Detection of 2019 novel coronavirus (2019-nCoV) by real-time RT-PCR

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Background: The ongoing outbreak of the recently emerged novel coronavirus (2019-nCoV) poses a challenge for public health laboratories as virus isolates are unavailable while there is growing evidence that the outbreak is more widespread than initially thought, and international spread through travellers does already occur. **Aim:** We aimed to develop and deploy robust diagnostic methodology for use in public health laboratory settings without having virus material available. **Methods:** Here we present a validated diagnostic workflow for 2019-nCoV, its design relying on close genetic relatedness of 2019-nCoV with SARS coronavirus, making use of synthetic nucleic acid technology. **Results:** The workflow reliably detects 2019-nCoV, and further discriminates 2019-nCoV from SARS-CoV. Through coordination between academic and public laboratories, we confirmed assay exclusivity based on 297 original clinical specimens containing a full spectrum of human respiratory viruses. Control material is made available through European Virus Archive – Global (EVAg), a European Union infrastructure project. **Conclusion:** The present study demonstrates the enormous response capacity achieved through coordination of academic and public laboratories in national and European research networks.

Introduction

According to the World Health Organization (WHO), the WHO China Country Office was informed of cases of pneumonia of unknown aetiology in Wuhan City, Hubei Province, on 31 December 2019 [1]. A novel coronavirus currently termed 2019-nCoV was officially announced as the causative agent by Chinese authorities on 7 January. A viral genome sequence was released for immediate public health support via the community online resource virological.org on 10 January (Wuhan-Hu-1, GenBank accession number MN908947 [2]), followed by four other genomes deposited on 12 January in the viral sequence database curated by the Global Initiative on Sharing All Influenza Data (GISAID). The genome sequences suggest presence of a virus closely related to the members of a viral species termed severe acute respiratory syndrome (SARS)-related CoV, a species defined by the agent of the 2002/03 outbreak of SARS in humans [3,4]. The species also comprises a large number of viruses mostly detected in rhinolophid bats in Asia and Europe.

As at 20 January 2019, 282 laboratory-confirmed human cases have been notified to WHO [5]. Confirmed cases in travellers from Wuhan were announced on 13 and 17 January in Thailand as well as on 15 January in Japan and 19 January in Korea. The extent of human-to-human transmission of 2019-nCoV is unclear at the time of writing of this report but there is evidence of some human-to-human transmission.

Among the foremost priorities to facilitate public health interventions is reliable laboratory diagnosis. In acute respiratory infection, RT-PCR is routinely used to detect causative viruses from respiratory secretions. We have previously demonstrated the feasibility of introducing robust detection technology based on real-time RT-PCR in public health laboratories during international

VIEWPOINT

Interpreting Diagnostic Tests for SARS-CoV-2

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The pandemic of coronavirus disease 2019 (COVID-19) continues to affect much of the world. Knowledge of diagnostic tests for severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is still evolving, and a clear understanding of the nature of the tests and interpretation of their findings is important. This Viewpoint describes how to interpret 2 types of diagnostic tests commonly in use for SARS-CoV-2 infections—reverse transcriptase–polymerase chain reaction (RT-PCR) and IgM and IgG enzyme-linked immunosorbent assay (ELISA)—and how the results may vary over time (**Figure**).

Detection of Viral RNA by RT-PCR

Thus far, the most commonly used and reliable test for diagnosis of COVID-19 has been the RT-PCR test performed using nasopharyngeal swabs or other upper respiratory tract specimens, including throat swab or, more recently, saliva. A variety of RNA gene targets are used by different manufacturers, with most tests targeting 1 or more of the envelope (*env*), nucleocapsid (*N*), spike (*S*), RNA-dependent RNA polymerase (*RdRp*), and *ORF1* genes. The sensitivities of the tests to individual genes are comparable according to comparison studies except the RdRp-SARSr (Charité) primer probe, which has a slightly lower sensitivity likely due to a mismatch in the reverse primer.[1]

In most individuals with symptomatic COVID-19 infection, viral RNA in the nasopharyngeal swab as measured by the cycle threshold (Ct) becomes detectable as early as day 1 of symptoms and peaks within the first week of symptom onset. The Ct is the number of replication cycles required to produce a fluorescent signal, with lower Ct values representing higher viral RNA loads. A Ct value less than 40 is clinically reported as PCR positive. This positivity starts to decline by week 3 and subsequently becomes undetectable. However, the Ct values obtained in severely ill hospitalized patients are lower than the Ct values of mild cases, and PCR positivity may persist beyond 3 weeks after illness onset when most mild cases will yield a negative result.[2] However, a "positive" PCR result reflects only the detection of viral RNA and does not necessarily indicate presence of viable virus.[3]

In some cases, viral RNA has been detected by RT-PCR even beyond week 6 following the first positive test. A few cases have also been reported positive after 2 consecutive negative PCR tests performed 24 hours apart. It is unclear if this is a testing error, reinfection, or reactivation. In a study of 9 patients, attempts to isolate the virus in culture were not successful beyond day 8 of illness onset, which correlates with the decline of infectivity beyond the first week.[3] That is in part why the "symptom-based strategy" of the Centers for Disease Control and Prevention (CDC) indicates that health care workers can return to work, if "at least 3 days (72 hours) have passed since recovery defined as resolution of fever without the use of fever-reducing medications and improvement in respiratory symptoms (e.g., cough, shortness of breath); and, at least 10 days have passed since symptoms first appeared."[4]

The timeline of PCR positivity is different in specimens other than nasopharyngeal swab. PCR positivity declines more slowly in sputum and may still be positive after nasopharyngeal swabs are negative.[3] In one study, PCR positivity in stool was observed in 55 of 96 (57%) infected patients and remained positive in stool beyond nasopharyngeal swab by a median of 4 to 11 days, but was unrelated to clinical severity.[2] Persistence of PCR in sputum and stool was found to be similar as assessed by Wölfel et al.[3]

In a study of 205 patients with confirmed COVID-19 infection, RT-PCR positivity was highest in bronchoalveolar lavage specimens (93%), followed by sputum (72%), nasal swab (63%), and pharyngeal swab (32%).[5] False-negative results mainly occurred due to inappropriate timing of sample collection in relation to illness onset and deficiency in sampling technique, especially of nasopharyngeal swabs. Specificity of most of the RT-PCR tests is 100% because the primer design is specific to the genome sequence of SARS-CoV-2. Occasional false-positive results may occur due to technical errors and reagent contamination.

