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Pathogenesis of COVID-19 and the quality control of nucleic acid detection

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ABSTRACT

The new coronavirus pneumonia (COVID-19) epidemic spread rapidly throughout the world. Considering the strong infectivity and clustering of COVID-19, early detection of infectious cases is of great significance to control the epidemic. Nucleic acid testing (NAT) plays an important role in rapid laboratory diagnosis, treatment assessment, epidemic prevention and control of COVID-19. However, since COVID-19 is caused by a new emerging virus and NAT for COVID-19 has not been clinically applied before, false negative results inconsistent with clinical diagnosis have appeared in clinical practice. Therefore, it is urgent to improve the sensitivity of NAT for COVID-19. This study aimed to summarize the current situation and prospect of NAT based on the latest findings on COVID-19 infection. Also, the quality control of sample collection was discussed. Hopefully, this study could help to improve the effectiveness of NAT for COVID-19.

1. Introduction

On January 30, 2020, WHO declared the outbreak of COVID-19 as a public health emergency of international concern. The genome of the new coronavirus has 29903bp and polycis-trans structure. It has been found that the virus specifically recognizes the receptor on host cell membrane, angiotensin-converting enzyme 2(angiotensin I - converting enzyme 2, ACE2), through the S protein on its surface [1,2], and mediates virus-host cell membrane fusion. The host cell protein translation system translates the 5 'end Orf1a/B gene and generate the RNA polymerase complex, takes the template of viral genome, generates negative chain, followed by a negative chain as templates are chain RNA genome, and thus realizes the replication of viral genome. During the early phase of COVID-19 infection, most of patients have normal or decreased white blood cell counts, decreased lymphocyte counts, increased C-reactive protein (CPR) level, normal PCT, and positive imaging findings. Patients with severe COVID-19 showed significant increases in proinflammatory cytokines such as IL-6, TNF-a, and IFN-r, with cytokine storm characteristics [3].So far, there is still no specific treatment for COVID-19, and thus, early diagnosis, timely prevention and control are key to containment of the epidemic. The diagnosis and treatment program of COVID-19 published by the National Health Commission of China specifies the positive result of nucleic acid testing (NAT) as a diagnostic criterion.

At present, eight nucleic acid detection reagents have been approved by the Food and Drug Administration of China for the diagnosis, prevention and control of COVID-19 [4]. However, in clinical practice, due to the insufficient performance verification of NAT reagents, they may generate false negative results that are inconsistent with clinical diagnosis. Recently, it has been reported that a confirmed case in China-Japan Friendship Hospital in China was tested negative in the previous three NATs, and another case in Hangzhou was reported to be negative for six times before the 7th NAT, which makes the clinicians to question the effectiveness of NAT for COVID-19 [5]. According to the “Biosafety Guidelines for Laboratory Testing of COVID-19” issued by the Laboratory Medicine Branch of the Chinese Medical Association [3], and “Risk Communication and Community Engagement (RCCE) Readiness and Response to COVID-19” issued by the World Health Organization

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(WHO) [6] combined with the latest research results and findings of COVID-19, the current situation and influencing factors of NAT were discussed [7]. Meanwhile, the quality control of NAT sample collection was investigated to improve the effectiveness of NAT.

2. Etiology, transmission pathogenesis and RT-PCR technique of COVID-19

Structurally, SARS-CoV-2 belongs to the family of coronaviruses and is genetically similar (over 85% similarity) to bat coronaviruses [8]. SARS-CoV-2 shares 78% and 50% genetic sequence with the viruses of SARS and MERS, respectively [9]. It is generally considered that Rhinolophus sinicus bat is the natural reservoir of SARS-CoV-2. A latest research proposed that pangolins may be the intermediate host that introduces COVID-19 to humans [10].

A novel study suggested that COVID-19 might spread through aerosol transmission under certain circumstances. Very recently, detection of SARS-CoV-2 in stool samples indicated the possibility of fecal-oral transmission [11]. So far, no evidence has supported COVID-19 infection is caused by contaminated food, but the possibility of aerosol or contact transmission via fecal excretion cannot be excluded [12]. The urinary system may also be a potential transmission route for COVID-19 infection [13]. ACE2, the SARS-CoV-2 receptor, has been demonstrated to be lowly expressed in various cell types of the human maternal-fetal interface [14], indicating that there might be no potential susceptible cell subpopulation of SARS-CoV-2 in the maternal-fetal interface and thus SARS-CoV-2 infection could not cause vertical transmission from mother to fetus. In addition, experimental evidence has proven that COVID-19 cannot be transmitted through skin contact.

