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REVIEW

Optimizing testing regimes for the detection of COVID-19 in children and older adults

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ABSTRACT

Introduction: Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) infection is a major pandemic and continuously emerging due to unclear prognosis and unavailability of reliable detection tools. Older adults are more susceptible to COVID-19 than children showing mature Angiotensin-Converting Enzyme 2 (ACE2), low concentration of immune targets, and comorbid conditions. Several detection platforms have been commercialized to date and more are in pipeline, however, the rate of false-positive results and rapid mutation of SARS-CoV-2 is increasing. Additionally, physiological, and geographical variations of affected individuals are also calling for diagnostic methods optimization.

Areas Covered: Extensive information related to the optimization and usefulness of SARS-CoV-2 diagnostic methods based on sensitivity and specificity as definitive and feasible investigative tools is discussed. Moreover, an option of combining laboratory diagnostic methods to improve diagnostic strategies is also proposed and discussed in the comparative section of optimization studies.

Expert Opinion: The review article explains the importance of optimization strategies for SARS-CoV-2 detection in children and older adults. There are advancements in COVID-19 detection including CRISPR-based, electrochemical, and optical-based sensing systems. However, the lack of sufficient studies on a comparative evaluation of standardized SARS-CoV-2 diagnostic methods among children and older adults, limit the authentication of commercialized kits.

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SARS-CoV-2; molecular diagnostics; immunodiagnostic; nanotechnology; sensors; point-of-care (POC) kits; children and older adults

1. SARS-CoV-2 outbreak: Introduction and commencement

This decade has witnessed sudden outbursts of epidemics and pandemics, for instance, Middle Eastern Respiratory Syndrome (MERS) in the Middle East 2012, Ebola virus disease (EVD) in West Africa in 2014, Zika virus disease in 2015 at various parts of Latin America, and COVID-19 pandemic in 2020 [1]. These outbursts of infectious diseases have imposed a great threat to human health and the global economy. Currently, the world is facing the impact of deathly novel coronavirus (nCoV-2019) which is also termed as Severe Acute Respiratory Syndrome Coronavirus-2 (SARS-CoV-2). Patient zero was first identified in Wuhan, Hubei province, China in December 2019, and rapid transmission of viral infections affected other countries in no time, and in the end, World Health Organization (WHO) declared a global public health emergency [2,3]. Since then, several epidemiological and clinical studies are conducted to understand pathogenesis and transmission of infection and understand how we can further focus on diagnostic and therapeutic interventions to pass the pandemic.

Infectious SARS-CoV-2 is an enveloped positive single-stranded RNA virus (+ssRNA) with 29,903 nucleotide RNA genome and ~100 nm in diameter along with four structural proteins, namely envelope (E), nucleocapsid (N), membrane (M), and spike (S) [4,5]. SARS-CoV-2 infection is mediated by the binding of S-protein to Angiotensin-Converting Enzyme 2 (ACE2) receptors on the host cell surface [6,7]. This leads to further replication of the viral genome and synthesis of structural proteins along with 25 different non-structural proteins including RNA-dependent RNA polymerase (RdRp) (Figure 1) [8,9].

The pathogenesis of SARS-CoV-2 involves two distinctive but synergistic mechanisms viz. (i) Viral replication during the incubation period in the initial 5–7 days and (ii) host immune response against localized lung inflammation [10,11]. An early study also indicated high virus transmission with a variable reproductive rate (R_0) of 2.2 (95% CI: 1.4–3.9) [12] and 2.68 [13], which indicates that a COVID-19 infected person can transmit the spread to an average of ~2.2 persons. Therefore, quarantine, self-isolation, repetitive hand washing, and wearing masks are suggested by the researchers, and government officials took strict decisions in this scenario. Despite all these efforts, 200 million active cases and 4.25 million deaths were reported in 218 countries and territories as of 05 August 2021. Figure 2 illustrates the progressive increment of COVID-19 cases worldwide and major milestones achieved from December 2019 to June 2021.

COVID-19 (Corona Virus Disease-2019) manifests a range of clinical symptoms including mild flu-like to life-threatening conditions, however, the major challenge is to identify asymptomatic cases especially in children and older adults. For instance, young children either experience mild or asymptomatic illness once infected with SARS-CoV-2, thus a lower prevalence of infection in young children

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Article highlights

- Focused on the limitations of laboratory-based SARS-CoV-2 diagnostic techniques in children and older adults.
- Optimization of COVID-19 detection assays and commercialized kits are highly recommended.
- Proposed strategies of optimized diagnostic methods for active COVID-19 cases in children and older adults.
- Promoted next-generation sensors and involvement of CRISPR-Cas-like techniques for differential detection mechanism for SARS-CoV-2 identification.

is observed. Though, in few cases of symptomatic children with SARS-CoV-2-specific antibodies showed negative RT-PCR test [14]. This can be justified by the possibility of an active immune system and pre-existing antibodies against other viral infections, for example, pneumonia in children. Additionally, the SARS-CoV-2 sample collection method and type of specimens collected from children are another concerns as the viral load may vary significantly. Furthermore, pathogenesis and transmissibility of COVID-19 may also differ in children and older adults, thus responsible factors of these differences include [15]:

(1) A Low number of ACE2 receptors in children, thus less mature enzyme protects against SARS-CoV-2 variants.
(2) Low inflammatory cytokines, which undergo substantial changes in adulthood. However, high levels of procalcitonin and interleukin-6 were reported in COVID-19 positive children [16,17].
(3) Variable protective nature of Th2 immune cells and associated eosinophilia.

Though, the exact pathogenesis of SARS-CoV-2 is unknown, yet severity variation in children and older adults calls for more investigation to validate detection methods. Therefore, the availability of accurate and quick COVID-19 detection assays and laboratory procedures are extremely valuable in clinical set-up due to:

(1) Viral genome amplification and sequencing-based complicated molecular diagnostic tests are available for COVID-19 confirmation. Real-time Reverse Transcriptase-Polymerase Chain Reaction (rRT-PCR)-based tests which rely on the identification and amplification of viral nucleotide, serological tests for host antibody testing, viral culture-based tests, and radiology-based diagnostic techniques are available for COVID-19 confirmation. But all these methods vary according to physiological environment and health of host cells along with the age factor, as older adults have activated ACE2 enzyme and are more prone to infection. Additionally, RT-PCR is a quantitative method, whereas COVID-19 is qualitatively measured as positive or negative, therefore pre-existing diagnostic techniques need to be modified as the semi-quantitative methods, especially in older adults.
(2) Additional parameters are also required because of the presence of higher mutational variability of the SARS-

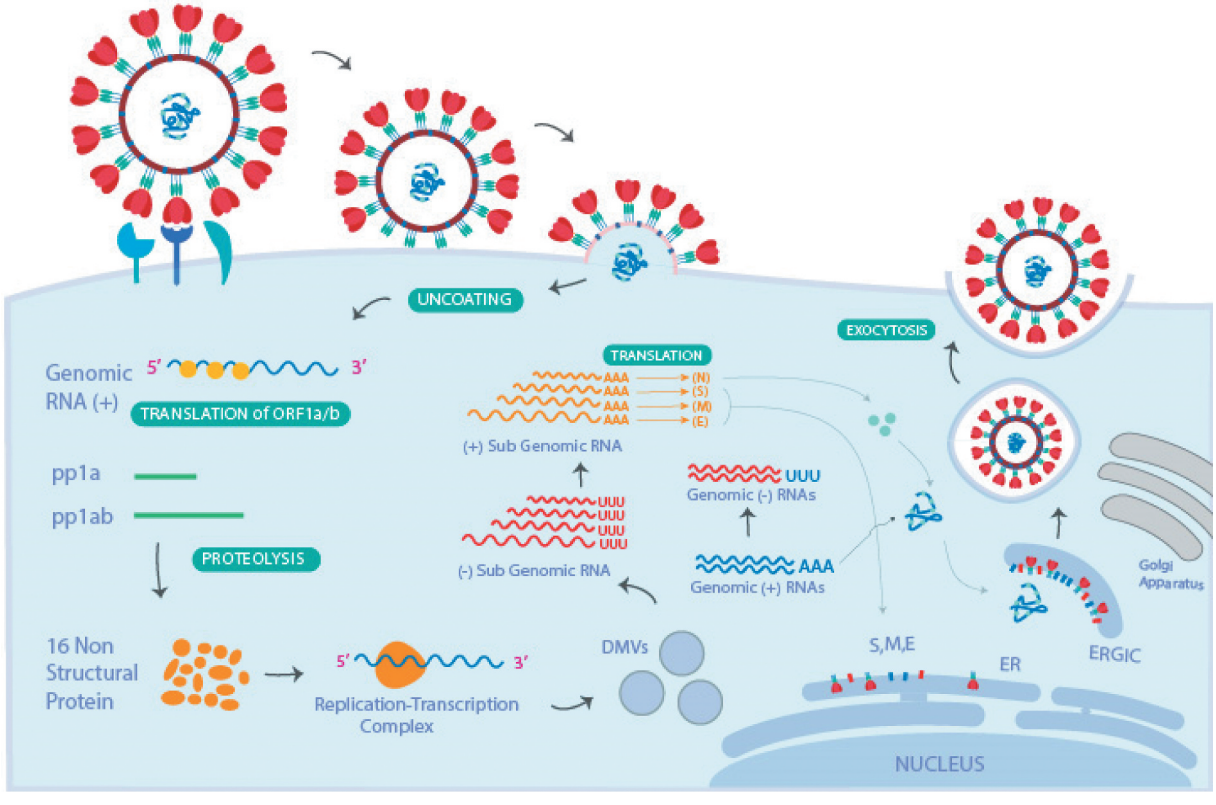

Figure 1. Representation of host cell interaction with SARS-CoV-2 and potential molecular mechanism of viral infection.