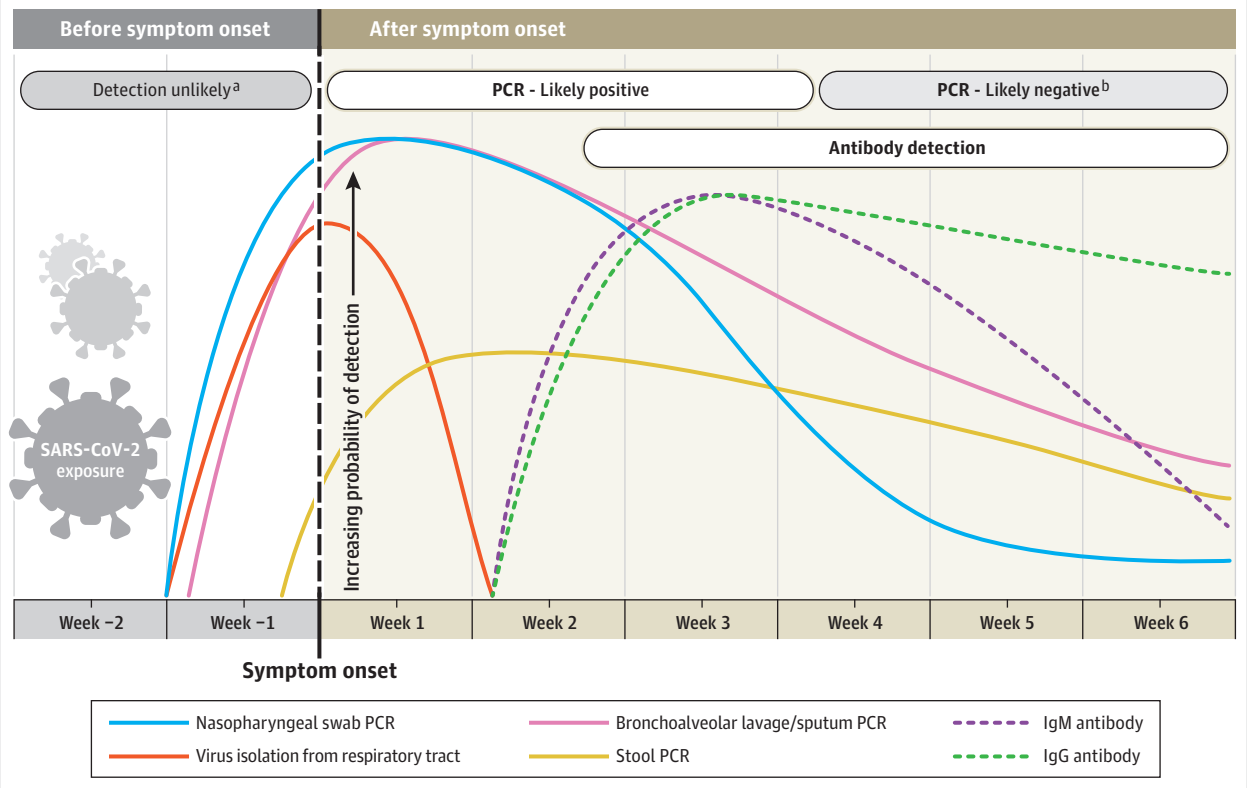
Detection of Antibodies to SARS-CoV-2

COVID-19 infection can also be detected indirectly by measuring the host immune response to SARS-CoV-2 infection. Serological diagnosis is especially important for patients with mild to moderate illness who may present late, beyond the first 2 weeks of illness onset. Serological diagnosis also is becoming an important tool to understand the extent of COVID-19 in the community and to identify individuals who are immune and potentially "protected" from becoming infected.

The most sensitive and earliest serological marker is total antibodies, levels of which begin to increase from the second week of symptom onset.[6] Although IgM and IgG ELISA have been found to be positive even as early as the fourth day after symptom onset, higher levels occur in the second and third week of illness.

For example, IgM and IgG seroconversion occurred in all patients between the third and fourth week of clinical illness onset as measured in 23 patients by To et al[7] and 85 patients by Xiang et al.[8] Thereafter IgM begins to decline and reaches lower levels by week 5 and almost disappears by week 7,

Figure. Estimated Variation Over Time in Diagnostic Tests for Detection of SARS-CoV-2 Infection Relative to Symptom Onset



Estimated time intervals and rates of viral detection are based on data from several published reports. Because of variability in values among studies, estimated time intervals should be considered approximations and the probability of detection of SARS-CoV-2 infection is presented qualitatively. SARS-CoV-2 indicates severe acute respiratory syndrome coronavirus 2; PCR, polymerase chain reaction.

[a] Detection only occurs if patients are followed up proactively from the time of exposure.

[b] More likely to register a negative than a positive result by PCR of a nasopharyngeal swab.

whereas IgG persists beyond 7 weeks.[9] In a study of 140 patients, combined sensitivity of PCR and IgM ELISA directed at nucleocapsid (NC) antigen was 98.6% vs 51.9% with a single PCR test. During the first 5.5 days, quantitative PCR had a higher positivity rate than IgM, whereas IgM ELISA had a higher positivity rate after day 5.5 of illness.[10]

ELISA-based IgM and IgG antibody tests have greater than 95% specificity for diagnosis of COVID-19. Testing of paired serum samples with the initial PCR and the second 2 weeks later can further increase diagnostic accuracy. Typically, the majority of antibodies are produced against the most abundant protein of the virus, which is the NC. Therefore, tests that detect antibodies to NC would be the most sensitive. However, the receptor-binding domain of S (RBD-S) protein is the host attachment protein, and antibodies to RBD-S would be more specific and are expected to be neutralizing. Therefore, using one or both antigens for detecting IgG and IgM would result in high sensitivity.[7] Antibodies may, however, have cross-reactivity with SARS-CoV and possibly other coronaviruses.

Rapid point-of-care tests for detection of antibodies have been widely developed and marketed and are of variable quality. Many manufacturers do not reveal the nature of antigens used. These tests are purely qualitative in nature and can only indicate the presence or absence of SARS-CoV-2 antibodies. The presence of neutralizing antibodies can only be confirmed by a plaque reduction neutralization test. However, high titers of IgG antibodies detected by ELISA have been shown to positively correlate with neutralizing antibodies.[7] The long-term persistence and duration of protection conferred by the neutralizing antibodies remains unknown.

Conclusions

Using available evidence, a clinically useful timeline of diagnostic markers for detection of COVID-19 has been devised (Figure). Most of the available data are for adult populations who are not immunocompromised. The time course of PCR positivity and seroconversion may vary in children and other groups, including the large population of asymptomatic individuals who go undiagnosed without active surveillance. Many questions remain, particularly how long potential immunity lasts in individuals, both asymptomatic and symptomatic, who are infected with SARS-CoV-2.

ARTICLE INFORMATION

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PRACTICE

PRACTICE POINTER

Interpreting a covid-19 test result

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What you need to know

- Interpreting the result of a test for covid-19 depends on two things: the accuracy of the test, and the pre-test probability or estimated risk of disease before testing
- A positive RT-PCR test for covid-19 test has more weight than a negative test because of the test's high specificity but moderate sensitivity
- A single negative covid-19 test should not be used as a rule-out in patients with strongly suggestive symptoms
- Clinicians should share information with patients about the accuracy of covid-19 tests

Across the world there is a clamour for covid-19 testing, with Tedros Adhanom Ghebreyesus, director general of the World Health Organization, encouraging countries to "test, test, test."[1] The availability of the complete genome of covid-19 early in the epidemic facilitated development of tests to detect viral RNA.[2] Multiple assays with different gene targets have been developed using reverse transcriptase polymerase chain reaction (RT-PCR).[3] These viral RNA tests use samples usually obtained from the respiratory tract by nasopharyngeal swab, to detect current infections. Serology blood tests to detect antibodies indicating past infection are being developed; these will not be considered in depth in this article.

Testing for covid-19 enables infected individuals to be identified and isolated to reduce spread,[4] allows contact tracing for exposed individuals,[5] and provides knowledge of regional and national rates of infection to inform public health interventions. However, questions remain on how to apply test results to make optimal decisions about individual patients.

Search strategy

This article was produced at speed to address an urgent need to address uncertainties in testing for covid-19. We searched Pubmed using the terms "covid", "SARS-CoV-2", "sensitivity", "specificity", "diagnosis", "test", and "PCR", and KSR evidence using terms for covid and test. This was supplemented by discussion with colleagues undertaking formal systematic reviews into covid-19 diagnosis.

How accurate are test results?

