Clinical Tests and COVID-19

COVID-19 Testing, How It Is Done and What It Means

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Paul W. Dillon, Ph.D.
Co-chair and Co-Program Director, Westchester Chemical Society
A subsection of the NY Section of the American Chemical Society
Patient Advisor, ILD Collaborative

ILD Collaborative

<u>Technical Discussion Overview about COVID-19</u>

- Availability of COVID-19 tests
- Understanding Clinical Testing
- COVID-19 Virus Structure
- Types of Clinical Tests (diagnostic-viral nucleic acid, viral antigen, and serological-antibodies to the virus)
- Qualitative Tests: sensitivity, specificity, predictive values, prevalence.
- Concerns about possible False Positives with antibody tests

Covid-19 Testing **<u>Deliverables</u>**:

Establish **<u>Genetic Sequence</u>** (mid January 2020)

Discover and define **<u>Analytic Tests</u>**

includes not only complex biologicals such as nucleic acids, antibodies and enzymes but also, ancillaries, such as solvents, buffers, detergents

Define **<u>Testing Procedure</u>**

For adding reagents, reaction times, sample collection, handling, storing and preparation, data capture and reporting

Determine **<u>Stability of Shipping and Storage</u>**

Validate and verify that test performance on Day 1 at manufacturing site compares to performance when sent cross country and used after a month

Evaluate **<u>Calibrators</u>** to convert test response to a medically meaningful value **<u>and Controls</u>** to ensure test system and reagents are working properly

Considerations why not enough tests and test materials are immediately available

COVID-19 is a new virus whose structure only became fully elucidated in January.

Usually it takes a year or more to develop new tests using standard formats and instruments.

Other major deliverables for COVID-19 tests were discussed above.

Manufacturers of tests and testing equipment, and laboratories also have a range of other urgent testing – people are still having, for example, heart attacks, that must be addressed. Every resource cannot be devoted to COVID-19.

My perspective as someone who has worked in the Clinical Diagnostics industry: We have done remarkably well in generating, getting (emergency) approvals, and producing the tests.

Because of the urgency of this pandemic, we are not sacrificing the good for the perfect. FDA has issued **Emergency Use Authorizations**, rather than requiring full, normal approval studies, for a range of tests starting February 4. Through June 1, it has issued:

Viral RNA tests –   69

plus   34 for private labs operating under the Clinical Lab Improvement Act

Antigen tests -   1

Antibody tests -   15

Understanding Clinical Testing

1. These lab tests are typically done on body fluids, most commonly blood but also urine, cerebrospinal fluid (spine tap), amniotic fluid, sputum, even breath, and others, less commonly.

2. Tissue samples, such as the nasopharygeal swabs used for much COVID-19 testing are dispersed in fluids (which often disrupt the virus).

3. Tests are usually performed on automated instruments. These conduct chemical reactions which generate a measurable signal: color change, light absorbance, light scattering, electrical potential, etc. The signal is converted to a concentration by measuring calibrators of know concentration.

Understanding Clinical Testing (continued)

4. Measured response is generally quantitative and not medically meaningful. The test is used on one or more artificial samples, calibrators, which contain medically meaningful amounts of the measurand (usually its concentration). This allows conversion of the test system's response to a medically meaningful value.

5. However, even with quantitative system responses, many tests are qualitative. Usually there is a cutoff value below which the test is Negative and above which it is Positive. Straddling the cutoff there may be an equivocal and/or retest range.

6. All of the COVID-19 tests are qualitative!

Virus Structure:

Like all viruses, it consists of

• nucleic acid - RNA in this case

• inside a protective protein capsule (the nucleocapsid protein)

• with proteins needed to take over its host cell, reproduce & escape

• plus a covering of "corona" - spike proteins which gain access to the host through a cell-membrane receptor (ACE2)