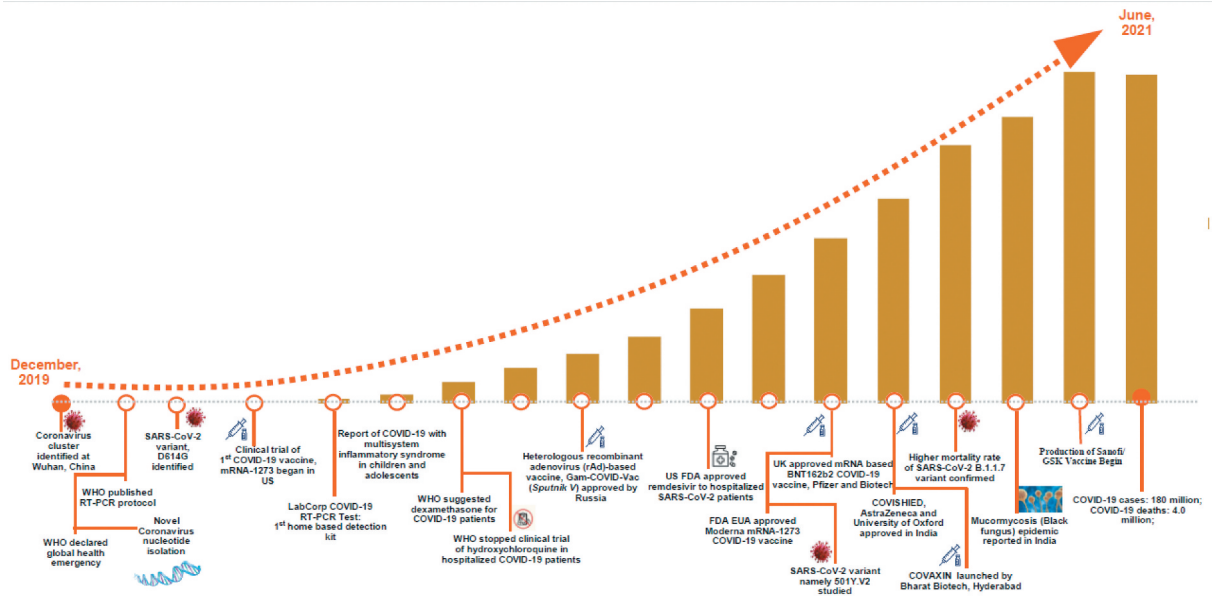

Figure 2. COVID-19 timeline with major events milestones.

CoV-2 virus and geographical variation of the host. As a result, many R&D research industries across different countries are racing to develop rapid testing kits, but most of them are approved for emergency use or may give false-positive results due to immunoglobulin cross-reaction.

Therefore, the specific objective of this review is to provide an overview of the need for optimizing different diagnostic methods for accurate COVID-19 detection in children and older adults. In the first section of this review article, we explain clinically prescribed latest innovative diagnostic techniques for COVID-19 detection along with their associated limitations. The next section of the article provides updates on diagnostic techniques and the need for further optimization in SARS-CoV-2 detection in detail. The last section of this narrative gives our opinion on US Food and Drug Administration (FDA) approved clinical diagnostic methods against SARS-CoV-2 and provides innovative ideas to design efficient detection strategies to establish globally standardized protocols in future reference.

2. Age-related impact of COVID-19

Understanding the role of age in the COVID-19 spread and severity is expository for evaluating the impact of precautionary measurements for decreasing transmission and estimating the potential burden of SARS-CoV-2 at the global level. In the following section, we hereby discuss the incidence, relevant parameters, and optimization of detection techniques for COVID-19 in comparison between children and older adults in detail.

2.1. Incidence of 2019-nCoV infection in children

Till date limited studies reported, in which younger age groups were infected with SARS-CoV-2, although infection was mild with the equivalent transmissibility [18,19]. A china-based study has shown 1.0% of children (<10 years) were found positive among 44,762 confirmed COVID-19 cases [20]. In another study, only 0.5% (0–4 years) and 1.3% (5–17 years) of people with lower age groups were tested positive out of 32,437 confirmed tests at public health laboratories in the US [21]. These studies stated lower frequency and severity of common symptoms in children compared to older adults.

Presently, there is only *in-vitro* evidence of low SARS-CoV -2-specific ACE2 receptors in children [22,23], however, several studies have suggested the value of innate immunity and occurrence of naïve T-cells responses in less severity of the SARS-CoV-2 infection in children [24,25]. Primary responder immune cells, especially monocytes, natural killer cells (NK), and dendritic cells are observed to act against the SARS-CoV-2 virus and resolve the infection [24]. However, asymptomatic children are difficult to be identified, as they cannot explain their health status or contact history with COVID-19 positive patients, thus they can be silent carriers of infection [26]. At the same time, children with comorbid conditions, such as respiratory diseases, immunodeficiency disease, chronic heart diseases, metabolic diseases, and tumors are extremely vulnerable to SARS-CoV-2 infection. In this scenario, >2000 children with COVID-19 in which 4.0% of children were asymptomatically positive, 5.0% had dyspnea or hypoxemia and 6.0% children progressed to Acute Respiratory Distress Syndrome (ARDS) were identified [27]. These clinical manifestations were more prominent in infants and pre-school children compared to older children. Besides, SARS-CoV-2 transmission from positive mothers to neonates was retrospectively analyzed where

premature labor, altered liver function, fetal and respiratory distress were also confirmed. However, all neonates who were tested COVID-19 negative, confirmed disability of had no confirmed vertical transmission. Though the children are vulnerable to SARS-CoV-2 infection, their clinical manifestations were considered less severe than that of older adults and have no significant influence on gender.

The major question the scientific community is asking is: why children have a milder SARS-CoV-2 infection once compared to older adults. Limited studies were performed in search of less COVID-19 severity in children; the major results justifying this reason are as follows:

(1) A major hypothesis supports the view that children have less mature ACE2 enzyme in the early stage of development, therefore the binding affinity between SARS-CoV-2 specific S-protein and ACE2 may be lower [28].
(2) The antibodies generated by repeated viral exposure by different pathogens may respond against SARS-CoV-2 and this tendency substantially changes from birth to adult age [29].
(3) The proportion of elevated inflammatory markers against SARS-CoV-2 is reported lower in children [16], yet few cases showed an increase in procalcitonin [30,31].
(4) SARS-CoV-2 coronavirus has to compete with other viruses at the epithelial lining of the lungs or airways in children in terms of growth and proliferation due to previous pathogenic infections [19].

Thus, finite information available on SARS-CoV-2 infection in children itself poses a challenge due to the absence of knowledge regarding clinical characteristics and the inability to identify asymptomatic features of infections. Additionally, recently identified SARS-CoV-2 variants such as B.1.1.7, B.1.526.2, B.1.151, and N501Y.V1 are responsible for severe suitability in children [32–35]. Therefore, reliable, accurate, and appropriate detection methods are required to establish and validate across the globe.

2.2. Incidence of 2019-nCoV infection in older adults

From the beginning of the COVID-19 outbreak, total numbers of positive cases were gradually increasing around the world and a high mortality rate was majorly found in older adults. COVID-19 patients of the older age group generally have comorbid conditions, such as hypertension, heart disease, and diabetes. Besides this, the quarantine period also contributed toward increased sarcopenia, loss of stress control, and physical and mental dependence in the elder population [36]. There is no significant change in the rate of fever, cough, and dyspnea in children and older adults; however, these are more severe in older adults [37,38]. High fever is associated and well-acknowledged with a higher level of inflammatory cytokines, and this can lead to death [39]. According to the Centers for Disease Control and Prevention (CDC), 80% of deaths in the USA were occurred among older patients by age 65–85 years. Therefore, it has become important to establish reliable diagnostic methods for older adults and care should be taken to avoid hospitalization.

(1) In terms of immune responses against SARS-CoV-2 infection, the human body shows B-/T-cell decrement with increasing age due to discontinuation of antigen stimulation and thymic involution [19], along with bone marrow and lymph nodes associated dysfunctions. The lymph node plays an active part in maintaining and coordinating new immune cells to control SARS-CoV-2 like viruses [36,40]. With increasing age, lymph nodes lower their functioning and are unable to maintain immune cells against emerging infectious diseases due to a reduction in proliferation and differentiation [41].
(2) From a molecular perspective, SARS-CoV-2 infection in the elderly can be explained by dysregulation in the transcriptome, proteome, or metabolome in associated genes or proteins [42,43]. As established earlier, viral S-protein binds with ACE2 receptors of host cells and endocytosis occurs for further replication. Therefore, ACE2 plays a key role in SARS-CoV-2 infection, yet the age-mediated regulation of ACE2 expression is still under investigation.
(3) Other laboratory parameters, for instance, lymphocytopenia, lower hemoglobin, and albumin level, elevated aspartate aminotransferase, creatine kinase, lactate dehydrogenase, and C-reactive proteins in older age groups compared to the younger group confirmed the severity of SARS-CoV-2 infection [44].

