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Mini Review

The Importance of Qualitative/Quantitative Analytical Tools in COVID-19 Vaccine Development

Behrouz Seyfinejad[1], Ommoleila Molavi[2,3], Abolghasem Jouyban[1,4*]

[1]Pharmaceutical Analysis Research Center and Faculty of Pharmacy, Tabriz University of Medical Sciences, Tabriz, Iran
[2]Biotechnology Research Center, Tabriz University of Medical Sciences, Tabriz, Iran
[3]Department of Pharmaceutical Biotechnology, Faculty of Pharmacy, Tabriz University of Medical Sciences, Tabriz, Iran
[4]Pharmaceutical Sciences Research Center, Shahid Beheshti University of Medical Sciences, Tehran, Iran

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Abstract

The COVID-19 pandemic has resulted in significant morbidity and mortality worldwide, emphasizing the need for effective vaccines. The development of a COVID-19 vaccine requires extensive analysis to ensure its safety and efficacy. In this review, the importance of analytical tools in COVID-19 vaccine development was discussed. A comprehensive literature search using PubMed and Scopus was conducted. Analytical tools play a crucial role in COVID-19 vaccine development, from the initial stages of antigen characterization to vaccine formulation and efficacy testing. The tools used include immunoassay-based methods, separation-based methods, and microscopy. These tools facilitate the characterization of antigens, selection of adjuvants, optimization of formulation, and stability testing. Moreover, analytical tools enable the evaluation of vaccine safety and efficacy, which are necessary for regulatory approval. Therefore, the implementation of analytical tools in COVID-19 vaccine development is crucial in ensuring the timely and successful development of a vaccine to combat the pandemic.

Introduction

The COVID-19 pandemic, caused by the SARS-CoV-2 virus, has had a profound impact on global health and the economy. One of the most promising approaches to control the spread of COVID-19 is the development of safe and effective vaccines. The speed at which COVID-19 vaccines have been developed and approved is unprecedented in the history of vaccine development. This was made possible, in part, by the use of advanced analytical tools and techniques.[1] The development of COVID-19 vaccines can be broadly categorized into four phases: discovery, preclinical testing, clinical testing, and approval. In the discovery phase, potential vaccine candidates are identified through various approaches, such as using viral vectors or messenger RNA (mRNA) technology. The preclinical testing phase involves animal studies to assess safety and efficacy. The clinical testing phase involves testing the vaccine in humans through a series of clinical trials. Finally, approval is granted by regulatory agencies based on safety and efficacy data.[2]

Analytical tools play crucial roles in vaccine development by enabling the identification of potential vaccine candidates, optimizing vaccine design, and ensuring vaccine quality and safety. In the discovery phase, analytical tools such genomics, proteomics, and structural biology are used to identify potential vaccine candidates by studying the structure and function of the virus. These tools can help to identify targets for vaccine development, such as viral proteins or nucleic acids.[3] In the preclinical testing phase, analytical tools are used to assess the safety and efficacy of vaccine candidates. These tools include animal models, cell culture systems, and assays that measure the immune response to the vaccine. Animal models are used to assess the safety and efficacy of the vaccine *in vivo*. Cell culture systems and assays are used to study the immune response to the vaccine *in vitro*.[4] In the clinical testing phase, analytical tools are used to assess the safety and efficacy of the vaccine in humans. These tools include various types of assays that measure the immune response to the vaccine, such as enzyme-linked immunosorbent assays (ELISAs), neutralization assays, chromatographic, and electrophoretic methods. These assays can help to determine the appropriate dose and schedule of the vaccine, as well as the duration of immunity.[5]

Since the identification of the SARS-CoV-2 virus in December 2019, multiple COVID-19 vaccines have been developed and approved around the world. These vaccines

***Corresponding Author:** Abolghasem Jouyban, Email: ajouyban@hotmail.com

have been developed using various approaches, such as inactivated or weakened viruses, protein subunits, viral vectors, and mRNA technology. Each of these approaches relies on a different set of analytical tools to identify potential vaccine candidates, optimize vaccine design, and ensure vaccine quality and safety. For example, the Pfizer-BioNTech and Moderna COVID-19 vaccines use mRNA technology, which relies on analytical tools such as high-throughput sequencing and bioinformatics to design the mRNA sequence that encodes the viral antigen.[5] The Oxford-AstraZeneca and Johnson & Johnson COVID-19 vaccines use viral vectors, which rely on analytical tools such as gene sequencing and protein structure analysis to design the vector construct.[6] Within just a few months after the development of world-leading vaccines, Iranian institutions and companies launched their own COVID-19 vaccine into the market. Despite being domestically developed, the developers of these vaccines vary significantly in their strategies, motivation, and capabilities. Therefore, nine vaccines with various platforms were developed in Iran. COVIran Barekat, FakhraVac, and OSVID-19 use inactivated viruses, SpikoGen, PastoCovac, Noora vaccine, and Razi Cov Pars use protein subunit, Corenapcin uses mRNA and HUM Immune Biotechnology Vaccine uses viral vector (non-replicating).[7] Given the wide range of platforms used in Iran for vaccine production, it is crucial to have a variety of analytical methods available. This ensures that all stages of production conform to both national and international standards.