No test gives a 100% accurate result; tests need to be evaluated to determine their sensitivity and specificity, ideally by comparison with a "gold standard." The lack of such a clear-cut "gold-standard" for covid-19 testing makes evaluation of test accuracy challenging.

A systematic review of the accuracy of covid-19 tests reported false negative rates of between 2% and 29% (equating to sensitivity of 71-98%), based on negative RT-PCR tests which were positive on repeat testing.[6] The use of repeat RT-PCR testing as gold standard is likely to underestimate the true rate of false negatives, as not all patients in the included studies received repeat testing and those with clinically diagnosed covid-19 were not considered as actually having covid-19.[6]

Accuracy of viral RNA swabs in clinical practice varies depending on the site and quality of sampling. In one study, sensitivity of RT-PCR in 205 patients varied, at 93% for broncho-alveolar lavage, 72% for sputum, 63% for nasal swabs, and only 32% for throat swabs.[7] Accuracy is also likely to vary depending on stage of disease[8] and degree of viral multiplication or clearance.[9] Higher sensitivities are reported depending on which gene targets are used, and whether multiple gene tests are used in combination.[3 10] Reported accuracies are much higher for in vitro studies, which measure performance of primers using coronavirus cell culture in carefully controlled conditions.[2]

The lack of a clear-cut "gold-standard" is a challenge for evaluating covid-19 tests; pragmatically, clinical adjudication may be the best available "gold standard," based on repeat swabs, history, and contact with patients known to have covid-19, chest radiographs, and computed tomography scans. Inevitably this introduces some incorporation bias, where the test being evaluated forms part of the reference standard, and this would tend to inflate the measured sensitivity of these tests.[11] Disease prevalence can also affect estimates of accuracy: tests developed and evaluated in populations with high prevalence (eg, secondary care) may have lower sensitivity when applied in a lower prevalence setting (eg, primary care).[11]

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One community based study of 4653 close contacts of patients with covid-19 tested RT-PCR throat swabs every 48 hours during a 14 day quarantine period. Of 129 eventually diagnosed with covid-19 by RT-PCR, 92 (71.3%) had a positive test on the first throat swab, equating to a sensitivity of 71% in this lower prevalence, community setting.[12]

Further evidence and independent validation of covid-19 tests are needed.[13] As current studies show marked variation and are likely to overestimate sensitivity, we will use the lower end of current estimates from systematic reviews,[6] with the approximate numbers of 70% for sensitivity and 95% for specificity for illustrative purposes.

What do clinicians need to know to understand a test result?

Sensitivity and specificity can be confusing terms that may be misunderstood[14] (see supplementary file 'Definitions and formulae for calculating measures of test accuracy'). Sensitivity is the proportion of patients with disease who have a positive test, or the true positive rate. Specificity is the proportion of patients without disease who have a negative test, or true negative rate. These terms describe the operating characteristics of a test and can be used to gauge the credibility of a test result. They can be combined to calculate likelihood ratios, which are dimensionless numbers that indicate the strength of a positive or negative test result.[15] For calculating probabilities, a likelihood ratio can be used as a multiplier to convert pre-test odds to post-test odds. Positive likelihood ratios greater than 1 are progressively stronger, with 10 representing a very strong positive test result. Negative likelihood ratios less than 1 are also progressively stronger, with 0.1 representing a very strong negative test result. In the case of the nasopharyngeal swab RNA test for covid-19, the positive likelihood ratio is about 14, which is excellent.[6] A positive covid-19 test result should be very compelling. The negative likelihood ratio is 0.3, which is a moderate result, but not nearly as compelling as a positive result because of the moderate sensitivity (about 70%) of the covid-19 test.

Interpretation of a test result depends not only on the characteristics of the test itself but also on the pre-test probability of disease. Clinicians use a heuristic (a learned mental short cut) called anchoring and adjusting to settle on a pre-test probability (called the anchor). They then adjust this probability based on additional information. This heuristic is a useful short cut but comes with the potential for bias. When people fail to estimate the pre-test probability and only respond to a piece of new information, they commit a fallacy called base-rate neglect. Another fallacy called anchoring is failing adequately to adjust one's probability estimate, given the strength of new information. Likelihood ratios can give a clinician an idea of how much to adjust their probability estimates. Clinicians intuitively use anchoring and adjusting thoughtfully to estimate pre- and post-test probabilities unconsciously in everyday clinical practice. However, faced with a new and unfamiliar disease such as covid-19, mental short cuts can be uncertain and unreliable and public narrative about the definitive nature of testing can skew perceptions.

Figure 1 shows how a clinician's thinking about a patient's probability should shift, based on either a positive or negative test result for covid-19. First, the clinician should estimate a pre-test probability, using knowledge of local rates of covid-19 infection from national[16] and regional[17] data and patients' symptoms and signs,[18] likelihood of alternative diagnoses, and history of exposure to covid-19. After choosing a pre-test probability on the x axis, one should then trace up to either the upper curve for a positive test result or the lower curve for a negative test result, then trace over to the y axis to read the estimate for post-test probability. The figure shows that the shift in the probability is asymmetric, with a positive test result having a greater impact than a negative test result, owing to the modest sensitivity and negative likelihood ratio of the RNA test.

The infographic (fig 2) shows the outcomes when 100 people with a pre-test probability of 80% are tested for covid-19 using natural frequencies, which are generally easier to understand. Online calculators are available which allow clinicians to adjust pre-test probability, sensitivity, and specificity to estimate post-test probability[19]

What else should clinicians consider when interpreting test results?

A single negative test result may not be informative if the pre-test probability is high

A 52 year old general practitioner in London develops a cough, intermittent fever, and malaise. On day 2 of his illness he receives a nasopharyngeal swab test for covid-19, which is reported as negative. His cough and low-grade fever persist but he feels systemically well enough to return to work. What should he do?

Pre-test probability is high in someone with typical symptoms of covid-19, an occupational risk of exposure, and working in a high prevalence region, and negative test results can therefore be misleading. Table 1 shows that for a pre-test probability of 90%, someone with a negative test has a 74% chance of having covid-19; with two negative tests this risk is still around 47%. If this doctor were to return to work and subsequently the test was confirmed as a false negative, then the decision to work would potentially have significant consequences for his patients, colleagues, and everyone with whom he came into contact. It is therefore safest for this GP with strongly suggestive symptoms to self-isolate in line with guidelines for covid-19, even though his test results are negative. This case illustrates the fallacy of base-rate neglect; it can be tempting to trust the results of an "objective" test more than one's own "subjective" clinical judgement. In general, during this pandemic, pre-test probabilities of covid-19 will be high, particularly in high prevalence secondary care settings.

A possible alternative diagnosis will reduce the pre-test probability

A 73 year old woman with severe chronic obstructive pulmonary disease (COPD) and a chronic cough develops acute shortness of breath and slight worsening of her non-productive cough. She reports no fever, has no known exposure to covid-19, and no recent travel. She presents to an emergency department where she is acutely short of breath. A chest radiograph shows possible infiltrates in the right upper and middle lung fields. She is admitted and placed in isolation on droplet precautions. She requires intubation for worsening respiratory distress. Initial nasopharyngeal covid-19 testing is negative. Should she remain in isolation on droplet precautions?

This patient has an alternative possible diagnosis: community-acquired pneumonia. Given her lack of other risk factors or clinical symptoms, and chest radiography findings we therefore estimate her pre-test probability at about 50%. One negative test reduces this risk to 24%, the patient therefore has an additional independently sampled nasopharyngeal swab RNA

test which was negative, giving a post-test probability after two negative tests of less than 10%. She is treated with antibiotics and continues to recover.

