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SARS-CoV-2 N-gene mutation leading to Xpert Xpress SARS-CoV-2 assay instability

To the Editor,
Real-time reverse transcription polymerase chain reaction (RT-PCR) assays have formed the backbone of COVID-19 diagnosis since the start of the SARS-CoV-2 pandemic. Assay performances have remained relatively stable over the course of the pandemic to date, but any genomic variants in the primer binding or probe regions of assays have the potential to compromise performance. One notable example is the spike protein H69/70del mutation, associated with the emergence of the alpha variant (B.1.1.7).[1] H69/70del results in false negative S-gene results in the Applied Biosystems TaqPath COVID-19 assay (ThermoFisher Scientific, USA), but the two other gene targets of the assay are not affected. This altered assay performance became a proxy for detection of the alpha variant.[1] Surveillance for mutations causing diagnostic assay failures for specific gene targets is crucial, especially if only one gene target is used in a particular assay.

There have been relatively few COVID-19 cases in Australia relative to global case numbers, but there is currently (1) a sustained outbreak of COVID-19 infections stemming from community transmission of the delta variant of concern (VOC) in June (Winter) 2021, and (2) rising case numbers of the omicron VOC since early December 2021. The Xpert Xpress SARS-CoV-2 assay ('GX'; Cepheid, USA) is one of several diagnostic assays currently used for detection of SARS-CoV-2 in Australia and globally. The GX assay has two targets within the envelope (E) and nucleocapsid (N) genes of SARS-CoV-2. If a sample is positive, the expected behaviour is for both gene targets to be detected with similar cycle threshold (Ct) values.[1] A change in the performance of the GX assay was recently observed across multiple assay cartridges from different cartridge lots, with large differences in cycle threshold (Ct) values between the E and N gene targets in a subset of delta SARS-CoV-2 samples. Samples were classified as possessing an altered GX profile (GX^{alt}) if there was a >3 Ct value difference between the E and N gene targets of the assay. A single category change to presumptive positive was adopted when a sample was E-gene positive (Ct value >30) but was N-gene negative.

The altered GX profile was first observed in August of 2021 in 9.5% (10/105) of cases, increasing to 20% (18/88) and 15% (3/20) of cases in September and October, respectively. Retrospective analysis of all positive cases from July 2021 confirmed that 0% (0/110) of cases exhibited the altered GX profile (GX^{alt}). Although the altered GX profile was defined as any sample with a greater than three Ct value difference between E-gene and N-gene targets, the differential was often far greater (median 10 Ct, IQR 9−11 Ct). Throughout the period of testing, no false negatives were observed.

Whole genome sequencing of isolates was undertaken as part of routine genomic sequencing, as previously described.[2] A candidate causative SNP for the GX^{alt} profile was identified at genomic position 29179 of the SARS-CoV-2 genome (G29179T) through comparative analyses. This SNP was not observed in any non-GX^{alt} samples.

Most commercial assays use closed-source, proprietary primer sequences. However, definitively linking SNPs to altered assay performance requires knowing the primer sequences, or precise target locations on the reference genome. Accordingly, we cloned the GX assay primers into a pcDNA3.1V5 His plasmid backbone (#K480001; ThermoFisher Scientific Australia), then sequenced purified clone products using Sanger sequencing. Thus, we confirmed that the G29179T point mutation is located 5 nt upstream from the 3′ end of the N-gene target region of the GX assay (Fig. 1). In correspondence, Cepheid agreed that 'the data appear to support this (altered assay performance)' (personal communication).