Spike protein

RNA

Cell membrane

ACE2
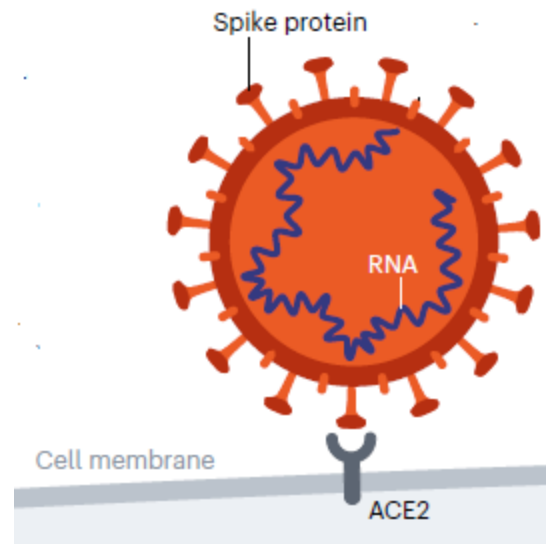
There are two types of **diagnostic tests** for COVID-19. The first step in either test type does is use chemicals to break apart the viral particles, accessing its nucleic acid and proteins.

Most are tests that find and quantify its nucleic acid (or certain COVID-19 specific sequences of it). These use an enzyme to translate its RNA into the complementary DNA. This is then multiplied, generally using a technique call **Polymerase Chain Reaction** (PCR).

This uses DNA sequences (often "Molecular Beacons") that contain a fluorophore (a chemical group that fluoresces) and a quencher (a group that suppresses the fluorescence). It also uses enzymes to extend these sequences, separating the fluorophore and quencher. The amount of viral DNA is then estimated by measuring **the fluorescence**.
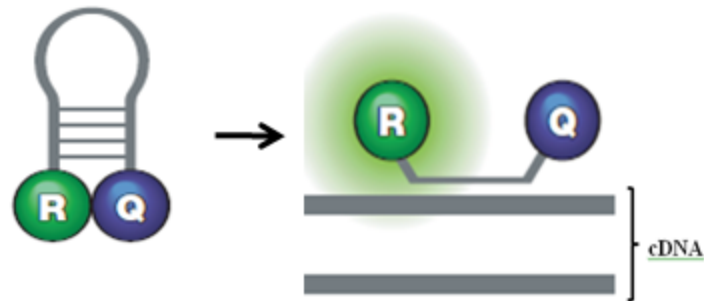
REVERSE TRANSCRIPTASE BUILDS DNA COMPLEMENATARY TO VIRAL RNA

cDNA

Viral RNA

POLYMERASE CHAIN REACTION AMPLIFIES cDNA

REPORTER (FLUOPHORE) AND QUENCHER PROBE BIND TO DNA AND ARE EXTENDED, SEPARATING THEM AND SIGNALLING
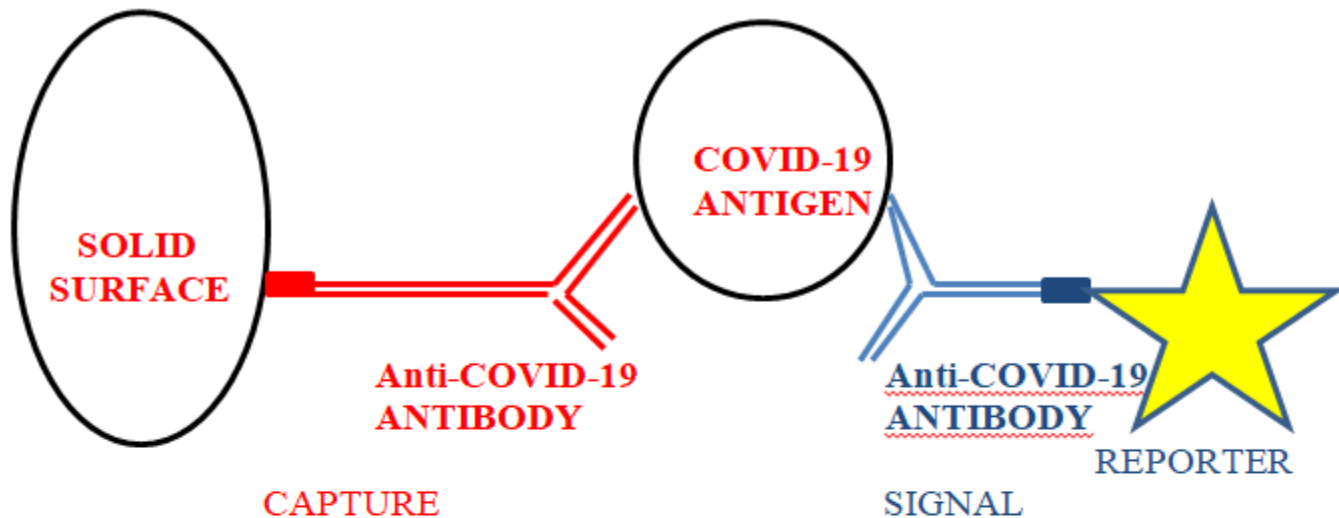