What are the implications for practice and policy?

While positive tests for covid-19 are clinically useful, negative tests need to be interpreted with caution, taking into account the pre-test probability of disease. This has important implications for clinicians interpreting tests and policymakers designing diagnostic algorithms for covid-19. The Chinese handbook of covid-19 prevention and treatment states "*if the nucleic acid test is negative at the beginning, samples should continue to be collected and tested on subsequent days.*"[20] False negatives carry substantial risks; patients may be moved into non-covid-19 wards leading to spread of hospital acquired covid-19 infection,[21] carers could spread infection to vulnerable dependents, and healthcare workers risk spreading covid-19 to multiple vulnerable individuals. Clear evidence-based guidelines on repeat testing are needed, to reduce the risk of false negatives.

Clinicians should ensure that patients are counselled about the limitations of tests (box 1). Patients with a single negative test but strongly suggestive symptoms of covid-19 should be advised to self-isolate in keeping with guidelines for suspected covid-19.

Box 1: Possible phrases for explaining covid-19 testing to patients

- No test is 100% accurate
- If your swab test comes back positive for covid-19 then we can be very confident that you do have covid-19
- However, people with covid-19 can be missed by these swab tests. If you have strong symptoms of covid-19, it is safest to self-isolate, even if the swab test does not show covid-19

What is the role of serology tests?

Serology tests, which detect immunoglobulins including IgG and IgM, are under development,[22-24] with the aim of detecting individuals who have had previous infection and therefore theoretically developed immunity. The time course and accuracy of serology tests are still under investigation, but the same principles of incorporating the test result with the clinical impression applies. False positive serology tests could cause false reassurance, behaviour change, and disease spread. If suitable accuracy can be established, the benefits of these antibody tests include establishing when healthcare workers are immune, helping to inform decisions about the lifting of lockdowns, and allowing the population to return to work.[25]

The WHO message "test, test, test"[1] is important from a population perspective; low sensitivity can be accounted for when assessing burden of disease. However RT-PCR tests have limitations when used to guide decision making for individual patients. Positive tests can be useful to "rule-in" covid-19, a negative swab test cannot be considered definitive for "ruling out."

How patients were involved in the creation of this article

Patients with covid-19 or possible covid-19 were not involved in the writing of this paper for practical reasons

Education into practice

What is the protocol for covid-19 testing in your organisation?

How do you explain covid-19 test results to patients?

Reflect on a recent clinical case of suspected covid-19—what was your estimated pre-test probability? How did this alter with the results of tests?

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Table

Table 1| Pre- and post- test probabilities for covid-19 RT-PCR tests, calculations based on a sensitivity of 70% and specificity of 95%

Pre-test probability	Post-test probability, negative test	Post-test probability, two independently negative tests	Post-test probability positive test
5	1.6	0.5	42
15	5	2	71
25	10	3	82
50	24	9	93
75	49	23	98
90	74	47	99

Figures

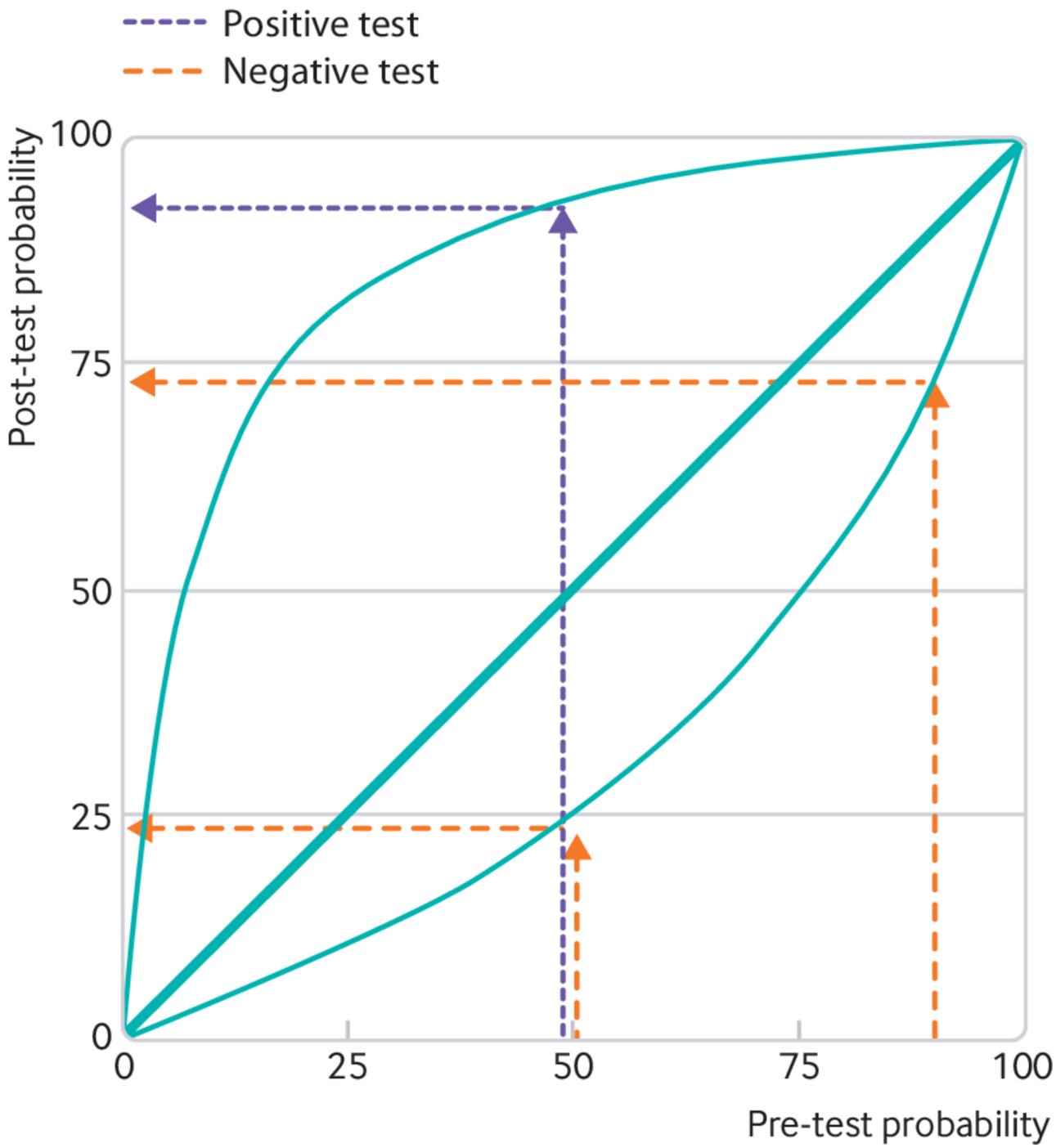


Fig 1 Leaf plot for covid-19 RT-PCR tests based on a sensitivity of 70% and specificity of 95%. The x axis gives the estimated pre-test probability of covid-19 based on the clinical details. The post-test probability is obtained by tracing up and across to the y axis from the lower curve for a negative test, or to the upper curve for a positive test result. The dashed lines illustrate pre-test probability of 90% (clinical case 1) and 50% (clinical case 2)