Pathological lesions in the lung are the major characteristics of COVID-19, and diarrhea is uncommon, suggesting that lung is the target organ of COVID-19 [15]. The entry of SARS-CoV-2 into host cells requires the binding of S protein and ACE2 on the cell membrane [16]. During viral replication, amplification and release, the body defense response is initiated. Most of COVID-19 infected patients can be cured by adjuvant therapy for virus clearance and inflammatory damage repairing through autoimmune function. However, over-activated inflammatory response and cytokine storm may trigger viral pneumonia. ACE2 is abundant in alveolar epithelium, intestinal epithelium and vascular endothelial cells. The release of cytokines (e.g. MCP-1, GM-CSF, M-CSF) which was induced by viral A single N501T mutation (corresponding to S487T mutation in SARS-CoV-2) may significantly enhance the binding affinity between COVID-19 RBD and human ACE2, Which activated after binding to the corresponding receptors on the surface of macrophages. The activated macrophages not only initiate the specific immune response by recruiting abundant mononuclear phagocytes, but also release inflammatory factors (e.g. IL-1β, TNF-α, IL-6, MCP-1) and induce tissue damages. MCP-1 could stimulate the synthesis of angiotensin II (Ang II), further aggravating the inflammatory response [17]. The latest research has proven the involvement of inflammatory cascade in pulmonary lesions, such as alveolar edema or inflammatory exudation [18]. Nevertheless, mechanisms underlying the development of inflammatory cascade following SARS-CoV-2 infection remain largely unclear. It is speculated that SARS-CoV-2 infection induces the release of TNF-α, IL-1, interferons and chemokines by activating immune cells, which then aggregate and infiltrate in the lung. Meanwhile, intracellular inflammation-related pathways are activated. The inflammation cascade is initiated and further stimulates the release of abundant cytokines to activate more inflammatory cells. Such a vicious circle finally leads to cytokine storm.

Real time fluorescence quantitative PCR (RT-PCR) is a major diagnostic method for COVID-19 in clinical practice. The basic principle is through the specificity of the fluorescent tag probe, based on the reaction PCR products tags in the process of tracking, real-time monitoring of the growth of the product quantity, according to the amplification curve calculated amount of initial template (Fig. 1). The SARS-CoV-2 genome contains the 5′-terminal replicase encoding gene [open reading frame 1 ab (ORF1ab)], the structural protein encoding gene [spike protein (S), envelope protein (E), membrane protein (M) and nucleocapsid protein (N). [19]. Comparing with other coronavirus nucleic acid sequences, ORF1ab fragment and N gene are specific genes of coronavirus and type. Specific primers were designed for these two genes to detect SARS-CoV-2, and S gene was added to some reagents to improve detection sensitivity. Compared with gene sequencing, the detection time is relatively short, the operation is convenient, and the specificity and repeatability are better [20]. Chu et al. [21] showed that the sensitivity of N gene detection was higher. Corman et al. [22] optimized the detection of SARS-CoV-2 and established a RT-PCR detection process. Positive case diagnosis requires two positive targets in the same specimen, or two positive targets in the same specimen, or two positive targets in the same specimen.

3. Quality control

So far, the approved reagent has not achieved desired performance verification, resulting in weakened detection capability. Li et al. detected 255 samples collected from COVID-19 cases twice using the same NAT kits, but only 77.25% of the cases received the same results in the two tests. They also proposed that different component solutions in sampling tubes may result in false negative results. Therefore, quality control should be carried out in NAT for COVID-19.

3.1. Quality control before analysis

(I) Sample collection: The quality of collected samples determines the efficiency of NAT for COVID-19. The lower respiratory tract is the major part attacked by SARS-CoV-2 infection. Theoretically, sputum from deep respiratory tract or bronchoalveolar lavage fluid (BALF) is the ideal sample for COVID-19 detection. However, to prevent nosocomial infection, only nasopharynx or oropharynx swabs are collected in clinical practice. Surface mucosal cells are collected by swabbing the posterior pharyngeal wall, crypt and lateral wall of tonsil beyond the root of tongue for 3–5 times. Meanwhile, multisite samples (e.g. oral and bilateral nasopharynx swabs) collected from the same case are recommended to improve the detection efficiency [24].