At the time of carrying out this study, the G29179T SNP had not yet been linked to assay instability. However, during the editorial process, three studies from Western Australia, South Korea, and the United States of America have found that the G29179T SNP leads to N-gene target detection failure in the GX assay,[3−5] strongly and independently
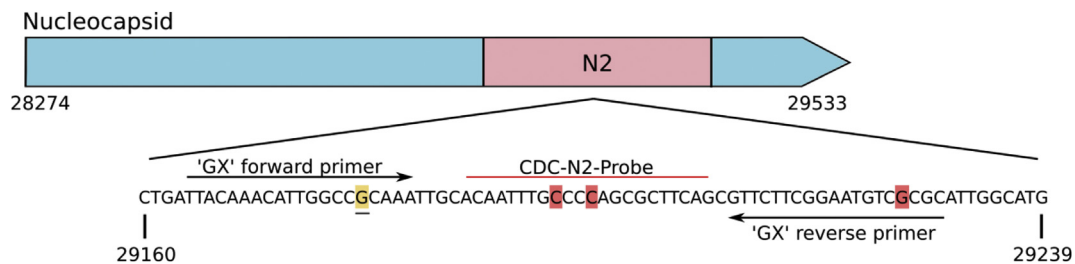
Fig. 1 Centers for Disease Control and Prevention (CDC) primer and probe targets of the nucleocapsid gene of SARS-CoV-2 and current identified mutations within these regions. The N2 target region for diagnostic assays of the nucleoprotein gene is depicted in pink, with genome positions numbered according to the reference genome (Wuhan-HU-1; NC_045512.2). The primers are shown for the Xpert Xpress SARS-CoV-2 assay (GX; Cepheid USA), as determined through molecular cloning. The GX primer targets are very similar to the CDC published PCR primer set,[6,10] so we show the CDC probe location. The location of the synonymous mutation on which the present study focuses (29179: G→T) is highlighted by the yellow box and underlined, five nucleotides upstream of the 3' end of the GX forward primer. Previously described nucleocapsid mutation locations are shown by the red boxes, within the probe (29197: C→T; 29200: C→T/A) and reverse primer (29227: G→T) regions, and have resulted in GX assay failure and instability, respectively.[3–5,7–9]

supporting our findings in the present manuscript. Instability of the GX assay caused by different point mutations has also been observed previously. For example, previously described mutations have resulted in GX N-target failure, with explanation centred on the primers/probes closely resembling the Centers for Disease Control and Prevention (CDC) primer and probe set.[6–10]

Given the impact of G29179T on the GX assay, and the assay's global popularity for diagnostics, we queried the global phylogeny of SARS-CoV-2 (https://hgdownload.soe.ucsc.edu/goldenPath/wuhCor1/UShER_SARS-CoV-2/, 20 December 2021) to determine the global spread and abundance of G29179T. By querying the phylogeny using matUtils,[11] we determined that 12,481 of 3,152,290 (0.4%) sequences in the global alignment from 27 countries (earliest occurrence 15 March 2020) contained the G29179T mutation. Given that the phylogeny is not exhaustive with respect to global sequencing efforts (for example by not including any sequences that are only available on GISAID; https://www.gisaid.org/), the true number of samples with G29179T is likely higher. Samples with G29179T were not monophyletic, emerging independently in multiple lineages, suggesting convergent acquisition of G29179T. The G29179T mutation is most prevalent in delta genomes, and no omicron samples have yet acquired the G29179T mutation. However, the independent acquisition of G29179T in a wide range of lineages suggests that omicron samples may also be affected over time.

Our analysis cannot suggest whether the repeated evolution of G29179T is adaptive or neutral, but, regardless, the mutation is clearly important for molecular diagnostics. Given the global distribution of G29179T, and independent confirmation of its negative impact on the GX assay in three different countries,[3–5] the global performance of the GX assay and other assays that target the N-gene [e.g., Mira-Mic SARS-CoV-2 (Bio Molecular Systems, Australia) and BD Max COVID-19 (BD Life Sciences, USA)] is likely to be affected.[12] Consequently, obtaining a positive result is reliant on the stability of the alternative target(s) of these assays, with pooling of samples potentially leading to incremental performance instability. Clinicians should be aware of this emerging problem in diagnosing delta SARS-CoV-2 and that ongoing assay performance monitoring is critical in the setting of rapid viral evolution.

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SARS-CoV-2 IgM testing for travellers: a private pathology perspective from New South Wales and the Australian Capital Territory, Australia

To the Editor,
We read with interest the letter by Hasan *et al.*[1] outlining the pitfalls of relying on SARS-CoV-2 IgM in a risk stratification matrix required prior to travel by some overseas countries (Table 1).[2,3]

The diagnosis of acute COVID-19 relies on SARS-CoV-2 nucleic acid amplification testing (NAAT).[4] However, since 8 November 2020, the Chinese Embassy has required both SARS-CoV-2 NAAT and SARS-CoV-2 IgM serology ('dual test') be performed within 48 hours of travel from Australia to China. We agree with Hasan and colleagues that IgM detection prior to travel has currently a low sensitivity of detecting cases that are polymerase chain reaction (PCR) negative yet potentially infectious. Conversely, IgM is frequently positive beyond the infectious period and the requirement for a negative 'dual test' prior to travel is unnecessary.