The purpose of this review is to discuss the importance of analytical tools in COVID-19 vaccine development. It also provides an overview of the current analytical tools being used, from vaccine design optimization to manufacturing and quality assurance, as well as immunogenicity.

The development of analytical tools in the midst of a pandemic

Whether a researcher is developing an *in vivo* preclinical model or an *in vitro* assay, the process of developing analytical tools for vaccine research is the same in both cases. To choose the best analytical technique or equipment, the first step in developing a novel assay is to specify the research question that has to be answered. During the design phase of the assay, the inputs are determined, key reagents are identified, an assay protocol is developed, the assay is validated, and the protocol is formalized into a standard operating procedure (SOP) specifying the necessary measurements, outputs, and interpretation.[8] A protocol must be validated after its establishment to determine its specificity and sensitivity, identify any potential reagent interference or cross-reactivity, and evaluate accuracy and reproducibility for the entire intended purpose. Assay evaluation is a continuous process that includes continuing quality control and validation.[9] Time is of the essence in a pandemic situation, so assay development must be speeded up to support vaccine research. It can be challenging to connect a certain immunological impact to particular product attributes, since there is frequently insufficient scientific understanding of a vaccine's precise protective effect. This makes it difficult to create adequate assays to check the product quality of a vaccine. The vast diversity of diseases also leads to a great deal of heterogeneity in vaccine production technologies.[10] As a result, in order to determine the quality of each vaccine, a unique set of tests is required. Three International Conference on Harmonization (ICH) guidelines, Q7, Q8, and Q9,[11-13] provide a comprehensive summary of the current regulatory requirements for a biopharmaceutical product.

Analytical methods for COVID-19 vaccine development

During the development and manufacturing stages of vaccines, a wide range of analytical techniques is used to assess the properties of candidate vaccines. The vaccine pipeline is using a range of approaches that have emerged from various backgrounds. Mass spectrometry (MS) and high-performance liquid chromatography (HPLC) have moved beyond protein-therapeutic characterization. Capillary electrophoresis (CE) is one of the most well-known electrophoretic procedures that has its roots in traditional molecular biology. Approaches for particle size that have been developed through polymer analysis or material lab use. Methods like CE or slab-gel electrophoresis are preferred for the mRNA component. LC-MS or HPLC with charged aerosol detection are typically the methods of choice for lipid nanoparticle (LNP) and lipid characterization. It is preferable to use methods like cryo-electron microscopy or particle sizing for complete mRNA-LNP complexes. During the development of mRNA vaccines, slab-gel electrophoresis or CE and polymerase chain reaction (PCR) continue to be the standard analytical techniques.[8]

Vaccine characterization

For COVID-19, a number of vaccine technology platforms, including mRNA, viral vector, DNA, subunit protein, and inactivated virus methods have been used.[14] Each platform uses a different strategy to provide two key signals required for activation of anti-COVID-19 immune responses. Indeed, an effective vaccine should provide two main signals for the cells of our immune system in order to induce protective immunity against an invader. The first signal comes from a non-self-antigen, which is mostly a structural protein in the infectious agent. Among the four structural proteins of SARS-CoV-2, the spike protein is the leading mediator of virus entry into human cells and the main determinant factor of virus virulence, therefore it has been used as antigen in COVID-19 vaccines.[15] The second type of signals required for induction of adaptive immune responses are called danger signals which are some molecular pattern found in invading pathogens, known as pathogen associated molecular patterns

