Vaccine development for zoonotic viral diseases caused by positive-sense single-stranded RNA viruses belonging to the *Coronaviridae* and *Togaviridae* families (Review)

SEYED SAJJAD BABAEIMARZANGOU[1], HIMASADAT ZAKER[2], EHSAN SOLEIMANNEZHADBARI[3], NAEIMEH SHAMSI GAMCHI[2], MASOUD KAZEMINIA[4], SHIMA TARIGHI[5], HOMAYON SEYEDIAN[6], ARISTIDIS TSATSAKIS[7], DEMETRIOS A. SPANDIDOS[8] and DENISA MARGINA[9]

[1]Division of Poultry Health and Diseases, Department of Clinical Sciences, Faculty of Veterinary Medicine, Urmia University, Urmia 5756151818; [2]Histology and Microscopic Analysis Division, RASTA Specialized Research Institute (RSRI), West Azerbaijan Science and Technology Park (WASTP), Urmia 5756115322; [3]Young Researchers Club, Urmia Branch, Islamic Azad University, Urmia 5716963896; [4]Department of Food Hygiene and Quality Control, Faculty of Veterinary Medicine, University of Tehran, Tehran 1417935840; [5]Veterinary Office of West Azerbaijan Province, Urmia 5717617695; [6]Faculty of Veterinary Medicine, Urmia University, Urmia 5756151818, Iran; [7]Laboratory of Toxicology, Department of Medicine, University of Crete, 71307 Heraklion; [8]Laboratory of Clinical Virology, School of Medicine, University of Crete, 71003 Heraklion, Greece; [9]Department of Biochemistry, Faculty of Pharmacy, 'Carol Davila' University of Medicine and Pharmacy, 020956 Bucharest, Romania

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Abstract. Outbreaks of zoonotic viral diseases pose a severe threat to public health and economies worldwide, with this currently being more prominent than it previously was human history. These emergency zoonotic diseases that originated and transmitted from vertebrates to humans have been estimated to account for approximately one billion cases of illness and have caused millions of deaths worldwide annually. The recent emergence of severe acute respiratory syndrome coronavirus-2 (coronavirus disease 2019) is an excellent example of the unpredictable public health threat causing a pandemic. The present review summarizes the literature data regarding the main vaccine developments in human clinical phase I, II and III trials against the zoonotic positive-sense single-stranded RNA viruses belonging to the Coronavirus and Alphavirus genera, including severe acute respiratory syndrome, Middle east respiratory syndrome, Venezuelan equine encephalitis virus, Semliki Forest virus, Ross River virus, Chikungunya virus and O'nyong-nyong virus. That there are neither vaccines nor effective antiviral drugs available against most of these viruses is undeniable. Therefore, new explosive outbreaks of these zoonotic viruses may surely be expected. The present comprehensive review provides an update on the status of vaccine development in different clinical trials against these viruses, as well as an overview of the present results of these trials.

Correspondence to: Professor Denisa Margina, Department of Biochemistry, Faculty of Pharmacy, 'Carol Davila' University of Medicine and Pharmacy, 6 Traian Vuia Street, 020956 Bucharest, Romania
E-mail: denisa.margina@umfcd.ro

Professor Aristidis Tsatsakis, Laboratory of Toxicology, Department of Medicine, University of Crete, Nisirou 11, 71307 Heraklion, Greece
E-mail: tsatsaka@uoc.gr

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Contents

1. Introduction

Coronaviridae, a family of positive-strand RNA viruses, are human pathogens that can cause a worldwide epidemic (1). Several fatal and novel strains of this family have been spread into the human population globally over the past decades.

The severe acute respiratory syndrome (SARS)-coronavirus (CoV) was the first lethal virus that infected 8,096 cases and caused 774 deaths in 2003 (2). In 2012, Middle East respiratory syndrome (MERS)-CoV led to an official 2,442 cases and led to the death of 842 individuals (3,4). Finally, SARS-CoV-2 was first reported in China and then caused the current SARS-CoV-2 pandemic in 2019, which markedly altered human life (5).

Alphaviruses, transmitted innately by mosquitoes, are other positive-strand RNA viruses belonging to the family *Togaviridae* that induce debilitating symptoms in humans. The distribution of mosquito-borne alphaviruses is substantially restricted in areas where vector hosts and reservoirs are present. However, the 2004-2019 Chikungunya virus (CHIKV) outbreak revealed the potency of this family to also affect non-endemic regions (6). The two recent worldwide outbreaks of CHIKV affected approximately eight million individuals in almost 50 countries worldwide (7).

While the world is still being affected by the recent SARS-CoV-2 pandemic, the crucial need for novel antiviral platform technology research in vaccine development is urgently required. As regards the unpredictable nature of viral epidemics, the Coalition for Epidemic Preparedness Innovations accelerates the expansion of various vaccine platforms against emerging infectious diseases, such as MERS-CoV and CHIKV before their epidemics appear (8).