Additionally, the recent epidemic of mucormycosis (*black fungus*) and relevant super-infections in older COVID-19 patients are another major concerns [45,46]. Total 80% of worldwide older adult patients are mainly affected with diabetic ketoacidosis and neutropenia, which were further increased by an inflammatory reaction and steroid treatment during SARS-CoV-2 co-infections [47]. Thus, following the surge of COVID-19 in older adults requires timely, accurate detection to control the mortality rate; therefore, clinicians need to standardize SARS-CoV-2 diagnostic methods at the geographical and genetic level.

3. Optimization of COVID-19 detection methods for children and older adults

3.1. Need of optimization

Till the year 2020, scientists and clinicians working on SARS-CoV-2 stated that children are not very prone to COVID-19 because they have less mature ACE2 receptor in comparison to adults [15], and immune cross-protection from other coronaviruses cause low susceptibility [37]. However, according to a study published in *The Lancet*, a frequent mutation in SARS-CoV-2 coronavirus, such as B.1.1.7 variant is reported to be more lethal to children, as 70% out of 80 pediatric patients were COVID-19 positive [32]. On the other hand, the immunocompromised pediatric COVID-19 patients in a case series demonstrated the origin of S:Δ141-143 deletion, yet a higher

level of RBD and S1- specific antibodies [48]. It creates the possibility of escape mutant generation, which can be induced by S-protein-based immune responses. Several reports on COVID-19 recovered children were also found with Multisystem-Inflammatory Syndrome (MIS), which was previously considered a rare disease [49–51]. Unlike children, the fully matured ACE2 receptor and immunocompromised state of older adults make them vulnerable to SARS-CoV-2 infection. Not limited to this, the year 2021 also faces another epidemic of Mucormycosis (black fungus) in COVID-19 adult patients, in which only India reported 28.4 million cases and 70% of them were older adults [52].

The above studies provide a platform for the possibility that children and older adults (>60 years) have different mechanisms of action against SARS-CoV-2 infection, which is yet to be explored. However, the emergence of escape mutants and infection rate has an equal probability of SARS-CoV-2 variants infection in children and older adults. Therefore, age-related studies at large populations need to be conducted to explore the exact mechanism of action of the SARS-CoV-2 virus and its variants. Additionally, working and target genes or proteins for each commercialized kit are different, therefore serological and immunological parameters are yet to be standardized before prescribing the COVID-19 test. Other parameters explaining the need for optimizing COVID-19 test regimes are listed in the following section:

(1) In earlier stages of the COVID-19 pandemic across the globe, clinicians prescribed diagnostic tests only for hospitalized patients at higher risk [53], therefore mildly ill or asymptomatic patients were missed out and older adults with comorbid conditions ended up with death.
(2) Another major concern for COVID-19 detection in symptomatic children included unrecognized or overlooked symptoms before confirmation; therefore, it is difficult to establish standard detection methods in younger patients.
(3) Additionally, variations in manufacturer's and laboratory's working processes are also critical points for generating consistent tests [54,55]. For instance, the CDC suggests primer targeting of N-gene at two sites, whereas CDC, China recommends ORF1ab and N-gene targeting, and Pasteur Institute, France focuses on RdRP gene primer targeting.
(4) Immunological testing also employed different proteins or associated fragments of the SARS-CoV-2 virus; thus, validation of these kits at a large population is an essential part of the current pandemic.

As of 22 July 2021, 603 immunodiagnostic and 388 molecular tests are under Emergency Use Authorization (EUA)-level around the world against SARS-CoV-2 detection, however, more than 98 kits are under development, and one kit has been withdrawn from the market [56]. Initially, inaccurate results from the above diagnostic methods did not rule out completely, as past infection and elevated immunoglobulin can interfere with the SARS-CoV-2 detection [57]. Not restricted to this, severe SARS-CoV-2 variants such as B.1.427, P.1, and 501Y.V2 were reported lethal in adults [58,59], and children are also at risk for recently identified delta pro-variant of SARS-CoV-2, namely B.1.617.2 [59,60]. Though, m-RNA vaccine BNT162b2, Pfizer Ltd. was reported effective against B.1.1.7 and B.1.1351 SARS-CoV-2 variants [61], yet a study published in *The Lancet* confirmed that B.1.617.2 variant of the coronavirus is immune to BNT162b2 vaccine [62], and B.1.617.2 indeed is variant of concern. Therefore, more studies need to conceptualize to avoid negative results, identify novel biomarkers, accurate detection, and more pharmaceutical intervention for COVID-19.

3.2. Optimization of COVID-19 testing regimes

To date, several studies have been performed to optimize the molecular diagnostic techniques for the recognition of SARS-CoV-2 infection. Age, gender, geographical environment, and physiological mechanism of host cells majorly affect COVID-19 detection, and therefore standardization of diagnostic techniques is very much required. For an accurate and reliable diagnosis, research groups around the globe, are performing comparative studies on commercialized SARS-CoV-2 kits to accomplish the requirement of reliable detection. For this, reaction volume, sample concentration, primer concentration, type of specimens, their transportation conditions, and amplification system (in case of PCR kits) need to be optimized. Figure 3 summarizes commercialized diagnostic kits for SARS-CoV-2 detection.

3.2.1. Oligonucleotide amplification based 2019-nCoV detection

Metagenomics next-generation sequencing (NGS) was the first method to identify COVID-19 positive suspects in the initial stage of the outbreak [4]. In this process, bronchoalveolar lavage (BAL) fluid sample was processed to extract total RNA followed by RT-PCR, and the amplified product was sequenced. After the publication of the SARS-CoV-2 genome sequence on 7 January 2020 (GeneBank accession number MN908947), more than 991,096 2019-nCoV genomic sequences were shared as the Global Initiative on Sharing All Influenza Data (GISAID) [4,63]. Although, the higher cost and time-consuming process of genome sequencing called upon RT-PCR as COVID-19 confirmatory test and by the end of January 2020, several primers and probe sequences were released, and once the first RT-PCR kit developed by 4 February 2020, several kits were later commercialized. Though, these kits received emergency use certificates from the FDA and are restricted to be used by only healthcare professionals all over the globe (Table 1).

In a study, rRT-PCR-based Xpert® 65 Xpress SARS-CoV-2/Flu/RSV test by Cepheid Inc. USA exhibited higher sensitivity and accuracy compared to other tests, namely Cepheid Xpert Xpress Flu/RSV and GenMark 33 ePlex respiratory panel [64]. They measured positive percent agreement (PPA) of 98.7% and negative percent agreement (NPA) of 100% against SARS-CoV -2-specific E- and N2 genes. Similarly, the analytical performance of rRT-PCR-based SARS-CoV-2 detection kits, Allplex™ 2019-nCoV Real-time PCR, Real-Q 2019-nCoV Real-Time Detection, StandardM nCoV Detection and PowerChek™2019-nCoV kits which are developed by Korea-based Seegene,

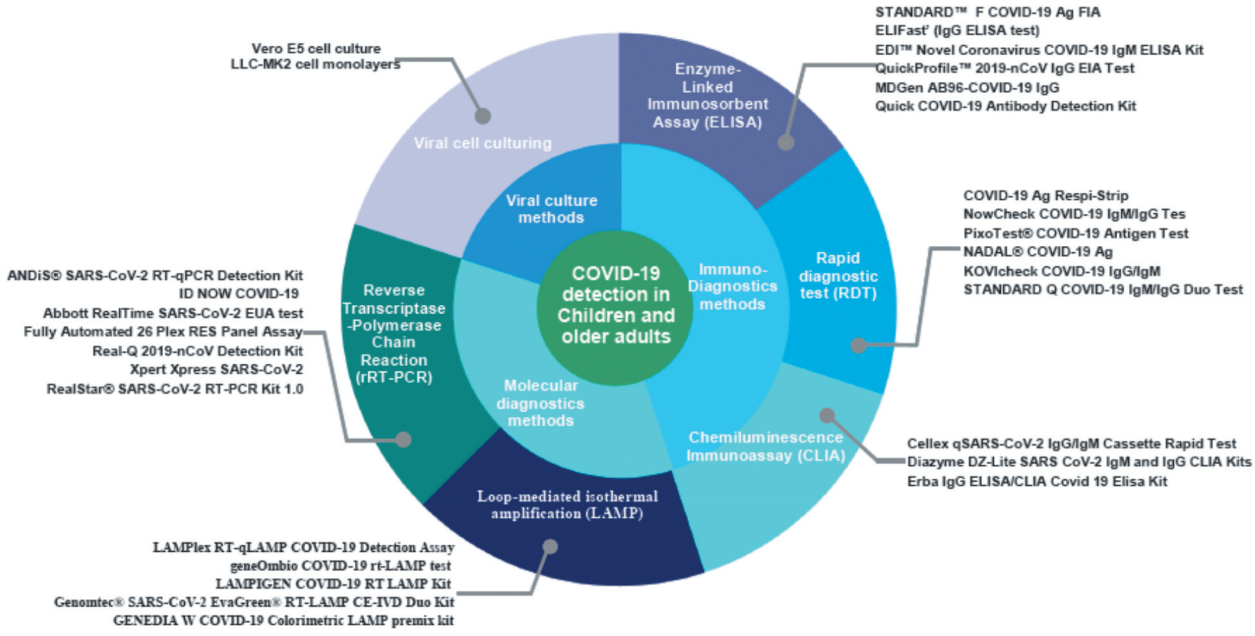

Figure 3. Summary of COVID-19 detection and commercialized diagnostic kits.