(PAMP). Danger signals function as adjuvant and the selection of the appropriate types of adjuvant significantly impact the type and magnitude of adaptive immune responses and its efficacy in combating the infectious agents.[16] Non-self-antigen included in COVID-19 vaccines is the full length spike protein or some epitopes of this protein. These protein/peptide-based antigens are included in the COVID-19 vaccines in the forms of either ready to use antigens or nucleic acid RNA or DNA coding sequences of the selected antigens. In DNA and mRNA vaccines, antigens is made intracellularly by body's own cells and DNA/RNA molecules function as adjuvants, thus they can strongly induce both antibody and cell mediated immune responses. In inactivated and subunit vaccines, viral antigen is included in vaccines along with an adjuvant. Since the antigens of these vaccines are considered exogenous proteins for antigen presenting cells of innate immunity, antibody responses will be the major type of immunity induced by these vaccines, unless a particular adjuvant capable of inducing cell mediated immunity is included in these vaccines. Therefore, in subunit and inactivated vaccines, the selection of the right type of adjuvant is highly important.[16-18] Once a vaccine antigen and adjuvant have been chosen and produced, the first step is to evaluate their physical qualities because these can have a big impact on the type and quality of the immune response. Assays are required to verify the identification and purity of the vaccine antigen, as well as the antigen's structure and chemical composition, and to look for harmful impurities. The numerous analytical techniques for characterizing vaccines are described in details here, with a critical evaluation of the benefits and drawbacks of each strategy, which are summarized in Table 1.

Identity and purity

Depending on the vaccine type, several analytical methods are used to determine the antigen's identity. To swiftly verify the identity of DNA, mRNA, or viral vector vaccines, molecular biology techniques such as PCR, reverse transcription PCR (RT-PCR), and sequencing can be utilized. [32] These methods can also be used to verify the introduction of antigen-coding sequences in the expression systems being utilized to produce recombinant

Table 1. Analytical assays for vaccine characterization.

Analytical technique	Application	Advantages	Disadvantages
ELISA[19]	– Determine amount and identity of antigen or detect potential contamination using pathogen-specific or antigen-specific antibodies	– Low cost – Antigen-specific staining – Less time consuming compared with WB – Quantitative method – Proteins are not denatured and in native form	– Harder to detect issues of cross-reactivity of antibodies compared with WB (which can be seen via difference in band sizes) – Dependent on quality and specificity of antibody – No information on size of product of degradation
PCR/RT-PCR[20]	– Confirm identity of vaccine expression systems and vaccine antigen – Detection of RNA/DNA of pathogens	– High sensitivity – Easy access to equipment – Rapid results	– Susceptible to interference from contamination, especially carryover from other PCR products – Requires knowledge of target for PCR primer design
WB [21]	- Determine identity, size and amount of vaccine protein content using antigen specific antibodies and band size - Detect host cell protein contamination	– Antigen-specific staining – Size of bands of interest can indicate quality, degradation, post-translation modifications	– Dependent on quality and specificity of antibody – Low throughput – Large amount of protein required for detection – Primarily qualitative/semi-quantitative method – Proteins in denatured form
Fluorescence/UV spectroscopy[22]	– Determine tertiary structure of vaccine and correct protein folding	– High sensitivity – Easy method	– Contamination can quench or produce autofluorescence and give false readouts
HPLC[23]	– Determine identity, purity, size, stability and amount of ingredients in vaccine	– Relative quick – High sensitivity and resolution – Highly automated systems require minimal training – Can be combined with other techniques such as MS	– Requires high sample input – Expensive
MS mapping[24]	– Determine identity and amount of vaccine – Assess post-modification to vaccine	– Small sample input required – High-throughput and can detect post-translational modifications	– Specialized equipment required which is not widely available – Expensive
Sterility test[25]	– Detects presence of microbial contamination	– Low cost – Simple procedure	– Detects only live microbial contamination and provides limited information on identity of contamination
General safety test (abnormal toxicity test) [26,27]	– Toxicity of vaccine/detect adverse events related to vaccine	– Simple procedure	– Ethical concerns related to use of animals – Several studies show poor predictor/correlate of toxicity in humans
Limulus amebocyte lysate[28,29]	– Detects presence of endotoxins in vaccine	– Low cost – Simple procedure	– Animal derived reagents – Batch-to-batch differences
High-throughput sequencing[30,31]	– Detect adventitious viruses	– Simple procedure	– Ethical concerns related to use of animals – Several studies show poor predictor/correlate of toxicity in humans

ELISA; enzyme-linked immunosorbent assay, PCR; polymerase chain reaction, RT-PCR; reverse transcription-PCR, HPLC; high performance liquid chromatography; MS; Mass spectrometry, WB; western blot.

proteins, as well as their identity, copy number, and genetic stability. The great sensitivity of PCR-based techniques makes them vulnerable to interference from contamination, especially carryover from other PCR products in the lab, which is one of their limitations.[33]

