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Technical guidance for laboratory testing of 2019-nCoV infection (Third Edition)

National Health Commission of the People's Republic of China

This technical guidance was created to instruct centers for disease control at all levels, and other competent bodies, on how to conduct laboratory testing of pathogens associated with the unexplained viral pneumonia in Wuhan, China.

1. Specimen collection

1.1. Specimens to be collected

Pathogens associated with suspected 2019-nCoV infections cases and clustering cases. Cases requiring diagnosis or differential diagnosis for possible 2019-nCoV infection, or environmental or biological materials requiring further investigation.

1.2. Specimen collection requirements

1.2.1 Technicians engaged in specimen collection shall be trained, and qualified, in biosafety and possess experience in the collection and detection of pathogens. Personal protective equipment (PPE) shall include items such as N95 mask or higher level of respiratory protection, goggle, double latex gloves, protective suit, waterproof boots. If contact with patient's blood, body fluids, secretions or excreta, replacing the outer glove in time.

1.2.2 Specimens collected from hospital inpatients shall be collected by qualified hospital medical staff.

1.2.3 Specimens from persons who were in close contact with patients diagnosed with 2019-nCoV will be collected by the local disease control center or medical institutions.

1.2.4 Laboratory testing may require the collection of multiple specimens from patients over the course of the disease.

1.3. Types of specimens to be collected

Upper and lower respiratory tract specimens shall be collected simultaneously for each case; however, priority shall be given to lower respiratory tract specimens, including, bronchial fluid and alveolar lavage fluid. If there is clinical evidence of a conjunctival or eye infection, eye conjunctival swab specimens shall be collected. Stool specimens are required in case of diarrhea. Specimens could be collected based on clinical manifestations and sampling intervals.

Other research materials could be collected according to research requirements.

1.3.1 Upper respiratory tract specimens: pharyngeal swabs, nasal swabs, nasopharyngeal extracts.

1.3.2 Lower respiratory tract specimens: sputum from deep coughs, respiratory tract extracts, bronchial lavage fluid, pulmonary alveolus lavage fluid, and pulmonary tissue biopsy specimens.

1.3.3 Blood specimens: gather anticoagulation in the acute phase within 7 days after the onset of disease. The collection volume is 5 ml and fasting blood specimens collected using a vacuum blood collection tube containing EDTA anticoagulant is recommended.

1.3.4 Serum specimens: collect a duplicate specimen of serum from the acute and the recovery phase. The first serum specimen shall be collected as soon as possible, preferably within 7 days of symptom onset. The second serum specimen shall be collected 3 to 4 weeks later. The collection volume is 5 ml and fasting blood specimens collected using an anticoagulant-free vacuum blood collection tube is recommended. Serum specimens are mainly used for the determination of antibodies, and the infection status of the case. Serum specimens were not tested for nucleic acids.

1.3.5 Conjunctival specimens: eye conjunctival swab specimens with symptoms of eye infection.

1.4. Specimen collection method

1.4.1 Pharyngeal swab: wipe the bilateral pharyngeal tonsil and the posterior pharyngeal wall with two sterile plastic rods, polypropylene fiber head swabs; immerse the swab head in a tube containing 3 ml of virus preservation solution (isotonic saline solution, tissue culture solution or phosphate buffer solution can also be used), discard the tail, and screw the tube cap tightly.

1.4.2 Nasal swab: gently insert a sterile plastic rod swab with a polypropylene fiber head into the nasopalatine duct of the nasal passage, hold, and then slowly rotate to withdraw. Repeat this procedure for the other nostril. Immerse the two swabs in one tube containing 3 ml of sampling solution, discard the tail, and screw the tube cap tightly.

1.4.3 Nasopharyngeal or respiratory tract extract: use a collector connected to a negative pressure pump to extract mucus from the nasopharynx or secretions from the trachea. Insert the head of the collector into the nasal cavity or trachea, switch on the negative pressure, rotate the head of the collector and slowly withdraw, collect the extracted mucus, and rinse the collector once with 3 ml of sampling solution. Alternatively, use a pediatric catheter connected to a 50 ml syringe collector.

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1.4.4 Deep cough sputum: ask the patient to cough deeply, collect the expelled sputum in a 50 ml plastic screw cap tube containing 3 ml of sampling solution.

1.4.5 Bronchial lavage fluid: insert the collector head into the trachea about 30 cm deep from the nostril or tracheal opening, inject 5 ml of physiological saline, switch on the negative pressure, rotate the collector's head and slowly withdraw. Collect the extracted mucus and rinse the collector once with sampling solution. Alternatively, use a pediatric catheter connected to a 50 ml syringe collector.

