Chapter

Prophylactic Ribonucleic Acid Vaccines to Combat RNA Viral Infections in Humans

Irina Vlasova-St. Louis and Jude Abadie

Abstract

Vaccines have evolved as widely applicable and available prophylaxes against infectious diseases. Advances in ribonucleic acid technologies revolutionized the biopharmaceutical field of vaccine manufacturing. Numerous novel mRNA-based vaccines that have been approved by the United States and European regulatory agencies are proven to be safe and effective in preventing disease. This chapter presents the history of RNA vaccine development in the context of preventing diseases caused by RNA viruses such as SARS-CoV-2, HIV, influenza, Chikungunya, Zika, RSV, PIV, HMPV viruses, Rabies, and Ebola. Advantages, disadvantages, and challenges in mRNA vaccine engineering, delivery, and safety are discussed. The formulation, safety, long-term effectiveness, and requirements for booster immunizations are presented using data from clinical trials. The results of these clinical trials highlight important milestones, setbacks, and ultimate advancements in vaccine development. mRNA vaccines have significantly impacted public health in a relatively short time, and they demonstrate great potential in serving as clinical public health prophylaxis against current and future pandemics. Future development is likely to include polyvalent, mosaic, and strain/lineage-specific individualized vaccines.

Keywords: prophylactic vaccines, mRNA vaccines, SARNA vaccines, RNA viruses, SARS-CoV-2, influenza, HIV, RSV, parainfluenza type 3 virus, human Metapneumovirus, chikungunya, Zika virus, COVID-19, Ebola, epidemic, pandemic, clinical trials, genomics surveillance, public health, emergency preparedness

1. Introduction

The history of vaccines development continually demonstrates their evolution as prophylaxes agents against the spread of disease. For example, as demonstrated with annual flu immunizations, vaccinations have been key in establishing herd immunity and preventing outbreaks of infectious diseases. The pandemic resulting from SARS-CoV-2 infections necessitated vaccine development in a fashion that was accelerated compared to standard regulatory approval processes. Biopharmaceutical companies initiated vaccine research and development as soon as SARS-CoV-2 sequencing data become available. This led to rapid and seamless transitions to clinical trials using conventional vaccine candidates, as well as mRNA vaccines.

Next-generation RNA sequencing continues to evolve as the primary method public health laboratories use to conduct genomic epidemiology surveillance. This is particularly important for novel zoonotic infections that can cross inter-species barriers with the potential to cause epidemics and, perhaps, pandemics. RNA viruses demonstrate continual genetic recombination, in conjunction with the rapid accumulation of mutations, due to the inefficient proofreading ability of viral replicases. Therefore, real-time viral genotyping is of critical importance to public health during outbreaks resulting from virulent RNA viruses. Genotyping data became the fundamental basis for vaccine design. Furthermore, it provided insight into vaccine breakthroughs and allowed vaccine optimization through transgene sequence modifications.

The four types of conventional vaccines include live-attenuated vaccines, whole-pathogen inactivated vaccines, toxoid vaccines, and recombinant protein vaccines [1]. Inactivated vaccines and live-attenuated vaccines contain the whole pathogen. Live-attenuated vaccines (for example, against yellow fever, chickenpox, rotavirus, smallpox, or combined vaccine against measles, mumps, rubella (MMR)), are produced through various attenuation procedures [2]. These vaccines are quite immunogenic, and they can induce long-lasting humoral (systemic or mucosal) and cellular immune responses. However, whole virion vaccines are costly because viruses must be grown in cell cultures during commercial production [3]. There is a risk of reversion of live attenuated vaccines to a wild form, and this is why they are contraindicated for immunocompromised individuals. Poliovirus, hepatitis A, influenza, and rabies are the most successful inactivated vaccines. They can be conveniently freeze-dried for transport; however, large doses of virion administration are required, which can cause unintended adverse events due to host immune reactions. Additionally, the inactivation process may alter immunogenic epitopes confirmation, which makes vaccines less effective [4]. Toxoid vaccines immunize against toxins, which are produced by some bacterial pathogens (e.g., tetanus).

Recombinant DNA technologies produced recombinant protein vaccines. These vaccines were considered safer, with fewer adverse events in clinical trials. However, the identification of the best immunogenic antigen and the complexity of manufacturing design lengthened pre-clinical studies from several years to decades [4]. Protein vaccines often require adjuvants or conjugates to improve immunogenicity, stabilizers to maintain antigen conformation, and other nanomaterials – to improve internalization by antigen-presenting cells (APCs) *in vivo* [4]. The examples of most recent protein vaccines are hepatitis B and human papillomavirus (HPV). Traditionally, the development and production of these conventional vaccine types have been laborious and costly; furthermore, many of them lacked the efficacy to attain post-market approval.

Advances in nucleic acid technologies revolutionized the biopharmaceutical field of vaccine manufacturing. The ability of two novel types of vaccines, mRNA and DNA-based, to produce protein inside the immunized organisms, opened a new era in vaccinology [5]. However, unlike protein vaccines that are formulated without cargo, the DNA and mRNA vaccines required vehicles so that they could be delivered into cells [6]. Upon immunization, DNA vaccines use either plasmid or viral vectors to deliver the transgene into cells. Various lipid nanoparticle cargos have recently been developed for mRNA vaccines to increase the efficiency of cytoplasmic delivery. The poor stability of mRNA molecules (*ex vivo* and in *vivo*) requires additional considerations for formulation and storage (**Table 1**) [7]. Several biochemical solutions for RNA chemistries and lipid nanoparticle design have been proposed and thoroughly reviewed [8–11]. The major challenge identified for mRNA-based vaccines is achieving

