Reto Lienhard[1,*], César M. J. A. Metzger[2], Jonas Sieber[2], Michael Bel[3], Lorenz Risch[4], Gilbert Greub[5], Laurent Kaiser[6,7], Adrian Egli[8,9]

What does the UK variant tell the clinical microbiologists?

Viruses mutate frequently during multiplication cycles in host cells. In conjunction with evolutionary selection, this high mutation rate allows for rapid adaptation. Three important selection processes are noted: (i) host adaptation, e.g. following a species jump when successful entry into cells of a new host equates with persistence of the virus, (ii) immune evasion due to selective pressure through the host's immune response, e.g. via neutralising antibodies, and (iii) diagnostic selective pressures from specific diagnostic gene targets. Due to cumulative or crucial mutations, new genotypic and potential phenotypic variants arise unpredictably in different countries around the globe[1-3].

While most mutations are deleterious, and the new variants will not successfully establish, some will maintain in the viral population[4]. Few may even have an evolutionary advantage and spread faster within the population. The accumulation of mutations may occur in several positions along the viral genome. When these occur within genomic regions targeted by PCR analytes, mutations may lead to partial or complete inhibition of the amplification of the target. In order to ensure an effective diagnostic strategy, the PCR targets used should be regularly monitored in order to avoid diagnostic gaps.

On 14 December, 2020 authorities in the United Kingdom announced the emergence of a variant of concern (VOC; Pango lineage B.1.1.7 or also known as N501Y.V1), suggesting an increased transmissibility[5,6]. This particular lineage showed 17 lineage defining mutations through the SARS-CoV-2 genome, eight of which are located in the Spike glycoprotein gene (S gene), an important immune and diagnostic target[7]. Questions arose about a possible change in pathogenicity, diagnostic failures or vaccine escape. In the past, modifications of the spike gene have already been linked to some performance problems of S gene-based PCR due to an S gene dropout in reactivity of the assay. Another VOC that emerged independently in South Africa (Pango lineage B.1.351 or also known as N501Y.V2) and in Brazil (P.1 or also known as N501Y.V3) also warrants attention[8]. These particular variants also have mutations in the S gene, however, a specific mutation (del 69-70) present in B.1.1.7 is not shared and the variant does not show an S gene dropout.

In Switzerland, during the review of diagnostic results for quality surveillance in the weeks preceding Christmas 2020, one laboratory detected an unexpected increase of S gene dropouts. This discovery led to the suspicion of the presence of the B.1.1.7 lineage in Swiss samples and prompted the laboratory, authorities and university laboratories to sequence S gene dropout samples to verify the hypothesis, that this VOC could be detected in samples from Switzerland. With the presence of the B.1.1.7 lineage confirmed in Swiss samples (in the beginning mostly in samples from tourists or in samples from recent returnees from the UK), the last weeks of December 2020 and in early 2021 the Thermo Fisher Taq Path assay was used to increase the pretest probability in a screening attempt searching for additional cases of B.1.1.7 isolates. Identified samples with the S gene dropout were then sequenced via amplicon-based either Sanger sequencing or whole genome sequencing. The emergence of the B.1.1.7 variant showed the high importance of the surveillance of diagnostic assays to discover new variants and of the screening for and early detection of diagnostic evasion events. Thereby avoiding potential underdetection of positive samples and the severe consequences thereof both for the diagnostic and the efforts to contain the spread of an infectious disease such as COVID-19.

The CCCM-SSM group together with the Federal Office for Public Health (FOPH), the National Reference Centre for Emerging Viruses and Spiez Laboratory (a branch of the Federal Office for Civil Protection [FOCP]) would like to discuss here important aspects revealed by this recent concern about the emergence of SARS-CoV-2 variants and their potential role in diagnostics especially against the backdrop of an epidemic situation such as the COVID-19 crisis. We will therefore illustrate and discuss three important points for diagnostics.

