Laboratory testing for COVID-19

Emergency Response Technical Centre,
NIVD under China CDC

March 15th, 2020
PART ONE

Overview
1.1 The journey of discovery

1. Infectious bronchitis virus

In 1933, asthma attacks in chickens were discovered by Baudette et al. Infectious bronchitis virus or IBV was first isolated from chickens in 1937. Porcine transmissible gastroenteritis virus (TGEV)

2. B814 and HCoV-229E

In 1965, Tyrrell and Bynoe first used the human fetal nose and tracheal in vitro culture to isolate a strand of virus from the nasal wash of a common cold patient, which was named B814;

In 1966, Hamre and Procknow isolated a similar virus from human embryonic kidney cells and named the strain 229E.

3. HCoV-OC43

In 1967, McIntosh et al. isolated a batch of viruses from the human embryo trachea organ culture from an adult with a cold. OC43 was the major strand among them (organ culture, OC)

4. Discovery of SARS-CoV

2002, SARS-CoV;
2004, NL-63;
2005, HKU1
2012, MERS-CoV
2019, 2019-nCoV
1.2 Naming

In 1968, June Almeida and Tyrrell performed morphological studies on these viruses. Electron microscopy observations revealed that the envelopes of these viruses had spikes that resemble the sun coronal, so they named these viruses coronaviruses.

In 1975, International Virus Nomenclature Committee or ICTV officially established Coronaviridae for the virus.
1.3 Classification

A. Order
   NIDOVIRALES

   Family
      Coronaviridae

   Subfamily
      Coronavirinae
      Torovirinae

   Genus
      Alpha coronavirus
      Beta coronavirus
      Gamma coronavirus
      Delta coronavirus
      Torovirus
      Bafinivirus

   Lineage
      a
      b
      c
      d
Figure 3: Phylogenetic analysis of full-length genomes of 2019-nCoV and representative viruses of the genus Betacoronavirus

1.4 Replication

**FIGURE 28.6.** Overview of coronavirus replication (see text for details).
1.5 The unique intracytoplasmic discontinuous transcription pattern of the coronavirus

1. RdRp
2. Replicative intermediate
3. The RNA virus with the largest genome

Prone to genome mutation and recombination
1.6 Coronavirus structure

China releases genetic sequence of newly discovered coronavirus from Wuhan

The Chinese Center for Disease Control and Prevention, the Chinese Academy of Science and the Chinese Academy of Medical Science released through the GISAID initiative the genome sequence of a newly discovered coronavirus. The virus was identified during an outbreak in the city of Wuhan, where patients were suffering from respiratory illnesses such as pneumonia since late December 2019.

The genome sequence of this betacoronavirus is crucial to develop specific diagnostic tests and to identify potential intervention options.

> read more and access the data

Courtesy: IVDC, China CDC
1.7 Symptoms of the diseases caused by human coronavirus

- Headache
- Fever
- Overall soreness and ache
- Flu symptoms
- Chills
- Dry cough
- Vomiting
PART TWO

Testing techniques
Laboratory testing techniques for COVID-19

- Nucleic acid testing
- Viral isolation
- Serological testing
Content

2.1 Specimen collection requirements
2.2 Nucleic acid testing
2.3 Antibody testing
2.4 Biosafety requirements
Part I

Specimen collection requirements

- Collection target
- Specimen categories
- Specimen packaging and preservation
- Requirements for the sampling personnel
- Specimen processing
- Specimen transportation
1. Specimen collection target

1. Suspected COVID-19 cases;

2. Others requiring diagnosis or differential diagnosis for SARS-CoV-2
2. Sample collection requirements

**Sampling personnel**

- 1. The SARS-CoV-2 testing specimens shall be collected by qualified technicians who have received biosafety training (who have passed the training) and are equipped with the corresponding laboratory skills. Personal protective equipment (PPE) is required for sampling personnel when performing the sampling.
- 2. Specimens of inpatient cases shall be collected by medical staff of the hospital where they are being treated.
- 3. Specimens of close contacts shall be collected by the designated local CDCs and medical institutions.
- 4. Multiple specimens may be collected in the course of the disease, depending on the need of laboratory testing.
3. Categories of specimen collected

Respiratory tract specimens in the acute phase (including upper or lower respiratory tract specimens) must be collected from each case; lower respiratory tract specimens shall be preferred for the collection from severe cases. Stool samples, urine samples, whole blood samples and serum samples can be collected according to clinical needs.