Vaccine type	Advantages	Disadvantages
Nonreplicating or non-amplifying mRNA vaccines	Non-viral cytosolic delivery with biodegradable lipo-particles Transient translation of mRNA improves the safety profile Large-scale vaccine production Elicit cell-mediated and humoral immune responses Easy to modify	RNase free conditions is required during manufacturing Low temperature storage is required Host immune response to exogenous mRNA results in inhibition of translation Transient translation might result in low amount of antigen production
Self-replicating or self-amplifying RNA vaccines	Non-viral cytosolic delivery with biodegradable lipo-particles Multiple rounds of replication in the cytoplasm (lower doses are needed) SARNAs are presented by both major histocompatibility complexes 1 and 2 (endogenous adjuvant) Elicit humoral and functional T cell responses against antigen Easy to modify	More complexed manufacturing is required due to two separate RNA formulations Low temperature storage is required Sensitive to RNases Elicit innate host immune response which may inhibit translation
DNA-based vaccines	Good thermostability Fast internalization into cells via fusion mechanism Efficient transcription of the transgene upon nuclear delivery Non-replicative virus design Transient episomal transcription from newer adenoviral vectors	Complexed manufacturing of viral particles Low success of plasmid-based delivery of viral components Risk of host genome integration and promoter-driven expression of host oncogenes Host immune response to the vector upon booster

See text for references.

Table 1.
Advantages and disadvantages of DNA and RNA-based vaccines.

effective *in vivo* translation and identifying the correct/optimal dose of immunogen [12]. Therefore, despite the cost-effectiveness of *in vitro* synthesized mRNA vaccines and the potential for attaining large-scale manufacturing, the formulation of mRNA vaccines for delivery was an obstacle for several decades that has recently been overcome [13]. The history of successes and failures in vaccine development against infections caused by RNA viruses is elucidated throughout the literature review for infections caused by the Ebola virus, SARS-CoV-2, rabies, Zika, HIV, influenza, and the respiratory syncytial virus (RSV).

2. SARS-CoV-2 RNA vaccines

Coronaviruses are enveloped and contain between 25 and 32 Kb of non-segmented positive-sense RNA. Before the emergence of SARS-CoV-2, coronaviruses caused sporadic epidemics around the world [14, 15]. As described in [16], early during the COVID-19 outbreak, next-generation sequencing (NGS) of SARS-CoV-2 RNA provided valuable data about viral genome, its molecular origin, and a deeper understanding of pathogenicity.

As the COVID-19 pandemic spread, the world anxiously anticipated vaccine countermeasures [17]. At that time, mRNA vaccine development was the scientific

leader in our fight to end the pandemic. It is nothing short of spectacular heroism and scientific acuity that novel, effective mRNA vaccines were developed in less than 1 year and awarded emergency use authorization (EUA) in the United States. EUA authority allows the Food and Drug Administration (FDA) to approve medical products in order to diagnose, treat, or prevent life-threatening diseases during times or circumstances when no viable alternatives exist during public health emergencies. The Secretary of the US State and Human Services declared the COVID-19 public health emergency on January 31, 2020.

The first batch of Moderna's mRNA-1273 was released for Phase 1 study in the United States in February 2020. This vaccine targeted the receptor binding domain of the Spike protein subunit and was encapsulated in lipid nanoparticles. The cytosolic delivery and temporary presence of mRNA in the cytoplasm improved the safety profile of these nucleic acid vaccines. To assess safety, Pfizer and BioNTech launched phases 1 and 2 clinical trials with the mRNA vaccine during the subsequent months. The primary goal for the phase 2 trial was to achieve *in vivo* protein translation and induction of humoral immune responses upon intramuscular injection. When phases 1 and 2 were successfully completed, the FDA approved phase 3 in conjunction with EUA-authorized vaccine use [18, 19]. While perhaps not expected, it was quickly realized that mRNA vaccines were neither 100% effective nor 100% safe. Subsequent infections, caused by SARS-CoV-2 lineage Omicron, were accompanied by numerous vaccine breakthroughs. Fortunately, novel variants have been associated with milder diseases demonstrated by lower rates of morbidity and mortality. Investigational findings after showed that the anti-SARS-CoV-2 neutralizing antibody titers declined about six months after initial vaccination, which supported recommendations for a booster vaccination. Booster vaccines, like the initial vaccinations, were neither fully effective nor safe. Adverse reactions reported among vaccinations include myocarditis, thyroiditis, systemic vasculitis, and vaccine-associated pulmonary immunopathology [20–22].

Another new type of vaccine known as self-amplifying RNAs (SARNA) has recently completed pre-clinical studies [23]. SARNAs are synthetic RNAs capable of *in vivo* self-amplification for 40 to 60 rounds, a feature supported by their delivery with an alphavirus replicase gene that encodes an RNA-dependent RNA polymerase (RdRP) [24]. SARNA and RdRP can be synthesized as two different amplicons or formulated as one *cis*-amplicon sequence in the lipid nanoparticle cargo. The ability to undergo several rounds of replication *in vivo* makes the SARNA vaccine more cost-effective than mRNA. However, SARNAs constructs are larger than those of mRNAs, and that feature may adversely alter the effectiveness of delivery. This concern is currently being addressed in phase 1, open-labeled trial NCT05155982. The study design includes 8 arms in which participants are administered 25 to 50 micrograms of SARNA-based COVAC-1 vaccine or placebo [25]. Two other SARNA vaccine candidates entered phase 2 clinical trials in the United Kingdom (randomized-controlled ISRCTN17072692, and NCT04758962) to assess the safety and measure the titers of vaccine-induced serum (IgG type) binding antibody responses to the SARS-CoV-2 S glycoprotein [26, 27].

