting for (49.4%) all positive cases.

This is consistent with the rates obtained from 135 countries, including UK and middle eastern countries such as Iran. In all these countries the delta variant or the lineage B.1.617.2 was the prevalent variant during the same period. [16].

The delta variant (B.1.617.2) was identified in India late in 2020. It was held accountable for the sweeping second wave of SARS-COV-2 in India. [17]

This could be attributed to the high transmissibility, resulting in its labeling as a Variant Of Concern (VOC)[14] According to Burki 2021, delta variant is sixty percent more infectious than the alpha variant. Its reproduction number is between 6 and 7. This means that one infected individual can disseminate the infection to 6 to 7 others. [18] In

addition to the higher transmissibility of Delta variant, it has been associated with higher rates of hospitalization, ICU admission, as well as higher mortality risk compared to non-VOC strains [19]. Another observation that could explain the vast predominance of Delta lineage is the occurrence multiple mutations in the receptor binding domain of the spike protein, rendering it more resistant to neutralizing antibodies, and hence to vaccine. [14]

The 'delta plus' variant is another variant of concern. It has evolved as a result of K417N mutation in the spike protein of the original delta strain [17]. This mutation may have aided antibody escape and further viral transformation [20].

After its detection in India, the delta Plus variant had propagated to many other countries, including the US [20]. Together with the delta variant, the delta plus variant has become the prevalent edition of the virus worldwide [21].

The delta plus variant was first observed in our study in March 2021. It peaked in June 2021 reaching 15% of all detected variants. It rapidly declined to only 0.7-2.3% in later months.

The P.1 or Gamma, variant of SARS-CoV-2 was first detected in Manaus, Brazil, in November 2020, when it started dissemination worldwide. It has been classified by the WHO as a VOC owing to its high transmissibility compared to previous lineages [22].

In our study, we detected 8 cases representing (0.3%) of positive cases. They were only detected from March till June 2021.

The beta strain was first reported in South Africa in October 2020. Later, it became the prevalent strain in Africa. Despite its widespread in many African countries, the beta variant was not detected in our study. This is consistent with the results obtained by [23] This could be attributed to the limited viral introduction from sub-saharan Africa to the middle East and North Africa compared to introductions from Europe and Asia [24].

The epsilon (B.1.429/B.1.427) lineage was first reported in California, USA in November 2020. It was classified as a variant of interest in March of 2021. It was soon replaced by newer and more transmissible variants [25]. This may justify the lack of detection of this variant in our study.

The 'other variants' category represented (43.6%) of all positive samples. The percentage was much higher at the beginning of the study,

accounting for more than 98% of the studied cases in December 2020. This percentage gradually declined throughout the period of the study, except for two small spikes (36.6%) in May 2021 and (38.6%) in August 2021.

Due to the use of Allplex SARS-CoV-2 assays in this study, which is directed at the detection of 6 selected variants, other variants may have been missed and therefore reported as other variants.

At the beginning of the pandemic, the majority of isolates recovered from Africa were originally introduced from America and Europe. Only one isolate was similar to the original isolate from Wuhan. [26] A sequencing study done by **Alotaibi et al.** on 18 isolates recovered from Egypt during 2020, has shown similarity with the reference Wuhan-Hu-1 strain. [27] which may justify the spike of 'other variants' at the beginning of our study.

In Egypt, most of the cases during the first wave belonged to lineage B (mainly B.1). [28] During the second wave, the B.1.1.1 lineages began to dominate over B.1. [29]

Genomic sequencing studies revealed the emergence of lineage C.36 (B.1.1.36) in samples collected in Egypt late in the second wave. [29] As specified by Pangolin, the C36 lineage was first reported in the United States in March 2020. It has been reported in more than 56 countries. However, with reference to the GISAID database, Egypt showed the highest occurrence. [30]

The second and third waves were dominated by C-like lineages (especially C.36). [28]

According to **Agwa et al.** the C.36 lineage represents 34% of all sequenced variants in Egypt. [30] It has predominated during over B.1.1.1 and B.1 lineages, yet with close clinical picture and mortality rates [29]. The peak of C.36 sub lineages was observed in May 2021 [20] which may explain the second peak of 'other variants' in our study during the same period.

Conclusions

To understand the epidemiology of the SARS-CoV-2 and its variants, continuous monitoring and rapid detection of variants are mandatory. While genomic sequencing remains the golden standard, multiplex PCR assays are helpful screening tools in settings with limited resources. AllPlex™ SARS-CoV-2 Master Assay and AllPlex™ SARS-CoV-2 Variant assays provide a rapid and more affordable alternative to genomic

sequencing. They are also highly sensitive to the detection of chosen mutations [31]. However, they miss the detection of other unselected variants such as C.36 or new variant such as B.1.1529 (omicron). Identifying different types of emerging SARS-CoV-2 variants will help clinicians for better diagnosis, therapy guidelines selections with better preventive measures and vaccination.

Conflict of interests

The authors report no conflicts of interest.

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Author contributions

All authors contributed to the study conception and design. They have all participated in material preparation, data collection and analysis. All authors have contributed in drafting and/or revising the previous versions of the manuscript. All authors read and approved the final manuscript.

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