

1 **The Laboratory Diagnosis of COVID-19 Infection: Current Issues and Challenges**

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12 Running head: COVID-19 laboratory diagnosis.

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17

18 **Abstract**

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20 The COVID-19 outbreak has had a major impact on clinical microbiology laboratories in the past
21 several months. This commentary covers current issues and challenges for the laboratory
22 diagnosis of infections caused by SARS-CoV-2. In the pre-analytical stage, collecting the proper
23 respiratory tract specimen at the right time from the right anatomic site is essential for a
24 prompt and accurate molecular diagnosis of COVID-19. Appropriate measures are required to
25 keep laboratory staff safe while producing reliable test results. In the analytic stage, real-time
26 RT-PCR assays remain the molecular test of choice for the etiologic diagnosis of SARS-CoV-2
27 infection while antibody-based techniques are being introduced as supplemental tools. In the
28 postanalytical stage, testing results should be carefully interpreted using both molecular and
29 serological findings. Finally, random access, integrated devices available at the point of care
30 with scalable capacities will facilitate the rapid and accurate diagnosis and monitoring of SARS-
31 CoV-2 infections and greatly assist in the control of this outbreak.

32

33 **Keywords:** COVID-19, SARS-CoV-2, specimen type, molecular testing, serology, result
34 interpretation

35

36 Introduction

37

38 The identification by United States public health officials of “presumptive” COVID-19 cases
39 believed to be due to community transmission of this infection, brings into sharp focus the
40 importance of the laboratory diagnosis of infection caused by SARS-CoV-2 (1-5). The current
41 recommendations for laboratory diagnosis of COVID-19 infection from the CDC are that
42 clinicians coordinate this testing with local public health authorities and/or the CDC. The
43 preferred testing method is the real-time reverse transcription-polymerase chain reaction (real-
44 time RT-PCR) test method (6-8) similar to that developed for the diagnosis of SARS-CoV (9, 10).
45 Viral cultures are not recommended. This commentary addresses current issues for the
46 laboratory diagnosis of COVID-19 that must be understood both by clinicians, clinical
47 microbiology laboratories, and public health authorities.

48

49 Pre-Analytical Issues

50

51 **Initial Respiratory Tract Specimen Collection for Diagnosis and Screening of Patients with**
52 **COVID-19 Pneumonia.** Within 5 – 6 days of the onset of symptoms, patients with COVID-19
53 infections have demonstrated high viral loads in their upper and lower respiratory tract (11-14).
54 A nasopharyngeal (NP) swab and/or an oropharyngeal (OP) swab are often recommended for
55 screening or diagnosis early infection (9, 12, 15). A single NP swab has become the preferred
56 swab, as it is tolerated better by the patient and is safer to the operator. NP swabs have an
57 inherent quality control in that they usually reach the correct area to be tested in the nasal

58 cavity. Wang et al have just reported that OP swabs (n=398) were used much more frequently
59 than in nasal swabs (n=8) in China during the COVID-19 outbreak; however, the SARS-CoV-2
60 RNA was detected only in 32% of OP swabs, which was significantly lower than that in nasal
61 swabs (63%) (16). While collection/testing of both nasal and OP swabs – either as independent
62 specimens or together within a single aliquot of viral transport media – might be an attractive
63 option under normal circumstances, institutions must also consider the potential stress that
64 this pandemic places on national/international supply-chains. In this light, another excellent
65 reason to limit testing NP swabs is to prolong supplies of flocked swabs and/or transport media.
66 However, as we understand more about respiratory and oral contact routes of transmission, we
67 may learn that patients with pharyngitis as a dominant initial presenting symptom can be
68 adequately sampled via the OP route.

