e-ISSN: 0976-822X, p-ISSN:2961-6042

Available online on http://www.ijcpr.com/

International Journal of Current Pharmaceutical Review and Research 2026; 18(7); 528-532

Original Research Article

A Study of COVID-19 Testing: Clinical Utilities, Performance Barriers and the Transition to Multiplex Surveillance

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Received: 01-04-2026 / Revised: 15-05-2026 / Accepted: 21-06-2026

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Conflict of interest: Nil

Abstract

The shifting status of SARS-CoV-2 from a pandemic emergency to an endemic respiratory threat has fundamentally altered global diagnostic requirements. This review article comprehensively examines the trajectory of COVID-19 testing protocols, contrasting the clinical efficacy and structural limitations of predominant diagnostic tools. Although quantitative real-time PCR (RT-qPCR) serves as the gold standard for analytical sensitivity, its utility is constrained by logistical delays, resource costs, and the post-infectious RNA retention paradox. In contrast, rapid antigen diagnostic tests (Ag-RDTs) facilitate decentralized screening but are hindered by viral-load dependencies and diminished accuracy in asymptomatic patients, requiring serial testing protocols. Furthermore, both modalities are vulnerable to sample degradation and variant-driven mutational escape. To navigate these limitations and differentiate overlapping clinical symptoms from co-circulating seasonal pathogens like Influenza A/B and RSV, this paper explores the vital evolution toward multiplex syndromic platforms alongside cutting-edge CRISPR and AI-driven screening techniques. Ultimately, consolidating integrated multiplex surveillance optimizes public health responses, enhances antimicrobial stewardship, and secures a scalable diagnostic network ready to confront future novel respiratory outbreaks.

Keywords: SARS-CoV-2, Multiplex Syndromic Testing, RT-qPCR, Rapid Antigen Diagnostic Tests (Ag-RDTs), Respiratory Pathogens, Public Health Surveillance, Diagnostic Barriers.

DOI: 10.25258/ijcpr.18.7.84

Introduction

The identification of Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2), the causative viral agent of Coronavirus Disease 2019 (COVID-19), necessitated the rapid development and scaling of diagnostic frameworks globally. In the initial phases of the public health emergency, diagnostic workflows were highly centralized, relying heavily on dedicated molecular laboratories. This structure frequently generated severe operational bottlenecks, long turnaround times, and extensive reagent supply shortages. [1]

As SARS-CoV-2 transitioned from an acute pandemic threat into a predictable, endemic respiratory pathogen, the core objectives of testing evolved. The priority shifted from strict containment and contact tracing to individualized patient triage, immediate clinical management, and continuous sentinel surveillance. In the current clinical landscape, testing strategies face new complexities. Co-circulating seasonal pathogens—including Influenza A, Influenza B, and Respiratory Syncytial Virus (RSV)—produce acute respiratory infection (ARI) symptomatology that overlaps heavily with COVID-19, making differential diagnosis impossible based on clinical evaluation alone. [2] Sustained control over the current endemic landscape relies heavily on our ability to constantly update and adapt our clinical understanding of SARS-CoV-2. Consequently, a systematic study of COVID-19 testing methodologies is essential to understand how clinical testing pipelines can remain accurate, resilient and cost-effective.

Clinical Utilities of Established Modalities: The Definitive Reference Standard (Molecular methods): Nucleic Acid Amplification Tests

(NAATs), predominantly executed via quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR), continue to serve as the benchmark reference standard for confirming an active infection.

