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Real-time PCR-based SARS-CoV-2 detection

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ABSTRACT

Introduction: reverse transcription-polymerase chain reaction (RT-PCR) is an accurate technique in coronavirus detection. It was selected as a confirmatory test for SARS-CoV-2 infection due to proven safety in the diagnosis of MERS-CoV and SARS-CoV strains.
Objective: to characterize the reverse-transcription polymerase chain reaction assay as the main diagnostic test used to confirm SARS-CoV-2 infection during the active period of viral replication.
Method: a literature review was conducted in articles published up to May 2020. The following databases were consulted: Scopus, Wiley Online Library, SciELO, DIALNET, EBSCO, MEDLINE, and PubMed. Articles in Spanish and English were retrieved, selecting 46 references.
Development-Results: reverse transcription-polymerase chain reaction (RT-PCR) assay to detect SARS-CoV-2, targets to ORF1ab fragments, RNA polymerase dependent (RdRp), the E gene, the N gene, and the S gene. Nasopharyngeal swab achieved better results than oropharyngeal swab and saliva. RT-PCR testing using rectal swab specimens appeared required on suspected false-negative cases. The Reverse Transcription Loop-mediated Isothermal Assay showed faster diagnosis compared to RT-PCR with equal sensitivity and specificity.
Conclusions: the availability of diagnostic tests is crucial for the isolation of positive cases and traceability of the transmission epidemiological chain. RT-PCR proved to be the test of choice during the active period of viral replication. The RT-LAMP assay is a rapid diagnostic alternative with similar principles to the RT-PCR technique.

Keywords: Polymerase Chain Reaction, Coronavirus Infections, Clinical Laboratory Techniques

INTRODUCTION

Coronaviruses belong to the family Coronaviridae of the order Nidovirales, subfamily Coronavirinae (1). They are large, positive-sense RNA viruses comprising four genera: alpha, beta, delta, and gamma (2).

Up to 2019, six human coronaviruses (HCoV) were responsible for respiratory diseases. Two of them, the Severe Acute Respiratory Syndrome Coronavirus (SARS-CoV) and the Middle East Respiratory Syndrome Coronavirus (MERS-CoV), represent viral strains capable of infecting the lower respiratory tract (2).

An outbreak of severe acute respiratory syndrome (SARS) in 2002, caused by SARS-CoV-2, threatened the lives of 8 098 people with a rate of 774 deaths. The disease epicenter was Guangdong, China; and it spread internationally to more than a dozen countries. Bats were assumed as natural hosts (3,4).

In 2012, the Middle East Respiratory Syndrome (MERS), caused by MERS-CoV, emerged in Saudi Arabia. Bats were considered as the natural hosts and the intermediate hosts were dromedary camels. A total of 2494 cases, with 858 deaths, were reported by rapid nosocomial transmission. MERS revealed clinical features similar to SARS with prominent gastrointestinal symptoms and acute renal failure (3,4).

The Chinese Government warned the international scientific community on 29 December 2019, the outbreak appearance in Wuhan of several sporadic cases presenting pneumonia with no known underlying pathology, which was epidemiologically associated with a wholesale seafood market (4). On 21 January 2020, Chang et al. (5) reported the first case in Taiwan of Coronavirus Disease 19 (Covid-19) in a 55-year-old woman, after testing positive by Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) using oropharyngeal swab samples, to a new strain of the genus betacoronavirus (6,7), the Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2). After the first confirmed case report, the virus spread successfully worldwide, being considered as a pandemic by the World Health Organization (WHO) in March 2020 (8).

RT-PCR is based on the amplification of viral RNA in several cycles until sufficient viral genetic material is obtained to be subsequently detected by the markers used. It is a real-time test that can effectively diagnose the disease in stages where the virus is replicating and there is sufficient viral load for its detection.

The global outbreak of SARS-CoV-2 has challenged humanity to find a rapid and safe solution for containing the accelerated spread of Covid-19. In the meantime, an effective diagnosis is intended to reveal the magnitude of the problem.

The prompt deployment of diagnostic tests and protocols used for virus detection, is a crucial contribution, especially RT-PCR, based on its proven safety detecting other coronavirus strains. The use of this method, together with several actions, proved the effectiveness of controlling the Covid-19 in Chinese territory. It was possible to reduce the peak of cases reached on 12 February 2020, with 14 840 daily cases confirmed in Hubey (9) to only one confirmed case in the whole territory of the Asian country on 2 May 2020 (10).

The purpose of this investigation is to characterize RT-PCR as the main diagnostic test used to detect SARS-CoV-2 during the active period of viral replication.

METHOD

A review was conducted on articles published up to May 2020. The following databases were consulted: Scopus, Wiley Online Library, SciELO, DIALNET, EBSCO, MEDLINE, and PubMed.

Data collection was carried out during April and May. The terms used in the search were: RT-PCR test, RT-PCR, Reverse Transcriptase Polymerase Chain Reaction, COVID-19, SARS-CoV-2, and its Spanish translations "RT-PCR test", "RT-PCR", "Reacción en Cadena de la Polimerasa con Transcriptasa Inversa", "COVID-19", "SARS-CoV-2".