Table 1. List of commercially available RT-PCR kits against SARS-CoV-2 virus and beneficial use in case of children and older adults.

	Kit Name	Manufacturer	Sensitivity	Remarks
E-gene	LightMix Modular SARS-CoV-2 (COVID19)	TIB/Roche Diagn, Switzerland	1.8×10^{-1} $TCID_{50}$/ml	Several research groups evaluated the efficiency of the kit for SARS-CoV-2 detection [136,137] and were highly recommended for clinical use.
	*ΠGeneFinder™ COVID-19 Plus RealAmp Kit	ELITech Group, Republic of Korea	10 copies/ reaction	Single-step, rapid, and in-house real-time PCR assay, which can be helpful in children and older adults.
Orf1ab	AQ-TOP™COVID-19 Rapid Detection Kit	Seasun Biomaterials Inc., South Korea	7.0 copies/µl	LAMP and PNA (Peptide Nucleic Acid) detection probe-based detection kit, may enhance sensitivity and slower reaction time
	*$ΠXpert Xpress SARS-CoV-2	Cepheid, USA	0.0200 PFU/ml	First Flu/RSV cartridge technology against multiple targets of the SARS-CoV-2 virus.
	*AtilaiAMP® COVID Detection Kit	Atila Biosystems Inc., USA	10 copies/µl	Fluorescence resonance energy transfer (FRET) based isothermal amplification method, hence beneficial at low viral load in children and older adults.
	*BIOMAXIMA S.A., Poland	SARS-CoV-2 Real-Time PCR LAB-KIT™	≥10 copies	The manufacturer provides a special viral transport medium (VTM) for sample transport and storage at room temperature. This can be beneficial for carrying children and older adult samples, as low volumes are available in these cases.
	$DiaPlexQ™ Novel Coronavirus (2019-nCoV)	Solgent Co. Ltd, Korea	200 copies/ml	Multiplex OneStep RT-qPCR with higher selectivity can be helpful in older adults with comorbid conditions.
	*EURORealTime SARS-CoV-2	EUROIMMUN, Germany	150 copies/ml	Manufacturers validated accurate results in low viral load, thus children and older adults can be diagnosed at an early stage of COVID-19 infection.
RdRp gene	1copy™ COVID-19 qPCR Kit	1drop Inc, Korea	4.0 copies/ reaction	Limitations: 1. Performance of kit is not evaluated in presence of vaccines and drugs, which is an important parameter in children and older adults. 2. Qualitative test, hence, limits the quantitative measurement of viral load which is required in children and older adults.
	Abbott RealTime SARS-CoV-2 test	Abbott Molecular Inc., USA	100 copies/ml	
	#RADI COVID-19 Detection Kit	KH Medical, Korea	0.66 copies/µl	Compatible with human feces samples, this promotes noninvasive sample collection from children and older adults.
	*SARS-COV-2 R-GENE®	BioMérieux, France	0.43 $TCID_{50}$/ ml	The most commonly used clinical RT-PCR kit and several studies have been conducted for validation.
	#$Allplex 2019-nCoV assay	Seegene, Inc., Korea	100 copies/ reaction	The test is designed as a viral genome extraction-free method that will be for children and older adults.

*2nd target: SARS-CoV-2 specific N-gene
#2nd target: SARS-CoV-2 specific S-gene
$2nd target: SARS-CoV-2 specific E-gene
Π2nd target: SARS-CoV-2 specific RdRP-gene

Kogene Biotech, BioSewoom, and SD BIOSENSOR respectively were analyzed [65]. The Allplex™, PowerChek™, and Real-Q demonstrated a limit of detection (LOD) of 153.9, 84.1, and 80.6 copies/ml respectively with a positive detection rate of more than 75%. In terms of molecular diagnostic methods, LOD is defined as the lowest target concentration that can be measured in ≥95% of repeated tests [66]. The LOD of molecular methods are reported in the following units: copies/ (copies of the viral genome per ml of transport media), copies/ reaction volume, $TCID_{50}$ copies/ml, and morality of target analyte, thus a comparison of different kits with different LOD unit is difficult [67]. A comparative study on Altona Diagnostic Germany, BGI Genomics Co. China, CerTest Biotec Germany, KH Medical Korea, PrimerDesign Ltd. England, R-Biopharm AG Germany, and Seegene Korea were also performed [68]. Among evaluated kits, PrimerDesign Ltd. England has a LOD of 23 copies/ml for ORF1ab/RdRP gene, while Altona Diagnostic Germany exhibited the lowest LOD of 3.8 copies/ ml for E- and S-gene. Besides this, they optimized the RT-PCR kits for different SARS-CoV-2 positive clinical specimens where R-Biopharm AG, Germany exhibited the highest sensitivity toward E-gene. In conclusion, each laboratory needs to optimize in-house E- and S-gene-specific PCR reactions, as different age groups have a different binding efficiency with ACE2 receptor (ACE2R). The above studies compared the RT-PCR kits based on sensitivity and specificity to justify their importance in terms of children and older adults. Though the standard protocol of rRT-PCR is demanding and time-consuming, therefore isothermal amplification methods and CRISPR-Cas-based diagnostic tools are entering into COVID-19 diagnostics.

Recent research led to the development of several RT-PCR kits, but these kits were developed and commercialized hurriedly, hastily, and without proper validation. Countries reported issues with the reliability and accuracy of available diagnostic kits in the initial period of the COVID-19 pandemic [57]. Therefore, researchers start to report and compare the commercially available diagnostic kits. For Instance, an optimized protocol for SARS-CoV-2 detection in asymptomatic cases was established [69]. In this study, CDC, USA, and Institute for Basic Science (IBS) virus facility-based primers sets for rRT-PCR SARS-CoV-2 detection were discussed; among them two of CDC, USA prescribed primer sets exhibited false-positive results due to the formation of short and long dimer bands. Therefore, a comparative study among rRT-PCR, conventional PCR, and multiplex PCR for 2019-nCoV detection via using 16 primer sets was further performed [70]. This three-step optimization protocol includes sample quality test, option for real-time detection, and confirmation of SARS-CoV-2's presence or absence using the above-mentioned PCR techniques. Sample collection in children and older adults is always a difficult task, therefore, RT-PCR for COVID-19 detection via stool samples was optimized and compared with pharyngeal swab specimens along with CT findings [71]. After the publication of similar studies, industries refused to disclose critical primer sequence information, thus verification and validation of primers' quality and sensitivity became difficult. Therefore, researchers start to optimize other parameters for developing correct COVID-19 detection approaches, especially in children and older adults. At first, it was noted that different buffer components in rRT-PCR could inhibit the amplification reaction in the process of SARS-CoV-2 viral RNA detection and still a major challenge in molecular diagnostics.

The higher cost, undisclosed information, and limited availability of these essential commercial reagents are also major concerns in effective COVID-19 detection. Therefore, recently, three commercial rRT-PCR kits, namely MutaPLEX® Coronavirus RT-PCR kit by Immundiagnostik AG Germany, GeneFinder COVID-19 Plus RealAmp kit by HISS Diagnostics, Germany, and COVID-19 genesig® Real-Time PCR assay kit [Z-PATH-COVID-19-CE] by Hain Life Science, Germany were compared [72]. This study demonstrated a 50% fluorescence reduction in an sample containing PBS, which further increased to 70% with the use of DL buffer w/o RNasin. In some studies, scientists are designing rRT-PCR protocols for 2019-nCoV viral genome detection via omitting RNA extraction steps. This methodology will reduce detection time without affecting the sensitivity of the kit, which can be beneficial for COVID-19 detection in children and older adults. To achieve this objective, Altona RealStar® SARS-CoV-2 rRT-PCR kit by Altona Diagnostics, Germany, and SeeGene Allplex 2019-nCoV rRt-QPCR assay by SeeGene Inc. South Korea were evaluated [73]. The study stated that RNA extraction is not required in SARS-CoV-2 detection if specimens were collected in UTM® viral transportor molecular water. However, if it is stored in saline water or Hank's medium, then viral RNA extraction is required before amplification. Similarly, TaqPath™ one-Step rRT-PCR kit for 2019-nCoV viral genome detection without the additional step of RNA extraction was also analyzed [74]. In this study, samples were incubated with TaqPath™ master mix 10 minutes before amplification and achieved LOD of 6.6×10^3 copies/ml, sensitivity, specificity, and accuracy of 95%, 99%, and 98.5% respectively. General extraction-free diagnostics methods show a higher rate of premature termination and constant diagnostic investigations are not feasible. Additionally, a lower amount of biological COVID-19 samples may cause false-negative results and increase the error rate as well as the cost of the test. Above mentioned viral genome extraction free methods involve measurable dilution of inhibitory substances along with minimizes viral RNA loss by lowering cell lysis temperature. This approach of RT-PCR may improve turnaround time and reduces the cost to enhance the applicability of optimized diagnostic methods in financially weak patients.