A variety of strategies can be used at the proteome level to validate protein-based vaccines and demonstrate that host cells correctly translate viral vectors, mRNA, and DNA into proteins. Protein size and purity can be semi-quantitatively confirmed by protein separation using gel electrophoresis, followed by staining or western blots. This technique is advantageous due to its relative simplicity and the fact that most laboratories already possess the necessary equipment, making it easily accessible and practical. The requirement to an antibody against the vaccine protein is a drawback of western blots.[34] The structure of the vaccine protein can be further analyzed using more sophisticated techniques including cryo-electron microscopy, HPLC, and monoclonal antibody surface mapping. The SARS-CoV-2 spike protein undergoes complicated structural changes that expose and conceal possible epitopes.[35,36] It has been demonstrated that locking the spike protein in a pre-fusion conformation for MERS and SARS-CoV-2 increases their capacity to elicit neutralizing antibodies[37,38] demonstrating the importance of protein structure for the vaccine's immunogenicity. Moreover, the protein's post-translational changes, such glycosylation, may be crucial for both immune identification and protein stability. Heavy glycosylation of the SARS-CoV-2 spike protein may serve to protect antibody and T-cell recognition sites.[39,40] The glycosylation process can be affected by the expression system used, such as whether mammalian or insect cells are used. The ability to identify post-translational changes of recombinant proteins and contrast them with the native protein is made possible by mass spectrometry techniques.[41] Most of these procedures may need specific tools and training, which is a drawback.

Vaccine purity and detection of contaminants

There is a risk of contamination for vaccines made by cell-based expression systems, including endotoxins, adventitious viruses, and host cell proteins. Researchers should refer to the standards and guidelines published by the appropriate regulatory agency, such as the United States Food and Drug Administration (US-FDA) or European Medicines Agency (EMA),[42,43] as regional regulatory requirements. Sterility testing can identify bacterial and fungal contaminations. All biological manufacturing facilities regularly carry out these tests, which typically entail inoculating sterile media with a vaccine sample and watching for growth via CO_2 production or visual changes for 14–28 days.[44] The assay is simple yet time-consuming, and it merely determines whether contamination is present or not. In recent years, high-throughput sequencing assays have been designed to screen for a broad repertoire of various adventitious viruses. Detecting adventitious viruses in cell substrates can be challenging, even with PCR, if the source is unclear. Chemical residues or microbiological byproducts (endotoxins) can be detected in the vaccine formulation using assays like the limulus amebocyte lysate assay[45] or the pH change/cell cytotoxicity assays, respectively. Several reagents/inputs (such as nucleosides, enzymes) are still created by microbial fermentation, therefore several of the above-mentioned assays are still valid. Novel types of vaccines, like as mRNA and DNA, are often chemically manufactured. The oligoribonucleotide impurities caused by RNA polymerases' abortive initiation events or double-stranded RNA produced by self-complementary extension are two contaminants peculiar to mRNA vaccines that are known to cause inflammation.[46] The purity and integrity of an mRNA COVID-19 vaccine were evaluated using conventional methods including microfluidic capillary electrophoresis (CE).[47] Furthermore, mRNA contamination can be found using HPLC and assays with double-stranded monoclonal antibodies that are specific to mRNA.[48]

Safety

Safety assessment of vaccines is extensive and given top attention in the design of vaccines. The safety assessment of vaccines begins with non-clinical examination of their individual ingredients in terms of purity, stability, potency, consistency, activity and sterility then continues throughout the clinical development phase and entire period of vaccine usage, including post-approval. Vaccines safety assessments are conducted at multiple levels by a range of independent organizations including academia, industry, regulatory agencies, the medical community and even the general public, which all play a role in monitoring vaccine safety. The general safety test (or abnormal toxicity test) involves giving a specific dose of a vaccine to an animal and measuring the animal's subsequent toxic effects through body weight loss, clinical scoring, temperature, and/or survival. It is a straightforward procedure for determining the toxicity of biological products. Since they are inbred, mice are frequently used for these tests, but their predictive power may be limited. [49] Several groups of therapeutic drugs are no longer required to pass the general safety test, according to regulators like the FDA.[49] *In vivo* toxicity testing assays are gradually being replaced by alternative *in vitro* based assays to evaluate cytotoxicity, genotoxicity, and carcinogenicity in which cell viability, mutations, and chromosomal damage are monitored in cells after injection of vaccine components.[50] The possibility of vaccine-enhanced disease, which has been documented for prior SARS,[51,52] dengue,[53] MERS,[54] and respiratory syncytial virus[55] vaccines, was one early worry for SARS-CoV-2 vaccines. Without access to animal challenge models, it is hard to assess the possibility of vaccine-enhanced disease, and this was a significant problem for the early SARS-CoV-2 vaccines because such models were not accessible at the time. Luckily, none of the SARS-