cDNA

Recently, FDA has approved an **antigen test**. Rather than finding and quantifying the nucleic acid, it measures the amount of one of the viral proteins.

This uses a classical **"sandwich" immunoassay**. An antibody (or other "capture" molecule) which bonds the antigen is chemically attached to a solid surface (this could be a magnetic particle). The sample is introduced, the antigen captured, and everything washed. Then a second antibody, chemically linked to some detector (like a fluorophore) is introduced, "sandwiching" the antigen between "capture" and "signal" antibodies. The signal is read.
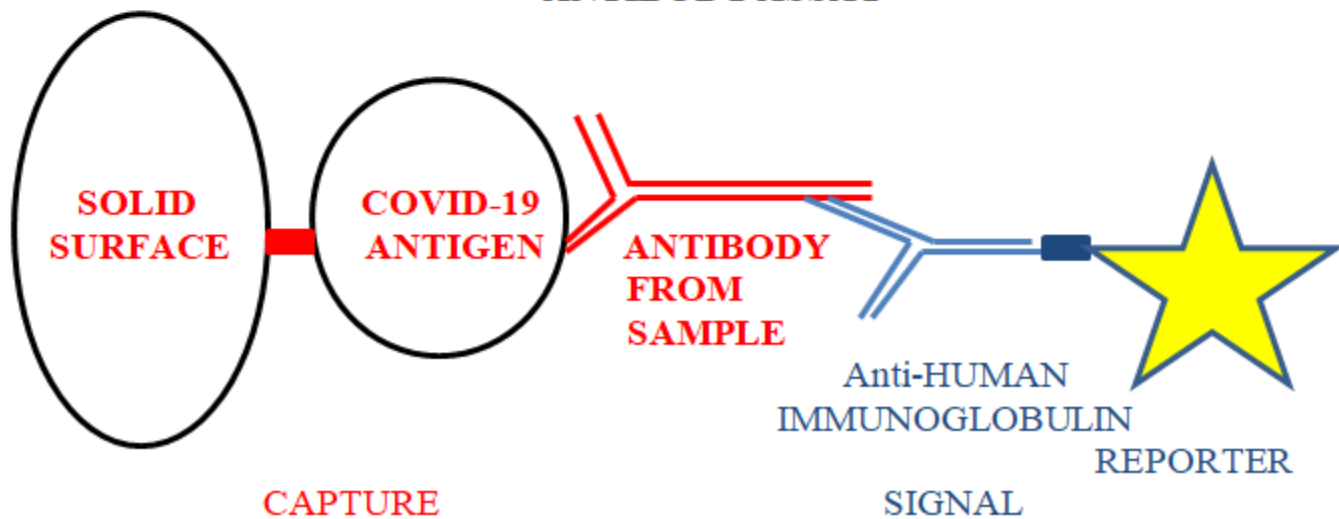
Finally are **serological** tests (tests for antibodies to COVID-19). These are like an inverse of a sandwich immunoassay.

The antigen is tethered to a solid surface. The sample is introduced, any antibody to the antigen captured, and everything washed. Then an antibody directed against human immunoglobulins, linked to the detector is introduced, "sandwiching" the antibody being tested for between "capture" antigen and "signal" anti-human-immunoglobulin antibodies. The signal is read.