3.2.1.1. Isothermal amplification based 2019-nCoV detection. Loop-mediated isothermal amplification (LAMP) is a novel and improved isothermal nucleic acid amplification assay, which has an exponential amplification feature for multiple target detection in the same reaction. A standard rRT-PCR takes 90–120 minutes to analyze the samples, whereas LAMP-based evaluation takes only 30 minutes at a constant temperature. This method utilizes 4–6 primers for six binding regions of the target viral RNA and since the SARS-CoV-2 RNA virus is of 30 kb size, a single RT-LAMP reaction may efficiently complete the task in a short time. One team from Oxford University has designed four sets of primers in which two sets target N-gene and the other two target S-gene and

ORF1ab [75]. They used FIP-6-carboxyfluorescein (FAM) conjugated primers for effective fluorescent results and colorimetric readout was performed using pH-sensitive dye (phenol red). In this study, the sensitivity of detection was reported to be 80 copies/ml.

Based on the LAMP principle, Seasun Biomaterials Inc., South Korea has developed a real-time LAMP-based AQ-TOP™ COVID-19 Rapid Detection Kit to detect 2019-nCoV-specific Orf1ab and human RNase P gene. The manufacturer reported 7.0 copies/µl of LOD along with a positivity rate of 95% in clinical samples. Similarly, LoopX© is also a collaborative effort of a France-based research team to detect 2019-nCoV specific RdRp-gene with 98.6% sensitivity and 91.5% specificity [76]. This all-in-one automated and reproducible RT-LAMP-based detection kit may also be useful for saliva samples, a noninvasive method, in the case of older adults and children. Recently, variplex™ RT-LAMP SARS-CoV-2 detection assay to identify E-gene was developed and sensitivity improvement from 76.3% to 92–100% was reported when RT-PCR is combined with LAMP assay [77]. This method exhibited LOD of 0.004 $TCID_{50}$/reaction in clinical samples and the use of saliva samples enhances accuracy in children and older adults. Thus, isothermal amplification kits proved beneficial in the age-related study as the process involve denaturation step omission and less time in the experimental procedure. The constant thermal condition also adds a value of high amplification efficiency even in the low viral load at an early stage of infection in children and older adults.

The LAMP method adopted a completely different approach in terms of variable target genes and effective reagents that affects LOD (viral copies detected per minute). Therefore, after extensive use of LAMP techniques in COVID-19 detection, several studies were conducted to modify LAMP assays for better and rapid detection in children and older adults. Furthermore, an RT-LAMP method for 2019-nCoV viral genome detection in nasopharyngeal swab samples without RNA extraction was reported [78]. After optimizing the primary swab sample of 1.0 µl, significant results were obtained with LucigenQE lysis buffer, and colorimetric readout showed LOD of $5x10^5$-$1x10^6$ RNA copies/ml. They also used fluorescent RT-LAMP instead of the colorimetric method, therefore real-time quantitative evaluation of Cq values can provide easy readouts and further utilize in mobile RT-LAMP workflow. Though, this method was less sensitive than conventional RT-PCR, yet sufficient range of LOD can be measured to detect 2019-nCoV in individuals with low viral load, such as children and sometimes, older adults.

Furthermore, a dual-target RT-LAMP, namely 2019-nCoV-specific S- and RdRp-gene with LOD of 25 copies per reaction was validated [79]. They exhibited higher sensitivity via the addition of guanidine hydrochloride (pH 8.0) in LAMP reactions. The only limitation of this study was the use of an artificial viral target. It needs further validation with different clinical samples of SARS-CoV-2. A study published in *Virology Journal* validated RT-LAMP based POC kit for SARS-CoV-2-specific ORF8 and N-gene detection [80]. The optimization of the study showed excellent signals at 67°C with a sensitivity of 100 copies/µl and significant specificity over 20 different respiratory samples. Similarly, ID NOW™ Instrument and ID NOW™ COVID-19 Test Kit (Abbott Inc., USA) were developed on the LAMP principle; in which ID NOW™ provides results in 5.0 min with LOD of 2.0×10^4 copies/ml. Though LAMP-based assays are sensitive implementation of these POCs in a remote location where laboratory setup is not possible, is difficult to manage.

3.2.2. Immunodiagnostic methods for SARS-CoV-2 detection

As COVID-19 moves from transition to flattening phase, the requirement of quick serological tests increases for viral antibody detection. The population-wide serological screening provides checking of the recovered as well as asymptomatic individuals to learn the accurate extent of the infections. The serum antibodies-based SARS-CoV-2 detection first identifies humoral response of IgM in the initial stage of infection, whereas IgG provides long-term immunological memory for adaptive immunity [81]. In immunodiagnostic tests, solely IgM antibody requires another confirmatory test, but the presence of both IgM and IgG antibodies is generated because of the previous infection. It was not an active infection; however, a negative result may occur due to seven days window period of SARS-CoV-2 infection [82]. Major commercial immunodiagnostic kits are mentioned in Table 2.

Among the other proteins, S-protein of SARS-CoV-2 has been reported to play an important role in binding and entry in host cells with the help of N-terminal S1 receptor-binding domain (RBD), N-terminal domain (NTD), and C-terminal S2 subunits [83,84], whereas while replicating, N-protein binds and covers the viral RNA into nucleocapsid [85,86]. Studies performed on recovered individuals from COVID-19 have shown that S- and N-proteins get primarily attacked by the host-neutralizing antibodies [87]. Therefore, serological immunoassay development majorly focuses on specific domains of SARS-CoV-2 antigen, which are mainly targeted by humoral immune responses.

Although, SARS-CoV-2 proteomes were reported to share a conserved region with the SARS-CoV coronavirus, however, recently antibodies response-based cross-reactivity was also observed. In this direction, an *in-vitro* antibody assay, namely PepSeq for epitope mapping and cross-recognized Spike S2 subunit epitopes-specific IgG antibodies against SARS-CoV-2 and SARS-CoV coronaviruses was developed [88]. The limitation of this study included: small population size of convalescent donors, therefore immunodominant epitopes might have been omitted. Additionally, PepSeq epitope mapping was restricted to up to 30 amino acids; therefore, it was unable to perform on post-translationally modified products. Another study published in *Cell Reports* also evaluated cross-reactivity of SARS-CoV-specific IgG and IgM-based 11 antibodies in SARS-CoV-2 positive patients [89]. They confirmed partial cross-neutralization of coronavirus-specific spike antibodies, specifically 240°C and 154°C, whereas 341°C and 540°C were reported to lose their neutralization capacity, when faced with COVID-19. This high evidence of cross-reactivity among different strains of coronaviruses may help in designing a diagnostic and therapeutic intervention for SARS-CoV-2 infection. In the case of older adults, antibodies against common coronaviruses are significantly elevated in comparison to the younger population and binding antibodies increases with

Table 2. List of commercially available immunodiagnostic kits against SARS-CoV-2 coronavirus and beneficial use in terms of children and older adults.

SARS-CoV-2 proteins	Immunoglobulin	Kit Name	Manufacturer	Sensitivity/ Agreement statistics	Cross reactivity	Remarks
N-protein	IgG/ IgM	STANDARD F COVID-19 Ag FIA	SD BIOSENSOR, Inc., Korea	PPA- 47.1% LOD- 2.0×10^6 copies/ml	–	The fluorescent property of the immunodiagnostic kit can detect COVID-19 infection in low viral load in children and older adults.
	IgM	EDI™ Novel Coronavirus COVID-19 IgM ELISA Kit	Epitope Diagnostics, Inc., USA	PPA- 73.1% NPA- 100%	–	IgM of suspected patient interacts with N-protein fabricated ELISA kit, thus rapid mutation in S-protein will not affect the test, and accurate diagnosis occurs.
S- protein	IgG/ IgM	*Cellex qSARS-CoV-2 IgG/IgM Cassette Rapid Test	Cellex Inc., USA	PPA- 93.75% NPA- 96.40%	–	Rapid (15–20 minutes) LIFA test, beneficial for age-related SARS-CoV-2 studies.
		*One Step Test for Novel Coronavirus (2019-nCoV) IgM/ IgG Antibody	Getein Biotech, Inc., China	Sensitivity- 76.7% PPA- 76.3% NPA- 98.8%	–	
		NADAL® COVID-19 IgG/IgM Test	nal von minden GmbH, Germany	Sensitivity- 94.1%	SARS-CoV, MERS-CoV, and rheumatoid factor positive samples	LIFA test with higher sensitivity and identify primary infection, beneficial in children and older adults.
	IgG	COVID-19 IgG, EIA-6146	DRG International, Inc., USA	Sensitivity- 99.7%	–	One step ELISA identifies the adaptive immune response means a prior infection that can help in the detection of asymptomatic COVID-19 in children and older adults.
	IgA	**OmniPATH™ COVID-19 Total Antibody ELISA Test	Thermo Fisher™ Scientific, USA	Sensitivity- 100%	HIV+ samples	Highly sensitive and selective ELISA test
		Anti-SARS-CoV-2 ELISA (IgA)	EUROIMMUN AG, Germany	Sensitivity: Variable Specificity: 98.3%	–	The sensitivity of the ELISA test varies with days and maximum in 11–60 days (96.9%).