Interestingly, both types of vaccines (mRNA and SARNA) elicited not only antigen-specific antibody responses but also antigen-specific T-cell responses, while SARNA elicited a stronger response at lower doses in mice [28]. A novel self-amplifying messenger ribonucleic acid (SAmRNA) trial by Gritstone Bio, Inc. is recruiting HIV-infected individuals to assess vaccine safety. Vaccines use a codon-optimized cassette covering multiple epitopes from the SARS-CoV-2 spike and non-spike proteins and additional T cell epitopes (NCT05435027) [29].

In lieu of fast changes in SARS-CoV-2 lineages, a variety of RNA vaccine reformulations may be needed to maintain emergency preparedness for future responses. A new development has recently been announced: the FDA granted emergency use authorizations (EUAs) to new formulations of both Pfizer-BioNTech and the Moderna COVID-19 vaccines. Authorized bivalent formulas, so-called "updated boosters" now contain two mRNA components of SARS-CoV-2 virus: the first is the originally approved (against lineage A of SARS-CoV-2); the second is common between the BA.4 and BA.5 lineages of the Omicron variant of SARS-CoV-2 [30]. Ongoing genomic surveillance of SARS-CoV-2 variants of concern allows real-time detection of immune escape mutations and prediction of vaccine breakthroughs [16].

3. Vaccines against human immunodeficiency virus infection

Human Immunodeficiency Virus (HIV) continues to present a serious global health threat since it made its appearance as a human-to-human transmitted pathogen, causing acquired immunodeficiency syndrome (AIDS) [31]. HIV1 and HIV2 are single-stranded positive-sense RNA retroviruses that are subdivided into several distinct classes [16]. HIV vaccine designs appeared to be the most challenging among other RNA viruses, due to frequent mutations, integration into the human genome, and a long latency phase [16].

There were more than two dozen HIV clinical trials conducted since early the 1990s that tested plasmid DNA-based protein vaccines as prophylactic or therapeutic types. These clinical trials were successful in phases 1 and 2; however, they were stopped in mid-phase 3 for futility by Data Safety Monitoring Board (DSMB) [32]. DSMB data analysis study did not find a statistically significant decrease in the number of HIV infections in the vaccine compared to placebo groups [33]. It was determined that the elicitation of humoral immune response was not robust enough to prevent infection or stop the progression of AIDS [34].

Numerous recombinant DNA-based vaccines were tested against several immunogenic epitopes (e.g., HIV protease, gag, env, gp120/140, or reverse transcriptase proteins) [35–37]. Various routes of administration (mucosal, intradermal, intravenous, and intramuscular) were also tested [33], and intramuscular injections were found to be the most efficacious. Recombinant DNA-based HIV vaccines generated only modest HIV-specific T cell and humoral responses, and that was insufficient for protection [38, 39]. Research studies on therapeutic vaccines continue to be performed. The randomized, double-blind, placebo-controlled dose escalation trimer-4571 vaccine (against HIV envelope protein) in combination with alum adjuvant has been the most widely reviewed study [40].

Ongoing challenges in HIV vaccine development include frequent HIV virus mutations that can lead to a glycan shield that covers HIV immunogens prompting Scripps Research Institute and Moderna's team to design a trimeric mRNA vaccine against HIV/AIDS (NCT05217641). The study focuses on recruiting participants who will be immunized with various doses of a modified trimeric vaccine composed of mRNA against glycan shields, CD4KO, and gp151 [41, 42]. Another phase 1 trial (NCT05001373) evaluates the safety and immunogenicity of two mRNA vaccine types after intraperitoneal administration. That trial aims to detect antigen-specific epitopes on CD4+ T cells and B cells in peripheral blood and in the germinal centers of secondary lymphoid organs [43]. Both mRNA trials are designed to induce Broadly Neutralizing Antibodies (BNAbs) in HIV-uninfected adult participants [44].

Different studies' interpretations differ in opinion with respect to the benefits and ability of HIV vaccines in activating endogenous single/double-stranded RNA sensing molecular machinery [45, 46]. It has been shown that in patients with advanced HIV infection, the immune system functions in absence of a sufficient amount of cytokine interferon-gamma (IFNG), and the innate immune branch often exhibits exaggerated responses to antigenic stimulation [47, 48]. Clinically, such responses are seen in the form of immune reconstitution inflammatory syndrome (IRIS) toward persistent antigens from previously treated opportunistic infections [49, 50]. Because they are not specific, vaccines can cause exaggerated systemic innate immune responses that lead to adverse events in immunocompromised individuals through activation and overexpression of TLR 3,7,8 OAS1–11, MDA1–5, IRFs, IFI, type 1 interferon genes, and the components of the inflammasome [49–52]. Adequate levels of interferon-gamma are necessary to establish appropriate virus-specific cytotoxic lymphocyte responses after therapeutic vaccination [53, 54]. IFNG is primarily produced by mature CD4+ T helper cells, which demonstrates low counts in immunocompromised individuals [55]. Therefore, the response to vaccines that are supplemented with adjuvants can be unpredictable [55]. The NCT04177355 trial evaluates the safety and immunogenicity of the HIV1BG505SOSIP.664gp140 vaccine in healthy HIV-uninfected adults [56]. This vaccine is formulated in combination with TLR-7/8 agonists and alum adjuvant (inflammasome activator). Yet, the safety of the vaccine/agonist/adjuvant combinations is needed to be assessed in HIV-infected populations to demonstrate clinical utility.