CoV-2 vaccines currently being used in clinical settings have been associated with vaccine-enhanced disease.[56]

Assessment of vaccine immunogenicity

Antibody-mediated humoral immunity and cellular (B and T cell) immunity are the two basic forms of vaccine-induced immune responses. As viruses are intracellular pathogens, both humoral (antibody) and cell mediated (cytotoxic T cells) immune responses are needed to be activated in response to a vaccine and work together to combat the virus. Anti-virus antibodies neutralize the invading virus and prevent its binding to the cells and resulting cell infection. Cytotoxic T cells of cell mediated immunity are needed to destroy virally infected cells thereby preventing the infection of neighboring cells and tissues. Thus a COVID-19 vaccine that strongly activates both antibody and cell-mediated immune responses is expected to efficiently induce protection against the viral infection.[18] Indeed, the validity of a COVID-19 candidate vaccine should be evaluated by an assessment of antibody, cellular, and functional immune responses to COVID-19 antigens. As it will be discussed later, the IgG and IgM antibody responses against COVID-19 antigens are usually quantified by ELISA. A number of cellular immune assays have been used for evaluation of cell-mediated immune responses against COVID-19. Detection of COVID-19 specific interferon-γ (IFN-γ) secreting T cells by ELISPOT is one the most recent method for assessment of the cellular immune response against COVID-19 after vaccination.[57] The functional activity of immune responses are assessed by neutralization assays using either wild-type virus or pseudovirus *in vitro*. Thus, to establish an immunological correlation of vaccine protection, which may then be utilized to direct subsequent vaccine development, immune assays are necessary. Having a reliable immunological correlate of vaccine protection allows for easy comparisons between different candidate vaccines, streamlines the approval process for future vaccines, and eliminates the need for costly and time-consuming phase III outcome trials. It is crucial for vaccine researchers to continue evaluating a wide variety of immunological measures while testing their vaccine candidates because although there is some link between serum-neutralizing antibodies and protection, no immune correlate of protection for SARS-CoV-2 has been established yet.[58]

Assays of humoral immunity

Vaccine antibody responses are mostly assessed using sera taken from venous blood. Unlike cell-based tests, sera are simple to inactivate, making it possible to securely remove post-infection samples from locations where virus challenges are carried out. In every antibody assay, it is crucial to run both negative (naive) and positive sera controls. With the global spread of SARS-CoV-2 and the rapid vaccine distribution, the supply of seronegative sera may decrease, leading certain groups to resort to archived sera collected prior to the pandemic as negative controls to ensure their seronegativity. Moreover, a number of investigations have demonstrated antibody cross-reactivity between seasonal human coronavirus antibodies and SARS-CoV-2,[59,60] which has the potential to induce background interference in SARS-CoV-2 serology assays.

Using binding assays like multiplex bead/protein microarrays or conventional ELISA, one may quantify antigen-specific antibodies. Although neutralization assays are generally more accurate predictors of protection than antibody-binding assays, they are also more labor-intensive, expensive, and time-consuming, have high levels of variability, and frequently require the use of high-level biosecurity facilities that may not be widely accessible. As a result, other more straightforward functional assays have been created as alternatives, such as virus pseudotyping assays that, for example, use a lentivirus backbone to express the SARS-CoV-2 spike protein and a reporter gene. These assays make it possible to more easily measure the ability of immune sera to inhibit the infectivity of the pseudotype lentivirus. In certain functional tests, the ability of immune sera to prevent the attachment of the spike protein to the angiotensin-converting enzyme 2 receptor bound to an ELISA plate is measured rather than the neutralization assay's requirement for the use of cells.

ELISA

In before ELISA technique, capture antibodies are used to either directly bind or immobilize an antigen, and then a tagged detection antibody is applied to measure the relative amounts of different antibodies. The simplicity and speed of ELISA are its advantages. IgM, IgG1, IgG2a/c, IgG2b, and IgG3 are examples of antigen-specific antibodies that can be detected by the assay. The quantities and ratios of these antibodies can reveal information about the type of immune response that the vaccine elicited. For instance, the synthesis of IgG2 and IgG1 antibody subclasses is selectively biased by the cytokines produced by T helper 1 (Th1) and T helper 2 (Th2) cells, respectively.[61] Several SARS-CoV-2 antibody ELISA studies have revealed low sensitivity (30-40%) and significant background levels, particularly at early post-immunization time periods (7–14 days).[62-65]