(II) Inflammatory cytokine storm may influence the result of NAT

Clinical diagnosis is required for cases with typical pulmonary symptoms but negative NAT results in regions where COVID-19 is endemic, so that missed diagnosis can be significantly avoided [25]. It is also suggested that patients with severe symptoms receive deep sputum or BALF test.

(III) Inspection time: Virus replication is rapid and nucleic acid positive rate is high when in the extreme stage of disease. Due to limited reagents and detection manpower, it is necessary to perform NAT for COVID-19 patients in their acute phase. According to the management of infectious diseases under Class A, samples (multisite samples) suspected of COVID-19 infection is better to be retained [26].

(IV) Storage conditions and transportation of sample: Recommended to be stored in a special virus preservation solution, samples containing RNA viruses must be stored in a

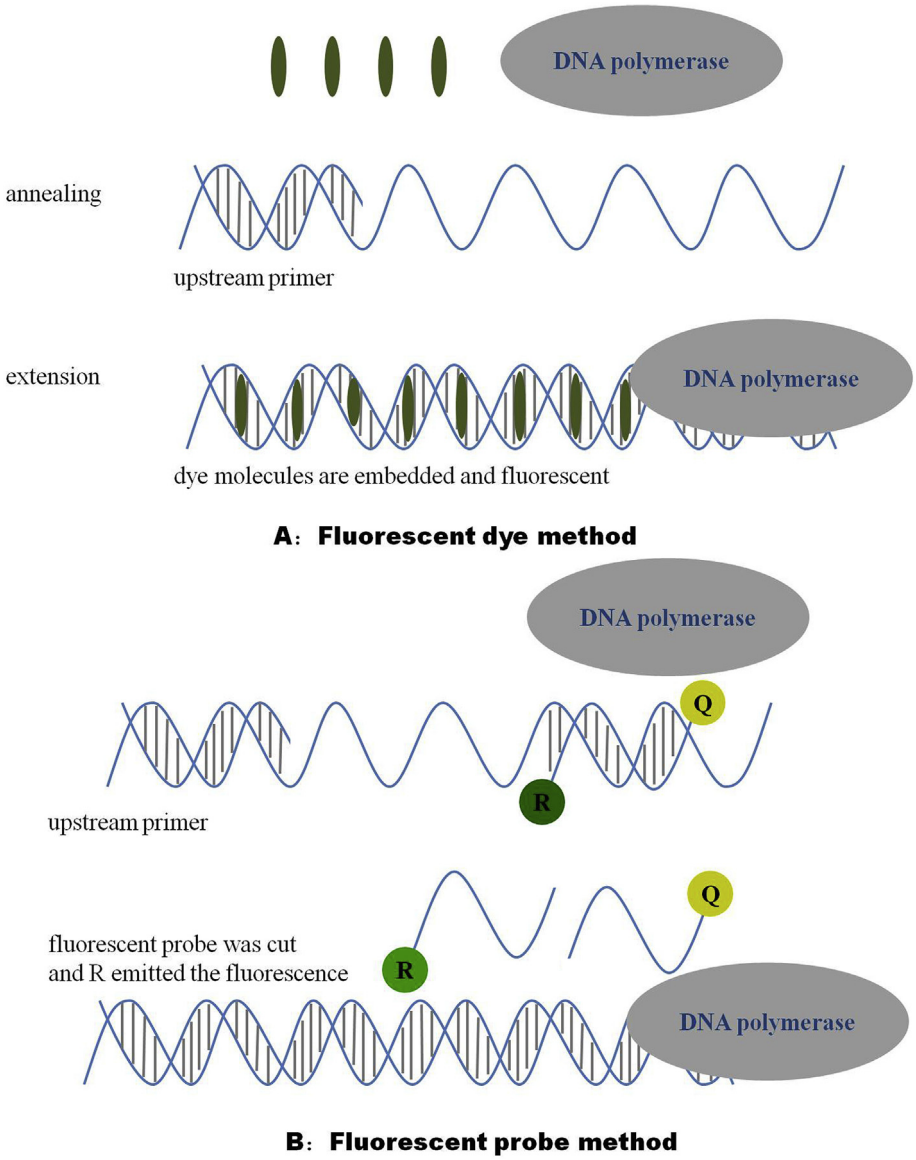

Fig. 1. LI Jia-jun, ZHENG Xiao, SHENG Jie, XU Yao. Novel Coronavirus and Research Progress of Related Clinical Detection [23]Schematic diagram of two fluorescence PCR.

refrigerated state (4 °C or lower) and transported for inspection as soon as possible. After 72 h, samples containing RNA viruses should be stored and transported at −70 °C.