The major disadvantage of *in vitro*-transcribed mRNA vaccines is the unstable nature of mRNA molecules which often leads to their degradation by intracellular enzymes ribonucleases (i.e., RNases) [57]. mRNA synthesized by *in vitro* preparations can generate a small percentage of double-stranded RNAs that trigger activation of pathogen-associated molecular pathways through induction of interferon response genes [11]. The end result is enhanced mRNA degradation and a decrease in antigen production [58]. This is the main reason why formulations that used naked mRNA were unsuccessful [55]. Additionally, the poor thermal stability of mRNA vaccines requires product refrigeration. Those logistical constraints can present with problems during the distribution of the product in resource-limited settings (**Table 1**).

Perhaps the vaccine formulation for prophylactic and therapeutic vaccination should be different as the goal of the latter is to prevent the infection via various routes, and the former is to control localized viral replication. Researchers remain hopeful that novel self-amplifying vaccine formulations will lead to effective mosaic anti-HIV vaccines that completely interrupt HIV transmission and prevent subsequent infection [11].

4. Influenza vaccines

Influenza viruses are negative-sense, enveloped, segmented single-stranded RNA viruses that are encapsidated by nucleoproteins [59]. Several approved influenza vaccines were developed through recombinant DNA technology. These vaccines are reformulated annually based on predicted hemagglutinin (HA) and neuraminidase (NA) gene mutations (drifts and shifts). Constructs are delivered with baculovirus vector into host cells and recombinant HA protein is manufactured as a vaccine [60]. Influenza type A HA is subdivided into heterosubtypic groups 1–18, and influenza B - into 9. Several vaccines from four main biopharmaceutical companies are cleared by FDA: Fluad Quadrivalent and Flucelvax Quadrivalent are inactivated vaccines (Seqirus), Fluarix Quadrivalent is also inactivated vaccine (GlaxoSmithKline

Biologicals), and Flublok Quadrivalent is a recombinant influenza vaccine (Protein Sciences Corporation).

Many more vaccines are in clinical trials measuring primary outcomes as the humoral immune protection against surface viral proteins of seasonal avian influenza strains/subtypes/groups [61].

Pre-clinical trials in 2009 tested DNA plasmid carriers that contained genes that express viral antigens [62]. The poor success of those DNA-based vaccines may have been due to inefficient delivery of nucleic acids to cell nuclei and subsequent failure of DNA amplification in those target cells. Replication-competent and non-replicating adenoviral vectors offered improved delivery platform for influenza vaccines and achievement of systemic and mucosal immunity [63, 64]. As for mRNA and self-replicating RNA vaccines, they are delivered into the cytoplasm of cells but do not require nuclear translocation [65]. When formulated into lipid nanoparticles, RNA vaccines are efficiently delivered into the cytoplasm.

The first human clinical influenza mRNA-based trial employed a non-chemically modified mRNA construct, where the intent was to induce antibody titers against multivalent targets of four different influenza strains [66]. ModernaTX, Inc. is in recruitment of participants to evaluate modified mRNA-1647 to assess sero-responses in comparison to adjuvanted inactivated, quadrivalent seasonal influenza vaccine [67]. Subsequent vaccine goals include the development of multiplexed vaccine candidates into one dose of SARS-CoV-2, respiratory syncytial virus, or other formulations. Pfizer led a clinical research study of six SARNAs preparations of hemagglutinin antigens that were designed against four influenza strains. The proportion of participants achieving hemagglutination inhibition titers for each strain had been measured in the context of secondary outcomes [68]. It remains to be established if RNA vaccines will provide long-term protection with an established frequency of booster administration.

The global initiative on sharing all influenza data (GISAID) established the first repository of shared influenza sequences in 2006. GISAID has been instrumental for WHO and National Influenza Centers in providing bi-annual recommendations on strain selection for influenza vaccines [69]. Moreover, GISAID provides bioinformatics workspaces like FluSurver to allow scientists to perform assessments of the positions of novel mutations, changes in antigenic properties or glycosylation, and even predict viral susceptibility to drugs [70]. The geographical assessment of currently circulating strains can be visualized, as well as the phylogeny of current clades and the molecular clock of viral evolution (**Figure 1a, b**) [70]. For present strains of epidemiological importance, the frequency projections of currently circulating A/H3N2 clades are calculated from a fitness model based on the current frequency and estimated fitness [71]. The strain fitness is estimated by a combination of antigenic novelty and mutational load. Antigenic novelty is based on inferred measurements of antigenic advance from hemagglutination inhibition assay [71]. Mutational load is calculated by the number of amino acid mutations each strain carries at putative non-epitope sites relative to its most recent ancestor from the previous season (see **Figure 1** and pull down menus under Black "X" in: https://www.gisaid.org/epiflu-applications/influenza-genomic-epidemiology/).

a. Real-time tracking of influenza A/H1N1 evolution.

Top left: Rectangular phylogenetic tree of influenza A/H1N1 shows color-coded clades (by genotype). The black line represents linear regression of divergence. Black **X** represents an interactive drop-down menu with information about the date,

specific nucleotides changes, amino acid changes, calculated divergence score, and potential vaccine selection. **Top right**: geographical distribution of A/H1N1 clades.