Cell-based ELISA (CELISA) to measure anti-spike antibody

Researchers created a spike protein CELISA assay to get around the high non-specific binding to sera associated with traditional ELISA with antigen-coated plates.[66] In this technique, SARS-CoV-2 spike-containing lentiviral vectors are transduced into HeLa cells to produce persistent spike-expressing cell clones (HeLa-Sp). The HeLa cells' surface expression of the spike protein is verified by flow cytometry and immunofluorescence labeling. Later, a cell fixation stage was skipped since it produced too much background. When employed with

human sera, the unfixed CELISA exhibits a considerably reduced nonspecific background than the traditional spike ELISA. Alternative transfected cell lines, such as HEK293 cells, are used in the CELISA assays described in the literature, and samples are analyzed using flow cytometry to represent antibody binding.[67] The batch-to-batch variability of typical cell-based assays, variation in technical procedures, and varying transfection rates are limitations of CELISA assays that make comparisons between laboratories challenging.

Multiplex immunoassays

Traditional ELISA assays have a significant drawback in that they only test one analyte at a time and call for large sample quantities. Antibodies and other analytes can be detected in a single sample using protein microarrays and other multiplexed assay platforms, such as bead-based immunoassays. For instance, the LabScreen COVID PLUS (Lambda) assay measures antibodies to MERS-CoV, SARS-CoV, and other common seasonal coronaviruses as well as to various SARS-CoV-2 fragments, including the full spike extracellular domain, S1, RBD, S2, and nucleocapsid (NP) protein.[68] This aids in locating any potential coronavirus cross-reactive antibodies. The IgA, IgG, and IgM antibody subclasses are measured using a chemiluminescent multiplex immunoassay (Vibrant America Clinical Laboratories) against various SARS-CoV-2 antigens (S1, RBD, S2, and NP).[69] These highly sensitive multiplex assays only need a small amount of serum sample, and they have the potential to describe the coronavirus antibody repertoire in vaccinated subjects or infected in greater detail.

Capillary electrophoresis

Another technique for analyzing the interactions of viruses and subviral particles (such as spike proteins) with antibodies and receptors is CE. Using CE, viruses and their sub-components can be distinguished by ionic mobility and size, as well as by charge.[70] The output data show distinct peaks based on various migration times, and the analytes separated by CE can subsequently be identified using UV absorbance, reactive dye, or intercalating fluorescent labels. The migration time and the peak of the virus or one of its components are shifted when antibodies bind to the intended viral protein.[71] The advantage of CE is that it just requires a small amount of samples. Moreover, CE is a sensitive method that has even been demonstrated to be able to distinguish between pathogenic SARS-CoV-2 subtypes.[72] The method has a drawback in that contaminants and buffer solutions may impair the assay's detection sensitivity,[73] and the sample (in this case, sera), may need further processing or an antibody purification step.

Cellular immune assays

Animal splenocytes and human peripheral blood mononuclear cells (PBMCs) serve as the main sources of information for studying antigenic-specific cellular immunological memory responses. The most widely used cellular assays for measuring antigen-specific B- and T-cell responses include: (1) measuring antigen-stimulated immune cell proliferation using methods like the 3H-thymidine incorporation assay, carboxyfluorescein succinimidyl ester (CFSE) dilution assay, and surrogate proliferation cell markers; (2) measuring cytokine production using ELISA, cytokine bead arrays (CBA), enzyme-linked immune absorbent spot (ELISPOT)/FluoroSpot, and intracellular cytokine staining (ICS); there is still a significant degree of variation between laboratories in both technical approaches and interpretation, and each method has its own pros and disadvantages.

T-cell proliferation assays

The level of T-cell memory, which is essential for antiviral recall responses, can be determined by observing the proliferation of PBMCs and T-cell subsets ($CD4^+$/$CD8^+$) after stimulation with antigen-specific stimulation (*e.g.*, live virus, recombinant SARS-CoV-2 spike protein, or vaccine antigen) in a cell culture setting at various time points.[74] T-cell proliferation can be assessed in a variety of methods, including by surrogate cell cycle markers or through the absorption or persistence of cell-permeant reagents. The fraction of actively proliferating cells in the sample can be measured by immunochemical or radiographic detection techniques. Synthetic nucleoside analogs, such as 3H-thymidine[75] and 5-bromo-2-deoxyuridine (BrdU)[76] are taken up and incorporated into the DNA of dividing cells. BrdU labeling is a more widely used approach since it is faster and more sensitive than 3H-thymidine assay, which is labor-intensive and has safety issues with the handling and disposal of radioisotopes.