Selection pressure on diagnostic targets

The high potential for mutations in viruses should remind us, that a diagnostic tool set up on the detection of a single sequence on a sole gene is at high risk for diagnostic evasion mutations and thereby of misdiagnosis. In 2006 we experienced a similar situation with two common commercial PCR assays for *Chlamydia trachomatis*, which missed a newly established Swedish variant (nvCT) due to a large 400 bp deletion encompassing the targeted DNA region [9]. Since then, diagnostic escape of *C. trachomatis* has been documented in different countries and diagnostic assays [10-12]. The fact that diagnostic escape may occur several times in a bacterial genome underlines that, with mass screening, a strong selection pressure is applied that may lead to mutations to spread even

1 ADMed Microbiologie, La Chaux-de-Fonds, Switzerland
2 Federal Office for Civil Protection, Spiez Laboratory, Spiez, Switzerland
3 Federal Office of Public Health, Bern, Switzerland
4 Labormedizinisches Zentrum Dr. Risch AG, Bern-Liebefeld, Switzerland
5 Institute for Medical Microbiology, University Hospital Lausanne, Lausanne, Switzerland
6 Laboratory of Virology, University Hospital Geneva, Geneva, Switzerland
7 National Reference Center for Emerging Viruses, University Hospital Geneva, Geneva, Switzerland
8 Clinical Bacteriology and Mycology, University Hospital Basel, Switzerland
9 Applied Microbiology Research, Department Biomedicine, University of Basel, Switzerland