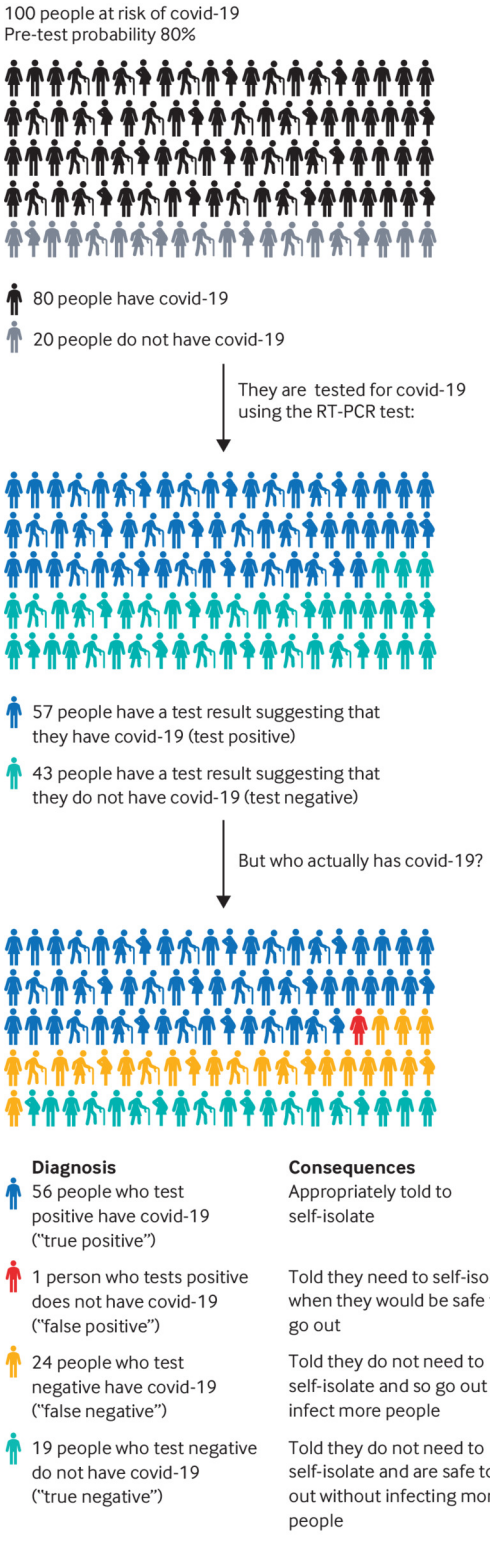


Fig 2 Infographic showing outcomes of 100 people who are tested for covid-19

Table 1

Primers and probes, real-time RT-PCR for 2019 novel coronavirus

Assay/use	Oligonucleotide	Sequence[a]	Concentration[b]
RdRP gene	RdRp_SARSr-F	GTGARATGGTCATGTGTGGCGG	Use 600 nM per reaction
	RdRp_SARSr-P2	FAM-CAGGTGGAACCTCATCAGGAGATGC-BBQ	Specific for 2019-nCoV, will not detect SARS-CoV. Use 100 nM per reaction and mix with P1
	RdRP_SARSr-P1	FAM-CCAGGTGGWACRTCATCMGGTGATGC-BBQ	Pan Sarbeco-Probe will detect 2019-nCoV, SARS-CoV and bat-SARS-related CoVs. Use 100 nM per reaction and mix with P2
	RdRp_SARSr-R	CARATGTTAAASACACTATTAGCATA	Use 800 nM per reaction
E gene	E_Sarbeco_F	ACAGGTACGTTAATAGTTAATAGCGT	Use 400 nm per reaction
	E_Sarbeco_P1	FAM-ACACTAGCCATCCTTACTGCGCTTCG-BBQ	Use 200 nm per reaction
	E_Sarbeco_R	ATATTGCAGCAGTACGCACACA	Use 400 nm per reaction
N gene	N_Sarbeco_F	CACATTGGCACCCGCAATC	Use 600 nm per reaction
	N_Sarbeco_P	FAM-ACTTCCTCAAGGAACAACATTGCCA-BBQ	Use 200 nm per reaction
	N_Sarbeco_R	GAGGAACGAGAAGAGGCTTG	Use 800 nm per reaction

[a] W is A/T; R is G/A; M is A/C; S is G/C. FAM: 6-carboxyfluorescein; BBQ: blackberry quencher.

[b] Optimised concentrations are given in nanomol per litre (nM) based on the final reaction mix, e.g. 1.5 µL of a 10 µM primer stock solution per 25 µL total reaction volume yields a final concentration of 600 nM as indicated in the table.

health emergencies by coordination between public and academic laboratories [6-12]. In all of these situations, virus isolates were available as the primary substrate for establishing and controlling assays and assay performance.

In the present case of 2019-nCoV, virus isolates or samples from infected patients have so far not become available to the international public health community. We report here on the establishment and validation of a diagnostic workflow for 2019-nCoV screening and specific confirmation, designed in absence of available virus isolates or original patient specimens. Design and validation were enabled by the close genetic relatedness to the 2003 SARS-CoV, and aided by the use of synthetic nucleic acid technology.

Methods

Clinical samples and coronavirus cell culture supernatants for initial assay evaluation

Cell culture supernatants containing typed coronaviruses and other respiratory viruses were provided by Charité and University of Hong Kong research laboratories. Respiratory samples were obtained during 2019 from patients hospitalised at Charité medical centre and tested by the NxTAG respiratory pathogen panel (Luminex, S´Hertogenbosch, The Netherlands) or in cases of MERS-CoV by the MERS-CoV upE assay as published before [10]. Additional samples were selected from biobanks at the Rijksinstituut voor Volksgezondheid en Milieu (RIVM), Bilthoven, at Erasmus University Medical Center, Rotterdam, at Public Health England (PHE), London, and at the University of Hong Kong. Samples from all collections comprised sputum as well as nose and throat swabs with or without viral transport medium.

Faecal samples containing bat-derived SARS-related CoV samples (identified by GenBank accession numbers) were tested: KC633203, Betacoronavirus BtCoV/Rhi_eur/BB98–98/BGR/2008; KC633204, Betacoronavirus BtCoV/Rhi_eur/BB98–92/BGR/2008; KC633201, Betacoronavirus BtCoV/Rhi_bla/BB98–22/BGR/2008; GU190221 Betacoronavirus Bat coronavirus BR98–19/BGR/2008; GU190222 Betacoronavirus Bat coronavirus BM98–01/BGR/2008; GU190223, Betacoronavirus Bat coronavirus BM98–13/BGR/2008. All synthetic RNA used in this study was photometrically quantified.

RNA extraction

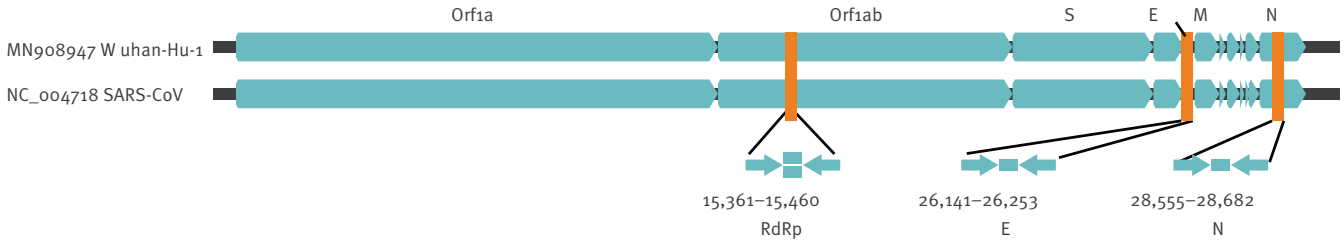
RNA was extracted from clinical samples with the MagNA Pure 96 system (Roche, Penzberg, Germany) and from cell culture supernatants with the viral RNA mini kit (QIAGEN, Hilden, Germany).