The terms were used in search formulas. The structures of the search formulas were database-specific. Forty-six articles published in health sciences journals were retrieved.

DEVELOPMENT

Comparisons between the genome of 1 008 types of SARS coronavirus in humans, 338 in bats, and 3 131 MERS coronaviruses allowed establishing a high similarity with SARS-CoV-2 with only 5 differences in nucleotides of approximately 29.8 kb (11). The complete genome sequence of the new coronavirus (WH-Human_1) was first published on 10 January 2020 (12). A review of the SARS-CoV-2 genetic code showed differences with the Severe Acute Respiratory Syndrome (SARS) or similar coronaviruses corresponding to 380 amino acid substitutions (11). SARS-CoV-2 has 14 ORFs encoding 27 proteins and is parallel to the SARS-like bat coronaviruses (11,13,14).

The complete sequencing of the viral code was a fundamental contribution to the development of diagnostic tests based on the principle of RT-PCR, given the need for primer synthesis that allowed correct identification of specific base-pair sequences associated with changes in the SARS-CoV-2 genome (15), to avoid cross-reaction with other viral strains from the same family or with respiratory pathogens.

The target regions for diagnostic kits using RT-PCR include reading the RNA polymerase dependent (RdRp), ORF1ab fragments, the envelope gene (E gene), the nucleocapsid protein gene (N gene) (15-20) and the S gene (13,16). To improve detection sensitivity, most manufacturers choose two or more target regions of the viral nucleic acid sequence (13,15,21). The diagnosis is confirmed in patients with positive results for both ORF1ab gene amplification and gene (N) or gene (E) amplification (18).

One-step RT-PCR targeting ORF1b fragments or the nucleocapsid gene (N gene) of SARS-CoV-2 was designed to react with SARS-CoV-2 and its closely related viruses, such as SARS coronavirus, which can result in false-positive reactions when identifying the virus causing Covid-19 (22).

The German company TIB MOLBIOL GmbH, in collaboration with several partners, developed a novel and robust real-time RT-PCR assay starting the second week of January 2020. The test detects viral RNA by RT-PCR assays targeting envelope genetic (E) and RNA-dependent polymerase (RdRp) (23). It was highly specific for SARS-CoV-2 RNA (Gene E: 3.2 RNA copies/reaction 95% CI: 2.2-6.8) (RdRP 3.7 RNA copies/reaction 95% CI: 2.8-8.0) and did not cross-react with other coronaviruses (24). In another approach, the researchers created single-step RT-PCR assays to detect the ORF1b and N-gene regions of SARS-CoV-2 in 1 hr 15 min (23).

Chan et al. (25) developed an RT-PCR test targeting SARS-CoV-2 RNA polymerase (RdRp)/helicasa (Hel), which did not cross-react with other coronaviruses and exhibited increased analytical sensitivity compared to the RdRp-P2 assay widely used in European laboratories (22).

High performance commercially available tests include the Xpert® Xpress SARS-CoV-2 assay by Cepheid, USA. This test provides results in only 45 minutes using the GenXpert benchtop system. It requires one minute for sample preparation and targets multiple regions of the viral genome (26,27). In clinical samples, Xpert Xpress SARS-CoV-2 achieved a 100 % match compared to other RT-PCRs developed, and the assay outperformed the diagnostic platforms commonly used in the sensitivity panel (28).

Nasopharyngeal swabs are usually the collection method used to diagnose viral presence by RT-PCR but may miss an infection in the early stages, in which case a deeper sample obtained by bronchoscopy is useful (29). A bronchial sample has the benefit of increased detection when testing for viral nucleic acid in the alveolar lavage fluid, followed by sputum, nasal and pharyngeal swabs (13). In a study of 4880 cases, Liu et al. (29) reported an alveolar lavage fluid with the highest 100 % positive rate for the ORF1ab fragment of SARS-CoV-2; sputum exhibited a positive rate of 49.12 %, and nasal and pharyngeal swab samples had a low positive rate of 38.25 %.

Wang et al. (31) reported that oropharyngeal swabs were used significantly more often than nasopharyngeal swabs during the Covid-19 outbreak in China; However, SARS-CoV-2 RNA was detected in only 32 % of the oropharyngeal samples, which resulted significantly inferior to the 63 % positivity on nasal swabs, to corroborate the results another study was carried out comparing both samples where 73,1 % of positive cases using nasopharyngeal swabs turned negative on the oropharyngeal swab (32), indicating that false negatives can occur using only samples from the oropharyngeal cavity (19,32).

In most individuals with symptomatic Covid-19 infection, the viral RNA in the nasopharyngeal swab is detected starting the first day of symptoms and peaks after one week (15). In a study by Wölfel et al. (33), swabs from all patients taken between day one and day five were positive for the virus, while none of the 27 urine samples and 31 serum samples were positive for SARS-CoV2-RNA. Another trial by Xiao et al. (19) reported an average time of 20 days from symptom onset to a negative SARS-CoV-2 RT-PCR test result. In some cases, viral RNA has been detected by RT-PCR 6 weeks after the first positive test (15).

