OCEBM levels-of-evidence pyramid for therapies; Fig. 1d)[3,4].

Newer forms of evidence are also being interrogated. For instance, in a program providing rapid access to compassionate use of the antiviral Veklury, ~60% of patients hospitalized for severe COVID-19 demonstrated improvement, a finding that was quickly disseminated by publication[26]. These findings also raise the possibility of implementing master observational studies for COVID-19, as has been proposed for cancer with clinical trials such as ROOT that plan large-scale structured data acquisition in an observational setting[14,19]. In addition, acquisition of real-world data by exploiting digital technology to download medical or insurance records or to mine clinical trial databases has also led to approvals in cancer[12,13] and may provide rapid access to important information related to COVID-19 therapeutic effectiveness. It is understood that some of the studies that are ongoing or proposed for COVID-19 are not RCTs and, therefore, while providing proof of concept, may still need to be confirmed by RCTs. Still, it is critical to appreciate how our response to COVID-19 has demonstrated that we do not need to become mired in old or misinterpreted dogma concerning levels-of-evidence rankings to advance a field where there is urgency.

It is also important to recognize that levels-of-evidence hierarchies have been extensively updated since their earliest renditions, 30 to 40 years ago[1,5]. Indeed, in 2009 and 2011, the OCEBM levels-of-evidence pyramid for treatment studies (Fig. 1c,d)[3,4] raised several forms of non-RCT with dramatic effects to the top evidence tiers. For these types of observations, therapeutic efficacy may be such that randomization to a control arm may not be ethical[30]. The key is to balance the risk of authorizing a therapy that may later be disproven versus that of delaying adoption of a life-saving therapy by requiring a RCT that would likely take years to perform[30]. Indeed, there are quantifiable threshold values above which it is highly likely that effectiveness seen in non-randomized trials will consistently translate to improved survival.

In summary, contemporary levels-of-evidence hierarchies have already been broadened to acknowledge the important role played by non-RCTs (Fig. 1). Furthermore, powerful digital and molecular technologies exist today that were inconceivable when the earliest levels of evidence were formulated, over 40 years ago[1]. Newer types of evidence are being exploited, including real-world data and the use of genomic sequencing and mechanism-based reasoning to select cancer patients for matched gene- and immune-targeted treatments. The COVID-19 pandemic has revealed that we can exploit novel types of evidence, including those generated by observational studies (Table 1) and by digital technologies, including downloadable apps. The latter can produce clinically relevant information self-reported by millions of individuals within a few weeks[20,21]. In all, the COVID-19 pandemic has shown that we must balance scientific rigor, reflected by classic levels of evidence, with the need for urgency. The lessons learned may expedite the discovery of important treatments for other deadly diseases. ❐

Jacob J. Adashek[1] and Razelle Kurzrock[2] ✉

[1]Department of Internal Medicine, University of South Florida, H. Lee Moffitt Cancer Center & Research Institute, Tampa, FL, USA. [2]Center for Personalized Cancer Therapy and Division of Hematology and Oncology, Department of Medicine, University of California San Diego Moores Cancer Center, La Jolla, CA, USA.
✉*e-mail: rkurzrock@ucsd.edu*

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Could mutations of SARS-CoV-2 suppress diagnostic detection?

To the Editor — The recent emergence of SARS-CoV-2 strains H69/V70[1,2], D796H[3] and D614G[4] in the United Kingdom and the N501Y strain in South Africa has prompted concerns as to their susceptibility to vaccine neutralization. I argue here another concern deserves equal attention: whether such strains can evade detection by diagnostics and compromise our ability to accurately track disease.

SARS-CoV-2 is arguably one of the most intensely studied viruses since the advent of HIV. Genotyping of the virus is occurring on a global scale and enabling nearly 'real-time' acquisition of viral genetic

Emerging SARS-CoV-2 strains (spike mutations)

```
Canonical   60    SNVTWFHAIHVSGTNGTKRFD 80
ΔH69/V70    60    SNVTWFHAI--SGTNGTKRFD 80
Canonical   780   EVFAQVKQIYKTPPIKDFGGF 800
D796H       780   EVFAQVKQIYKTPPIKHFGGF 800
Canonical   600   PGTNTSNQVAVLYQDVNCTEV 620
D614G       600   PGTNTSNQVAVLYQGVNCTEV 620
Canonical   490   FPLQSYGFQPTNGVGYQPYRV 510 (RBD)
N501Y       490   FPLQSYGFQPTYGVGYQPYRV 510
```