Real-time reverse-transcription PCR

A 25 µL reaction contained 5 µL of RNA, 12.5 µL of 2 × reaction buffer provided with the Superscript III one step RT-PCR system with Platinum Taq Polymerase (Invitrogen, Darmstadt, Germany; containing 0.4 mM of each deoxyribont triphosphates (dNTP) and 3.2 mM magnesium sulphate), 1 µL of reverse transcriptase/Taq mixture from the kit, 0.4 µL of a 50 mM magnesium sulphate solution (Invitrogen), and 1 µg of nonacetylated bovine serum albumin (Roche). Primer and probe sequences, as well as optimised concentrations are shown in Table 1. All oligonucleotides were synthesised and provided by Tib-Molbiol (Berlin,

Figure 1

Relative positions of amplicon targets on the SARS coronavirus and the 2019 novel coronavirus genome



E: envelope protein gene; M: membrane protein gene; N: nucleocapsid protein gene; ORF: open reading frame; RdRp: RNA-dependent RNA polymerase gene; S: spike protein gene.

Numbers below amplicons are genome positions according to SARS-CoV, GenBank NC_004718.

Germany). Thermal cycling was performed at 55 °C for 10 min for reverse transcription, followed by 95 °C for 3 min and then 45 cycles of 95 °C for 15 s, 58 °C for 30 s. Participating laboratories used either Roche Light Cycler 480II or Applied Biosystems ViiA7 instruments (Applied Biosystems, Hong Kong, China).

Protocol options and application notes

Laboratories participating in the evaluation used the TaqMan Fast Virus 1-Step Master Mix (Thermo Fisher) with the same oligonucleotide concentrations and cycling conditions. The QIAGEN One-Step RT-PCR Kit was also tested and found to be compatible.

The intended cross-reactivity of all assays with viral RNA of SARS-CoV allows us to use the assays without having to rely on external sources of specific 2019-nCoV RNA.

For a routine workflow, we recommend the E gene assay as the first-line screening tool, followed by confirmatory testing with the RdRp gene assay. Application of the RdRp gene assay with dual colour technology can discriminate 2019-nCoV (both probes positive) from SARS-CoV RNA if the latter is used as positive control. Alternatively, laboratories may choose to run the RdRp assay with only the 2019-nCoV-specific probe.

Ethical statement

The internal use of samples for diagnostic workflow optimisation was agreed under the medical ethical rules of each of the participating partners.

Results

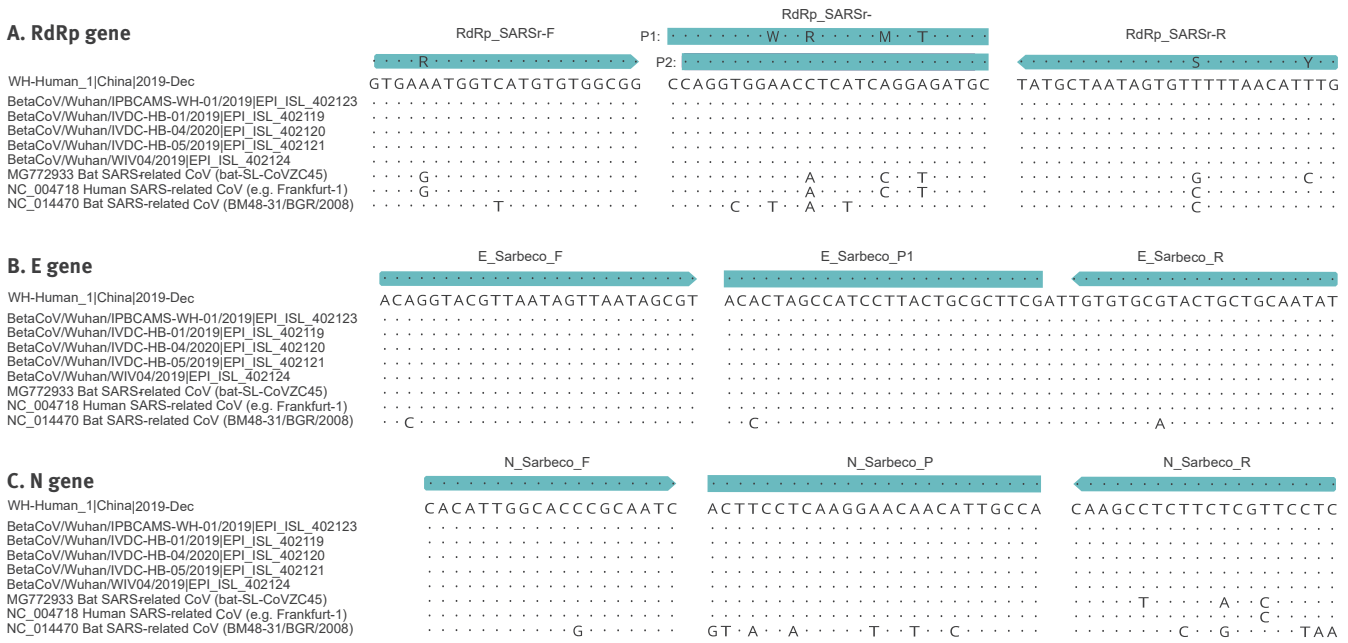
Before public release of virus sequences from cases of 2019-nCoV, we relied on social media reports announcing detection of a SARS-like virus. We thus assumed that a SARS-related CoV is involved in the outbreak. We downloaded all complete and partial (if>400 nt) SARS-related virus sequences available in GenBank by 1 January 2020. The list (n=729 entries) was manually checked and artificial sequences (laboratory-derived, synthetic, etc), as well as sequence duplicates were removed, resulting in a final list of 375 sequences. These sequences were aligned and the alignment was used for assay design (Supplementary Figure S1). Upon release of the first 2019-nCoV sequence at virological.org, three assays were selected based on how well they matched to the 2019-nCoV genome (Figure 1). The alignment was complemented by additional sequences released independently on GISAID (https://www.gisaid.org), confirming the good matching of selected primers to all sequences. Alignments of primer binding domains with 2019-nCoV, SARS-CoV as well as selected bat-associated SARS-related CoV are shown in Figure 2.

Assay sensitivity based on SARS coronavirus virions

To obtain a preliminary assessment of analytical sensitivity, we used purified cell culture supernatant containing SARS-CoV strain Frankfurt-1 virions grown on Vero cells. The supernatant was ultrafiltered and thereby concentrated from a ca 20-fold volume of cell culture supernatant. The concentration step simultaneously reduces the relative concentration of background nucleic acids such as not virion-packaged viral RNA. The virion preparation was quantified by real-time RT-PCR using a specific in vitro-transcribed RNA quantification standard as described in Drosten et al. [8]. All assays were subjected to replicate testing in order to determine stochastic detection frequencies at each assay's sensitivity end point (Figure 3A and B). All assays were highly sensitive, with best results obtained for the E gene and RdRp gene assays (5.2 and 3.8 copies per reaction at 95% detection probability, respectively). These two assays were chosen for further evaluation. One of the laboratories participating in the external evaluation used other basic RT-PCR reagents (TaqMan Fast Virus 1-Step Master Mix) and repeated the sensitivity study, with equivalent results (E gene: 3.2 RNA copies/reaction (95% CI: 2.2–6.8); RdRP: 3.7 RNA copies/reaction (95% CI: 2.8–8.0). Of note, the N gene assay also performed well but was not subjected