Supplier in Switzerland	Test kit	Targets						Reported impact of VOC on signal detection of the S gene target. B.1.1.7 aka N501Y.V1
		ORF1ab	RdRp	S	E	M	N	
Abbott	Abbott RealTime SARS-CoV-2 (#09N77-095)	0	1	0	0	0	1	No S gene target
Abbott	ALINITY m SARS-COV-2 ASSAY (#09N78-095)	0	1	0	0	0	1	No S gene target
Altona	Altona «RealStar® SARS-CoV-2 RT-PCR» (#821005)	0	0	1	1	0	0	No impact
Axon Lab / Cepheid	Gene Xpert® Xpress SARS-CoV-2 (#12039255)	0	0	0	1	0	1	No S gene target
Axon Lab	BioGX SARS-CoV-2 kit (#444213)	0	0	0	0	0	2	No S gene target
Axon Lab	EASY® SARS-CoV-2 Kit (#RT020)	0	1	0	0	0	1	No S gene target
Axon Lab	Hi-PCR® Coronavirus (SARS-CoV-2) Probe PCR Kit (#MBPCR242)	0	1	0	0	0	0	No S gene target
Axon Lab	«NaGene Multiple Real-Time PCR kit for Detection of 2019-nCoV»	1	0	0	0	0	1	No S gene target
Axon Lab	Progenie Coronavirus 2019-nCoV Assay (#CORO-UX 5.2)	0	0	0	0	0	1	No S gene target
Becton Dickinson	ViaSure SARS-CoV-2 (N1+N2) Real Time PCR (#444215)	0	0	0	0	0	2	No S gene target
Becton Dickinson	ViaSure SARS-CoV-2 S gene Real Time PCR (#444212)	0	0	1	0	0	0	Detection compromised*
Becton Dickinson	ViaSure SARS-CoV-2, Flu (A+B) & RSV Real Time PCR Detection Kit (#444217)	0	0	0	0	0	2	No S gene target
bioMérieux	BIOFIRE® COVID-19 test (#423744)	1	0	0	0	0	0	No S gene target
bioMérieux	BIOFIRE® FILMARRAY® Respiratory Panel 2.1+ (RP2.1+) (#423740)	0	0	1	0	1	0	No impact
bioMérieux	SARS-COV-2 R-GENE® (#423720)	0	1	0	1	0	1	No S gene target
Bühlmann	Seegene Allplex™ 2019-nCov (#RP10243X) (older Kit)	0	1	0	1	0	1	No S gene target
Bühlmann	Seegene Allplex™ SARS-CoV-2 (#RV10248X) (newer kit)	0	1	1	1	0	1	2x 1-mer mismatch**
Bühlmann	Seegene Allplex™ SARS-CoV-2/Flu A/Flu B/RSV Assay (#RV10259X)	0	1	1	0	0	1	1x 1-mer mismatch**
DiaSorin	Simplexa™ COVID-19 Direct (#MOL4150)	1	0	1	0	0	0	No impact
Hologic	Panther® Fusion SARS-CoV-2 (#AW-21159-001)	2	0	0	0	0	0	No S gene target
Hologic	Aptima® SARS-CoV-2 Assay (#PRD-06419)	2	0	0	0	0	0	No S gene target
Hyris	SARS-CoV-2 human diagnostics (#bKTH-SCV2.02)	0	0	0	0	0	2	No S gene target
FRIZ Biochem	COVID-19 direct RT-PCR (#FBC101)	0	0	0	1	0	1	No S gene target
Lubioscience	2019-nCoV CDC EUA Kit (#10006606)	0	0	0	0	0	2	No S gene target
Qiagen	QiaStat-Dx Respiratory SARS-CoV-2 Panel (#691223)	0	1	0	1	0	0	No S gene target
Qiagen	NeuMoDx SARS-CoV-2 Assay (#300800)	1	0	0	0	0	1	No S gene target
Qiagen	SARS-CoV-2 N1+N2 Assay Kit (#222015 or #222017)	0	0	0	0	0	2	No S gene target
Roche Diagnostics	cobas® 6800/8800 SARS-CoV-2 (#09175431190)	1	0	0	1	0	0	No S gene target
Roche Diagnostics	cobas® 6800/8800 SARS-CoV-2 & Influenza A/B (#09233474190)	1	0	0	1	0	0	No S gene target
Roche Diagnostics	cobas® Liat SARS-CoV-2 & Influenza A/B (#09211101190)	1	0	0	0	0	1	No S gene target
Sansure	Novel Coronavirus (2019-nCoV) Nucleic Acid Diagnostic Kit (#S3104E)	1	0	0	0	0	1	No S gene target
Siemens Healthineers	FTD SARS-CoV-2 qPCR Test (#11416302)	1	0	0	0	0	1	No S gene target
TECOmedical	PathoFinder RealAccurate® Quadruplex SARS-CoV-2 PCR Kit (#PF0971C-R)	0	1	0	0	0	1	No S gene target
TECOmedical	Eurobio Scientific EurobioPlex SARS-CoV-2 Multiplex (#EBX-041-192)	0	2	0	0	0	1	No S gene target
Thermo Fisher	TaqPath™ COVID-19 Combo Kit (#A48067)	1	0	1	0	0	1	S gene drop out
Thermo Fisher	TaqPath™ COVID-19, FluA/B, RSV (#A49867)	0	0	1	0	0	1	No impact
TibMolBio	LightMix® Modular Sarbecovirus SARS-CoV-2 (#50-0776-96)	0	1	0	1	0	1	No S gene target

*PCR kit not available anymore on the Swiss market.
**Mismatch not detectable because both the S gene and the RdRp gene targets are detected using the same fluorescence channel.

Table 1.

in the genome of pathogens with stable DNA genomes. Thus, the strong selection pressure due to the worldwide concerted effort to detect SARS CoV-2 was likely to lead to mutations in the RNA genome of this pandemic virus. Similarly, a PCR assay based on the S gene alone will likely provide a false negative result with a variant of SARS CoV-2 having the del 69-70 mutation. Adding one or two additional gene targets to the PCR assay largely prevents problems when facing new mutations or variants. This is the safest way to avoid new variants being invisible to diagnostic tools.

