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Research Article

Circulation of Newly Emerged SARS-CoV-2 Variants in Central India

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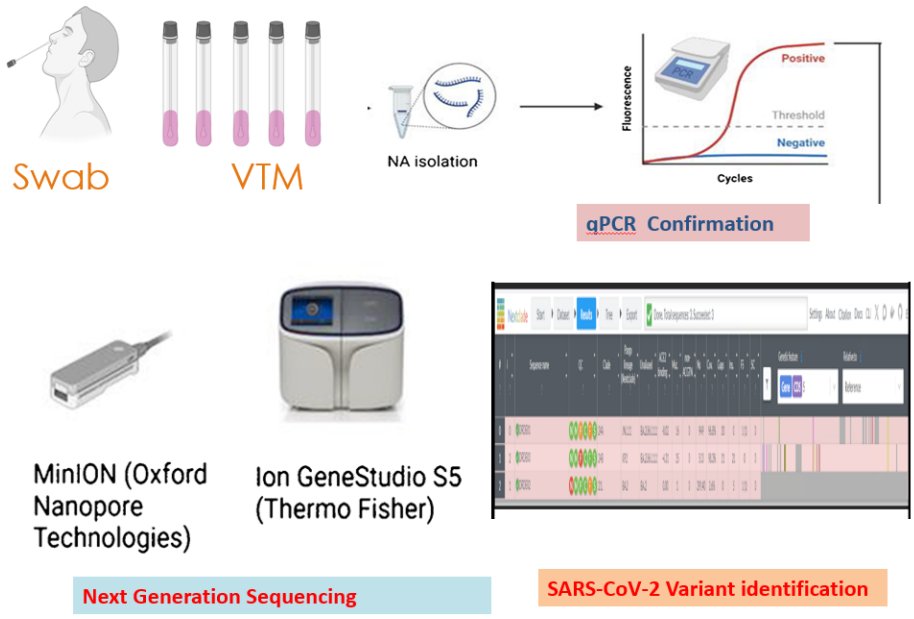
Abstract

Background: Evolution of SARS-CoV-2, etiology of COVID-19 pandemic is characterized by emergence of novel variants at regular interval. Around March 2024, a new variant of SARS-CoV-2 (KP.2) was reported at global scale. This notable shift was also observed during SARS-CoV-2 genome characterization in India. Along with this new variant, two recently emerged variants were also added to JN.1 and KP.2 lineages as a predominant SARS-CoV-2 variant. No other variant apart from these lineages were observed. **Methods:** NGS based whole genome sequencing of SARS-CoV-2 positive viral RNA was carried out. Further, variant analysis was performed using various algorithms employing NGS system softwares. **Results:** Genome analysis revealed unique amino acid substitutions in viral spike protein compared to other SARS-CoV-2 variants and identified the emergence and circulation of a new variant of SARS-CoV-2 from samples investigated in central India. **Conclusions:** This study indicates the necessity for continuous monitoring of circulation of these new variants of SARS-CoV-2 for management of COVID-19.

Graphical Abstract



Keywords: SARS-CoV-2; NGS; Variants; Lineages

Introduction

Since emergence of SARS-CoV-2 in December 2019, a large number of variants were reported across the globe [1]. However, emergence of Delta variant inflicted the most serious threat to public health and economy. This has alarmed for continuous monitoring for newly emerged variants for better preparedness and minimizing such catastrophic losses [2--6]. Thereafter, the other variant Omicron posed a serious threat to people and vaccine efficacy. The viral genetic makeup revealed unique changes in spike protein, which is the most important interacting viral protein with human receptors. The unique genetic patterns of spike protein are attributed to the enhancement in viral transmission patterns and evasion of human immune system [7-10]. Another important Omicron subvariant XBB was studied in depth by researchers due to its higher transmission among large population across the globe, evading the host immune response and failure of reported therapeutics [11].

The future challenges of emerging variants are not easy to tackle until continuous and detailed deciphering patterns of unique behaviours of each strain would be studied in depth to facilitate challenging public health interventions at global pace [12]. The next generation sequencing based continuous monitoring and deciphering unique viral characteristics with evolutionary dynamics can only help us to understand the behaviour of virus and changes required in existing diagnostic and therapeutic interventions. Here, we have reported the laboratory investigation of clinical samples, during the year 2024 and observed emergence of newer SARS-CoV-2 variants in central India.