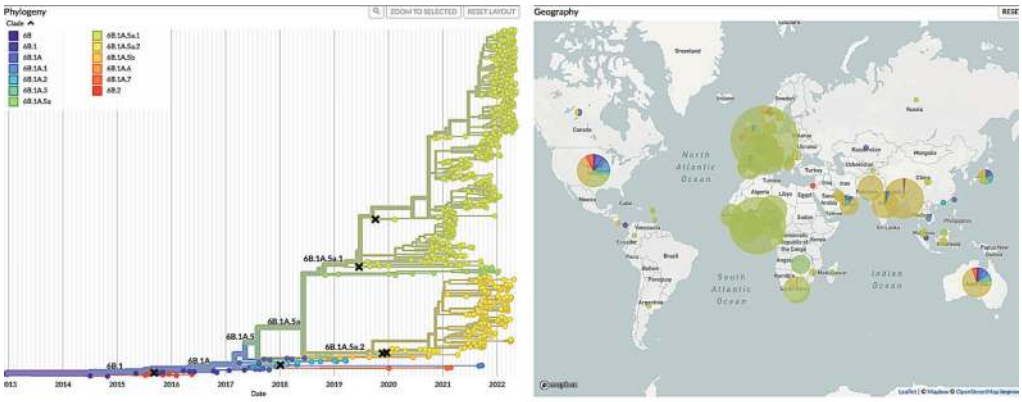
Bottom: Molecular clock representation of clades divergence since 2013, with an estimated rate of $3.7x10^{-3}$ substitutions per site per year.

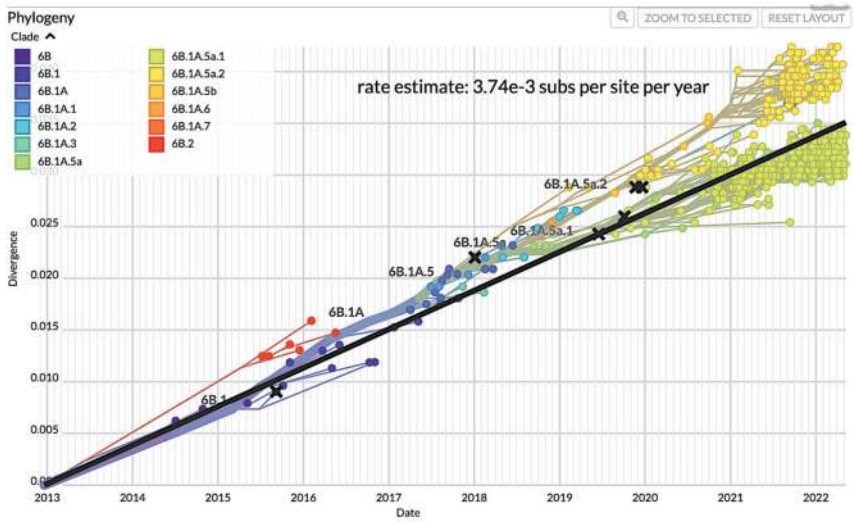
b. Real-time tracking of influenza A/H3N2 evolution.

Top left: Rectangular phylogenetic tree of influenza A/H3N2 shows color-coded clades (by genotype). The black line represents linear regression of divergence. Black **X** represents interactive drop-down menu with information about the date, specific nucleotides changes, amino acid changes, calculated divergence score, and potential vaccine selection. **Top right**: geographical distribution of A/H3N2 clades.

Bottom: Molecular clock representation of clades divergence since 2013, with an estimated rate of $4.06x10^{-3}$ substitutions per site per year.

As more and more public health laboratories upload the sequencing results to GISAID, the global real-time tracking of influenza became possible. As a result, RNA vaccines can be re-designed just in a few days, and produced in just a few weeks.





(a)