1) Upper respiratory tract specimens: including nasopharyngeal swabs, pharyngeal swabs etc.
2) Lower respiratory tract specimens: including deep-cough sputum, alveolar lavage fluids, bronchial lavage fluid and respiratory tract extracts.
3) Fecal specimens: Fecal samples are about 10 g (peanut size). If it is not convenient to collect fecal samples, an anal swab can be collected.
4) Blood specimens: One should, as much as possible, collect anticoagulated blood in the acute phase within 7 days after the onset of the disease. 5 ml of blood is required for each collection. Vacuum tubes containing EDTA anticoagulant are recommended in blood collection.
5) Serum specimens: Both acute-phase and convalescent serum specimens should be collected as much as possible. The first serum specimen should be collected as soon as possible (preferably within 7 days after the onset of illness), and the second specimen should be collected during 3-4 weeks after the onset of illness. 5 ml of blood is required for each specimen and vacuum tubes without anticoagulant are recommended. Serum specimens are mainly used for measuring antibodies, rather than nucleic acid testing.
6) Urine specimens: Collect 2-3ml of mid-stream urine sample in the morning. (原文没有，请确认)
Pharynx structure

Applied anatomy of pharynx
I. Sections
1. Nasopharynx
2. Oropharynx
3. Laryngopharynx
Collection of nasopharyngeal swab

The sampler gently holds the person's head with one hand, the swab in another, insert the swab via nostril to enter, slowly get deep along the bottom of the lower nasal canal. Because the nasal canal is curved, do not force too hard to avoid traumatic bleeding. When the tip of the swab reaches the posterior wall of the nasopharyngeal cavity, rotate gently once (pause for a moment in case of reflex cough), then slowly remove the swab and dip the swab tip into a tube containing 2-3ml virus preservation solution (or isotonic saline solution, tissue culture solution or phosphate buffer), discard the tail and tighten the cap.
Collection of pharyngeal swab

The sampled person first gargles with normal saline, the sampler immerses the swabs in sterile saline (virus preservation solution is not allowed to avoid antibiotic allergies), holds the head of the sampled person up slightly, with one’s mouth wide open, making a sound "ah" to expose the lateral pharyngeal tonsils, insert the swabs, stick across the tongue roots, and wipe both sides of the pharyngeal tonsils with pressure at least 3 times, then wipe on the upper and lower walls of the pharynx for at least 3 times, and dip the swabs in a tube containing 2-3ml storage solution (or isotonic saline solution, tissue culture solution or phosphate buffer solution), discard the tail and tighten the cap. The pharyngeal swabs can also be placed in the same tube together with the nasopharyngeal swab.
Deep cough sputum:
Ask the patient to cough deeply, and collect the sputum coughed up in a 50-ml screw-capped plastic tube containing 3 ml of sampling solution. If the sputum is not collected in the sampling solution, 2-3 ml of the sampling solution can be added into the tube before testing, or add sputum digestion reagents of equal volume of sputum.

Phosphate buffer containing 1 g/L of protease K
Phosphate buffer containing 0.1 g of dithiothreitol and 0.78 g of sodium chloride
Fecal specimen processing

Take 1ml sample processing solution, pick up a little sample about the size of a soybean and add it into the tube, gently blow for 3-5 times, set aside at room temperature for 10 minutes, centrifuge at 8,000rpm for 5 minutes, absorb the supernatant for detection.

Treatment solution for the fecal specimen
211g tris,
8.5g sodium chloride,
1.1g calcium chloride anhydrous or 1.47g calcium chloride containing crystalline water, dissolved into 800 ml deionized water, with the pH adjusted to 7.5 with concentrated hydrochloric acid and replenishing with deionized water to 1000 ml.
Anal swab

Gently insert the disinfectant cotton swab into the anus for 3-5cm in depth, then gently rotate and pull out, immediately put the swab into a 15-ml screw-capped sampling tube containing 3-5ml virus preservation solution, discard the tail and tighten the tube cover.
4. Specimen packaging and preservation

1. Collected specimens shall be packaged separately in a biosafety cabinet of a BSL-2 laboratory.
2. All specimens should be placed in an airtight freeze-tolerant sample collection tube of appropriate size, with a screw cap and a gasket inside. The sample number, category, name and sampling date should be indicated on the outside of the container.
3. Specimens kept in an airtight container should be sealed in a plastic bag of appropriate size, with each bag containing one specimen.