3.2. Quality control during analysis

(V) It is recommended to use the COVID-19 NAT kit recommended by the National Health Commission of China for nucleic acid amplification. One weak positive quality control and three negative quality controls are recommended for each batch, and the three negative quality controls should be randomly placed in different positions. To improve the accuracy and sensitivity of the detection, each laboratory should explore the PCR amplification system, and expand the reaction system to 40 μL or 50 μL. The remaining nucleic acid should be stored at −70 °C for standby to avoid RNA degradation in case that the extracted nucleic acid cannot be amplified in time.

(VI) The National Health Commission of China also implements laboratory quality evaluation of COVID-19 NAT, in which bacteriophage virus-like particles are developed in the sample without biological infection risk to evaluate the accuracy, specificity and analytical sensitivity of laboratory test results [27].

3.3. Interpretation of NAT results

At present, most COVID-19 NAT kits are designed to detect either three gene fragments (ORF1ab gene, core-shell protein gene N fragment and enveloped protein gene E fragment), or two gene fragments (ORF1ab and core-shell protein gene N fragment) in the open reading frame. Because COVID-19 NAT kit only has negative control and positive control, the test results should be carefully interpreted. According to the recommendation of the National Health Commission of China, the results should be interpreted as follows [28]. In the open reading frame, for the detection results of

two gene fragments (specific ORF1ab and core-shell protein gene N) detection kit: (1) positive when both ORF1ab and N genes are positive. (2) re-sampling if either the ORF1ab gene or N gene is positive; positive if the ORF1ab gene or N gene is still positive after review. (3) re-sampling if the ORF1ab or N gene test results are suspicious (in the gray area); positive if the results are still in the gray area and a significant peak is found in the amplification curve, otherwise negative (Table 1).

In the open reading frame, for the detection results of three gene fragments (ORF1ab, N fragment of core-shell protein gene and E fragment of envelope protein gene) detection kit: (1) positive when two or three ORF1ab and N genes are positive; (2) re-sampling if a single gene fragment is positive; positive if the result is still positive in the reexamination; (3) re-sampling if the detection result of a single gene fragment is suspicious (in the gray area); positive if the result is still in the gray area and a significant peak is found in the amplification curve, otherwise negative; (4) for the other possible results, the processing and result interpretation is the same as (2) or (3) (Table 2).

3.4. False negative results of NAT

At present, the false negative result of NAT has aroused much concern. The case may manifested as highly suspected COVID-19 based on clinical symptoms and imaging results, but with "negative" results in NAT. The false negative result of NAT could be explained as follows by the Clinical Laboratory Center of National Health Commission of China.

Existing data showed that after infected by the virus, the virus passes through the nose and mouth into the throat, trachea and bronchus, and then reaches the alveoli. The people infected could experience the incubation period, and then develop mild to severe symptoms. The viral load of different cell types are as follows: alveolar epithelial cells (lower respiratory tract) > airway epithelial cells (upper respiratory tract) > fibroblasts, endothelial cells and macrophages. The viral load of different specimen types are as follows: alveolar lavage fluid (optimal) > deep cough sputum > nasopharyngeal swab > oropharyngeal swab > blood [29,30]. Therefore, in some patients, the amount of virus in oropharyngeal or nasopharyngeal cells might be low or extremely low. If only the oropharyngeal or nasopharyngeal specimens are taken, the viral nucleic acid cannot be detected. Hence, samples of cases with suspected infection need to be taken in different periods and in different parts of the body to avoid false negative result. For example, if no virus is found in the pharyngeal sample, bronchial lavage or feces samples should be considered.

The improper sampling and sample management may also cause "false negative" result. As mentioned in this study, the improper collection site or the wrong usage of swab should be avoided as far as possible. The virus preservation tubes should also be managed properly. For example, the misuse of polypropylene or polyethylene plastic tubes which readily adsorb nucleic acids (DNA/RNA) could lead to a decrease in nucleic acid concentration in the preservation solution. In practice, polyethylene-propylene polymer plastic or specially-treated polypropylene plastic containers are recommended to store viral nucleic acids. Most of the above problems can be solved by effective training of specimen collectors.

Currently, the quality of RNA extraction cannot be fully guaranteed since there lacks a clear explanation for methods of RNA extraction. The sample size of amplification used in the system is usually determined by volume of sample rather than RNA amount, which may lead to varied quality of RNA extracted by different methods, thus affecting the final amplification effect. It is suggested that the amplification primers be increased in the samples to control the efficiency of RNA extraction, so as to avoid the false negative result caused by the unqualified nucleic acid extraction.