*2nd biomarker: SARS-CoV-2 specific N-protein
**2nd biomarker: SARS-CoV-2 specific IgG/IgM
PPA: Positive Percent Agreement
NPA- Negative Percent Agreement

respiratory illness whereas neutralization antibodies may decrease [39,90]. These pre-existing antibodies are not necessarily protective against incessant coronavirus infection and low neutralizing antibody stimulation may cause susceptibility to re-infection in older adults, even after vaccination. Therefore, highly specific diagnostic methods need to be developed to rule out the cross-reactivity of different strains of coronaviruses. In this direction, the following immunodiagnostic methods have been formulated for SARS-CoV-2 detection to date:

3.2.2.1. Rapid diagnostic test (RDT) or Lateral Flow Immunoassays (LFIA). Rapid diagnostic tests (RDTs) principally depend upon lateral flow immunoassay (LFIA) technology, which is a simple and quick method and can be potentially used as a point-of-care (POC) device. It is the most common, low-cost diagnostic method which is designed as paper substrate with wax printed channels to allow sample flow over the testing strip and both qualitative, and quantitative analysis can be performed [91,92]. Therefore, the current scenario of this pandemic calls for large-scale production of reliable RDT due to lower cost and off-the-shelf components.

In this direction, Cellex Inc. USA was the first to develop and secure EUA approval for SARS-CoV-2 IgG/IgM RDT [93]. This LFIA method is used to detect IgG and IgM against coronavirus in serum, plasma, or whole blood samples and gives results in 15–20 minutes. This kit exhibited 93.8% sensitivity and 96.0% specificity in 128 COVID-19 positives and 250 control patients. In continuation, Autobio Diagnostics, China, and Chembio Diagnostic, USA developed Autobio Diagnostics Anti-SARS-CoV2 RDT and Chembio Diagnostic System's DPP® COVID-19 IgM/IgG system respectively and received emergency use approval. These tests are superior in comparison to other LFIA kits because, instead of relying on visual detection, these methods analyze with DPP microreader for qualitative measurement of IgG/IgM and further avoid biases or misinterpretation. Diagnostic System's DPP COVID-19 IgM/IgG system reported specificity of 97.6%, 96.8%, and 94.4% for IgM, IgG, and combined IgG/IgM respectively. Similarly, the performance of STANDARD F COVID-19 Ag FIA (SD BIOSENSOR, Inc., Korea) on SARS-CoV-2 cell lines was studied and reported LOD of 2.0×10^6 copies/ml [94]. The fluorescent property of this kit can detect COVID-19 infection in low viral load in children and older adults, as the manufacturer also showed the effectiveness of the kit with Ct value below 25. Other than these kits, One Step Test for Novel Coronavirus (2019-nCoV) IgM/IgG Antibody (Getein Biotech, Inc., China), Cellex qSARS-CoV-2 IgG/IgM Cassette Rapid Test (Cellex Inc., USA), and NADAL® COVID-19 IgG/IgM Test (nal von minden GmbH, Germany) are other SARS-CoV-2 specific S-protein mediated IgG/IgM antibodies based immunodiagnostic kits. These kits are effective for both primary and secondary immune responses at every stage of infection that can be beneficial for diagnosis in children and older adults.

3.2.2.2. Enzyme-Linked Immunosorbent Assay (ELISA). Recently, several immunodiagnostic assays have been developed and validated, which have specific and unique performance characteristics for SARS-CoV-2 detection. However, very few tests showed quantitative results in differentiating positive/ negative cases, which also exhibited quantitative measurement of humoral responses. Moreover, scaling up these tests was at the extremely critical stage as COVID-19 cases are gradually increasing; therefore, clinicians are suggesting ELISA-based COVID-19 detection to identify IgG, IgM, and IgA against SARS-CoV-2 specific RBD. This simple qualification plan opens new paths to develop rapid detection kits for on-spot COVID-19 detection. To date, more than 100 ELISA kits have been developed for both SARS-CoV-2 specific proteins and antibodies for coronavirus detection.

Recently, an innovative multiplex immunoassay, namely CoViDiag assay was designed to detect antibodies against N, S1, S2, RBD, and NTD proteins [95]. This multiplex method improved the sensitivity in the range of 92–100% within 14 days of infection onset and that completely depended upon antibody. Besides, a conventional immunodiagnostic method via developing a paper-based ELISA for SARS-CoV-2 humanized antibodies detection was modified [96]. This assay detected 0.124 IU/ml of SARS-CoV-2 humanized antibodies within 30 min and the estimated cost of the device was 1.45–1.65 USD. Similarly, the sensitivity of INNOVITA Biological Technology Co., China, Zhejiang Orient Gene Biotech Co., China, and Hangzhou All Test Biotech Co., China-based IgG/IgM ELISA kits were evaluated [97]. They evaluated the efficiency of the kits at a different time points, and among them 2019-nCoV IgG/IgM Rapid Test Cassette (Hangzhou AllTest Biotech Co., China) showed poor sensitivity. Along with this, EDI™ Novel Coronavirus COVID-19 IgM ELISA Kit (Epitope Diagnostics, Inc., USA), COVID-19 IgG, EIA-6146 (DRG International, Inc., USA), and OmniPATH™ COVID-19 Total Antibody ELISA Test (Thermo Fisher Scientific, USA) are other immunodiagnostic kits for 2019-nCoV detection. OmniPATH™ COVID-19 Total Antibody ELISA Test detects RBD of S1 subunit during an adaptive immune response, thus kit can be used in COVID-19 detection at a different time point in older adults.

3.2.2.3. Chemiluminescence Immunoassay (CLIA). The chemiluminescence immunoassay (CLIA) works on a similar principle as ELISA where binding affinity between SARS-CoV-2 viral antigen and host antibodies were evaluated based on chemical reaction among tagged probes and the yield of the emitted light is recorded [98,99]. Ortho Clinical Diagnostics, USA designed the first CLIA test namely, VITROS® Anti-SARS-CoV-2 antibody test which takes 50 minutes to detect both IgG/IgM collectively. This automated device showed 83% sensitivity and 100% specificity in clinical samples. The further effort of Roche's technology results in the development of electro-chemiluminescent immunoassay (ECLIA) namely, Elecsys® Anti-SARS-CoV-2 test to detect total antibody against SARS-CoV-2 specific N-protein within 18 minutes. This method works on the principle of electrochemical reaction mediated chemiluminescent and exhibited a sensitivity of 100% in ≥14 days after PCR confirmation and specificity of 99.81% in clinical samples. Similarly, Bio-Rad Laboratories and Abbott

Inc. developed Bio-Rad's Platelia SARS-CoV-2 Total Ab test and Abbott's SARS-CoV-2 IgG Assay, which detect antibodies against the SARS-CoV-2 N-protein.

Recently, LFIA and ELISA-based COVID-19 detection assays are commercialized at a large scale and clinicians are prescribing these kits as a screening test. These immunodiagnostic techniques can provide the feasibility of on-spot rapid detection of SARS-CoV-2 in children and older adults. Some of the immunodiagnostic kits include Cellex's qSARS-CoV-2 IgG/IgM Rapid Test, Chembio Diagnostic System's DPP COVID-19 IgM/IgG system, DiaSorin's LIAISON SARS-CoV-2 S1/S2 IgG system, Bio-Rad Laboratories Platelia's SARS-CoV-2 Total Ab assay, and Autobio Diagnostics Anti-SARS-CoV2 Rapid Test for active viral proteins in different age group patients [82,100]. These tests majorly target IgM and IgG antibodies in patients that could have been the evidence of the previous infection which older adults have in numbers.

Additionally, negative results of blood-based immunodiagnostic tests do not confirm active 2019-CoV infection as the window period of antibodies production can be delayed, especially in children and older adults. Therefore, a titer of IgA class antibodies is recently launched to detect 2019-nCoV coronavirus in respiratory tract secretions. Moreover, these RDT kits have demonstrated analytical sensitivity in the range of 69–88% and 90–99% for IgM and IgG, respectively [101,102], yet delayed antibodies production rendered immunodiagnostic testing questionable.