69
70 In order to properly obtain an NP swab specimen, the swab must be inserted deeply into the
71 nasal cavity and should elicit “tears”. Patients will likely flinch, but that means the swab has hit
72 the target. The OP swab should elicit a gag reflex, but there is much person to person variability
73 in the gag response. Swabs should be kept in place for 10 seconds while twirling the swab three
74 times. Swabs should have flocked non-toxic synthetic fibers such as polyester as well as
75 synthetic nylon handles (17). Collecting a NP/OP swab specimen may carry a theoretical risk of
76 transmitting the SARS-CoV-2 particularly if airborne transmission should be demonstrated as
77 the investigation of the COVID-19 outbreak continues (18). If personal protective equipment
78 (PPE) can’t be utilized due to scarcity of such PPE, other means of collecting upper respiratory
79 tract specimens will be needed (18). One alternative option for collecting an upper respiratory

80 tract specimen to evaluate patients with suspected COVID-19 pneumonia is a self-collected
81 saliva specimen (19-22). From a practical perspective, should the supply of swabs become
82 scarce, self-collected saliva and nasal washes may be the only specimens available. Should the
83 supply of swabs become scarce, other non-flocked swabs and transport media have been
84 cleared equivalently by the Food and Drug Administration (FDA) under the emergency use
85 authorization (EUA), but head to head comparisons are lacking currently.

86

87 After collection, swabs should be placed in viral (universal) transport medium for rapid
88 transportation to the clinical microbiology laboratory, ideally under refrigerated conditions
89 (17). It should be noted, however, that in some cases, saliva/NPS/OPS may miss early infection,
90 and, repeated testing or obtaining lower respiratory tract specimens may be required.

91 Moreover, other respiratory viral pathogens such as influenza and respiratory syncytial viruses
92 must be ruled out. In many ways, COVID19 highlights the key difference between ‘analytic’ and
93 ‘clinical’ sensitivity – that is, the ability of an assay to detect pathogen when present in a clinical
94 specimen *versus* the ability of a test to identify a patient’s overall infected status. The latter, of
95 course, reflects various other factors that include the specimen-site and method of collection,
96 in conjunction with the burden of organism as a function of anatomic location, disease severity,
97 and time symptomatic (and variability of these factors from individual to individual). Repeated
98 testing may particularly be important if a patient has a clinical picture of viral pneumonia, a
99 potential exposure history, and/or radiographic findings (chest CT or MRI scan) consistent with
100 COVID-19 pneumonia. Equally challenging are how the results of a single undetected result
101 should impact decisions regarding patient quarantine and social distancing, in particular when

102 the patients themselves are healthcare providers (including clinical laboratory staff). Serology,
103 as discussed in the post-analytical section, may assist in such situations.

104

105 **Late Detection and Monitoring of Patients with Severe COVID-19 Pneumonia.** Ideally, sputum
106 or bronchoalveolar lavage should be used for collecting lower respiratory tract specimens as
107 they yielded highest viral loads for the diagnosis of COVID-19 (18, 23). A recent study revealed
108 that bronchoalveolar lavage yielded the highest SARS-CoV-2 RNA rate, although this study did
109 not compare/evaluate NP swabs (16). Patients who present with severe pneumonia and acute
110 respiratory distress syndrome may require emergent intubation as well as respiratory isolation
111 in a negative pressure room. If possible, a lower respiratory tract sputum specimen should be
112 collected during the intubation procedure. Alternatively, sputum and/or bronchoalveolar lavage
113 specimens can be collected after intubation (9, 11).

114

115 However, patients with COVID-19 pneumonia have demonstrated high viral RNA of SARS-CoV-2
116 in fecal material (24, 25) as well as delayed shedding from the respiratory tract (4, 18) late in
117 their clinical course. Enteric involvement previously has been seen in patients with severe novel
118 coronavirus infections (9, 26-32). In four such studies, SARS coronavirus was isolated from stool
119 cultures (26, 28, 31). In another study, SARS coronavirus was demonstrated inside enterocytes
120 by electron microscopy (30). Thus, aside from direct respiratory sampling, the preferred
121 method for detecting SARS-CoV-2 in advanced COVID-19 cases may be a rectal swab by real-
122 time RT-PCR (9, 26-28, 30-32).