Biophysical Mechanism: Molecular assays operate by isolating and exponentially copying highly stable, conserved segments of the SARS-CoV-2 single-stranded RNA genome. Modern multiplex RT-PCR setups minimize the risk of diagnostic failure due to viral evolution by testing for two or more distinct genetic sequences simultaneously. The most common diagnostic targets include: the Nucleocapsid (N) gene, the Envelope (E) gene, the Open Reading Frame 1ab (ORF1ab) sequence and the RNA-dependent RNA polymerase (RdRp) region. During the thermal cycling process, fluorescently labeled oligonucleotide probes generate a signal that scales with the amplification of the target DNA. The cycle at which this fluorescence crosses a designated background threshold is defined as the Cycle Threshold (Ct) value, which serves as a semi-quantitative inverse proxy for viral load. [3] Because RT-qPCR cycle threshold (Ct) values are heavily influenced by the chosen detection platform, targeted genes, sample origin, and RNA integrity, a universal interpretation framework does not exist. Generally, nasopharyngeal Ct thresholds are stratified as: <20 (very high viral load), 20–25 (high load), <30 (moderate-to-low load), and 30–33 (minimal transmission risk). [4]

Clinical Advantages and Operational Realities: The definitive advantage of molecular testing is its unmatched analytical sensitivity. Most authorized reference assays maintain a lower limit of detection (LoD) down to 100 to 500 genomic copies per milliliter of sample matrix. [5] However, this hyper-sensitivity creates a distinct clinical paradox: the Post-Infectious Recovery Paradox. RT-PCR can easily detect residual, non-viable, or highly fragmented viral RNA strands for several weeks after an individual has clinically recovered and ceased shedding live, transmissible virus. [6] Therefore, a prolonged positive molecular result does not necessarily indicate active infectivity. [7]

Operationally, laboratory-based RT-PCR remains restricted by high cost per test, specialized hardware requirements, and unavoidable logistical delays (12 to 48 hours for external transport and processing). While isothermal alternative technologies, such as Loop-Mediated Isothermal Amplification (LAMP), allow for rapid processing without advanced thermal cyclers, they exhibit a notable drop in sensitivity when dealing with low-concentration specimens (Ct >30). [8]

Rapid Antigen Diagnostic Tests (Ag-RDTs): Point-of-Care Breakthroughs: To relieve pressure on public health laboratories and provide actionable, real-time data, rapid antigen diagnostic tests (Ag-RDTs) became crucial community screening tools.

Biochemical Principles: Unlike molecular assays designed to isolate genetic codes, Ag-RDTs employ lateral flow immunochromatography to detect viral structural proteins. The vast majority of commercially licensed kits utilize monoclonal antibodies targeted to capture the viral nucleocapsid (N) protein, rather than the highly mutable spike (S) protein. The N-protein is highly abundant during active viral replication and maintains structural stability across different lineages. [9]

During a test, the patient's nasal or nasopharyngeal swab fluid migrates across a nitrocellulose membrane via capillary action. If viral proteins are present, they attach to colored or fluorescently tagged conjugate antibodies, forming a visible target band at the test line zone within 15 to 30 minutes.

Real-World Performance Variations: Independent validation studies demonstrate that while rapid antigen tests deliver exceptional clinical specificity (98 to 99), their overall diagnostic sensitivity ranges from 65% to 85% due to variables in viral kinetics. [10]

1. Viral Load Dependency: When viral load is at its peak (Ct < 25), rapid antigen tests reliably identify active cases, matching molecular detection rates (> 95%). However, during early incubation or late-stage resolution (Ct >30), the antigen concentrations frequently fall below the test strip's lower limit of detection, causing false negatives. [10]

2. Symptomatic vs. Asymptomatic Variations: Sensitivity is considerably higher in patients experiencing active symptoms (typically 80% to 88%) than in asymptomatic screening cohorts (50% to 75%). Asymptomatic individuals are frequently tested outside the peak viral shedding window, meaning their samples lack the minimum protein density needed to trigger a visible line. [10]

3. The Serial Testing Mandate: To counteract lower analytical sensitivity during the initial viral incubation phase, public health regulatory bodies implemented sequential diagnostic mandates for over-the-counter rapid antigen test kits. Under these guidelines, symptomatic individuals who receive an initial negative rapid test are required to perform a second confirmatory test 48 hours later to capture potential late-rising viral loads. Conversely, because asymptomatic individuals exhibit significantly lower viral shedding kinetics, they must complete three consecutive tests over a total 96-hour window (at 48-hour intervals) to

reliably rule out an active, transmissible SARS-CoV-2 infection. [11]

Real-World Performance Barriers: Despite rapid deployment, both molecular and immunological testing pathways encountered severe real-world operational and biological friction.