Materials and Methods

Clinical samples: The clinical samples (Oro-pharyngeal/ Naso-pharyngeal) were referred to Defence Research & Development Establishment (DRDE), Gwalior from sentinel District Hospitals of Madhya Pradesh, through the Indian SARS-CoV-2 genomic Consortium (INSACOG). Four referred positive SARS-CoV-2 clinical sample from Gwalior region of Madhya Pradesh state during the period of January to May 2024 were analyzed in this study.

Extraction of viral RNA and Confirmation of by SARS-CoV-2 specific RT-qPCR

The clinical swab samples were processed for extraction of viral nucleic acid employing viral RNA extraction kit (Qiagen, Germany) following the manufacturer's instructions. Subsequently, the eluted viral RNA was used to perform SARS-CoV-2 gene specific multiplex RT-qPCR (Thermo, USA).

Next GenerationWhole Genome Sequencing (NG-WGS)

Library Preparation and nanopore sequencing

Firstly, SARS-CoV-2 positive RNA were processed for conversion of complimentary DNA (cDNA) employing Luna One-Step RT-qPCR Kit (Sigma Aldrich, USA) followed by DNA amplification PCR using SARS-CoV-2 specific two sets of primer pools. Here the nanopore SARS-CoV-2 protocol with 'midnight' primer sets was carried out in two primer pools A and B respectively for each of amplified SARS-CoV-2 complementary DNA amplified through multiplex PCR reactions. These two pools were processed for multiplex PCR in a single tube with a reaction volume 10 µl (consisting of 1.5µl (100 M) primer pools in respective tubes, 8 µl of positive SARS-CoV-2 template RNA) further generated a tiled and non-overlapping amplicons of 1200 bp. The reaction of multiplex PCR was carried out through a reverse transcription (55 ^{0}C, 30 minutes), denaturation (95 ^{0}C, 1 minute) and a continuous annealing- extension of 34 cycles for each of primer pools A and B (denaturation (95 ^{0}C, 20 sec) and annealing combined extension (60^{0}C, 210 sec) followed by a final extension (65 ^{0}C for 10 minutes). The amplified amplicons were pooled after multiplexing PCR represents the SARS-CoV-2 whole genome, which was processed for nanopore library preparation following the ONT Manufacturer's instructions. We used a Rapid Barcoding Sequencing Kit protocol (ONT, Oxford, UK). Finally, sequencing was carried out in ONT MinION MK1B nanopore sequencer following manufacturers instructions under the MinKNOW software (ONT, UK). The COMMANDER software (Genotypic Technology, India) was used for bioinformatic analysis to generate the output files and interpret the results.

Library Preparation and Ion AmpliSeq sequencing

The SARS-CoV-2 whole genome sequencing was also carried out employing Ion Ampliseq technology of Ion Torrent S5 sequencer (Thermo, USA) following the manufacturer's instructions. The SARS-CoV-2 whole genome Ion Ampliseq research panel was used to generate the 125-275bp amplicons designed against whole genome of SARS-CoV-2 virus. The Ion Ampliseq research insight primer pools contains 247 primer pairs to cover the whole genome of novel SARS-CoV-2. Firstly, the eluted viral RNA were converted to cDNA employing the SuperScript IV cDNA Synthesis Kit (Thermo Fisher Scientific,USA). Whole genome of SARS-CoV-2 was further amplified through PCR followed by ligation of adaptors. The ligated products were then purified using magnetic beads (Beckman Coulter, US) following the manufacturer's instruction of library purification. These libraries were then quantified with the help of Qubit 4.0 Fluorimeter (Thermo Fisher, USA) employing DNA quantitation kits following the manufacturer's instructions. Finally next generation sequencing of these samples

were carried out in Ion Torrent S5 FNA sequencer (Thermo Fisher Scientifc, USA) in an Ion 540 sequencing chip to generate up to 1 million reads of paired-end per samples. The analysis of the results obtained trough Ion Torrent S5 sequencer were processed and analysed in Ion Torrent suite software version 5.2 (Thermo Fisher, USA) following the manufacturers instructions.

Mutation and evolutionary analysis of spike protein

A separate mutation and evolutionary pattern analysis of next generation sequencing amplified whole genome consensus fasta files were carried out using online software tools like Nextclade version 3.31 and Pangolin version 4.3.1 (https://clades.nextstrain.org). Here a comparative variation obtained against the different genes of SARS-CoV-2 were comparatively evaluated. We also did the divergence analysis manually employing MEGA 5.0 software. Further, we conducted a comparative mutation and phylogenetic analyses using open source Pangolin, Nextclade, and the Ion Reporter (Thermo Fisher, USA) softwares. The unique mutations were compared with the prototype Wuhan Hu-1 and other important SARS-CoV-2 variants. The phylogenetic tree was constructed for circulating SARS-CoV-2 to see viral evolutionary patterns with these unique variants circulating in India in 2024 employing the NextClade online software v.3.2.0. (https://clades.nextstrain.org/).