Take-home message: Never use a single target PCR for detection of emerging pathogens.

Knowledge on PCR targets

To prevent such errors, diagnostic assays should be designed accordingly with a focus on conserved sites (i.e. the genomic sequences least likely to accumulate mutations over time). The diagnostic targets should be regularly controlled by manufacturers. Clear declarations of target primers and probes used in commercial assays in their documentation inserts allows for well-informed users of such kits and therefore a better understanding and monitoring of the diagnostic tool. Unfortunately, this is not a CE-IVD requirement and is often missing or deeply buried within the documentation. Clinical microbiologists should always ask for this information before implementing a new assay in laboratory routine diagnostics. This point is indirectly included in the accreditation norm ISO 17025 as part of the risk analysis or the assessment that the assay chosen is able to fulfil its diagnostic requirements.

Such information is often not easily available or not provided with much precision due to commercial confidentiality clauses. Most commercial SARS-CoV-2 PCR tests target between one and four target analytes. In Switzerland, the 37 most frequently used SARS-CoV-2 PCR tests target one or more sequences such as the ORF1ab region (N=14), the RdRp gene (N=13), the S gene (N=8), the E gene (N=10), the N gene (N=32) and in one case the M gene.

Table 1 provides the PCR target analytes of commonly used SARS-CoV-2 specific assays in Switzerland and the currently available information concerning the impact of the B.1.1.7 variant on detection success of the target analytes.

Take-home message: Control and request all necessary information, including target analytes, for the commercial kits you plan to use.

Surveillance of targets in the assay

The last point we discuss concerns the required attention for the interpretation of multi-target results. The B.1.1.7 lineage presents an S gene dropout on assays targeting the S gene such as the Thermo Fisher Taq Path SARS-CoV-2 PCR. If this is a recognised reaction on this three-gene target assay, any other non-conventional reaction could also be an indication of a possible new variant or mutation. Table 2 illustrates four hypothetical cases that can be interpreted as a positive diagnostic result. Cases B and C reflect the problem of a low quantity of virus within a sample. Case D illustrates an anomaly by not detecting any signal (or a much weaker signal) on target 2 even though the signal for the two other targets reveals a high quantity of genetic material in the sample. This could provide evidence of a mutation in the diagnostic target site. Cases C and D should immediately be re-run on another diagnostic platform. In case D, a whole genome sequencing or at least the sequence of the target region 2 should be performed.
Take-home message: Keep an eye on and further investigate unusual reactions and mismatches.

Case	Target 1	Target 2	Target 3	Interpretation
A	16	17.1	17.5	Positive
B	nd	33.5	36.0	Weak positive
C	nd	nd	38.5	Weak positive or not conclusive
D	20	nd / 31.0	21	Possible mutation on target 2

Table 2. Ct values and interpretation of different combinations of a hypothetical multi-target SARS-CoV-2 PCR test. The gap of certain targets may provide evidence for mutant strains. (nd = not detectable)

The presented points and the corresponding proposed take-home messages are highly important in diagnostics. Diagnostic laboratories using highly sensitive PCR technologies need to be aware that viral evolution may impact diagnostic performance, as was demonstrated by the emergence of the B.1.1.7 lineage. Unexpected discrepancies and the emergence of variants should be confirmed ideally using whole genome sequencing in order to adapt diagnostic assays and to maintain a high performance of diagnostics during public health crises. While the processing of otherwise unusually high numbers of samples for the SARS-CoV-2 diagnostic allowed for the rapid identification of a target evasion on a multi-target PCR assay and its unambiguous interpretation as a possible new variant. The correct identification of lower amplification results, mismatches or complete dropout of targets as the spread of a new variant of a pathogen during routine diagnostic work outside of an intense pandemic (such as the COVID-19 pandemic) may not be readily obvious. When the herein presented diagnostic mismatches were correctly identified and interpreted by one laboratory within a few weeks, which then could sound the alarm for others to join in and further investigate the observations, one may wonder how long it may take to realise a similar situation, when much less samples are being processed by unit of time.