Pre-Analytical and Sampling Barriers

Matrix Variations: Anatomical collection site variance (e.g., anterior nares vs. nasopharyngeal vs. saliva) significantly altered viral yield. Nasopharyngeal swabs preserved the highest sensitivity but suffered from poor compliance due to patient discomfort.

Degradation and Transport: Cold-chain failures or delays in viral transport media (VTM) led to RNA degradation, causing false-negative RT-qPCR results.

Analytical Barriers and Viral Evolution

The "Target Blindness" Phenomenon: Genetic drift in the SARS-CoV-2 genome regularly threatened assay reliability. For instance, deletion mutations in the spike (S) gene of certain variants caused S-gene target failure (SGTF) in prominent commercial PCR assays.

Kinetic Delays in Antigens: During early-stage infections or low-viral-load infections (common in highly vaccinated populations), RATs exhibited a prolonged false-negative window. Research confirms that at low viral concentrations, positive bands frequently emerge only at the absolute end of the designated 15-to-30-minute reading window, leading to premature negative interpretations by users.

Genetic Drift and Antigen Stability: A primary vulnerability facing all forms of COVID-19 testing is the continuous evolution of the SARS-CoV-2 genome. As new sub lineages accumulate amino acid mutations within their structural coding regions, single-target tests face the risk of diagnostic escape or performance drift.

If a novel variant modifies the precise epitope structure recognized by a test's monoclonal antibody, the binding affinity can decrease dramatically, leading to an increase in false negatives.

To preserve long-term diagnostic resilience, assay manufacturers must regularly evaluate their antibody designs against emerging strains and prioritize multi-target configurations.

The Contemporary Transition: Syndromic Multiplex Testing: With the stabilization of SARS-CoV-2 into seasonal endemic patterns, singleplex testing (testing for one virus at a time) creates a distinct diagnostic bottleneck. Clinical presentation alone cannot differentiate between COVID-19, Influenza A, Influenza B, or RSV, given their overlapping symptom profiles (fever, cough, myalgia, and respiratory distress). In primary care and home settings, clinicians and consumers now prefer multiplex antigen panels that offer simultaneous, differential diagnosis from a single specimen collection.

[Patient Presents with Acute Respiratory Infection Symptoms]