123

124 **Safety Measures for Specimen Processing for PCR Processing and Testing.** Processing of
125 respiratory specimen should be done in a class II biological safety cabinet (6, 9, 10). Ideally,
126 some would argue that biosafety level three (BSL3) work procedures should be used and that
127 the safety cabinet should be in a negative pressure room within the laboratory such as done for
128 mycobacterial cultures. For nucleic acid extraction before real-time RT-PCR is performed, the
129 specimen should be transferred to lysis buffer under this BSL-2 cabinet. This lysis buffer should
130 contain a guanidinium-based inactivating agent as well as a non-denaturing detergent. Indeed,
131 the buffers included in common commercial extraction platforms, such as the bioMerieux
132 easyMAG or QIAGEN EZ1, do contain guanidium/detergents and are able to inactivate any
133 viable coronavirus (33-35). Likewise, universal transport medium including guanidinium salt is
134 available at
135 http://www.chinamerlin.com/en/index.php?p=products_show&id=166&s_id=&c_id=68&lanmu
136 [=2](#) (Accessed 3 March 2020). Because this test is a reverse transcription method, the
137 saliva/swabs used to collect the clinical specimens should be quickly added to lysis buffer to
138 disinfect the specimen as well as to stop degradation of the coronavirus RNA (6, 9, 10). The
139 clinical specimens/swabs should not be heated to 56°C for 30 minutes as evidence suggests
140 that this process may also degrade the coronavirus RNA, even as it inactivates viable
141 coronavirus (9, 36).
142
143 Moreover, self-enclosed systems integrating nucleic acid extraction, amplification and
144 detection such as ID NOW™ (Abbott, San Diego, CA) (37, 38), cobas® Liat® (Roche Molecular
145 Systems, Pleasanton, CA) and GeneXpert® (Cepheid, Sunnyvale, CA) (39) when available and

146 meeting local regulatory requirements for SARS-CoV-2 testing will be very useful. Once the
147 clinical specimen in viral transport medium is transferred into a cartridge in a class II biosafety
148 cabinet, the cartridge is “sealed”. Many of these random-access “sealed” devices are suitable
149 for point-of-care testing for local hospitals and clinics without biosafety cabinets. In this
150 situation, the specimen collector in appropriate protective gear (splash guard/goggles, mask,
151 gloves, and disposable laboratory coat) could directly transfer the specimen into detection
152 cartridges at bedside or in a location without a class II biosafety cabinet, and the closed
153 cartridge will be safely placed on instrument for testing. However, spills of transport solution
154 during transfer to these cartridge-based tests should be avoided, and if they occur,
155 decontaminated as appropriate.

156

157 **Analytical Issues**

158

159 **Assay Selection.** Immunoassays have been developed for rapidly detection of SARS-CoV-2
160 antigens or antibodies. These rapid point-of-care immunoassays are generally lateral flow
161 assays. Such lateral flow assays have been developed for detecting antigens such as the SARS-
162 CoV-2 virus or for detecting antibodies (IgM and IgG) against COVID-19 infections.

163

164 Rapid antigen lateral flow assays would theoretically provide the advantage of fast time to
165 results and low-cost detection of SARS-CoV-2 but are likely to suffer from poor sensitivity based
166 on the experience with this method for influenza (Flu) viruses (40-44). Monoclonal antibodies
167 specifically against SARS-CoV-2 have been under preparation, and several rapid antigen assays

168 are being developed (<https://www.medrxiv.org/content/10.1101/2020.03.07.20032524v1>.
169 Assessed 13 March 2020). There is concern that given the variability of viral loads in COVID-19
170 patients, antigen detection may miss cases due to low infectious burden or sampling variability.
171
172 Serology measures the host response to infection and is an indirect measure of infection that is
173 best utilized retrospectively. Serological methods are rapidly being developed and have proven
174 to be useful in confirming COVID-19 infection (25). Serology previously has had an important
175 role in the epidemiology of SARS (45) and other coronavirus outbreaks (46). Rapid lateral flow
176 assays for both IgM and IgG antibodies undoubtedly will play an important role in COVID-19
177 and should allow the burden of infection, the role of asymptomatic infections, the basic
178 reproduction number, and the overall mortality to be determined. However, IgM responses are
179 notoriously non-specific, and given the weeks required to develop specific IgG responses,
180 serology detection is not likely to play a role in active case management except to
181 diagnose/confirm late COVID-19 cases or to determine the immunity of health care workers as
182 the outbreak progresses. Cell culture is not recommended for diagnostic purposes.
183
184 **Assay Selection for Molecular Detection of SARS-CoV-2.** Random-amplification deep-
185 sequencing methods played a major role in the initial identification of SARS-CoV-2 (47-51).
186 Deep sequencing molecular methods such as next generation sequencing and metagenomic
187 next generation sequencing will continue to be needed to determine future mutations of SARS-
188 CoV-2 but are currently impractical for diagnosing COVID-19 infections. Most of the molecular
189 diagnostics being developed for the diagnosis of COVID-19 infections involve real-time RT-PCR