1.4.6 Pulmonary alveolus lavage fluid: after giving the patient local anesthesia, insert a bronchoscope through the mouth or nose, through the pharynx, into a branch of the right lung middle lobe or the left lung lingula, and wedge the tip of the bronchoscope into the bronchial branch opening. 30 ml sterilized physiological saline was rapidly injected into the tracheal biopsy hole each time with a total perfusion volume of 60–100 ml, and then alveolar lavage fluid was immediately sucted by 70mmhg negative pressure.

1.4.7 Blood specimens: collect 5 ml of blood specimens using a vacuum iliac vessel containing EDTA anticoagulant, allow the specimen to rest at room temperature for 30 min and then centrifuge at 1,500 to 2,000 rpm for 10 min. Collect plasma and blood cells in sterile screw plastic tubes.

1.4.8 Serum specimens: collect 5 ml of blood using a negative pressure vacuum blood collection tube, allow the specimen to rest at room temperature for 30 min and then centrifuge at about 120 $\times g$ for 10 min and collect the serum in a sterile plastic screw cap tube.

1.4.9 Stool specimens: collect 3–5 ml stool samples when diarrhea occurs early in the onset of the disease.

1.4.10 Eye conjunctival swab specimens: gently wiping the conjunctival surface of the eye with a swab, put the swab head into the sampling tube, discard the tail, and screw the tube cap tightly.

1.4.11 Other materials: collect according to research requirements.

1.5. Specimen packaging

After collection, specimens are to be packed into three sections within a biosafety cabinet in a biosafety level 2 (BSL-2) laboratory.

1.5.1 All specimens shall be placed in a suitable, securely sealed, freeze-resistant collection tube and the screw cap shall be equipped with a gasket. The specimen number, type, name, and sampling date must be indicated on the outside of the container.

1.5.2 Place the hermetically sealed specimens in an appropriately sized plastic bag, with one specimen per bag. Packaging shall comply with the packaging requirements of ICAO Document *Technical Instructions for the Safe Transport of Dangerous Goods by Air*.

1.5.3 For the transportation of external specimens, three-layer packaging shall be performed according to the type of specimens and infectious substances of type A or B.

1.6. Specimen preservation

Specimens used for viral isolation and nucleic acid detection shall be tested as soon as possible. Specimens that will be tested within 24 h shall be stored at 4 °C. Specimens that cannot be tested within 24 h shall be stored at −70 °C or below. Where −70 °C storage is not possible the specimens shall be temporarily stored in the freezer section of a refrigerator at −20 °C. Serum can be stored at 4 °C for 3 days. Repeated freezing and thawing shall be avoided during specimen transport. Specimens shall be stored separately from other specimens in a dedicated cabinet or storage space.

1.7. Specimen submission

Specimens shall be sent to a laboratory as soon as possible after collection. It is recommended that specimens be stored in dry ice where available when long-distance transport is required.

1.7.1 Specimen submission: in provincial centers for disease control, the specimens of clustering case shall be sent to the Chinese Center for Disease Control and Prevention for review and further testing as soon as feasible. Specimens shall be sent with Specimen Submission Form.

1.7.2 Pathogens and specimen transport

1.7.2.1 Domestic transport: transport packaging for 2019-nCoV viral strains or other potentially infectious biological materials is in Class-A (UN2814). Packaging shall comply with the PI602 classification packaging requirements of ICAO Document Doc9284 *The Safe Transport of Dangerous Goods by Air*. Packaging for environmental samples is in Class-B (UN3373), shall comply with the PI650 classification packaging requirements of ICAO Document Doc9284. Packing can be conducted in accordance with the standards above when transported via other modes of transport.

Certificate of transport is required according to *Regulations for Transport Management of Infectious and Highly Pathogenic Human Microorganisms, Bacteria, Viruses, or Specimens* (Decree No. 45 of the Former Ministry of Health of the People's Republic of China).

1.7.2.2 International transport: transportation of 2019-nCoV viral strains and other potentially infectious biological materials requires relevant procedures in accordance with *Regulations for Management and Health Quarantine of Import and Export of Special Products*. Packages shall be standardized and comply with applicable international regulations.

1.7.2.3 Management of viral strains and specimens: the 2019-nCoV virus strains and related specimens shall be managed by designated staff members. An accurate record of the source, type, quantity, and registration number is required for each viral strain and specimen. Effective measures must be in place to ensure the biosecurity of all viral strains and specimens. Any misuse, malicious use, thefty, robbery, loss, or leakage events shall be strictly prevented.

2. Laboratory testing of pathogens

Pathogens associated with 2019-nCoV shall be identified by real-time reverse transcription PCR. Laboratory testing of 2019-nCoV must be performed in laboratories with appropriate protocols and equipment by personnel with biosafety training.