ANTIGEN ASSAY

SOLID SURFACE

Anti-COVID-19 ANTIBODY

CAPTURE

COVID-19 ANTIGEN

Anti-COVID-19 ANTIBODY

SIGNAL

REPORTER

ANTIBODY ASSAY

SOLID SURFACE

COVID-19 ANTIGEN

ANTIBODY FROM SAMPLE

CAPTURE

Anti-HUMAN IMMUNOGLOBULIN

SIGNAL

REPORTER

In developing qualitative tests, 2x2 tables are determined. These are tables indicated the numbers of patients in the study with and without disease and with positive or negative tests:

FP = Number of False Positives (Test +, no disease)

TP = Number of True Positives (Test +, diseased)
TN = Number of True Negatives (Test -, no disease)

FN = Number of False Negatives (Test -, diseased)

D = Number diseased = TP + FN

T = Number testing positive = FP + TP

N = Total number = FP + TP + TN + FN

Sensitivity = Probability of getting a positive test from a diseased patient = TP/D = TP/(TP+FN)

Specificity = Probability of getting a negative test from a non-diseased patient

= TN/(N-D) = TN/(TN+FP)

These are often estimated during the evaluation of a test. A number of patients previously diagnosed with the disease are tested to estimate sensitivity. Similarly, a number of patients previously diagnosed to be disease free are tested to estimate specificity.

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Probably more important to physicians, but not as easily determined, are **predictive values**. These depend on the actual prevalence of the disease in the target population:

Prevalence = Probability of a patient having the disease
$$= D/N = (TP+FN)/(TP+FN+TN+FP)$$

CAUTION! In a study where the numbers of diseased and non-diseased patients are decided in advance, the calculated "prevalence" is only an "apparent prevalence". To get a true estimate of prevalence, patients must be chosen without regard to diagnosis, tested and then diagnosed.

With an good estimate of prevalence, we can then calculate:

Positive Predictive Value = PPV = Probability that a patient testing positive actually has the disease

Negative Predictive Value = NPV = Probability that a patient testing negative is disease-free

Given the prevalence, the sensitivity and the specificity, we can calculate the predictive values:

$$PPV = \frac{Sens \times Prev}{(Sens \times Pev) + [(1-Spec) \times (1-Prev)]}$$

$$NPV = \frac{Spec \times (1-Prev)}{[(1-Sens) \times Prev + [Spec \times (1-Prev)]}$$

Where: Sens=Sensitivity, Spec=Specificity, and
Prev=Prevalence

Note: for serological tests "diseased" is taken as "has antibodies".

I and some friends who are in clinical diagnostics had concerns about serological tests, namely that if many tests turn out to be positive, could they be false positives? I have looked at the data in the Instructions For Use for all the serological tests in FDA's Emergency Use Approvals database.

Data from 13 manufacturers were included. There were many, presumed negative (e.g., drawn before Sept. 2019, blood bank donors) as well as samples from patients who had diseases with potentially cross-reacting antibodies (e.g., influenzas). Altogether 14,949 samples from presumed negatives were assayed. Of these, only 113 tested positive (this includes any unresolved equivocal or retest samples), a false positive rate of 0.76% (95% confidence interval, 0.62-0.91%, specificity = 99.24%).

False positives are NOT a problem with the serological tests. One manufacturer, Roche, has almost one third (5,402) of the presumed negatives.

Interpretation

But, we do not know the prevalence of antibodies in the population. This means that we cannot specify the PPV of a positive test.

For example, to be conservative, let's assume that the specificity (and sensitivity) are each only 95%. Let's suppose that a population of 1,000 is investigated and that the prevalence is only 2%. Then 0.02x1000 = 20 cases are positive. With 95% sensitivity, only 19 tests are positive. With a 5% false positive rate, the 980 negative cases will have 49 positive tests. This gives a PPV of 19/68 = 28%. Thus, if you have a positive test, you are almost 3 times as likely to NOT have antibodies as to actually have them!

Interpretation (Continued)

Diagnostic tests are used in various ways, including:

- Diagnosis – Identifying the cause or nature of a patient's symptoms.

- Prognosis – Predicting the expected course or outcome of a disease.

- Screening – Checking for a disease marker in asymptomatic individuals.