Current diagnosis protocols on symptomatic individuals cannot determine the difference between SARS-CoV-2 and common cold infection. Additionally, a 5–7 days virus incubation period is also required before screening or confirmatory immunodiagnostic tests [103,104]. Hence, the following parameters need to be optimized for an efficient disease prophylaxis in vulnerable children and older adults [105,106]: Repetitive testing time for COVID-19 management at home and hospital set-up, pre-analytical parameters such as storage condition and media for samples, optimization of antigen or antibody targets as target changes with age and most importantly, if multiple antibodies can be detected, immunodiagnostic tests must be highly specific for every single antibody. If successful, immunodiagnostic tests can be beneficial for seroprevalence studies and generated data can act as a standardized scale for effective detection methods. But immense variation in time duration to seroconversion among individuals needs for systematic and periodic studies on RDT COVID-19 detection methods.

3.2.3. Virus culture-based COVID-19 detection

Though RT-PCR is considered as the gold standard method for SARS-CoV-2 detection, where Ct values can correlate symptom onset to test (STT) date with infectivity potential [107]. Whole-genome or any part of it does not determine provenance or infection initiation time, thus a correlation between transmissibility and clinical progress can be missed out. Therefore, few studies were attempted to standardize SARS-CoV-2 viral culture to evaluate transmission modalities. A study published in the *Journal of Clinical Microbiology* evaluated the relation between detectable SARS-CoV-2 RNA and culturable viruses [108]. They collected throat, nasopharyngeal (NPS), and sputum samples from positive COVID-19 patients to determine *E, N*, and *nsp12* genes, among them *E*-genes showed the high culturable copy number (6.0 $\log_{10}$ genome copies/ml sample). ACE2 and Cluster of differentiation 26 (CD26) have been identified as associated with senescence and immunoregulation in COVID-19 infected older adults [109,110], therefore, higher viral load can be reported in these patients.

To prepare and optimize SAS-CoV-2 viral transport medium (VTM) for culture test, three methods were selected to sterilize Anderson's modified Hanks Balanced Salt Solution and antibiotics-based medium [111]. This study suggested using filters and autoclave-based sterilization methods for efficient viral load detection. The proposed culture medium proved to work efficiently after 4.0°C storage and if used within 48 h. To date, no diagnostic method showed the necessity of virus replication; however, prepared VTM can help with drug or antibody susceptibility of re-isolated COVID-19 virus in children and older adults. Recently, the population of older adults was found positive for SARS-CoV-2 infection after vaccination [112–114]. This can occur if SARS-CoV-2 mutants were escaped during vaccination. In this scenario, high-quality VTM will support the next lethal phase of the COVID-19 pandemic. Italy-based case report optimized cell culture method for SARS-CoV -2 infection diagnosis in seven-week-old infant [115]. Initially, immunofluorescence and nucleic acid amplification assays were found negative with coronavirus in nasopharyngeal samples, however, the cytopathogenic agent was later found in the cell culture of infant COVID-19 specimen. Although, validation of SARS-CoV-2 specific cytopathogenic agent and their infectivity still need to be performed at a large population of children and older adults.

The above studies proved that viral culture-based detection methods can be an effective indicator of SARS-CoV-2 infection, although it is restricted in current clinical studies due to the following reasons:

(i) Requirement of level III viral testing laboratory and technical expertise.
(ii) Type and quality of collected biological samples.
(iii) Financial availability for SARS-CoV-2-specific chemical and culture media purchase to avoid the presence of other viral strains.
(iv) Most important, biosafety concerns are highlighted by WHO and national health authorities while working on the viral culture method.
(v) Results are generally available after several weeks, which is not feasible for the current pandemic situation.
(vi) Cell cultures are susceptive to bacterial contamination and toxic materials in viral biological samples, especially in children where several healthy bacteria are present in their system.

3.2.4. External parameters

A serious concern in COVID-19 detection is the transportation of samples, and therefore pre-analytical parameters need to be optimized in each laboratory. To follow the protocol, clinical laboratories in the United State need to perform 'bridging

studies' on FDA-approved 2019-nCoV diagnostic kits [116]. In this scenario, the effect of different sample types stored in variable transport/collection media on the performance of SARS-CoV-2 RT-PCR kits was evaluated [117] and concluded that transport media, 0.9% NaCl or amies media are compatible for nasopharyngeal, E-Swab or 3D-printed swabs. These pre-analytical parameters also need to be optimized in children and the older adult's samples, as both hosts may either show mild viral load or limited life span respectively.

4. Concluding remarks

Latest SARS-CoV-2-based investigations and modern technologies representing a captivating road in diagnostics that may differentiate between children and older adults are discussed in this review. Though conventional methods are reliable, frequent mutations in the SARS-CoV-2 virus and the possibility of false-positive results in immunodiagnostics are affecting the timely treatment of COVID-19. Limitations of SARS-CoV-2 biomarkers and distinct physiology in a different age group are also major factors in clinical diagnostics. Currently, commercialized and under-process COVID-19 kits majorly target SARS-CoV-2-specific S, S1, N, and RBD proteins, among them S1 is more specific for virus detection but N-protein is more reliable for the accuracy of the kits [118]. Additionally, delayed adaptive immunity was also reported in older adults with a comorbid condition where malfunctioning of T- and B-cells along with excess type 2 cytokines production cause defects in viral replication and prolonged proinflammatory responses [119]. A study published in *The Lancet* also confirmed the lowering of procalcitonin and white blood cells (WBC) in hospitalized COVID-19 older adults, however, no bacterial infection (e.g sepsis) was reported [120]. Thus, more studies on comorbid conditions, such as cardiovascular and septic shock in COVID-19 positive patients need to be evaluated. Because, delaying the accurate identification causes increasing proinflammatory cytokines and inflammatory cells collectively target the lungs to damage tissues without providing control over the infection, thus leading to the death of SARS-CoV-2 infected individuals.

To date, highly specific prescribed RT-PCR and immunodiagnostic kits target a single gene or protein at one time. For example, immunodiagnostic tests such as NADAL® COVID-19 IgG/IgM Test (nal von minden GmbH, Germany) and STANDARD F COVID-19 Ag FIA, (SD BIOSENSOR, Inc., Korea) are specific for IgG/IgM against S protein and IgM against N protein respectively. However, the presence of target biomarkers varies with anatomical (i.e ACE2R binding affinity) and physiological (i.e different immune response) variations in children and older adults [121], hence this difference significantly affects the detection mechanism. On the other hand, RT-PCR kits based on direct identification of SARS-CoV-2 genes which have a different binding affinity with receptor and geographical variation of host cells also affect receptor polymorphism in children and older adults [122,123]. Therefore, we suggest performing tests of genomic variations identification which will promote the use of personalized kits. Targets for the tests also have to be identified by comparative screening for genomic regions that have a low mutation frequency to avoid primer and antibody mismatches in immunodiagnostic and molecular tests for SARS-CoV-2 detection and enhance test quality and stability [124]. In summary, the different specificity of each kit disables the establishment of correlation among serological, radiological, and molecular diagnostic methods. These techniques can be considered as codependent, rather than co-linked, hence optimization is necessary for the present scenario of frequent SARS-CoV-2 mutation.

Currently, rRT-PCR is the gold standard technique to detect SARS-CoV-2, but unfortunately, problems in sample collection, transportation, RNA extraction procedure, and RT-PCR amplification without any optimization can lead to false-positive results. Other than this, RT-PCR requires expertise in performing and analyzing the results along with sensitive equipment with specific operational conditions [57]. Similarly, several COVID-19 detection kits are designed based on immunodiagnostic tests, for instance, ELISA, LFIA, etc., and studies supported sensitive results when it is combined with nucleic acid tests (NATs) for children and older adults [125]. However, these methods are biased due to the time lapse between initial exposure and sample collection and assays to confirm the presence of SARS-CoV-2. Consequently, very limited data are available to date if researchers consider different isotypes and compare dual positivity of dissimilar isotype antibodies in immunodiagnostic tests.

The current race to develop reliable, cost-effective on-spot POC kits, and optimized laboratory techniques to confirm SARS-CoV-2 infection has boosted the development of innovative diagnostic kits. At present, several RT-PCR and immunodiagnostic kits are under development or already commercialized to detect coronavirus [56]. These assays are playing an important part in COVID-19 detection in children and older adults, as they are at higher risk for the infection. Therefore, optimization of these techniques based on age factors is required for the reliable development of COVID-19 detection kits. Several studies have been performed to date to optimize NAT, immunodiagnostic, and commercialized SARS-CoV-2 detection kits; however, these are not conclusive because of the lack of data to differentiate age factors.

5. Expert opinion

From the beginning of the COVID-19 outbreak, clinicians are prescribing to take precautionary measurements to avoid coronavirus exposure. The appropriate diagnostic strategy relies on rRT-PCR and auxiliary serological tests at the first stage when symptoms appear or direct exposure to COVID-19 positive patient happens. 2019-nCoV infected children are either mildly symptomatic or asymptomatic, whereas it is difficult to differentiate among other comorbid conditions in older adults. Age-related health conditions are interfering with reliable identification of 2019-nCoV as microbiota present in children from other medical conditions might develop a barrier for direct coronavirus detection. For older adults, a weak immune system and limited methods for sample collection might cause difficulty in COVID-19 detection on time.