Results

Clinical samples and SARS-CoV-2 RT- qPCR

The ongoing SARS-CoV-2 genomic surveillance activities at DRDE Gwalior under INSACOG network received four SARS-CoV-2 positive clinical samples for sequence-based confirmation and whole genome molecular characterization during the period January to May 2024. The age profile of patients ranged above 50 years and with almost equal proportion of male and female. These samples were confirmed by SARS-CoV-2 specific qPCR with a Ct value ranging from 14 to 35 (Table 1).

Table 1: Details of SARS-CoV-2 positive samples

Sample ID	Patient Gender	Patient Age	District Name	State Name	Collection Date	Ct value (O/N/S)
DRDE/01	Male	58	Gwalior	Madhya Pradesh	01/01/2024	14/14.5/15
DRDE/02	Female	78	Gwalior	Madhya Pradesh	13/02/2024	14.5/14/14.6
DRDE/03	Male	75	Gwalior	Madhya Pradesh	19/03/2024	31/31.6/32
DRDE/04	Female	20	Gwalior	Madhya Pradesh	13/02/2024	35/36/35

NGS based genome characterization and Mutational profiling of spike protein

We deciphered whole genome of SARS-CoV-2 (n=3) newly circulating variants in this particular part of India. The detailed genome coverage, generated SARS-CoV-2 genome reads were analyzed by variant calling on two different NGS sequencing platforms. Further, we assessed quality mapping scores, to find out the unaligned reads across the reference prototype novel 2019 SARS-CoV-2 genome. The genomic sequences deciphered by both Next Generation Sequencer revealed a high accuracy of approximately 99.99%. The details of genome wide coverage of each samples was listed in Tables 2. Here the samples with Ct values below 31 were successfully sequenced. However, we were unable to sequence the fourth sample of very high Ct value of 35. The amino acid substitutions of each isolate compared to prototype strain was depicted in Table 3.

Table 2: Genome analysis of SARS-CoV-2 sequenced in this study

Sample ID	High Quality Reads	Mapped Reads	Genome Coverage	Mean Depth	Mean Base Quality	Mean Map Quality	A%	T%	G%	C%	N%
DRDE/01	2913	2913	96.8	43.7	16.8	55.7	29.1	18.93	31.21	17.59	3.18
DRDE/02	4857116	2770	37.7654	7.79701	26.4	37.8	0.68	0.54	0.85	0.48	97.45
DRDE/03	357826	347549	99.5184	2253.06	24.9	37.9	29.36	19.29	31.63	17.93	1.79

Table 3: Amino acid substitutions in new SARS-CoV-2 variants.