- Monitoring – Following the course of a disease by repeated assays for specific markers. May be used to modify therapy.

The objective of a test may alter estimates of clinical relevance, such as predictive values. Thus, the PPV of a given test used in screening is probably less (because of lower prevalence) than diagnostic use of that test in a symptomatic patient.

PPV (%)
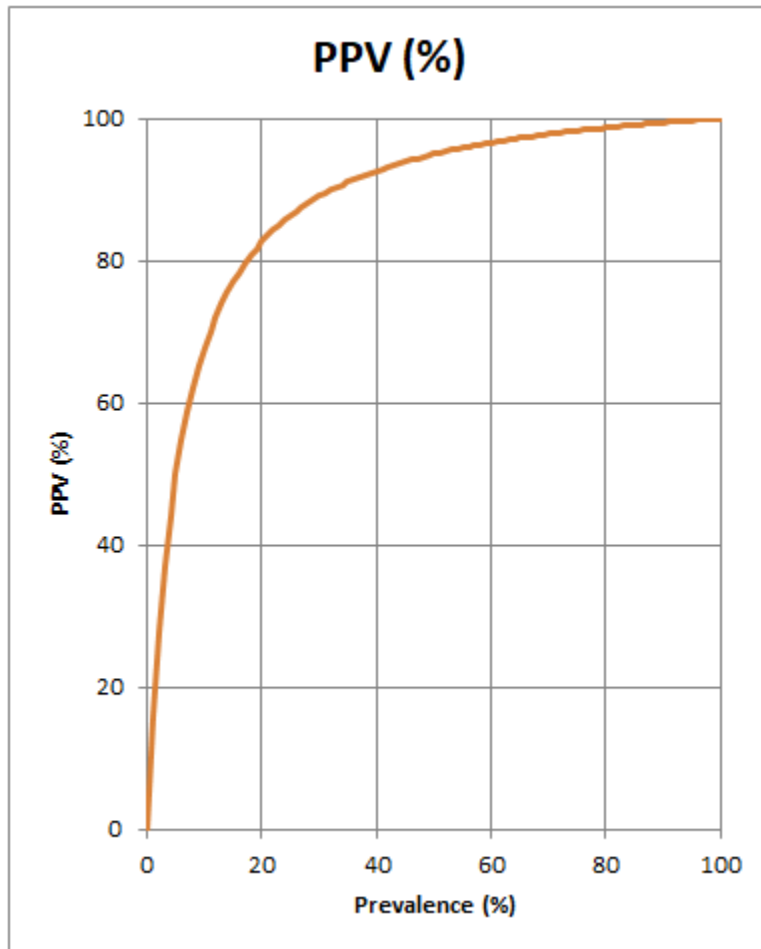
Note: A positive test has a 50:50 or greater chance of being correct if the prevalence is 5% or more.

A positive test has at least a 90% chance of being correct if the prevalence is greater than 32%.

However, even if predictive values for individual patients cannot be made without prevalence estimates, antibody testing of large populations can generate reasonable estimates of prevalence, as seen in the following slide.