At present, according to the instructions of the NAT kit, samples from one site in a person are tested and only one response is made for a patient. So the lack of sample duplication may also cause false negative result. Therefore, an increase in the sampling sites in a patient (e.g. taking nasal and pharyngeal swab samples simultaneously) and parallel testing using different kits are suggested to effectively control false negative results caused by lack of duplication.

Standardizing the transport and storage of specimens, the operation of clinical laboratories, and the interpretation of results are also key to the accuracy and reliability of test results. Improvement can be made in the training of competent laboratory personnel, the optimization of the laboratory quality management system, and proper apparition of the working zone.

Table 1
Interpretation of two gene fragment detection kit.

ORF1ab gene	N gene	interpretation
+	+	positive
+ or suspicious	-	Resampling for review
-	+ or suspicious	Resampling for review
Suspicious	suspicious	Resampling for review
-	-	negative

Table 2
Interpretation of three gene fragments detection kits.

ORF1ab gene	N gene	E gene	Interpretation
+	+	+	positive
+	+	-	positive
+	-	+	positive
-	+	+	positive
+ or suspicious	-	-	Resampling review
-	+ or suspicious	-	Resampling review
-	-	+ or suspicious	Resampling review
-	-	-	negative

3.5. Positive NAT results in patients discharged from the hospital

Recently, a small number of patients have been tested positive for COVID-19 again after being discharged from the hospital.

A possible explanation could be that the antibodies produced in response to the original virus in the recovered patients might be ineffective against the mutated virus. Positive results may appear in the patient infected with the mutated virus [31]. Recently, an analysis of 103 genomes of SARS-CoV-2 from different countries and regions found 149 mutations, and evolved S and L (Orflab:T8517C,ORF8:C251T) subtypes, S and L subtypes whose mutation sites(generally located in N and S proteins) are not commonly used in COVID-19 NAT and antigen design [32]. Therefore, the pathogenesis, disease overview and course characteristics of the disease need to be further understood. Besides, the patients discharged from the hospital need a 14-day medical observation, during which follow-up, health monitoring and guidance should be carried out.

Another cause of this condition could be the NAT kits, which may create problems in the following aspects: the selection of gene sequence, composition of reagents and sensitivity of the method [33]. After treatment, the virus in the patient's body is reduced, and the negative result will occur when the viral load in the tested sample is below the detection limit. However, this result does not mean that the virus in the body completely disappears because the virus may "rekindle" after treatment cessation.

The COVID-19 NAT only tells the presence or absence of viral RNA, but cannot prove the viral activity or the transmissibility of the virus. In this sense, it is necessary to conduct virus culture on the clinical specimens to detect whether there exists a "live" virus.

Nucleic acids, as the genetic material of the virus, can be exterminated after antiviral treatment, but the remaining RNA virus genetic material (DNA) pieces are still in the body, which cannot be discharged entirely from the body. In certain circumstances, they can be kept longer, and become the culprit for the “transient” positive result of NAT. As the patient’s recovery progresses [34], after the residual RNA fragments in the body being gradually exhausted, the patient’s NAT result can turn negative.

4. Conclusion

As SARS-CoV-2 is a new pathogen, common clinical methods, such as routine culture and serology, can hardly be applied to clinical detection of it. Therefore, viral NAT is an optimal method to diagnose, monitor, prevent and control COVID-19. But the pathological process and clinical mechanisms of the disease have not been fully elucidated. At present, most COVID-19 NAT kits in use have not undergone comprehensive clinical evaluation, and there are also problems in standardization of laboratory testing. In clinical practice, the operating procedures and quality control protocols should be strictly followed. When the NAT result is not consistent with the clinical manifestations, imaging detection (CT) and laboratory examination (NAT + virus specific antibody detection) should be combined for further diagnosis. Obtaining the gene sequence of COVID-19 and using it for effective detection is just the first step. More efforts are needed to explore the virus traceability, the viral transmission routes, the pathogenesis, and the effective targeted therapeutic drugs and vaccines.

Authors’ contributions

MXD and LKS wrote the main parts of this manuscript; XZR wrote other parts and designed the figures; LTP improved the language of the manuscript; MXD and LKS conceived the structure and revised the manuscript; All authors read and approved the final manuscript.

Declaration of competing interest

The authors declare that they have no competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrc.2020.12.094.

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