CFSE, cell trace violet, or violet proliferation dye 450 are examples of cell-permeant dyes that can be diluted and used to measure proliferation (VPD-450). In many laboratories, monitoring lymphocyte division using CFSE is a common practice. Cells are dyed with cell-permeant dyes prior to antigen stimulation, and flow cytometry measurements show that the dye intensity in daughter cells decreases by half with each cell division.[77] The assay's only drawback is that, at high concentrations, CSFE can be hazardous to lymphocytes and that, after eight daughter cell divisions, its fluorescence is too dim to discriminate from background auto-fluorescence. The expression of surrogate proliferation cell markers, with Ki67 being the most widely utilized marker in the literature,[78] is a final method for determining lymphoproliferation. Ki67 only expresses in cells that are actively proliferating at the time of the assay, as opposed to other assays which continuously track proliferation cells, providing just a snapshot at a certain time point. Finally, surface markers (such as $CD4^+$, $CD8^+$, or CD62L) can be employed in conjunction with all proliferation assays, with the

exception of radioactive labeling, to pinpoint distinct subpopulations among proliferating or non-proliferating cells.

Cytokine production assays

The degree and nature of the immunological response elicited by vaccine candidates can be assessed using the immune cells' cytokine production profile. CBA, ELISPOT, and ICS tests are the three most common assays used to evaluate immune cell activity. There are numerous other techniques for evaluating cytokine production. ELISA is a common technique for quantifying cytokines secreted by cells, although it has a very low throughput and needs a lot of samples. For the simultaneous assessment of up to 30 secreted analytes from small amounts of biological materials such as supernatant, serum, and cell lysate, CBA assays employ spectrally different beads covered with capture antibodies.[79] With the use of new upgraded CBA kits that are capable of detecting analyte concentrations as low as one picogram, it is possible to precisely quantify the analyte in the sample and compare results across experiments and labs. Any typical flow cytometer with three lasers (488 nm/532 nm/633 nm) may read the cytometric bead array. This technique's primary drawback is that it measures cytokines in bulk from a population of cells and is unable to provide information on the cells that produce cytokines. It is possible to quantify the prevalence of particular cytokine-secreting cells using ELISPOT assays.[80] Immune cells grow on cytokine-coated membranes that capture and bind cytokines secreted by stimulated cells. A second enzymatically conjugated cytokine-specific antibody that can generate a colored substrate is used to identify cytokine spots after washing the cells out of the way. The number of cytokine-producing cells and their amount of production are indicated by the size, intensity, and frequency of the dots.

Due to the assay's great sensitivity and repeatability, ELISPOT assays are frequently used in human clinical studies to evaluate T-cell responses.[81] Although the frequency of SARS-CoV-2-specific T cells was similar across symptomatic and asymptomatic patients, ELISPOT investigation on a large cohort of COVID-19 patients recently showed that asymptomatic individuals produced more interleukin-2 (IL-2) and IFN-γ (Figure 1).[82] The inability of an ELISPOT to directly get information on the phenotype of the cytokine-producing cells is a drawback of the tests. Moreover, only a small number of analytes can be evaluated at once, and the assay is unable to identify polyfunctional cells that produce a variety of cytokines. Several cytokines can be detected using the same ELISPOT assay thanks to a newly developed modified method called FluoroSpot assays, in which fluorescent rather than enzymatic conjugates are employed for the second cytokine-specific antibody.[83]

Regulatory process for the use of COVID-19 vaccines in human

Following to the completion of laboratory and preclinical testing of a vaccine, the company/researcher has to submit the tests results to FDA and get approval to begin studies in human. The main goal of nonclinical studies of a COVID-19 vaccine candidate is to characterize the vaccines for its immunogenicity and safety. The appropriate dosing regimen and proper route of vaccines