Figure 2

Partial alignments of oligonucleotide binding regions, SARS-related coronaviruses (n = 9)



The panels show six available sequences of 2019-nCoV, aligned to the corresponding partial sequences of SARS-CoV strain Frankfurt 1, which can be used as a positive control for all three RT-PCR assays. The alignment also contains a closely related bat virus (Bat SARS-related CoV isolate bat-SL-CoVZC45, GenBank accession number MG772933) as well as the most distant member within the SARS-related bat CoV clade, detected in Bulgaria (GenBank accession number NC_014470). Dots represent identical nucleotides compared with the WH_Human_1 sequence. Nucleotide substitutions are specified. Blue arrows: oligonucleotides as specified in Table 1. More comprehensive alignments can be found in the Supplement.

to intensive further validation because it was slightly less sensitive (Supplementary Figure S2)

Sensitivity based on in vitro-transcribed RNA identical to 2019 novel coronavirus target sequences

Although both assays detected 2019-nCoV without polymorphisms at oligonucleotide binding sites (Figure 2), we additionally generated in vitro-transcribed RNA standards that exactly matched the sequence of 2019-nCoV for absolute quantification and studying the limit of detection (LOD). Replicate reactions were done at concentrations around the detection end point determined in preliminary dilution experiments. The resulting LOD from replicate tests was 3.9 copies per reaction for the E gene assay and 3.6 copies per reaction for the RdRp assay (Figure 3C and D). These figures were close to the 95% hit rate of 2.9 copies per reaction, according to the Poisson distribution, expected when one RNA molecule is detected.

Discrimination of 2019 novel coronavirus from SARS coronavirus by RdRp assay

Following the rationale that SARS-CoV RNA can be used as a positive control for the entire laboratory procedure, thus obviating the need to handle 2019-nCoV RNA, we formulated the RdRp assay so that it contains two probes: a broad-range probe reacting with SARS-CoV and 2019-nCoV and an additional probe that reacts only with 2019-nCoV. By limiting dilution experiments, we confirmed that both probes, whether used individually or in combination, provided the same LOD for each target virus. The specific probe RdRP_SARSr-P2 detected only the 2019-nCoV RNA transcript but not the SARS-CoV RNA.

Detection range for SARS-related coronaviruses from bats

At present, the potential exposure to a common environmental source in early reported cases implicates the possibility of independent zoonotic infections with increased sequence variability [5]. To show that the assays can detect other bat-associated SARS-related viruses, we used the E gene assay to test six bat-derived faecal samples available from Drexler et al. [13] und Muth et al. [14]. These virus-positive samples stemmed from European rhinolophid bats. Detection of these phylogenetic outliers within the SARS-related CoV clade suggests that all Asian viruses are likely to be detected. This would, theoretically, ensure broad sensitivity even in case of multiple independent acquisitions of variant viruses from an animal reservoir.

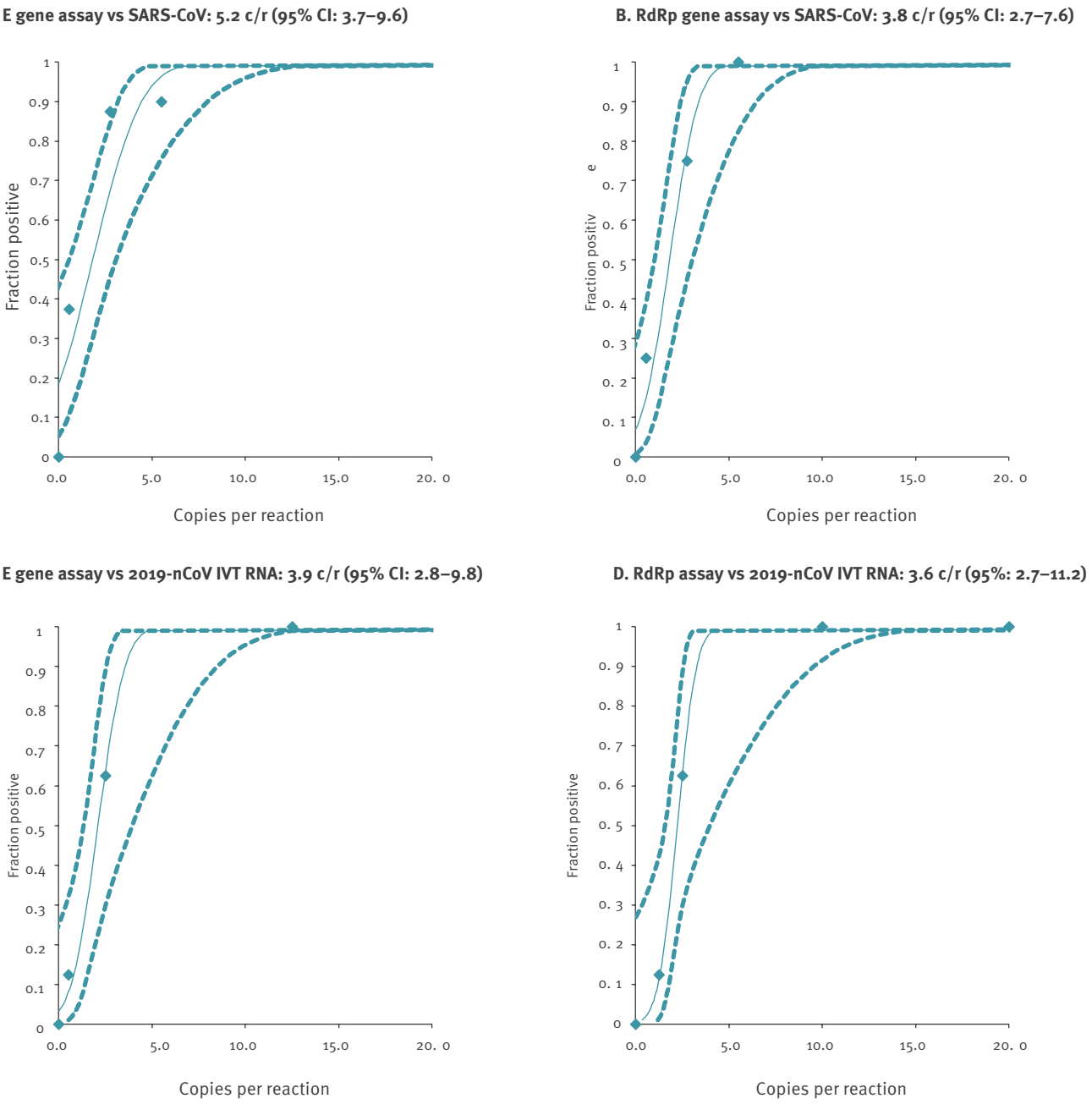
Specificity testing

Chemical stability

To exclude non-specific reactivity of oligonucleotides among each other, causing artificial fluorescent

Figure 3

Determination of limits of detection based on SARS coronavirus genomic RNA and 2019 novel coronavirus-specific in vitro transcribed RNA



CI: confidence intervals; c/r: copies per reaction; IVT: in vitro-transcribed RNA.

A: E gene assay, evaluated with SARS-CoV genomic RNA. B: RdRp gene assay evaluated with SARS-CoV genomic RNA. C: E-gene assay, evaluated with 2019-nCoV-specific in vitro-transcribed RNA standard. D: RdRp gene assay evaluated with 2019-nCoV-specific in vitro-transcribed RNA standard.

The x-axis shows input RNA copies per reaction. The y-axis shows positive results in all parallel reactions performed, squares are experimental data points resulting from replicate testing of given concentrations (x-axis) in parallels assays (eight replicate reactions per point).