Specimens for virus isolation and nucleic acid detection purposes should be tested as soon as possible. Specimens to be tested within 24 hours can be stored at 4 °C; those that cannot be tested within 24 hours should be stored at -70 °C or below (specimens may be temporarily stored in -20 °C refrigerators in the absence of -70 °C storage condition). Serum can be stored at 4 °C for 3 days and below -20 °C for a longer period. A special depot or cabinet is required to store specimens separately.
5. Specimen transportation

1. SARS-CoV-2 strains or other potentially infectious biological substances are subject to the packaging instructions for Category A substances assigned to UN2814, and the PI 602 of the Technical Instructions For The Safe Transport of Dangerous Goods by Air (Doc 9284) issued by ICAO.

2. Environmental samples, assigned to UN3373, shall be transported in Category B packaging in accordance with the PI 650, Doc 9284; one may refer to the aforementioned standards for specimens to be transported in other modes of transportation.

3. A Permit of Transport is required for the transportation of the SARS-CoV-2 strains or other potentially infectious substances, according to the Transport Regulations on the Highly Pathogenic Microorganism (Virus) Strains and Specimens that are Pathogenic to Humans (Order No. 45, former Ministry of Health).
Part II

Nucleic acid testing

- Technique
- Primer and probe
- Confirmation of positive specimens
- Principle
- Judgment of the testing results
1. Nucleic acid testing techniques

1. RT-PCR
2. Real time RT-PCR
3. Sequencing
2. Real time RT-PCR

- Forward primer
- Probe
- Reverse primer
- Reporter
- Quencher

Strand displacement

Lysis

End of amplification
1. Baseline: In the first few cycles of the PCR amplification reaction, the fluorescence signal is close to a straight line as it does not change significantly. Then, such a straight line is the baseline;

2. Fluorescence threshold: Generally, the fluorescence signal of the first 15 cycles of PCR reaction is used as the fluorescence background signal. The fluorescence threshold is 10 times the standard deviation of the fluorescence signal of the first 3-15 cycles. The fluorescence threshold is set in the exponential phase of PCR amplification.

3. Ct value: indicates the number of cycles that the fluorescence signal in each PCR reaction tube undergoes when the threshold is met. The Ct value of each template has a linear relationship with the logarithm of the initial copy number; a standard curve can be developed based on the known initial copy number, the x coordinate represents the logarithm of the initial copy number, and the y coordinate represents the Ct value.
Primer and probe of the SARS-CoV-2 nucleic acid assay

**Target 1 (ORF1ab):**
- Forward primer (F): CCCTGTGGGTGTTTACACTTTAA
- Reverse primer (R): ACGATTGTGCATCAGCTGA
- Fluorescent probe (P): 5'-FAM-CCGTCTCGGTATGGAAGTTATGG-BHQ1-3'

**Target 2 (N):**
- Forward primer (F): GGGGAACCTTCTCCCTAGAAT
- Reverse primer (R): CAGACATTTTTGCTCTCAAGCTG
- Fluorescent probe (P): 5'-FAM-TTGCTGCTTGACAGATT-TAMRA-3'
1.5 The unique intracytoplasmic discontinuous transcription pattern of the coronavirus

1. RdRp
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Prone to genome mutation and recombination
5. Judgment of the fluorescence quantitative RT-PCR assay results

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature</th>
<th>Time</th>
<th>Cycle</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reverse transcription</td>
<td>42 ℃</td>
<td>5 min</td>
<td>1 cycle</td>
</tr>
<tr>
<td>Initial denaturation</td>
<td>95 ℃</td>
<td>10 s</td>
<td>1 cycle</td>
</tr>
<tr>
<td>PCR</td>
<td>95 ℃</td>
<td>10 s</td>
<td>40 cycles</td>
</tr>
<tr>
<td></td>
<td>60℃ (Collect fluorescence)</td>
<td>45 s</td>
<td></td>
</tr>
</tbody>
</table>

1. Negative: no Ct value or Ct value is 40.
2. Positive: Ct value <37.
3. Repeated experiments are recommended should Ct value range between 37 and 40. If the Ct value reads <40 and the amplification curve has obvious peaks, the sample should be considered being tested positive, otherwise it should be considered as negative.
Confirmation of COVID-19 positive cases

To confirm a case as positive in the laboratory, one of the following criteria shall be met:

1. The real-time fluorescence-based RT-PCR assay of the 2019-nCoV in the same specimen shows that the two targets, ORF1ab and Protein N, are both positive. In case of the result showing positive for one target, then samples shall be re-collected for another test. If it is still positive for a single target, it is determined to be positive.

