Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

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Sequence mismatch in PCR probes may mask the COVID-19 detection in Nepal

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Dear Editor,

The end of 2019 marked the beginning of the COVID-19 (Coronavirus Disease 2019) pandemic [1]. COVID-19 is a respiratory disease caused by a novel coronavirus that has been named SARS-CoV-2 (Severe Acute Respiratory Syndrome Coronavirus 2). The World Health Organization (WHO) reported more than three million confirmed infections with over two hundred thousand deaths worldwide by the end of April 2020 [2]. At the same time, WHO Nepal reported just over 50 cases (with no deaths in Nepal), albeit more than 10 000 RT-PCR (Reverse Transcription Polymerase Chain Reaction) tests had been performed [3]. The WHO has recommended nucleic acid amplification-based assaying for the diagnosis of SARS-CoV-2 [4]. Although the National Public Health Laboratory (NPHL), the authoritative SARS-CoV-2 diagnostic lab in Nepal, has not disclosed which kit is made use of, their official website listed the use of WHO-recommended RdRp, N and E genes for PCR detection [5]. The protocols were optimized by Corman et al. (2020) at the Charité, Berlin, Germany [6] and recommended by the WHO [7]. The NPHL has also listed the N1, N2 and N3 regions of the nucleocapsid N gene for testing, which has been optimized by the US Centre for Disease Control and Prevention (US-CDC) and recommended by the WHO [7]. More recently, NPHL has been using PCR kits provided by the Nepal Government, and the target genes vary according to the kits (personal communication, see acknowledgement). The Global Initiative on Sharing All Influenza Data (GISAID) database [8] has provided data on variations in the WHO-recommended primer binding regions for the SARS-CoV-2 genomes [9] with the so-called Charité primers situated in the most conserved regions and the China-CDC primers in variable sites.

Nepal has a very large working migrant population in the Middle-East, India, Malaysia and South Korea among others [10]. Nepal shares a very generous open border with India, and the enormous migrational flux remains unrecorded as there is no requirement for passports, VISAs or any official documentation [10]. Most of the COVID-19 cases reported in Nepal have been traced back to Nepalese migrating from India during the pandemic (Supplementary Table 1). We analysed primer and probe binding sites in the genomes of SARS-CoV-2 deposited in the GISAID originating from Indian labs. Genomic sequence data for all Indian SARS-CoV-2 samples collected on or before 29 March 2020 were recovered from the GISAID database on 7 May 2020. Nepal launched a nationwide lockdown on 23 March 2020 and prohibited any type of land and air travel to and from the country, but few people crossing borders to Nepal were reported despite of the travel restrictions [11]. Thus, we chose a date one week after the lockdown for analysis. Ninety-four viral nucleotide sequences were compared with the Wuhan-Hu-1 strain (EPI_ISL_402125). One sequence was short (EPI_ISL_414515) and excluded from analysis. The sequences were submitted for Clustal-Omega multiple sequence alignment in the online webserver of European Molecular Biology Laboratory, European Biotechnology Institute (EMBL-EBI) [12]. Thirty-eight (41%, 38/93) viral genomes had mutations, one variation in each genome (Table 1). One genome had a single point mutation (A to G) at the probe binding region, for probes P1 (9.th

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Table 1
Positions of variations in primer and probe binding positions in different viral genomes.