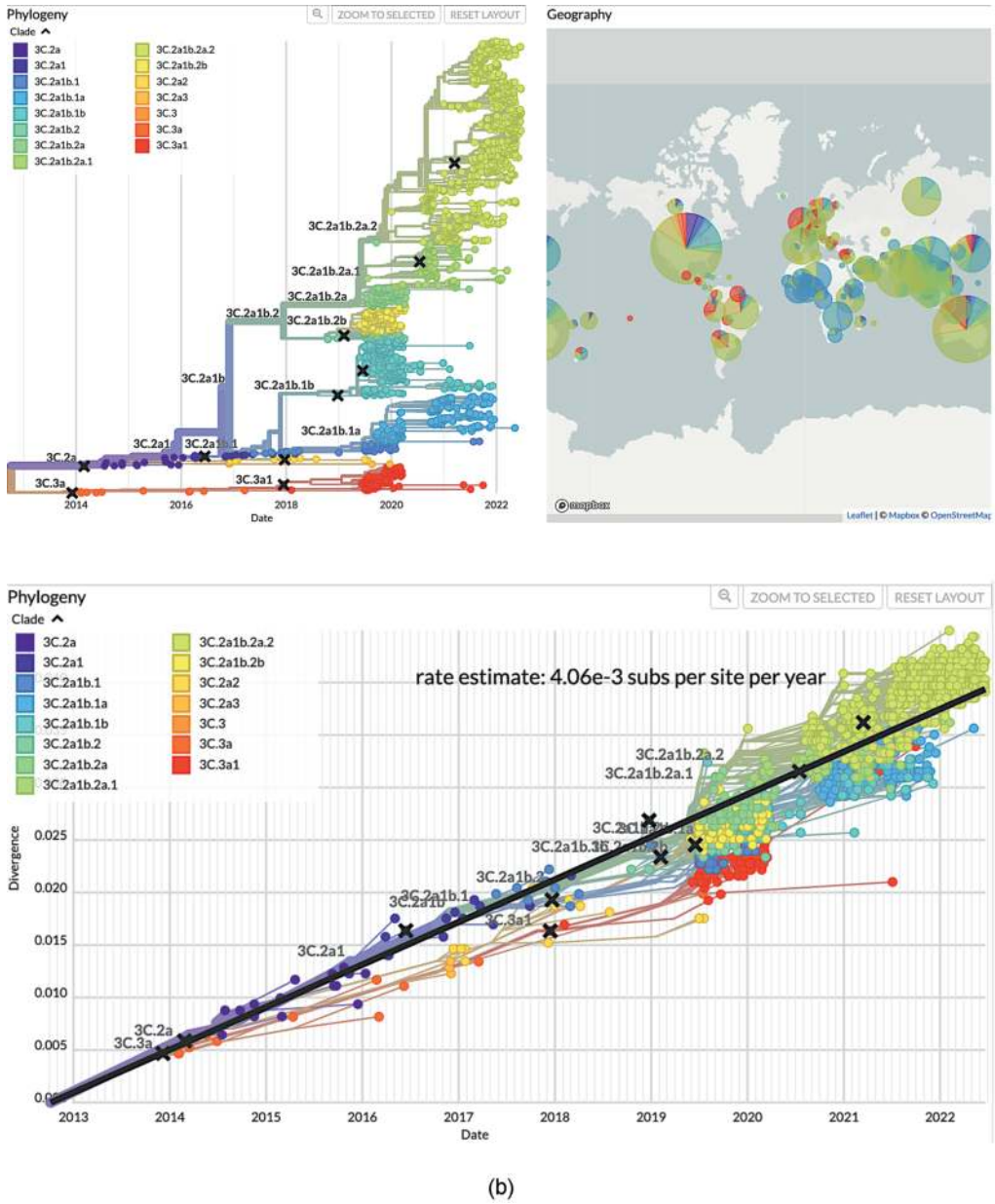


Figure 1.
Visualization of influenza phylogeny, geographical distribution, and divergence of clades.

5. Respiratory syncytial virus vaccines

The human respiratory syncytial virus (RSV) is a negative-strand RNA, enveloped, non-segmented virus of the order Mononegavirales, genus Pneumovirus and family Paramyxoviridae [72]. The human respiratory syncytial virus represents a significant public health burden in two main populations that includes young children and older adults. Previously, only passive immuno-prophylaxis with neutralizing antibodies was considered minimally protective against severe disease. The RSV-live attenuated vaccines did not prevent subsequent RSV disease [73]. Moreover, whole-virus inactivated vaccines were associated with enhanced

respiratory disease in the lungs, presenting with monocytic eosinophilic pulmonary inflammation on histologic evaluation [74].

Despite the diversity of antigens, human RSV infection produces some serotypes that can be divided into two antigenic subgroups, with the RSV A being more diverse than B subgroup [75]. Elucidation of the atomic structure in conjunction with the identification of the fusion (F) glycoprotein was of critical importance for vaccine development and clinical trials. Unfortunately, these protein vaccines did not meet clinical expectations in robustness for preventing subsequent disease progression [76]. The development of a new generation of RSV-F protein, stabilized in the perfusion conformation, allowed GlaxoSmithKline and Medimmune to launch four phase-3 clinical trials testing pregnant mothers and infants [77]. Within 6 months after immunization, these vaccines were found to be protective against severe lower respiratory tract infections in infants and mothers [77].

An RSV-targeting recombinant virus-like particle vaccine trial (NCT04519073) conducted in Belgium demonstrated promising preliminary results of increased titers of micro-neutralizing antibodies against RSV A and B [78]. Additionally, a Phase 3 randomized, observer-blinded study evaluated the safety, tolerability, and immunogenicity of the mRNA-1345 (RSVictory) vaccine targeting RSV (NCT05127434) [79]. The vaccine was successfully tested in adults ≥50 years of age when administered alone or when co-administered with inactivated quadrivalent influenza vaccine (Afluria®) [80]. Outcome data will evaluate the percent of participants with sero-responses, who are defined by a ≥ 4-fold increase in RSV-A neutralizing antibody titer between one and six months after vaccination. This study has been conducted by ModernaTM, and the main outcome goal is to achieve long-term immunity to both infections. These vaccines are yet to show reliable prophylactic and therapeutic efficacy.

6. Combination vaccines against parainfluenza type 3 virus (PIV3) and human Metapneumovirus (HMPV)

Like RSV, PIV is also a negative-strand RNA virus from the Paramyxoviridae family and Paramyxovirinae subfamily. Bi-directional high-throughput RNA sequencing technology elucidated several types of parainfluenzas (1–5), with PIV3 as most predominant [81]. Another, more recently identified member of the order Mononegavirales, family Paramyxoviridae, subfamily Pneumovirinae is a human metapneumovirus (HMPV) [82]. HMPV became a part of infectious disease genomic surveillance after development of whole-genome tiled amplicon sequencing technology. This methodology allowed the identification of two major phylogenetic subtypes of HMPV, each containing two sublineages (A1, A2, B1, B2) [83, 84]. The use of this new knowledge in vaccine manufacturing led to multi-viral vaccine research and development.

Human subfamilies (Paramyxovirinae and Pneumovirinae) are known to cause hospital-acquired infections, infections in young and elderly adults, and pneumonia in immunocompromised individuals [85]. Antiviral medication or vaccinations against these globally spread viral infections, including multiple re-infections that occur throughout life, did not exist until recently. Two clinical trials conducted by Moderna TX are recruiting participants to assess the safety, reactogenicity, and Immunogenicity of the mRNA-1653 vaccine. This is a combined design against PIV and HMPV, which will be tested in healthy adults (NCT03392389) and children 12

to 59 months of age (NCT04144348) [86, 87]. If these trials are successful, other Paramyxoviridae infections can be targeted with the same polyvalent vaccine design.

7. Chikungunya and dengue viruses' vaccine trials

Chikungunya, Dengue, and Zika viruses are transmitted by mosquitos of the *Aedes* genus. These infections had little attention in Western World prior to travel-related epidemics spreading from tropical countries of equatorial Africa, South America, India, or the Polynesian region.