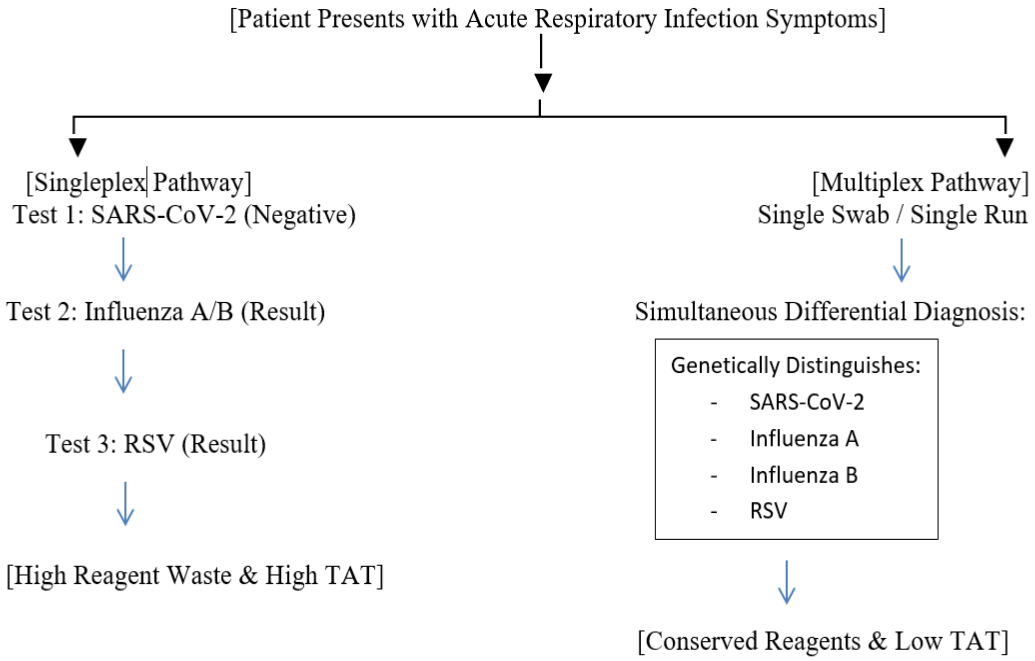

Figure 1:

These advanced diagnostic platforms integrate distinct, separated rows of monoclonal antibodies on a single test strip. This design safely isolates and identifies specific nucleocapsid or matrix proteins for multiple target viruses simultaneously. During winter seasons, these multiplex kits allow individuals to rapidly pinpoint the precise cause of their respiratory symptoms, guiding time-sensitive antiviral interventions (e.g., Oseltamivir for Influenza) within narrow therapeutic eligibility windows.

Emerging Diagnostic Frameworks: The pandemic accelerated several niche bio analytical techniques into mainstream medical science:

- **CRISPR Diagnostics:** Utilizing Cas12 or Cas13 enzymes targeted to clear SARS-CoV-2 RNA sequences, these platforms achieve the sensitivity of a PCR with the speed of a lateral flow strip.
- **AI and Machine Learning:** Computer Vision models trained on chest CT scans and X-ray datasets became highly reliable secondary diagnostic tools in triaging severe respiratory distress cases when laboratory reagent lines were entirely blocked.

Public Health and Epidemiological Impacts

The integration of multiplex networks yields profound structural benefits for public health surveillance:

- **Antimicrobial Stewardship:** Rapidly defining a viral etiology prevents the empirical, unnecessary prescription of antibacterial agents for viral respiratory syndromes.
- **Syndromic Mapping:** Aggregated data from automated closed multiplex platforms (e.g., Cepheid GeneXpert, BioFire) feeds directly into public health databases, generating real-time heat maps of co-circulating seasonal respiratory pathogens.
- **Healthcare Optimization:** Early identification allows hospitals to implement cohort-specific isolation protocols (e.g., separating RSV-positive pediatric patients from influenza-positive cohorts), reducing nosocomial (hospital-acquired) transmission.

Conclusion

The ongoing study of COVID-19 testing demonstrates that diagnostic technologies must continuously adapt to match the epidemiological status of the virus.

While centralized laboratory RT-PCR remains the irreplaceable reference standard for high-sensitivity diagnostic validation and complex clinical cases, point-of-care rapid antigen tests and multiplex home panels provide the processing speed and scalability required for routine population screening. Successfully balancing the analytical accuracy of molecular diagnostics with the speed, convenience and lower cost of lateral flow testing remains essential for managing respiratory health networks efficiently. The study of COVID-19 testing highlights that no singular assay is a silver bullet; rather, an optimal diagnostic framework requires an intentional mosaic of high-sensitivity molecular confirmation and high-frequency, low-barrier rapid screening.

Moving forward, the primary objective for the global scientific community is to maintain the molecular infrastructure built over the last several years. Repurposing these open diagnostic networks for other high-risk pathogens (such as avian influenza or novel coronaviruses) guarantees that the global healthcare network will not need to build its primary line of defense from scratch when the next pandemic arises.

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