Mutated positions	Viral Genomes with Mutations
Charité RdRp Probe P1: CCAGGTGGWACRTCATCMGGTGATGC Probe P2:. CAGGTGGAACCTCATCAGGAGATGC Mutation: CCAGGTGGGACCTCATCAGGAGATGC	EPI_ISL_431101
US-CDC N1 Probe P: ACCCCGCATTACGTTTGGTGGACC Mutation: ACTCCGCATTACGTTTGGTGGACC	EPI_ISL_435089, EPI_ISL_435083, EPI_ISL_435084, EPI_ISL_435074, EPI_ISL_435090, EPI_ISL_435086, EPI_ISL_435098, EPI_ISL_435085, EPI_ISL_435092, EPI_ISL_431103, EPI_ISL_435112, EPI_ISL_435081, EPI_ISL_435095, EPI_ISL_435099, EPI_ISL_435100, EPI_ISL_435093, EPI_ISL_435091, EPI_ISL_435097, EPI_ISL_435094, EPI_ISL_435096, EPI_ISL_435082, EPI_ISL_435088
US-CDC N3 Primer F: GGGAGCCTTGAATACACCAAAA Mutation: GGGAGCCCTGAATACACCAAAA	EPI_ISL_421668, EPI_ISL_421663, EPI_ISL_421662, EPI_ISL_421665, EPI_ISL_421666, EPI_ISL_421664, EPI_ISL_421671, EPI_ISL_421669, EPI_ISL_421672, EPI_ISL_424363, EPI_ISL_435104, EPI_ISL_421670, EPI_ISL_435101, EPI_ISL_421667, EPI_ISL_424361,

nucleotide) and P2 (8.th nucleotide), both binding in the same region of the RdRp gene. This position was “W" (A or T) for probe P1 designed to detect both SARS-CoV and SARS-CoV-2. The viral genome was a sample collected in the first week of March in Hyderabad, India. A second point mutation (C to T) was seen in 22 viral genomes at the third position in the probe binding site of N1 region (N gene) PCR assay. Most of the samples had been collected on March 28th or 29th, one of the samples was collected from a Nepalese in India. A third variation (T to C) was found in 15 genomes in the 8th position of forward primer binding site of N3 region (N gene) PCR assay. Thirteen out of fifteen of these samples were collected from Indian nationals in Iran.

While the NPHL has not officially reported of any change in PCR assay strategies, they have been using different PCR kits provided to them by the Nepal government which may vary in target genes, and thus mutations reported in this study may not be relevant. In this study, 23 viral genomes showed differences in the probe binding regions, and 15 viral genomes had mutations in the primer binding regions. Variations in the primer binding regions may not significantly affect the efficiency of PCR amplification as probe-free SYBR green based amplification could successfully amplify disease-confirmed PCR negative samples [13]. Mutations in 5th (T to G) and 20th (C/T to A) positions in forward primer for pandemic influenza A/H1N1/2009 did not impact the PCR efficiency [14]. While mutations at the 5′ end of primers may not impact PCR results, variations in the 3′ sequences of the primers may hamper PCR efficiency, as a stable double-helix is required by the Taq polymerase at the 3′ end [15]. In the same study [14], mutation in 3.rd position (G to T) of probe affected the PCR efficiency, but mutation in the 16.th position (G to A) did not. Another study [16] revealed mutations in the 14th or 17th position of probes designed for detection of respiratory syncytial virus group B caused immunologically positive clinical samples to be PCR negative. In this study, the mutations in Charité RdRp probes P1 and P2 binding regions are in positions 9 and 8, and mutations in the US-CDC N1 probe binding region is in the 3rd position. Further experimental validation is required to assess the impact of these mutations, since changes in different bases at identical positions may impact the efficiency of PCR amplification in a differential manner [13].

Acknowledgement: We sincerely want to avoid any misinterpretations that may arise based on the content of this letter with respect to the Indian origin of COVID-19 patients in Nepal. Cross border travel between India and Nepal has been going on for millennia and is proof of close-knit relationships between these two nations. We would like to thank Dr. Shravan Kumar Mishra, NPHL (Nepal), for describing the more recent use of COVID-19 diagnostic kits in NPHL. We would like to thank Dr. Anil Giri (Finland) and Dr. Reid Oldenburg (USA) for reading and commenting on this letter. Our sincere gratitude goes to all the scientists and submitting laboratories (Supplementary Acknowledgement Table 2) involved in collection, processing and deposition of SARS-CoV-2 sequences as well as the meta-data in GISAID without which this work could not have been done.

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CRediT authorship contribution statement

Divya RSJB Rana: Conceptualization, Data curation, Formal analysis, Writing - original draft, Writing - review & editing. **Nischal Pokhrel:** Data curation, Writing - original draft, Writing - review & editing.

Declaration of competing interest

None.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.mcp.2020.101599.

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