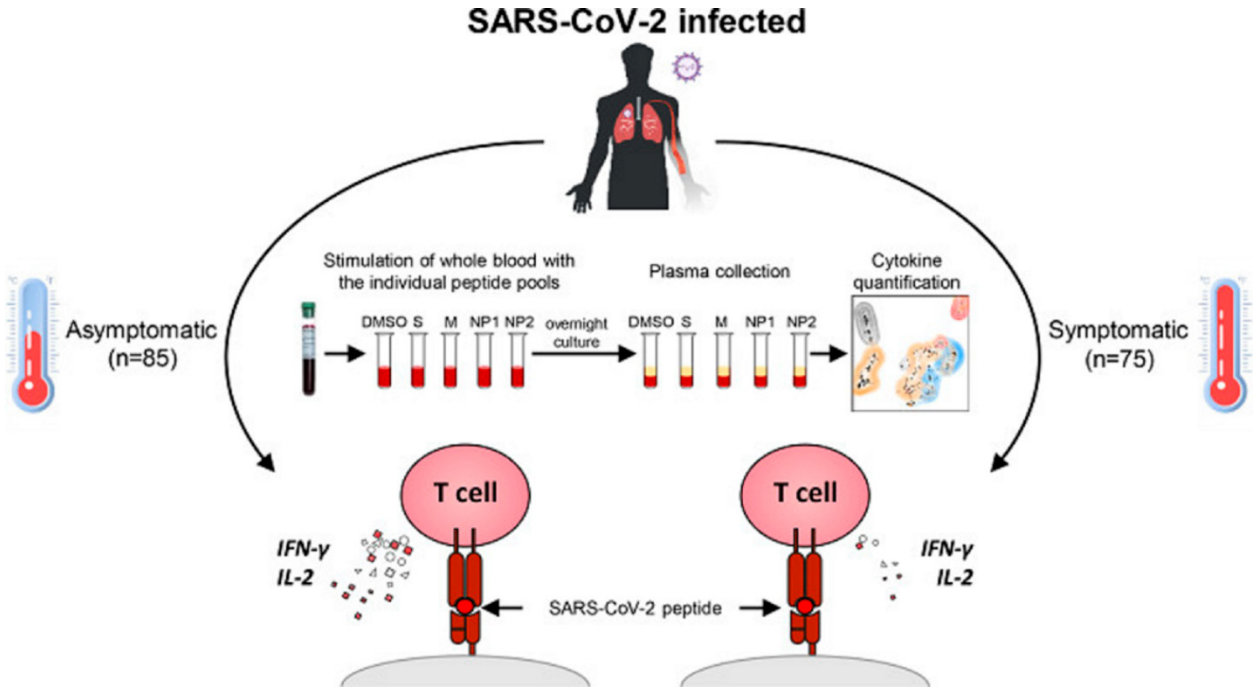

Figure 1. Study on SARS-CoV-2–specific T cells in a cohort consisting of asymptomatic individuals (n=85) and symptomatic COVID-19 patients (n=75) following seroconversion. The T cells responsive to structural proteins (M, NP, and Spike) were quantified using ELISPOT, and cytokine secretion was evaluated in whole blood.[82] (Creative commons CC BY-NC-SA 4.0 license)

administration for clinical studies, are also addressed by nonclinical studies animal models. Therefore, data from all key tests for vaccine purity, identity, stability, potency, dosing and immunogenicity should be included in the application for initiation of clinical studies. Based on the FDA most recent guideline, for immunogenicity tests should include the evaluation of humoral, cellular, and functional immune responses, as appropriate to each of the included COVID-19 antigens in the vaccines. ELISA is the preferred assay to characterize the humoral response. Sensitive and specific assays such as ELISPOT should be performed for the examination of $CD8^+$and $CD4^+$T cell responses. The functional activity of immune responses should be evaluated *in vitro* in neutralization assays using either wild-type virus or pseudo-virion virus. The assays used for immunogenicity evaluation should be demonstrated to be suitable for their intended purpose.[84]

Conclusion

In conclusion, the COVID-19 pandemic has highlighted the critical role of the analytical tools in vaccine development. From the identification of the virus to the development and evaluation of vaccines, a variety of analytical tools have been used to accelerate the pace of vaccine development. This review has provided an overview of the different analytical tools used in COVID-19 vaccine development, including quantitative methods such as immunoassays, chromatography, and electrophoretic methods as well as qualitative methods such as cryo-electron microscopy. As the pandemic continues to evolve and new variants emerge, the use of analytical tools will continue to be essential in the development and evaluation of new vaccines.

Authors' Contribution

Conceptualization: Behrouz Seyfinejad.
Supervision: Abolghasem Jouyban.
Writing–original draft: Behrouz Seyfinejad.
Writing–review & editing: Abolghasem Jouyban; Ommoleila Molavi.

Competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper. Moreover, Abolghasem Jouyban is one of the chief editors of the *ImmunoAnalysis journal*. He was not involved in any editorial decisions related to the publication of this article, and all author details were blinded to the article's peer reviewers as per the journal's double-blind peer review policy.

Data Availability Statement

Data sharing is not applicable to this article as no datasets were generated or analyzed during the current study.

Ethical Approval

Not applicable.

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