190 assays including those from the US Centers for Disease Control and Prevention (52), The Charité
191 Institute of Virology in Berlin, Germany (7, 53), and the Hong Kong University (21, 54). Other
192 molecular methods are being developed and evaluated worldwide and include loop-mediated
193 isothermal amplification, multiplex isothermal amplification followed by microarray detection ,
194 and clustered regularly interspaced short palindromic repeats (55).

195

196 **Target Selection for Real-Time RT-PCR Assays.** A real-time RT-PCR method is recommended for
197 molecular testing (6, 8-10). A major advantage of real-time RT-PCR assays is that amplification
198 and analysis are done simultaneously in a closed system to minimize false-positive results
199 associated with amplification product contamination. There are a number of coronaviruses that
200 cause respiratory and intestinal infections in humans (8, 56). Among these coronaviruses are a
201 group of SARS-like bat coronaviruses, including both SARS-CoV and SARS-CoV-2, that comprise a
202 unique clade under the subgenus *Sarbecovirus* (56, 57). Coronaviruses have a number of
203 molecular targets within their positive-sense, single-stranded RNA genome that can be used for
204 PCR assays (6, 7, 56, 57). These include structural proteins, including envelope glycoproteins
205 spike (S), envelope (E), transmembrane (M), helicase (Hel), and nucleocapsid (N) (56-58). In
206 addition to these genes that encode structural proteins, there are species-specific accessory
207 genes that are required for viral replication. These include RNA-dependent RNA polymerase
208 (RdRp), hemagglutinin-esterase (HE), and open reading frames ORF1a and ORF1b (7, 52-54, 56,
209 57). In the United States, the CDC recommends two nucleocapsid protein targets [N1 and N2]
210 (52) while WHO recommends first line screening with the E gene assay followed by a
211 confirmatory assay using the RdRp gene (7). Chan et al has just developed and compared the

212 performance of three novel real-time RT-PCR assays targeting the RdRp/He1, S, and N genes of
213 SARS-CoV-2. Among them, the COVID-19-RdRp/He1 assay had the lowest limit of detection in
214 vitro and higher sensitivity and specificity (58). However, it is likely that well-optimized targets
215 will arise from a number of viral genomic locations since assay performance is usually dictated
216 by the reagent design, not the target itself, since the viral genes are present in equal copy
217 number.

218

219 To avoid potential cross-reaction with other endemic coronaviruses as well as potential genetic
220 drift of SARS-CoV-2, at least two molecular targets should be included in the assay. Various
221 investigators in different countries have used a number of these molecular targets for real-time
222 RT-PCR assays. In the United States, the CDC has selected two loci in nucleocapsid gene as the
223 two-target assay appears to be performing well (52). One study utilized two sequence regions
224 (open reading frame 1b and a nucleocapsid protein) that are highly conserved amongst
225 *Sarbecoviruses* for initial real-time RT-PCR testing (6). Another study in Hong Kong, China used
226 two targets for their RT-PCR assay; the first used the nucleocapsid for screening followed by
227 confirmation by the open reading frame 1b (54). In Germany, two molecular targets (envelope
228 and RNA-dependent RNA polymerase) have been selected (7). In China, at the time of
229 preparation, several molecular devices have received urgent approval (8). To date, there has
230 been no indication that any one of these sequence regions used offer an advantage for clinical
231 diagnostic testing. However, the ideal design would include at least one conserved region and
232 one specific region to mitigate against the effects of genetic drift, especially as the virus evolves
233 within new populations.