2. The real-time fluorescence-based RT-PCR assay of two types of specimens show one single target positive at the same time, or one target positive in two samples of the same type, it could be determined as positive.
Part III

Antibody testing

- Antibody testing methods
- ELISA’s principle
- Colloidal gold antibody testing
- How to choose the nucleic acid test against the testing window
1. Antibody testing assays
Radioimmunoassay

Enzyme-linked immunosorbent assay

Luminescence

1960’s

1970’s

1990’s
2. Principle for indirect ELISA

- **Solid-phase antigen**
  - Incubation
  - Plate washing

- **Antibody to be tested**
  - Incubation

- **Enzyme-labeled antibody**
  - Incubation
  - Plate washing

- **Substrate**
  - Incubation

**Indirect antibody testing**

**Colour development**
3. Principle for colloid gold testing

- Sample pad
- Colloidal Gold Bonding Pad
- Detection line T
- QC line C
- Water absorbent materials
- Chromatography membrane (NC nitrocellulose membrane)
- PVC board
- Sample flow direction
- Negative
- Positive
- Null

Diagram shows the components and flow of a colloid gold testing device.
Serum antibody tests for SARS-CoV-2

- Serum antibody tests are used as supplementary tests for cases of negative 2019-nCoV nucleic acid tests, and used in conjunction with nucleic acid tests in the diagnosis of suspected cases, or used in serological surveys and past exposure surveys of concerned population groups. Laboratory confirmed positive cases need to meet one of the following two conditions:

1. Serum IgM antibodies and IgG antibodies to 2019-nCoV are positive;
2. Serum IgG antibodies to 2019-nCoV turn from negative to positive or the IgG antibody titers of recovery period are 4 times or more higher than that of acute phase.
Figure 1. Schematic diagram of the course of virus shedding and detection in body fluids during SARS illness and recovery. Onset of illness is taken to be the onset of symptomatic fever.

SARS发病(以发热为首发症状)后，病毒排出的时间分布，及各种标本检测出病毒的时间分布

Bermingham A, et al. 2004
# Overview of the laboratory testing methods for SARS-CoV

<table>
<thead>
<tr>
<th>Common testing method</th>
<th>Time required</th>
<th>Interpretation of the testing results and their implications</th>
</tr>
</thead>
<tbody>
<tr>
<td>ELISA test for the virus nuclear protein (N) antigen</td>
<td>4 hours</td>
<td>Serum samples collected during 1—10 days after the disease onset (3-5 days most sensitive). The positive test result carries diagnosis significance. It is a sensitive test used for early diagnosis, with stable results and minor impacts from the quality of the specimen.</td>
</tr>
<tr>
<td>Real-time PCR assay for the viral nucleic acid</td>
<td>6 hours</td>
<td>Pharynx and anal swabs and serum specimens collected during 3-10 days since the disease onset. The positive results from multiple specimens have diagnosis significance. The method is applied to the early diagnosis, with stable results and good sensitivity. However, the specimens’ quality exerts a major impact on the results.</td>
</tr>
<tr>
<td>Routine PCR assay for the viral nucleic acid</td>
<td>6 hours</td>
<td>Ditto, can be used for nucleic acid sequencing which has diagnosis significance. The method is applied for early diagnosis and is a stable and sensitive test. However, it can be impacted by the specimen’s quality.</td>
</tr>
<tr>
<td>ELISA and fluorescence detection for IgG and IgM</td>
<td>4 hours</td>
<td>Only the serum collected during 10 days after the disease onset can yield results. The 4-times increase or the result turning positive carry diagnosis significance. The method is applied to mid and late stage diagnosis with stable results.</td>
</tr>
<tr>
<td>Neutralizing antibody assay</td>
<td>3-5 days</td>
<td>Only the serum collected during 10-22 days after the disease onset can yield results, with 4-time increase having diagnosis significance. The method should be applied to the mid and late stage diagnosis, with stable results.</td>
</tr>
<tr>
<td>Viral isolation</td>
<td>5-10 days</td>
<td>The pharynx and anal swabs and serum specimens collected during 1-10 days after the disease onset have diagnosis significance. The method is used for the mid and late stage diagnosis with major impacts from the samples’ quality.</td>
</tr>
</tbody>
</table>
SARS-CoV post-infection test markers