Technical limits of detection are given in the panels headings. The inner line is a probit curve (dose-response rule). The outer dotted lines are 95% CI.

Table 2

Tests of known respiratory viruses and bacteria in clinical samples and cell culture preparations for cross-reactivity in 2019 novel coronavirus E and RdRp gene assays (n = 310)

Clinical samples with known viruses	Clinical samples[a]	Virus isolates[b]
HCoV-HKU1	14	1[c]
HCoV-OC43	16	2[d]
HCoV-NL63	14	1[e]
HCoV-229E	18	2[f]
MERS-CoV	5	1[g]
Influenza A(H1N1)pdm09	17	1
Influenza A(H3N2)	16	1
Influenza A (untyped)	11	NA
Influenza A(H5N1)	1	1
Influenza A(H7N9)	0	1
Influenza B (Victoria or Yamagata)	31	1
Rhinovirus/enterovirus	31	NA
Respiratory syncytial virus (A/B)	33	NA
Parainfluenza 1 virus	12	NA
Parainfluenza 2 virus	11	NA
Parainfluenza 3 virus	14	NA
Parainfluenza 4 virus	11	NA
Human metapneumovirus	16	NA
Adenovirus	13	1
Human bocavirus	6	NA
Legionella spp.	3	NA
Mycoplasma spp.	4	NA
Total clinical samples	**297**	**NA**

[a] For samples with multiple viruses detected, the virus with highest concentration is listed, as indicated by real-time PCR Ct value.

[b] Directly quantified or spiked in human negative-testing sputum.

[c] 1×10^5 RNA copies/mL, determined by specific real-time RT-PCR. Isolated from human airway epithelial culture.

[d] 1×10^{10} RNA copies/mL, determined by specific real-time RT-PCR of one isolate. The other isolate was not quantified but spiked in human negative-testing sputum.

[e] 4×10^9 RNA copies/mL, determined by specific real-time RT-PCR.

[f] 3×10^9 RNA copies/mL, determined by specific real-time RT-PCR of one isolate. The other isolate was not quantified spiked in human negative-testing sputum.

[g] 1×10^8 RNA copies/mL, determined by specific real-time RT-PCR.

signals, all assays were tested 120 times in parallel with water and no other nucleic acid except the provided oligonucleotides. In none of these reactions was any positive signal detected.

Cross-reactivity with other coronaviruses

Cell culture supernatants containing all endemic human coronaviruses (HCoV)229E, NL63, OC43 and HKU1 as well as MERS-CoV were tested in duplicate in all three assays (Table 2). For the non-cultivable HCoV-HKU1, supernatant from human airway culture was used. Viral RNA concentration in all samples was determined by specific real-time RT-PCRs and in vitro-transcribed RNA standards designed for absolute quantification of viral load. Additional undiluted (but not quantified) cell culture supernatants were tested as summarised in Table 2. These were additionally mixed into negative human sputum samples. None of the tested viruses or virus preparations showed reactivity with any assay.

Exclusivity of 2019 novel coronavirus based on clinical samples pre-tested positive for other respiratory viruses

Using the E and RdRp gene assays, we tested a total of 297 clinical samples from patients with respiratory disease from the biobanks of five laboratories that provide diagnostic services (one in Germany, two in the Netherlands, one in Hong Kong, one in the UK). We selected 198 samples from three university medical centres where patients from general and intensive care wards as well as mainly paediatric outpatient departments are seen (Germany, the Netherlands, Hong Kong). The remaining samples were contributed by national public health services performing surveillance studies (RIVM, PHE), with samples mainly submitted by practitioners. The samples contained the broadest range of respiratory agents possible and reflected the general spectrum of virus concentrations encountered in diagnostic laboratories in these countries (Table 2). In total, this testing yielded no false positive outcomes. In four individual test reactions, weak initial reactivity was seen but they were negative upon retesting with the same assay. These signals were not associated with any particular virus, and for each virus with which initial positive reactivity occurred, there were other samples that contained the same virus at a higher concentration but did not test positive. Given the results from the extensive technical qualification described above, it was concluded that this initial reactivity was not due to chemical instability of real-time PCR probes but most probably to handling issues caused by the rapid introduction of new diagnostic tests and controls during this evaluation study.

Discussion

The present report describes the establishment of a diagnostic workflow for detection of an emerging virus in the absence of physical sources of viral genomic nucleic acid. Effective assay design was enabled by the willingness of scientists from China to share genome information before formal publication, as well as the availability of broad sequence knowledge from ca 15 years of investigation of SARS-related viruses in animal reservoirs. The relative ease with which assays could be designed for this virus, in contrast to SARS-CoV in 2003, proves the huge collective value of descriptive studies of disease ecology and viral genome diversity [8,15-17].

Real-time RT-PCR is widely deployed in diagnostic virology. In the case of a public health emergency, proficient diagnostic laboratories can rely on this robust technology to establish new diagnostic tests within their routine services before pre-formulated assays become available. In addition to information on

reagents, oligonucleotides and positive controls, laboratories working under quality control programmes need to rely on documentation of technical qualification of the assay formulation as well as data from external clinical evaluation tests. The provision of control RNA templates has been effectively implemented by the EVAg project that provides virus-related reagents from academic research collections [18]. SARS-CoV RNA was retrievable from EVAg before the present outbreak; specific products such as RNA transcripts for the here-described assays were first retrievable from the EVAg online catalogue on 14 January 2020 (https://www.european-virus-archive.com). Technical qualification data based on cell culture materials and synthetic constructs, as well as results from exclusivity testing on 75 clinical samples, were included in the first version of the diagnostic protocol provided to the WHO on 13 January 2020. Based on efficient collaboration in an informal network of laboratories, these data were augmented within 1 week comprise testing results based on a wide range of respiratory pathogens in clinical samples from natural infections. Comparable evaluation studies during regulatory qualification of in vitro diagnostic assays can take months for organisation, legal implementation and logistics and typically come after the peak of an outbreak has waned. The speed and effectiveness of the present deployment and evaluation effort were enabled by national and European research networks established in response to international health crises in recent years, demonstrating the enormous response capacity that can be released through coordinated action of academic and public laboratories [18-22]. This laboratory capacity not only supports immediate public health interventions but enables sites to enrol patients during rapid clinical research responses.

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Conflict of interest

None declared.

Authors' contributions

VMC: Planned and conducted experiments, conceptualised the laboratory work

OL: Planned and conducted experiments, conceptualised the laboratory work

MK: Planned and conducted experiments

RM: Planned and conducted experiments, conceptualised the laboratory work

AM: Planned and conducted experiments, conceptualised the laboratory work

DKWC: Planned and conducted experiments

TB: Planned and conducted experiments

SB: Planned and conducted experiments

JS: Planned and conducted experiments

MLS: Planned and conducted experiments

DGJCM: Planned and conducted experiments

BLH: Planned and conducted experiments

BvdV: Planned and conducted experiments

SvdB: Planned and conducted experiments

LW: Planned and conducted experiments

GG: Planned and conducted experiments

JLR: Contributed to overall study conceptualization

JE: Planned and conducted experiments, conceptualised the laboratory work

MZ: Planned laboratory work, contributed to overall study conceptualization

MP: Planned laboratory work, contributed to overall study conceptualization

HG: Contributed to overall study conceptualization

CR: Planned experiments, conceptualised the laboratory work

MPGK: Planned experiments, conceptualised the laboratory work

CD: Planned experiments, conceptualised the laboratory work, conceptualised the overall study, wrote the manuscript draft.

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