234

235 In the United States, regulatory issues have complicated the development and implementation
236 of laboratory-developed molecular tests for the diagnosis of COVID-19. On February 29, 2010
237 FDA issued new guidance for laboratories to be able to develop and implement COVID-19
238 molecular diagnostic tests prior to obtaining EUA. Laboratories are required to submit an EAU
239 to the FDA within fifteen business days after validation. Moreover, the validation must include
240 the specimen types (e.g., nasopharyngeal, oropharyngeal, saliva) that are to be used clinically.
241 Although these new regulatory burdens did not prohibit the development of molecular
242 laboratory testing for the diagnosis of COVID-19, they did create a lot of extra work. At the time
243 of writing, the US FDA has granted quite a few EUAs ([https://www.fda.gov/medical-](https://www.fda.gov/medical-devices/emergency-situations-medical-devices/emergency-use-authorizations#coronavirus2019)
244 [devices/emergency-situations-medical-devices/emergency-use-](https://www.fda.gov/medical-devices/emergency-situations-medical-devices/emergency-use-authorizations#coronavirus2019)
245 [authorizations#coronavirus2019](https://www.fda.gov/medical-devices/emergency-situations-medical-devices/emergency-use-authorizations#coronavirus2019). Accessed 28 March 2020).

246

247 **Post-Analytical Issues**

248

249 **Interpretation of Molecular Results.** In the United States, initially if all two targets
250 (nucleocapsid proteins N1 and N2) test positive, a case is considered to be laboratory-
251 confirmed (52). A cycle threshold value (Ct-value) less than 40 is defined as a positive test, while
252 a Ct-value of 40 or more is defined as a negative test. A Ct-value <40 for only one of the two
253 nucleocapsid protein [N1 and N2] is defined as indeterminant and requires confirmation by
254 retesting (52). Currently in China for the assays with three targets, positives for two or more
255 targets are considered positive

256 ([http://www.nhc.gov.cn/yzygj/s7653p/202003/46c9294a7dfe4cef80dc7f5912eb1989/files/ce3](http://www.nhc.gov.cn/yzygj/s7653p/202003/46c9294a7dfe4cef80dc7f5912eb1989/files/ce3e6945832a438eaae415350a8ce964.pdf)
257 [e6945832a438eaae415350a8ce964.pdf](http://www.nhc.gov.cn/yzygj/s7653p/202003/46c9294a7dfe4cef80dc7f5912eb1989/files/ce3e6945832a438eaae415350a8ce964.pdf), in Chinese. Accessed 12 March 2020). Although
258 correlations were revealed, viral loads determined by real-time RT-PCR assays should not be yet
259 used to indicate COVID-19 disease severity or to monitor therapeutic response (11-13, 59, 60).
260 However, low Ct values indicating high viral loads may be used as an indication of
261 transmissibility (18, 61).

262

263 **Test of Cure and Test of Infectivity.** Monitoring patients with resolution of COVID-19
264 pneumonia may also be important in terms of when they should be released from isolation and
265 discharged. If discharged patients are still shedding viable coronavirus, they are likely to infect
266 others (27). Therefore, self-quarantine for up to one month may be advisable. NP and OP swabs
267 are not sufficient for either test of cure or test of infectivity (62). The optimal method for test of
268 cure most likely will be two consecutive negative real-time RT-PCR tests from rectal swabs; this
269 suggestion is based on the fact that SARS-CoV-1 has been cultured from stool during the 2002-
270 2003 SARS outbreak (26, 28, 31) and SARS-CoV-2 has been culture from stool during the COVID-
271 19 outbreak (16). Thus, a rectal swab that is positive by real-time PCR testing suggests that this
272 patient may be shedding viable SARS-CoV-2 in their stools thereby remain infectious (16, 24-28,
273 30-32). A very recent study on 20 serial COVID-19 patients indicated that infectious virus was
274 not isolated from stool samples in spite of high virus RNA concentration (14). The correlation of
275 RT-PCR positivity in stool with recovery of live virus from the same samples remains to be fully
276 investigated.