Several screened cases reported the detection of viral RNA in lower respiratory tract samples (sputum or endotracheal aspiration) in 100% of cases, nasal mucosa (81%), stool (69%), oropharynx (63%), gastric content (46%), anal mucosa (25%), conjunctiva (6.7%) and urine (6.2%) (34). Wang et al. (31) identified that bronchoalveolar lavage fluid specimens had the highest positive rates (14 of 15; 93%), followed by sputum (72 of 104; 72%), nasal swabs (5 of 8; 63%), fibrobronchoscopic brush biopsy (6 of 13; 46%), pharyngeal swabs (126 of 398; 32%), stool (44 of 153; 29%) and blood (3 of 307; 1%).

Patients with advanced-stage Covid-19 pneumonia have exhibited a high viral RNA load for SARS-CoV-2 when testing stool samples, as well as a lower presence of the virus in the respiratory tract. In previous outbreaks of Coronavirus causing epidemic events, an enteric involvement in transmission was established, therefore, apart from the collection of direct respiratory samples, the analysis of rectal swab samples should be considered as a method of choice for detecting SARS-CoV-2 in advanced cases of Covid-19 (26,35,36).

In another study, Wang et al. (37) reported three cases discharged meeting all the criteria approved by the National Health Commission of the People's Republic of China and were subsequently readmitted as positive cases to the virus. The three patients who exhibited mainly gastrointestinal symptoms such as diarrhea and changes in bowel habits, tested positive for fecal specimens after found to be negative by testing respiratory samples. In our opinion, it is necessary to include diagnostic tests using rectal swab collections in cases where respiratory specimens are negative and the patient maintains suggestive symptoms of Covid-19.

The salivary glands express the surface receptor for the angiotensin II converting enzyme (ACE2), the entry into the cell of SARS-CoV-2 depends largely on its binding to this receptor (38). In a research carried out in Italy, Azzi et al. (39) collected salivary samples from 25 patients affected by COVID-19, the samples were analyzed by RT-PCR, resulting in positive for all patients; another similar study conducted by Williams et al. (40) in Australia, positive samples were found in 33 of 39 patients infected by the virus. Although there are still insufficient studies for the detection of SARS-CoV 2 using saliva as a sample, increased sensitivity has been found using nasopharyngeal swabs.

The dependence on manual settings in the RT-PCR test is one of the fundamental limitations during the molecular diagnosis of SARS-CoV-2 when it comes to scalability and speed in outbreak scenarios. Therefore, alternative workflows are required to allow rapid tracking of high-priority samples. A fully automated RT-PCR platform, providing extraction, amplification, and signal detection without the need for human interaction could be the solution, such as the NeuMoDx 96 system (41) or the Cobas 6800 SARS-CoV-2 test (42).

Although the automation of the whole process is not feasible in all countries, mainly due to its high price, adapting strategies to speed up the manual analysis of samples would be a useful alternative in these cases.

Another way to achieve an effective identification and isolation of SARS-CoV-2 would be through a rapid and robust diagnostic test, which can be performed in the field and local care centers, without the need for specialized equipment or highly trained professionals to interpret the results. This is the case of the RT-LAMP (Reverse Transcription Loop-mediated Isothermal) test (43-45).

This novel test presented positive diagnostic results within 26.28 ± 4.48 min (46), while the RT-PCR assay requires 1-2 h after viral RNA preparation to obtain a result. It uses the same principle of polymerase chain reaction (PCR) but RT-LAMP does not require the thermal cycles that favor DNA replication used in RT-PCR and has the advantage of running at a constant temperature between 60 and 65ºC. In a report by Lin et al. (46) using 130 swabs and bronchoalveolar lavage fluid samples, the assay showed 58 confirmed individuals and no cross-reactivity with other respiratory pathogens, sensitivity was 100% with 100% specificity.

CONCLUSIONS

SARS-CoV-2 is a highly contagious virus that remains difficult to control. The availability of diagnostic tests is crucial for the isolation of positive cases and traceability of the transmission epidemiological chain. RT-PCR has proven to be a test of choice during the active period of viral replication. The testing samples may include oropharyngeal, nasopharyngeal, sputum, bronchial, and rectal swabs. The RT-LAMP assay is an effective alternative for the identification and isolation of the new coronavirus, with similar principles to RT-PCR.

CONFLICTS OF INTEREST

The authors declare that there is no conflict of interest.

AUTHOR'S CONTRIBUTION

José Francisco Cancino Mesa: Conceptualization, Data curation, Resources (Equal), Visualization (Equal), Writing original draft, Writing – review & editing (Equal).
Adrián Alejandro Vitón Castillo: Methodology, Supervision, Resources (Equal), Visualization (Equal), Writing – review & editing (Equal).

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