The mosquito-borne Chikungunya fever is caused by RNA arbovirus that belongs to the alphavirus genus of the family Togaviridae. Patients usually present with relatively mild disease; however, debilitating chronic arthritis has been reported in some patients who recover from the infection [88].

Phase 1 and 2 clinical trials of Chikungunya virus-like recombinant protein vaccines (aluminum hydroxide-adjuvanted) have been completed [89–91]. One study, conducted by Emergent BioSolutions (PXVX0317) reported promising results related to satisfactory safety outcomes and sufficient neutralizing antibody titer responses (NCT0348369; NCT03992872) [92]. Phase 3 was initiated in August 2022, and the focus was to test PXVX031 in adults ≥65 years of age [93].

DNA-based vaccines have been designed and tested (based on mumps and rubella viral vectors), but those vaccines failed to demonstrate long-term immunogenicity [94]. Two years before the COVID-19 pandemic, Moderna launched the first Phase 1 trial of the mRNA-1388 vaccine and subsequently the second trial of mRNA-1944 [95, 96]. Although these trials were interrupted by the COVID-19 pandemic, preliminary results showed favorable tolerability of mRNAs in healthy volunteers. mRNA-1388 is a prophylactic vaccine that consists of a single mRNA encoding the full native structural polyprotein (C-E3-E2-6k-E1 peptides). This polyprotein is naturally processed into C and E structural viral proteins that assemble into viral-like particles before being released from cells [97]. mRNA-1944 encodes the heavy and light chains of the Chikungunya antibody formulated in proprietary lipid nanoparticles and can be used as biotherapeutics [97]. More information about these vaccines and trial designs can be found in the archives of the United States Security and Exchange Commission reports [97].

Sequencing of the full 10 kB Chikungunya virus genome is important for epidemiological investigation and genomic surveillance; however, few Public Health Laboratories are pursuing these investigations [98]. Understanding genetic diversity and rates of *de novo* mutations will allow estimates of higher and lower fitness of circulating variants (those that have sufficient fitness to cause epidemics and those that can be naturally purified during transmission bottlenecks) [98, 99]. Similar analogies can be made with the 10.7-kB ribonucleic acid virus Dengue. The incidence of Dengue disease is increasing globally and is attributed to the exportation of the disease from tropical countries via tourism and inefficient mosquito controls. Significant concerns about the spread of this emerging disease, as well as potential solutions are elucidated in comprehensive reviews on dengue vaccine development [100–103]. The development of effective vaccines and mandatory vaccination of international travelers has already proven to be the most effective way in preventing the transmission of vector-borne diseases like yellow fever [104]. Thus, vaccination certificates may be required in the future for travelers as a condition of entry to specific countries, and this would facilitate safer international travel.

8. Zika virus vaccines

Zika is an eleven-kilobases-long single-stranded positive-sense RNA virus. Zika's genome encodes for one open-reading frame, which is translated into 20 functional proteins. There are seven nonstructural and 13 structural proteins, including pre-membrane, envelope, and capsid. Like most flaviviruses, Zika is transmitted by mosquitos. Intercontinental travel has facilitated Zika virus spread out of Africa, as well as it is being spread from human to human via sexual contact. Pregnancy, in conjunction with gestational Zika infection, is strongly associated with microcephaly and other congenital abnormalities in newborns. Preventing congenital Zika infections has been the subject of vaccine research in animal models [105].

Pre-clinical Zika studies with the modified-nucleoside mRNA vaccines have been designed to target the envelope and pre-membrane proteins [106]. Recently initiated Moderna's phase 1 and 2 human clinical trials have shown a near 90% seroconversion rate in adult participants after booster vaccination [107]. Phase 2 of this study is expected to be completed in 2024, with the primary outcome measure focusing on systemic reactogenicity while reducing adverse events, and achieving measurable serum-neutralizing antibodies against Zika virus [108].

Various formulations of SARNA vaccine studies in animal models have been compared with the efficacy of DNA and mRNA vaccines [109]. SARNAs have shown to be more effective in smaller doses compared to the other vaccines. One reason is attributed to the double-stranded SARNA being able to induce innate immune interferon type 1/2 responses, which serve as an endogenous adjuvant. This has been proposed to eliminate the administration of a second dose that is required for mRNA vaccines. In comparison, the second and third exposures to DNA vaccines elicit host immune response against the vectors that contain the vaccines' DNA (**Table 1**). Conversely, this is not known side-effect for mRNA or SARNAs because the majority of those vaccines are encapsulated into non-immunogenic neutral or charged lipid nanoparticles [110]. Seventy other DNA, RNA, and conventional Zika-vaccine studies are currently registered with clinicaltrials.org in the assessment of safety and preliminary efficacy (phases 1 and 2). Future studies are required to demonstrate which vaccine could be more robust, providing longer-lasting immunity against Zika infection.