Conclusion

Regular monitoring of analytical output and quality is the only direct control to avoid failure in diagnostics as well as in vaccination programs and (if available) therapeutic treatments losing their efficacy. Thus, it is especially important to have a national surveillance programme for the emergence of variants. Such a programme may be especially useful if it targets all patients hospitalised in university hospitals where most severe and unusual cases will finally get hospitalised. To be able to retrospectively sequence SARS-CoV-2 genomes of strains causing major unusual late complications of COVID-19, it is very important that clinical samples taken at the time of screening (when the viral load is high) are stored, since sometimes the nasopharyngeal viral load at time of hospitalisation will be too low (or even absent) to get sequenced. Thus, a move back towards PCR-based surveillance should be considered, especially with the current availability of salivary RT-PCR.

Acknowledgements

The authors would like to thank the companies and suppliers of PCR tests who provided additional information about their targets and the impact on the B.1.1.7 and/or the B.1.351 in conjunction with their PCR kits.

Correspondence
reto.lienhard@ne.ch

References

1. De Maio N, Walker CR, Turakhia Y, Lanfear R, Corbett-Detig R, Goldman N. Mutation rates and selection on synonymous mutations in SARS-CoV-2. bioRxiv 2021.
2. Petrova VN, Russell CA. The evolution of seasonal influenza viruses. Nat Rev Microbiol 2018; 16(1): 47-60.
3. Revill PA, Tu T, Netter HJ, Yuen LKW, Locarnini SA, Littlejohn M. The evolution and clinical impact of hepatitis B virus genome diversity. Nat Rev Gastroenterol Hepatol 2020; 17(10): 618-34.
4. Kajan GL, Doszpoly A, Tarjan ZL, Vidovszky MZ, Papp T. Virus-Host Coevolution with a Focus on Animal and Human DNA Viruses. J Mol Evol 2020; 88(1): 41-56.
5. Galloway SE, Paul P, MacCannell DR, et al. Emergence of SARS-CoV-2 B.1.1.7 Lineage - United States, December 29, 2020-January 12, 2021. MMWR Morb Mortal Wkly Rep 2021; 70(3): 95-9.
6. Leung K, Shum MH, Leung GM, Lam TT, Wu JT. Early transmissibility assessment of the N501Y mutant strains of SARS-CoV-2 in the United Kingdom, October to November 2020. Euro Surveill 2021; 26(1).
7. Lauring AS, Hodcroft EB. Genetic Variants of SARS-CoV-2-What Do They Mean? JAMA 2021; 325(6): 529-31.
8. Tang JW, Toovey OTR, Harvey KN, Hui DDS. Introduction of the South African SARS-CoV-2 variant 501Y. V2 into the UK. J Infect 2021.
9. Smid JH, Althaus CL, Low N, Unemo M, Herrmann B. Rise and fall of the new variant of Chlamydia trachomatis in Sweden: mathematical modelling study. Sex Transm Infect 2020; 96(5): 375-9.
10. Hadad R, Jensen JS, Westh H, et al. A Chlamydia trachomatis 23S rRNA G1523A variant escaping detection in the Aptima Combo 2 assay (Hologic) was widespread across Denmark in July-September 2019. APMIS 2020; 128(6): 440-4.
11. Johansen TB, Klovstad H, Rykkvin R, et al. The 'Finnish new variant of Chlamydia trachomatis' escaping detection in the Aptima Combo 2 assay is widespread across Norway, June to August 2019. Euro Surveill 2019; 24(42).
12. Roberts DJ, Davis GS, Cole MJ, et al. Prevalence of new variants of Chlamydia trachomatis escaping detection by the Aptima Combo 2 assay, England, June to August 2019. Euro Surveill 2019; 24(38).