277

278 **Serology of COVID-19.** Members of the coronavirus family have four structural proteins: the
279 spike [S], membrane [M], envelope [E], and nucleocapsid [N] proteins. Two of these proteins
280 appear to be important antigenic sites for the development of serological assays to detect
281 COVID-19. Serological methods have focused on detecting serum antibodies against S-proteins
282 from the coronavirus spike (46). The coronavirus envelope spike is responsible for receptor
283 binding and fusion and determines host tropism and transmission capability (56, 57). S-proteins
284 are determined by the S gene and are functionally divided into two subunits (S1 and S2). The S1
285 domain is responsible for receptor binding while the S2 domain is responsible for fusion. SARS-
286 CoV and SARS-CoV-2 bind to human angiotensin-converting enzyme 2, which is found on
287 human respiratory cells, renal cells, and gastrointestinal cells (56, 63, 64). The other proteins
288 that appear to be important antigenic sites for the development of serological assays to detect
289 COVID-19 is the N protein, which is a structural component of the helical nucleocapsid. The N
290 protein plays an important role in viral pathogenesis, replication, and RNA packaging.
291 Antibodies to the N protein are frequently detected in COVID-19 patient (65, 66), suggesting
292 that the N protein may be one of the immunodominant antigens in the early diagnosis of
293 COVID-19 (67).
294
295 Rapid lateral flow assays for antibodies (IgM and IgG) produced during COVID-19 infection have
296 been developed (68). Seroconversion occurred after 7 days in 50% of patients (14 days in all),
297 but was not followed by a rapid decline in viral load (14). Serological methods, when available,
298 will play an important role in the epidemiology of COVID-19 and in determining the immune
299 status of asymptomatic patients, but are unlikely to play any role in screening or for the

300 diagnosis of early infections (14, 65, 66). However, serology may be useful for confirming the
301 diagnosis of COVID-19 infection (25).

302

303 **Concluding Remarks**

304

305 The ongoing, unprecedented outbreak of COVID-19 infections globally has emphasized the
306 importance of the laboratory diagnosis of human coronavirus infections in order to limit the
307 spread as well as appropriately treat those patients who have a serious infection. This
308 commentary has addressed current issues regarding such testing for SARS-CoV-2. For example,
309 a NP rather than OP swab is recommended for early diagnosis or screening because it provides
310 higher diagnostic yields, is better tolerated by the patient and is safer for the operator. A NP
311 swab can be combined with an OP swab to increase sensitivity but requires twice the number
312 of swabs. Should the NP swabs become scarce, self-collected saliva or nasal washes could be
313 used as an alternative specimen type for epidemiological screening and the “worried well”,
314 which are asymptomatic persons with no exposure history who wish to be tested “just to be
315 sure they aren’t infected”. NP swabs would then be reserved for hospitalized patients; those
316 who test negative may need deep sputum or BAL samples collected. This importance of
317 repeated testing or the use of bronchoscopy in patients with severe illness should the first
318 screening test be negative must be understood. The role of rectal swabs in testing patients with
319 late infection or as a test of infectivity/cure is currently not well studied but needs urgent
320 attention. Equally unappreciated is the need for broad screening/testing with molecular testing
321 and/or serological testing in order to determine the true mortality rate as well as other

322 epidemiological markers. Finally, the importance of rapid development of integrated, random-
323 access, point-of-care molecular devices for the accurate diagnosis of SARS-CoV-2 infections
324 cannot be overemphasized. These STAT tests will be very important for real-time patient
325 management and infection control decisions, especially when other less infectious forms of
326 pneumonia are present and respiratory isolate resources are scarce. These assays are safe,
327 simple, and fast and can be used in local clinics and hospitals who already have the needed
328 instruments and who are responsible for identifying and treating such patients.

329

330 Disclosure statement

331

332 YWT and DHP are employees of Cepheid, the commercial manufacturer of the Xpert Xpress
333 SARS-CoV-2 test.

334

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