9. Rabies virus vaccines

The rabies virus is an RNA virus transmitted through mammalian vectors. The genome of the rabies virus encodes 5 proteins (N, P, M, G, and L), and the sequencing of the single-stranded RNA genome classified the viral structure within the Lyssavirus family. Due to the neurotropic properties of the virus and a lack of effective treatment, rabies exposure, if not immediately addressed, is lethal in humans and other mammals within three weeks from infection [111]. Furthermore, vaccine portfolios have not significantly advanced, and that may be in part due to the endemic and sporadic nature of the disease. While DNA vaccines against rabies have been developed, they have proven to be poorly immunogenic in humans [112]. Thus, conventional types of inactivated rabies virus vaccines (RabAvert, Rabipur, Imovax, etc.) are most common for vaccination of individuals in specific professions who are at high risk of rabies exposure [111].

mRNA rabies vaccines CureVac and CV7201 entered phase 1 clinical trial in order to assess their safety and reactogenicity [113, 114]. These mRNA vaccines also encode rabies virus glycoprotein G and have shown promise to be safe and effective

as a pre- and early post-exposure prophylactic vaccine for humans (NCT03713086; NCT02241135). Several novel self-amplifying RNA (SARNA) have been tested in combination with diverse nanoparticle formulations. Preclinical studies of proprietary cationic nanoemulsion-formulated glycoprotein G-encoding self-amplifying RNA (RG-SAM [CNE]) showed that the vaccine was well tolerated following multiple intramuscular injections in animals [112]. The rabies SARNA is a virus glycoprotein G RNA that showed promising results in phase 1 clinical studies through protecting neutralizing antibody responses (IgM and IgG) against viral glycoprotein. SARNA vaccines are well tolerated and cause mild side effects comparable to those in conventional vaccines trial (GlaxoSmithKline, NCT04062669) [115]. Establishing clinical efficacy is the next step for this type of SARNA vaccines, as they hold great promise to become valuable pre-exposure prophylactics. SARNA technology offers distinct advantages because they are highly amenable to mass- *in vitro*- transcription in GMP-level facilities.

10. Ebola virus disease vaccines

Ebola is a single-stranded, negative-sense RNA virus that causes the Ebola virus disease. Ebola is subdivided into five immunologically different subspecies based on surface envelope glycoprotein spikes and the virion proteins of nucleocapsid [116]. UCSC Genome Browser and GISAID contain the most comprehensive genomic information on Ebola subspecies sequence variations and phylogeography [117, 118].

More than four dozen vaccine trials were initiated after the Ebola outbreak of 2014 [119]. At least half of them were DNA-based transgenes, delivered with non-replicative viral vectors like Venezuelan equine encephalitis virus, human replication-defective adenovirus, recombinant chimpanzee adenovirus type 3, modified vaccinia strain Ankara, or Kunjin replicon virus-like particle vaccine. The other vaccine trials utilized replicative vectors, including human parainfluenza virus type 3-based vaccine, recombinant vesicular stomatitis virus-based vaccine (rVSV-EBOV), recombinant rabies virus, or recombinant cytomegalovirus. All of these vaccines were designed against envelope spike glycoproteins [120].

The first Ebola vaccine (rVSV-ZEBOV, Merk) was approved in the United States in 2019 and had been used in the 2018 Ebola epidemic in the Democratic Republic of the Congo as part of clinical trials. Subsequently, it had been used under criteria for compassionate use that included children and pregnant women [121]. rVSV-ZEBOV showed the ability to generate protective immunity in a form of anti-glycoprotein immunoglobulin G antibody titers that lasted at least 2 years of observation [122]. Several other DNA-based vaccines are being tested by Inovio Pharmaceuticals via routes of prime intramuscular injection with subsequent boost electroporation [123]. Electroporation is less invasive; however, it requires a specialized medical device to deliver brief electrical pulses during intradermal gene transfer [124]. Challenges remain with DNA vaccine platforms. These challenges include immune response to viral vectors after booster vaccination, safety concerns about replication-capable viral cargo (e.g., human genome integration), and slow optimization of antigen sequences to make multivalent vaccines against all sub-strains of the Ebola virus (**Table 1**).

mRNA vaccines can respond to these challenges quicker because the manufacturing process and formulations allow multi-sequence delivery and, therefore, avoids safety issues associated with booster immunization. The lipid nanoparticle (LNP) encapsulation technology and the design of glycoprotein mRNA with post-transcriptional modifications have the potential to exhibit durable immune responses

in pre-clinical and phase 1 studies [125]. Due to lower doses requirement, and lower cost, SARNA vaccines may have a higher potential to rapidly respond to future Ebola outbreaks. Like DNA vaccines, SARNAs are stable and can be delivered intradermally via electroporation. Non-human primate experiments showed that SARNA induces sufficient protective responses against Ebola after a single primed immunization [126]. Future expectations are that SARNA vaccines will be successfully delivered with electroporation (intradermal) and will not require boost immunization.

11. Future directions

Epidemics caused by genetically recombined or mutated RNA viruses will continue to evolve and pose health threats locally and globally. Because of this, RNA vaccinology will continue to strive to develop new manufacturing processes to improve RNA transcript stability by incorporating modified synthetic nucleotides during *in vitro* transcription, optimizing delivery formulations, and adjusting the adjuvants' potency. Additionally, next-generation viral genotyping conducted by CDC and Public Health Laboratories will provide real-time pathogen surveillance data for rapid modifications and manufacturing of RNA vaccines. Mosaic vaccines against multiple viral strains or multi-pathogen vaccines are a goal that needs to be achieved to prevent pandemics, epidemics, and endemic infections.

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Conflict of interest

None declared.

Abbreviations

GISAID	global initiative on sharing all influenza data
TLR	Toll-Like Receptor
IRF	Interferon regulatory factor
IFI	Interferon alpha inducible protein
OAS	2′-5′-Oligoadenylate synthetases
GMP	Good manufacturing practice
MDA5	melanoma differentiation-associated protein 5
UCSC	University of California Santa Cruz

Author details

Irina Vlasova-St. Louis[1,2]* and Jude Abadie[1,3]

1 El Paso Public Health Laboratory, USA

2 Johns Hopkins University Advanced Academic Program Individualized Genomics and Health, USA

3 Texas Tech University Health Sciences Center El Paso, USA

*Address all correspondence to: stlouis.irina@gmail.com

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