B.1.1.7 (8 spike mutations)

```
Canonical   60    SNVTWFHAIHVSGTNGTKRFD 80
ΔH69/V70    60    SNVTWFHAI--SGTNGTKRFD 80
Canonical   130   VCEFQFCNDPFLGVYYHKNNK 150
ΔY144/Y145  130   VCEFQFCNDPFLGV--HKNNK 150
Canonical   490   FPLQSYGFQPTNGVGYQPYRV 510
N501Y       490   FPLQSYGFQPTYGVGYQPYRV 510 (RBD)
Canonical   560   LPFQQFGRDIADTTDAVRDPQ 580
A570D       560   LPFQQFGRDIDDTTDAVRDPQ 580
Canonical   670   ICASYQTQTNSPRRARSVASQ 690
P681H       670   ICASYQTQTNSHRRARSVASQ 690
Canonical   710   NSIAIPTNFTISVTTEILPVS 730
T716I       710   NSIAIPINFTISVTTEILPVS 730
Canonical   980   ILSRLDKVEAEVQIDRLITGR 1000
S982A       980   ILARLDKVEAEVQIDRLITGR 1000
Canonical   1110  YEPQIITTDNTFVSGNCDVVI 1130
D1118H      1110  YEPQIITTHNTFVSGNCDVVI 1130
```

Fig. 1 | Multiple sequence alignments of SARS-CoV-2 S protein. Relative portions of the sequence alignments of S are shown. The mutated positions in variants are highlighted in yellow.

composition[5]. The mutation rate of the virus is ~2 nucleotides (nt) per month, which is considerably less than that of influenza (4 nt/month) or HIV (8 nt/month)[6]. Mutations put at risk detection strategies that do not accommodate changes in the viral genome.

The observed mutations in SARS-CoV-2 are not predicted to affect the utility of currently deployed vaccines[7]; however, changes in the viral nucleic acid and protein sequences put at risk the utility of certain in vitro diagnostic assays if the mutation occurs in an area critical for primer or antibody binding in RT-PCR and immunoassays. In addition, a particular concern is antibody-based COVID-19 diagnostic tests that assess the presence and concentration of SARS-CoV-2 viral proteins in biofluids (mainly lysates from nasopharyngeal, oropharyngeal or saliva extracts). The most commonly deployed immunoassays for detection of SARS-CoV-2 viral proteins include enzyme-linked immunosorbent assays (ELISAs) and lateral flow assays (LFAs). The targeted analytes in these assays are predominantly spike (S) or nucleocapsid (N) proteins, the two most abundant and immunogenic viral proteins present in the SARS-CoV-2 genome.

S protein is a seductive viral antigen. It is highly immunogenic and contains sequences unique to SARS-CoV-2[8], thereby potentially minimizing cross reactivity to sequences present in other known human coronaviruses, such as SARS-CoV, Middle Eastern respiratory syndrome (MERS) virus and human coronaviruses 229E, OC43, HKU-1 and NL63[9]. However, it comes with risks. S protein is the most likely viral protein to undergo mutation, especially mutations that may affect viral function, including infection rate[10], transmissibility[11,12] and the ability to infect individuals younger in age[13] (for example, a mutation near the receptor binding domain may affect entry into the host cell). As mutations occur, immunoassays that detect S protein are more susceptible to an increasing rate of false-negative results, and it is essential to obtain sufficiently accurate testing results to detect the virus during the pandemic.

Conversely, point mutations in the N protein are less likely to occur and less likely to affect viral function. Thus, N protein is considered the best target for in vitro diagnostic detection and vaccine development for COVID-19 because of the conservation of the N protein sequence, the expanding knowledge of its genetics and biochemistry, and its strong immunogenicity[14]. The N protein, however, is also not invulnerable to mutation, and in vitro diagnostic and vaccine design must account for potential and inevitable mutations.

Regarding in vitro diagnostic immunoassays, an assay design that includes polyclonal antibodies has distinct advantages over assays that rely on the detection of a single epitope using a monoclonal antibody. A polyclonal antibody recognizing multiple epitopes present on or within the N protein is most likely to continue to detect the protein, despite the presence of multiple mutations in the target analyte. Where a mutation occurs within an epitope, a monoclonal antibody reactive to only that single epitope may become ineffective in detecting the viral protein. 'Escape variant' detection is among the several well documented benefits of polyclonal antibodies in applications where multiepitope binding properties represent clear advantages[15].

In terms of the emerging SARS-CoV-2 strains — N501Y in South Africa[9], H69/V70[1,2], D796H[3] and D614G[4] — none represent mutations that would hinder the ability of a diagnostic polyclonal antibodies to N protein to detect SARS-CoV-2. Even the strain B.1.1.7 (Fig. 1), which was identified to have 17 mutations, would be detected using such antibodies.

With this in mind, and as new variants of SARS-CoV-2 are identified, it is critical that diagnostic tests for the virus in wide use are regularly reconfigured. In particular, diagnostic tests configured to use a single monoclonal antibody, especially those targeting S protein, must revalidate the performance of the test against emerging strains of SARS-CoV-2 or consider adapting the assay to the detection of N protein using high-affinity polyclonal antibodies as critical detection reagents. ❐

Carl A. Ascoli ✉

Rockland Immunochemicals Inc., Limerick, PA, USA.
✉*e-mail: carl.ascoli@rockland-inc.com*

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