Additionally, collecting and maintaining an appropriate environment for sample processing, the requirement of expertise in result analysis, time-consuming molecular diagnostic

tests, and gradually increasing rate of false-positive results limit the clinicians for starting the therapy. Clinicians are also facing the inability of children to explain the symptoms or situations if they have been exposed to 2019-nCoV, and on the other hand, older adults be at higher risk to even go to the hospital for a checkup. Their weak and delayed immune responses along with other medical conditions such as heart disease, diabetes, or other respiratory diseases can cost their life. By May 2020, WHO and CDC also confirmed the origin of hyper-inflammatory syndrome or Multisystem Inflammatory Syndrome in Children (MIS-C). This syndrome is also characterized by fever, gastrointestinal disorders, and multiorgan failure along with some auxiliary symptoms. Therefore, separate identification of 2019-nCoV in this time became difficult for clinicians. Table 1 is the representation of some molecular diagnostic assays that run on diverse platforms and some of them have been integrated with automated analytical devices along with high-throughput testing platforms, such as Roche cobas 6800/8800, Indianapolis, IN, and Hologic Panther/Fusion systems, San Diego, CA.

Lately, distinctive consideration is being given to S-protein antigen to confirm COVID-19 infection and decrease the chances of mass transfer, though single analyte detection cannot be effective in the current pandemic situation [126]. In this direction, RADI COVID-19 Detection Kit (KH Medicals, Korea) and Allplex 2019-nCoV assay (Seegene, Inc., Korea) designed dual analyte, S- and RdRP proteins-based RT-PCR kits. Whereas Cellex qSARS-CoV-2 IgG/IgM Cassette Rapid Test (Cellex Inc., USA) and One Step Test for Novel Coronavirus (2019-nCoV) IgM/IgG Antibody (Getein Biotech, Inc., China)-based immunodiagnostic assays are very specific to S- and N-proteins. Comparative analysis of commercialized COVID-19 diagnostic kits are also summarized in Table 3, to provide insights into the sensitivity and applicability of the kits in a practical environment. On the other hand, B.1.1.7, B.1.617.2, B.1.351, and P.1 are major SARS-CoV-2 specific S-protein mutants that emerged around the globe and were reported to affect the site of B-cell epitopes [127,128]. S-protein is the key factor of vaccine and detection platform development for COVID-19; therefore, existing diagnostic methods need to be optimized in such a way that it does not miss any mutants. In this direction, recently Thermo Fisher's new version of TaqPath™ COVID-19 detection kit, which was validated to detect orf1ab and N-gene of UK variant of SARS-CoV-2, namely B.1.1.7 (69–70del S gene mutation) is launched.

Furthermore, most methods are laborious and need technical expertise and laboratory infrastructure, and therefore there is a high need for shortlisting novel reliable detection tools. Among them, Droplet Digital PCR (ddPCR), RT-LAMP, Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)-Cas and electrochemical and optical sensors are getting attention. Besides, involvement of nanotechnology in 2019-nCoV detection, development of reliable POC, and commercialization on large scale is the next major tasks for the scientific community.

(1) SHERLOCK (Specific High Sensitivity Enzymatic Reporter UnLOCKing) and DETECTR are two promising CRISPR-based SARS-CoV-2 detection techniques. SHERLOCK is developed by the Massachusetts Institute of Technology (MIT) and Harvard University-based research team, which is reported to detect 2019-nCoV-specific S- and Orf1ab-gene in <1 h with LOD of 10–100 copies/µl [129]. However, this method requires sequential isothermal amplification and CRISPR reaction for SARS-CoV-2 identification, therefore this research team further omitted these steps in an improved version of SHERLOCK, namely STOP (SHERLOCK Testing in One Pot)-
COVID based detection assay [130]. Not limited to this, the use of magnetic beads for purification also lowered the detection time to 15–45 min with 93.1% sensitivity and 98.5% specificity [131].

(2) In addition, sensing diagnostic methods have also emerged as sensitive analytical tools against SARS-CoV -2 detection. To promote the development of sensitive and rapid biosensors, an anti-SARS-CoV-2 antibody immobilized on gold nanoparticles (AuNPs) fabricated fluorine-doped tin oxide sensing platform was initially designed for on-spot COVID-19 detection along with LOD of 120 fM in spiked saliva samples [132]. Similarly, a SARS-CoV-2 antibody fabricated field-effect transistor (FET)-based sensing platform was also developed [133]. This sensor exhibited LOD of 1.0 fg/ml in PBS and 100 fg/ml in nasal swab samples without sample pre-treatment or labeling. On other hand, a plasmonic photothermal (PPT) effect and localized surface plasmon resonance (LSPR)-based dual-functional optical DNA sensing platform was reported for 2019-nCoV multigene detection with LOD of 0.22 pM [134]. This optical sensor has higher sensitivity than another SPR sensor developed previously, where they used thiol-conjugated antisense oligonucleotides fabricated AuNPs sensing platform for SARS-CoV-2-specific N-protein detection with LOD of 0.18 ng/µl [135].

(3) Though the biosensors are sensitive toward SARS-CoV-2 detection, yet several concerns may limit the commercialization at a large scale. The first issue is to establish sophisticated instrumentation facilities to calibrate and validate the sensor at the laboratory level, which are expensive and time-consuming. Time is a critical component in this pandemic situation, where clinicians prefer to perform diagnostic assays as rapidly as possible on existing facilities. For instance, high throughput automated *in-vitro* diagnostic systems, for example, ABBOTT™ Architect™ and Roche cobas® will be more pragmatic in SARS-CoV-2 detection. R&D laboratories can also implement commercially available dipsticks as lateral flow assays in an on-spot auxiliary test for children and older adults.

Previous studies performed on SARS-CoV-2 diagnostic tests may influence procedures and sensitivity of detection methods for both children and older adults. Therefore, optimization of diagnostic techniques for future effective clinical strategies and strengthening the health system in the COVID-19 pandemic situation needs to be our primary concern. To date,

Table 3. Summary of comparative studies on commercialized rapid detection kits for reliable SARS-CoV-2 detection.

SARS-CoV-2	biomarker	Comparatively reliable kits Name/ Manufacture	Sensitivity or LOD	Conclusion	Remarks
IgG/ IgM	IgG/IgM antibody ELISA kits/ Zhu Hai Liv Zon Diagnostics Inc., China [138]	87.3%	Sensitivity: IgG/IgM antibody ELISA kit (Zhu Hai Liv Zon Diagnostics Inc., China)>IgG/IgM antibody GICA kit (Zhu Hai Liv Zon Diagnostics Inc., China) >qRT-PCR	The higher sensitivity of the ELISA is rare to find in terms of infectious disease, therefore, the proposed kit may be beneficial to children and older adults.	
N-protein specific IgG/ IgM	COVID-19 IgM/IgG Duo/ SD BIOSENSOR, Korea [139]	–	Delectability for IgM: COVID-19 IgM/IgG Duo (SD BIOSENSOR, Korea) = 2019-nCoV IgG/IgM Rapid Test Cassette (Hangzhou AllTest Biotech Co., Ltd., China)> 2019-nCoV IgG/IgM Detection Kit (Vazyme Biotech Co., Ltd., China)	These kits exhibited a consistent positive rate (%) for IgM and comparable differences for IgG at different time points of infection. Hence, these kits may avoid age-related factors in asymptomatic patients.	
–	Standard Q COVID-19 Ag/ SD Biosensor, Korea [140]	Excellent LOD at 10^{-5}dilution	LOD: Standard Q COVID-19 Ag (SD Biosensor, Korea) >COVID-19 Ag Respi-Strip (CorisBioconcept, Belgium) = NADAL COVID-19 Ag Test (Nal Von Minden GmbH, Germany)	The study validated the rapid antigen kits for different SARS-C0V-2 specimens, thus avoid the demand of the specific sample type in children and older adults.	
SARS-CoV-2 specific E- and S-gene	RealStar® SARS-CoV-2 RT-PCR Kit/ Altona Diagnostics, Germany [141]	LOD: 625 copies/ml	Sensitivity 97.6% and specificity 97.3% without any cross-reactivity.	The higher sensitivity of the kit in comparison to qRT-PCR may improve the delectability of SARS-CoV-2 in children and older adults.	
Orf1ab and N-gene	Novel coronavirus 2019-nCoV nucleic acid detection kit/BioGerm, Shanghai BioGerm Medical Co., Ltd., China [142]	Excellent LOD at 1:10 (Orf1ab) and 1:40 (N-gene) dilution	Novel coronavirus 2019-nCoV nucleic acid detection kit(BioGerm, Shanghai BioGerm Medical Co., Ltd, China) showed higher LOD in comparison to the other 4 rapid kits.	A study suggested validating the kit every time in the individual laboratory, thus standardized data can be generated for children and older adults.	

there is no absolute end point in advanced SARS-CoV-2 diagnostic strategies across the globe that could have brought us closer to controlling the infection in high-risk children and older adults. Therefore, more clinical and cohort studies need to be conducted to standardize detection methodologies to identify the age-related impact on SARS-CoV-2 detection. The future results may hold propitious development in the area of healthcare intervention for other infections that may occur in near future. The current establishment of advanced diagnostic methods along with their age-related optimization can be further explored for enduring more feasible and rapid results in next-generation analytical devices.

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Declaration of interest

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