Antigen assay
IgM antibody assay
IgG antibody assay
Fluorogenic primer-based quantitative PCR

Days after the infection

Positive result rate (%)
Analysis of the test results

General trend for the antibody generation during the primary response and secondary response.
Negative result from the nucleic acid assay

- Early infection
- Active infection
- Early infection
- Recovery period

- 1: IgM+ IgG-
- 2: IgM- IgG+
- 3: IgM+ IgG-
- 4: IgM+ IgG+
Patients may be during the "window period" of nCoV infection in 2019, typically 2 weeks.

May be at 2019-nCoV early infection.

May be during the mid and late infection stage or recurrent infection. When the IgG antibody in the recovery period increases by 4 times or more compared with the acute phase, a recurrent infection can be diagnosed.

The patient is during the active infection, but its body has already developed a certain immunity to 2019-nCoV.

The patient’s likely to be in the acute phase of 2019-nCoV infection. At this time, you need to question the results of nucleic acid testing. Other diseases such as rheumatoid factors have been found to cause weak IgM positive or positive tests.

May have been infected with 2019-nCoV in the past, but the patient has been recovered or the virus has been cleared. The IgG produced by the immune response is maintained for a long time and is still detected in the blood.

First infection with a very low viral load, and during an early stage. Thus, the viral load is lower than the lower limit of nucleic acid detection. The body produces a small amount of IgM antibodies, and has not yet produced IgG; a false positive result is caused by the patient’s own rheumatoid factor.

Recently infected 2019-nCoV and are in the recovery period. The internal virus is cleared, but the IgM has not been reduced to the lower limit of detection; or the nucleic acid test result is false negative, with the patient being in the active infection period.

For reference only. The clinical judgment should prevail.
Part IV Bio-safety requirements

- General introduction
- Animal infection experiments
- Operations of inactivated materials
- Viral culture
- Operations of the uncultured infectious substances
Bio-safety requirements for the COVID-19 laboratory activities

According to the biological features, epidemiological characteristics, clinical data and other available information concerning the SARS-CoV-2, the pathogen shall be temporally managed as **Category B pathogens** and microorganisms based on its hazards.
1) Viral culture

Viral culture refers to operations such as virus isolation, culture, titration, neutralization test, purification of live virus and its protein, lyophilization of virus, and recombination test to produce live virus. The above operations should be performed in a biosafety cabinet of a BSL-3 laboratory. When viral medium is used to extract nucleic acid, the addition of lysing agent or inactivating agent must be performed under the same level of laboratory and protective conditions as viral culture. Laboratories shall report to the National Health Commission for approval and obtain relevant qualifications before carrying out the corresponding activities.
2) Animal infection experiment

Animal infection experiment refers to operations such as infecting animals with live viruses, sampling of infected animals, processing and testing of infectious samples, special test for infected animals, disposal of infected animal excrement, etc., which should be performed in a biosafety cabinet of a BSL-3 laboratory. Laboratories shall report to the National Health Commission for approval and obtain relevant qualifications before carrying out the corresponding activities.
3) Operation of uncultured infectious substances

The operation of uncultured infectious substances refers to viral antigen detection, serological testing, nucleic acid extraction, biochemical analysis, inactivation of clinical samples and other operations performed on uncultured infectious substances before inactivation through a reliable method. The operation should be performed in a BSL-2 laboratory, with personal protective equipment subject to BSL-3 laboratory protection requirements.
4) Operation of inactivated substances

After reliable inactivation of infectious substances or live viruses, operations such as nucleic acid testing, antigen testing, serological testing and biochemical analysis should be performed in a BSL-2 laboratory. Molecular cloning and other operations not involving live pathogenic viruses may be carried out in a BSL-1 laboratory.
Experts team reached Wuhan, unusual pneumonia was associated with virus infection.

The first full-genome of 2019-nCoV was born.

CPE on HAE cells was observed.

Detection reagents were send to Hubei CDC and technical training performed.

National Health Commission of China declared the 2019-nCoV was the etiological agent of NCP.

Commercial rRT-PCR detection Kits of 2019-nCoV were distributed to Hubei.

hACE2 transgenic mice were challenged with 2019-nCoV.

Challenged mice showed typical pneumonia pathology, fulfilling with Koch’s postulates.