Our laboratories currently use two assays that have the ability to detect IgM: the Roche Elecsys Anti-SARS-CoV-2 assay that detects nucleocapsid IgM, IgA and IgG antibody (Roche Total; Roche, USA), and the Abbott Architect SARS-CoV-2 IgM assay that detects spike antibody (Abbott IgM; Abbott, USA). These two commercial in-laboratory tests (as opposed to rapid lateral flow tests) in our in-house validation studies were found to have similar high specificities (99.5% and 98.9%, respectively) to those stated by the manufacturer (99.8% and 99.56%). Sensitivities were found to be lower (67% for both Roche Total and Abbott IgM for samples that were 8−14 days post onset of symptoms in confirmed COVID cases) than those stated by the manufacturer (85%

and 86%). This may relate to the greater proportion of mildly symptomatic patients that were included in our analysis when compared to that of the manufacturer and has been described previously.[5,6] The positive and negative concordance of the IgM also correlated reasonably well to immunofluorescence assay (IFA) IgM (78%) and Euroimmun IgA (68%) assays. While the Abbott IgM assay was positive in three cases where the IFA was negative, these were deemed to be true positives based on timing of confirmed infection. Therefore, both the Abbott IgM and Roche Total assay were found to have a low risk of false positive IgM results.

We undertook a retrospective audit of all serology performed in our two laboratories between 9 July 2020 and 19 September 2021 with a particular focus on those requiring evaluation prior to travel.

A total of 5831 samples had COVID serology performed through our laboratories with 545 (9%) specifically for pre-travel testing. Sufficient history was provided in 504/545 (92%) to determine the country of destination. Of these, 224/504 (44%) were travelling to China. The next largest groups were flying to Samoa (*n*=59, 12%), USA (*n*=40, 8%), UK (*n*=26, 5%) and India (*n*=11, 2%).

The Abbott IgM assay was performed on 444 samples, while the Roche Total was performed on 152 samples; 101 of these samples were tested only with the Roche Total assay while the remaining 51 samples had Roche Total performed in addition to the Abbott IgM assay.

There were 45/444 (10%) positive Abbott IgM samples (with 44/44 returning a negative SARS-CoV-2 NAAT result from simultaneously collected nose and throat swabs), and there were 4/152 (3%) positive Roche Total samples, with 3/4 positive Roche samples also positive on the Abbott IgM assay, consistent with recent past infection with or without recent vaccination.

Of the 224 travellers to China, 15/190 (8%) were found to have positive Abbott IgM results with one of these also positive on the Roche assay and having confirmed infection overseas 6 months prior. This patient was eventually cleared for travel when a negative IgM result by IFA was obtained. Of the remaining 14, 10 were cleared to travel following reporting of the subsequent reflex Roche Total result which returned negative results for all of these samples. The remaining four that did not undergo reflex testing were reported as positive with further discussion with the Chinese Embassy allowing clearance due to negative PCR and recent vaccination in the previous 6 weeks considered the likely cause of the positive Abbott IgM result. Retrospective reflex testing found these four samples to also be negative on the Roche assay excluding recent infection. The remaining 34 travellers who were tested by the Roche Total alone were all negative. Since review of this dataset, an additional asymptomatic traveller to China was found to be positive by both Abbott IgM and Roche Total during the Delta outbreak in Sydney. He had not been vaccinated and, while PCR was negative, he was judged to have had recent infection. Travel was deferred and he was required to undergo repeat PCR testing (result on day 2 and day 22 negative), chest imaging (result normal) and repeat serology (3 weeks later: rising IgM and IgG levels) and travel was further delayed until his 'dual test' was negative.[7]

Of the 59 travellers to Samoa, 2/42 (5%) and 0/19 were positive on the Abbott IgM and Roche Total assays, respectively, with all reported as negative (following reflex

Table 1 Criteria for boarding[2,3]

Destination country	PCR	Serology	Further testing may be required
China	Negative PCR	IgM −	IgM +
Samoa	Negative PCR	IgM −	IgM +/IgG −
		IgG +	IgM +
		IgM −/IgG −	IgG −
		IgM +/IgG +	
		IgM −/IgG +	