Sample ID	Spike protein mutation	Amino Acid Substitutions
DRDE/01	(C44T,T3565C,A6183G,C7113G,G11727A,C11747T,C12815T,G17334T,C18894T,T22795G,T22926C,C22928T,G24872T,T27810T,G29871T	S:A27S S:L212I S:L455S S:F456L S:V1104I ORF1a:K1973R ORF1a:T2283I ORF1a:R3821K ORF7b:F19L
DRDE/02	(C44T,A897C, T2790C,C4965T,G4985C,A4989T, C4990A,A4992T,A4994C,T7359A,T8293C,A8393G,G9424A,A10447G,A10449C,T11042G,G11727A,T12789C,T12880C,C13339T,C13536T,A14856G,G15451A,A15582C,T15939C,T17410C,G17523T,G18163A, G18492A,A19326G, T19733,T1995C,G20055A,G20398A,T21618C,T21622C,C21624G,T21711C,A21987G,G22200T,T22208C,A22295C,G22317T, A22353C,G22556A,C22577G,A22578G,C22679T,T22682C, T22683C,T22684A,T22685C, T22686C, G22688A, T22813G, G22882T, C22895G, A22896T, A22898G, G22910A, T22916C, G22917A, A22942T, A22992G	S:I19T,S:T21R, S:L50S, S:D142G, S:G213V, S:F216L, S:N245H, S:G252V,S:D264A, S:V332I, S:H339G, S:P373S, S:F374P, S:F375P, S:A376T, S:N417K, S:K440N,S:H445V, S:S446G, S:D450N,S:W452Q, S:K460N, S:N477S, S:K478T, S:K481N, S:K484Q, S:P486F, S:K554E, S:V570A, S:S621P, S :Y655H, S:Y796D,S:F939S, S:K969N, S:Q1071H, S:L1143P, S:N1192I, N:K229Q, M:E19Q, M:A30T, M:T63A, M:I82S, M:V104A, M:P165L, E:I9T, ORF1a:D211A, ORF1a:I842T, ORF1a:T1567I, ORF1a:V1574L, ORF1a:D1575V, ORF1a:N1576I, ORF1a:I1577L, ORF1a:M2365K, ORF1a:T2710A, ORF1a:H3395P, ORF1a:F3593V, ORF1a:R3821K, ORF1b:G662S, ORF1b:K705N, ORF1b:C1315R, ORF1b: M1352I, ORF1b:V1566I, ORF1b: V2089A, ORF1b: I2163T
DRDE/03	A1236C, T1237C, A3603T, T3605A, G3606T, T3607G, T4689A, T4691A, C4692G, T4693A, A6613G, A8393G, G10380T, A10657C, T14408C, T15882G, T17857G, A17858T, A17861C, C17862A, T17863C, T20502G, A20503C,T20505C, A21987G, A22353C, C22679T, T22682C, T22683C, T22684A, T22685C, T22686C, G22688A, T26060C, G26610A, T26858C, A27636C, G27637A, G29527C	S:D142G, S:D264A, S:P373S, S:F374P, S:F375P, S:A376T, N:Q418H, M:A30T, ORF1a:D324A, ORF1a:H1113L, ORF1a:C1114M, ORF1a:V1475D, ORF1a:S1476R, ORF1a:T2710A,ORF1a:G3372V, ORF1b: L314P, ORF1b:Y1464V, ORF1b:D1465A, ORF1b:Y1466H, ORF1b:I2346L, ORF3a:I223T, ORF7a: V82I

Figure 1: Next Clade analysis.

Molecular and evolutionary analysis of circulating lineages

Here we have conducted a performance assessment through variant calling analysis in both next generation sequencer as compared to the reference strain of novel 2019 Wuhan Hu1 (MN908947.3) SARS-CoV-2. We have also studied the molecular signatures of circulating viral strains and the phylogenetic analysis revealed the important amino acid shifts across the genome as an etiology JN.1 and KP.2 variants of SARS-CoV-2. Apart from this, the detailed molecular analysis deciphered the spike protein with unique genome signatures over the circulating JN.1 variants identified the samples as JN.1.11 (n=2), KP.2 (n=1) and BA.2 (n=1) (Table 3).

Discussion

In 2021 a descendant JN.1 (BA.2.86.1.1) was identified with association to increased number of COVID cases. Similarly, another offspring KP.2 (JN.1.11.1.2) was also recorded for rise in COVID cases in 2021 [13]. Around 17 August 2023, BA.2.86 was initially categorized as variant under monitoring and in quick succession it was reclassified as variant of interest (VOI). In India, KP.2 was first reported from Odisha in 2nd January 2024. On 3rd May 2024, it was declared as variant under monitoring [14-15]. In this study we reported circulation of newly emerged SARS-CoV-2 variants of Omicron as JN.1.11 and KP.2 with a unique genomic signature circulating particularly in this part of central India. Few of these genomic signatures deciphered in JN.1 and KP.2 variants have not been reported till date. The validation of our sequencing results was found in concordant to an independent analysis of generated consensus fasta files of all the three variants of SARS-CoV-2 (JN.1.11, KP.2 and BA.2) by National Genomic Surveillance activity through INSACOG at the IBDC analysis. However, the unique mutations reported in the JN.1.11 and KP.2 strain particularly circulating in this region is of interest and need to be monitored to track the viral evolutionary patterns among SARS-CoV-2 variants.

In April, 2024 the World Health Organization (WHO) recommended the use of monovalent JN.1 lineage antigen for better neutralizing antibody responses against circulating strains. This led to manufacturing of monovalent JN.1 or KP.2 COVID-19 vaccine formulations by several industries and subsequent approval by regulatory authorities. This study clearly indicates the necessity of continuous monitoring to track rapidly emerging variants of SARS-CoV-2, which is crucial to manage the diagnostic as well the prophylactic interventions.

Conflict of Interest

None of the authors has a conflict of interest.

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Ethical Approval Statement

This study was performed after seeking approval by DRDE-Institutional Biosafety Committee vide the no. IBSC/VIRO-02/2020/PKD. The Experimental protocols were executed under the study according to all proper biosafety and the regulatory guidelines. The study Ethical approvals was done via Vidhya Ethics Clearance vide the no.VCH/VEC/Feb-2023/01.

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