Note: the models used to generate the previous and following slides are detailed, for those interested, at the end of this presentation,
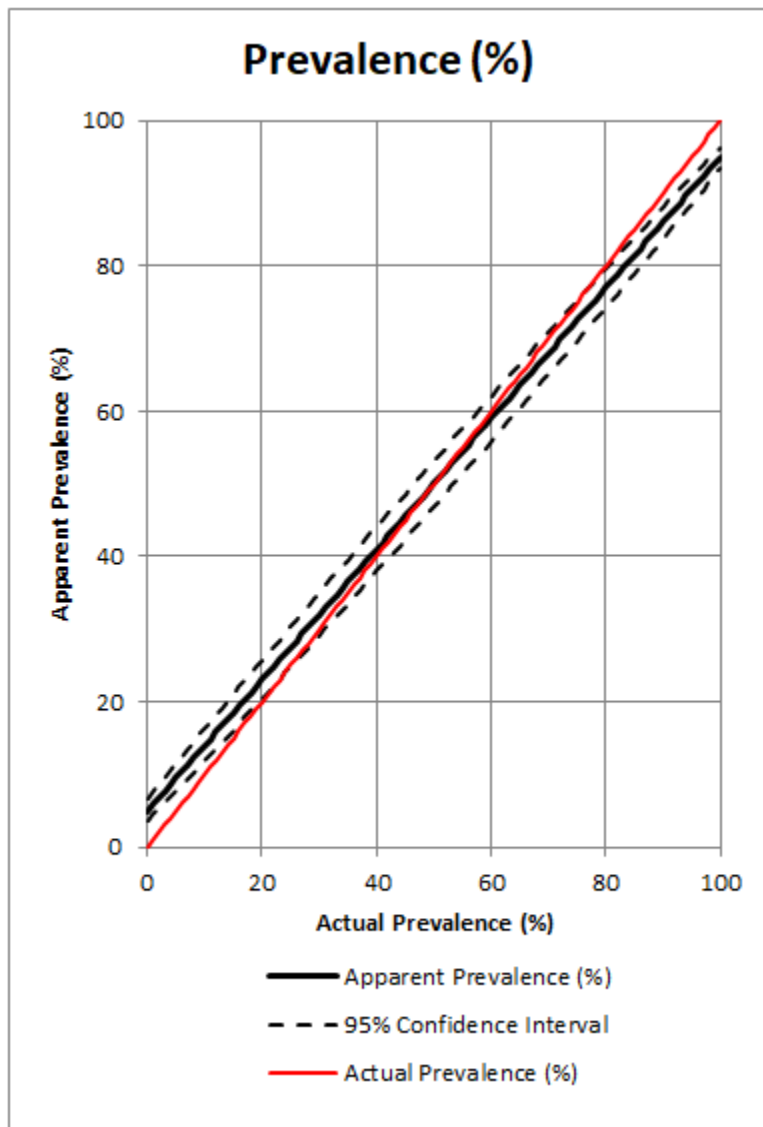
The apparent and actual prevalences are reasonably close to each other.

At lower actual prevalences, the apparent prevalences are modestly higher. Alternatively, at higher actual prevalences, the apparent prevalences are modestly lower.

Conclusions

1. I discuss the availability of COVID-19 tests.
2. I give an overview of clinical testing.
3. I present a brief look at the COVID-19 virus.
4. I discuss types of clinical tests (diagnostic-viral nucleic acid, viral antigen, and serological-antibodies to the virus) and briefly consider how they work.
5. I discuss qualitative tests (like those for COVID-19): sensitivity, specificity, predictive values, prevalence.
6. I discuss concerns about possible false positives with antibody tests but show that for those having FDA EUAs, this should not be a problem.
7. I note, that for an individual a positive test may have low predictive value (unless the prevalence is reasonably high).
8. Finally, I show that, on the other hand, antibody testing of larger populations would generate reasonable estimates of antibody prevalence.

Models for the PPV and Prevalence Plots

Common to both:

1. Assume that the population consists of 1000 people.
2. Actual prevalences from 0 to 100% in 1% increments are calculated.
3. Assume Sensitivity = Specificity = 95%.
4. This gives 1000 x Prevalence actual positive patients and
   1000 x (1-Prevalence) actual negative patients.
5. Number of positive tests for positive patients = 0.95 x number of positive patients =
   950 x Prevalence = True Positives
6. Number of positive tests for negative patients = 0.95 = 0.05 x number of negative patients =
   50 x (1-Prevalence) = False Positives
7. Total number of positive tests = sum (#5 and #6)

PPV Model:

Apparent PPV = Number of True Positives / Total Number of Positives

Prevalence Model:

a. Apparent Prevalence = Total Number of Positive Tests / Total Number of Tests = #7 above / 1000
b. Lower Confidence Limit =   BetaInv[(1-0.95)/2,Number of Positive Tests,1000-Number of Positive Tests+1)]
c. Upper Confidence Limit =   BetaInv[(1+0.95)/2,Number of Positive Tests+1,1000-Number of Positive Tests)]
d. BetaInv(…, …, …) is the Microsoft Excel Beta Inverse function.