Current viral nucleic acid detection methods mainly detect the open reading frame 1a/b (ORF1ab) and nucleocapsid protein (N) in the 2019-nCoV genome.

3. Standard operating procedures for RT-PCR detection of nucleic acids

3.1. Objective

To ensure the accuracy and reliability of results through the standardization of RT-PCR methods.

3.2. Scope

Suitable for detection of 2019-nCoV viral nucleic acids by RT-PCR.

3.3. Duties and responsibilities

Testing personnel: responsible for testing specimens in accordance with the applicable rules and regulations.

Test reviewers: responsible for determining the reliability and validity of test results and ensuring proper testing protocols were adhered to and performed without error.

Department heads: responsible for departmental oversight and reviewing testing reports.

3.4. Sample receipt, preparation, and storage

Ensure each specimen collected has the name, gender and age of the patient as well as a serial number; any abnormality in the specimen shall be noted, and the specimen shall be stored at −70 °C.

3.5. Test items

3.5.1. Nucleic acid detection of 2019-nCoV (RT-PCR method)

Primers and probes targeting the ORF1ab and N gene regions of 2019-nCoV are recommended.

Target ORF1ab:

Forward primer (F): CCCTGTGGGTTTTACACTTAA

Reverse primer (R): ACGATTGTGCATCAGCTGA

Fluorescent probe (P): 5′-FAM-CCGTCTGCGGTATGTGGAAAGGTTATGG-BHQ1-3′

Target N:

Forward primer (F): GGGGAACTTCTCCTGCTAGAAT

Reverse primer (R): CAGACATTTTGCTCTCAAGCTG

Fluorescent probe (P): 5′-FAM-TTGCTGCTGCTTGACAGATT-TAMRA-3′

Please refer to the relevant kit instruction for nucleic acid extraction protocol and RT-PCR reaction system.

3.5.2. Analysis of results

Negative: no Ct value or Ct = 40.

Positive: a Ct value <37.

A Ct value between 37 and 40 is indeterminate. It is recommended that the experiment be repeated. If, when repeated, the Ct value is <37, the specimen is positive, otherwise, it is negative.

The following conditions are sufficient to confirm a positive result:

Both target-specific RT-PCR results (ORF1ab and N) are positive in the same specimen. If only one target-specific RT-PCR result is positive, resample and retest are required.

Negative results do not rule out infection. False negatives can be caused by the poor quality of specimens, such as respiratory tract specimens collected from the oropharynx; collection that is too early or late in the progression of the disease; specimens that have not been properly stored, transported, or processed; technical factors, including virus mutation and PCR inhibition.

4. Laboratory biosafety

Based on the current biological characteristics, transmission characteristics, clinical data and other information of 2019-nCoV, 2019-nCoV is provisionally classified as the Risk Group 2 pathogenic microorganism. Detail requirements are as follows.

4.1. Virus culture

Refer to procedures including virus isolation, cultivation, titration, neutralization assay, purification of live virus and its proteins, virus freeze-drying, and recombination experiment generating live viruses. The above-said operations shall be performed in a biosafety cabinet of a biosafety level 3 (BSL-3) laboratory. When extracting nucleic acid from the virus culture, the steps of adding lysis or inactivator agents must be performed in a laboratory which has the same biosafety level and protective conditions as for virus culture. After lysis or deactivation, practices shall be conducted at the same biosafety and personnel protective levels as for non-cultured infectious materials. Before carrying out these activities, the laboratory shall submit a request to the National Health Commission of the People's Republic of China for approval, in order to get the qualifications required for conducting the corresponding activities.

4.2. Animal-infection experiments

This procedure refers to experiments that involved in infecting animals with live virus, sampling infected animals, processing and testing infectious specimens, specialized examination of infected animals, and processing of excreta from infected animals etc.. Animal-infection experiments shall be performed in qualified animal BSL-3 (ABSL-3) laboratory. Before carrying out these activities, a request shall be made to the National Health Commission of the People's Republic of China for approval by the laboratory, in order to be qualified for performing the corresponding activities.

4.3. Non-cultured infectious material operation

These procedures refer to practices using non-cultured infectious materials before proper deactivation, such as virus antigen detection, serological assay, nucleic acid detection, biochemistry analysis, and the deactivation of clinical specimens. These operations shall be performed in a biosafety cabinet of a biosafety level 2 (BSL-2) laboratory, but personal protective equipment is subject to BSL-3 laboratory protection requirements.

4.4. Inactivated material operations

These procedures refer to practices using either infectious materials or live viruses after proper deactivation, which involving nucleic acid detection, virus-antigen detection, serological assay and biochemistry analysis. These activities shall be performed in a BSL-2 laboratory. Other operations such as molecular cloning without involving pathogenic live viruses may be performed in a biosafety level 1 (BSL-1) laboratory.