Accordingly, the present comprehensive review aimed to provide an in-depth insight into the various vaccine technologies against the most significant zoonotic viral infection of Coronaviruses and Alphaviruses in different phases of human clinical trials.

2. Middle East respiratory syndrome coronavirus

MERS-CoV first emerged in Saudi Arabia in 2012 (9). It expanded to 27 other countries, and according to the World Health Organization, as of September, 2019, a 34.40% mortality rate was estimated (10). Among the genome encoding four structural proteins [envelope (E), membrane (M), spike (S), nucleocapsid (N)], the S protein, as a receptor identification and viral entrance into host cells, is the primitive target for effective immune response induction against MERS-CoV infection (11). During the period of infection, host furin protease splits the S protein into two subunits known as the receptor-binding subunit S1 and the membrane-fusion subunit S2 (12). MERS-CoV differs from SARS-CoV and SARS-CoV-2, as it is in lineage C, whilst SARS-CoV and SARS-CoV-2 are in lineage B, of β-CoV (13). MERS-CoV can identify dipeptidyl peptidase 4 (hDPP4, CD26) as its receptor (14), while SARS-CoV-2 and SARS-CoV enter host cells through their receptor, angiotensin-converting enzyme 2 (ACE2) (15). Evidence has indicated that similar to SARS-CoV, MERS-CoV has its origin in bats, as they are the natural reservoir (16). Moreover, bats and European hedgehogs are the other natural host (17). Dromedary camels have been recognized as an intermediate host for MERS (18), so the transmission possibility of MERS-CoV from camels to humans is well established, as well as human-to-human transmission (Fig. 1). It leads to clinical symptoms like fever, diarrhea, and mild to severe respiratory symptoms (Fig. 1). Various analyses have been conducted based on various vaccine candidates; the present review provides a summary of current MERS vaccines under preclinical development (19).

Preclinical MERS-CoV vaccines based on the viral S structural protein. S-protein-targeted vaccines are under preclinical development with some studies being performed on different animal models (11,20).

As regards the generation of MERS-CoV DNA vaccines, multiple designs of DNA vaccines encoding the MERS-CoV S protein or its S1 fragment have been tested. GLS-5300, a DNA vaccine expressing a full-length MERS CoV S-glycoprotein antigen has revealed a high immunogenic effect in mice, camels and non-human primates; thus, in 2016, a phase I clinical trial was commenced to examine the efficacy in humans and accomplished (NCT02670187, NCT03721718) (Table I) (21,22).

In addition, various iral-vectored vaccine developments have commenced: Various viral vectors have been formulated dependent on MERS-CoV S and/or its fragments to examine the immunogenicity against MERS-CoV infection in animal models, such as mice, camels and non-human primates. The viral-vectored vaccines include the modified vaccinia virus Ankara (MVA), adenovirus, vesicular stomatitis virus (VSV) and measles virus (MV) (23-25). MVA encodes full-length MERS-CoV S and was tested in dromedary camels with the result of inducing neutralizing antibody (NAb) (26). Extensive investigations have performed based on different recombinant adenovirus (rAd)-based MERS vaccines expressing full-length S protein, S1, N-terminal domain (NTD) and the recombinant receptor-binding domain (RBD) at the preclinical level (27,28). Human adenovirus serotype 5 (Ad5) is the most common Ad vector applied to develop MERS vaccines among the other types. Adenovirus type 41 (Ad41) as an enteric pathogen has potential for application as a vaccine. Both of these vaccines, Ad5-MERS-CoV S and Ad41-MERS-CoV S, have been investigated in preclinical studies (23,29). Moreover, chimpanzee adenoviruses (including AdC68 and ChAdOx1) have been assessed as viral vectors for MERS vaccines (30).

Another preclinical study, attributed to VSV-vectored MERS vaccines, which express full-length S protein and RBD, has also been performed (31). MV-based MERS-CoV vaccines which express MERS-CoV full-length S protein (MVvac2-MERS-S) and reduced S protein, a soluble form (MVvac2-MERS-solS), have been established (32).

Venezuelan equine encephalitis virus (VEEV) replicon particles (VRPs), a type of alphavirus-based platform as an encoding MERS-CoV S, can elicit NAb in both young and aged mice (33-35).

Newcastle disease virus (NDV) has been examined as a vaccine vector in non-human primates (26). An NDV vector expressing MERS-CoV S protein was revealed to have a long-lasting induction of NAb titers in camels (36).

To investigate other platforms, an inactivated dual rabies/MERS vaccine has been suggested in which the MERS-CoV S1 domain fused to rabies virus G-protein on the rabies virus virion (34). The inspiration for a rabies virus vector derived from studies uniting rabies and Ebola vaccine platforms (37,38).

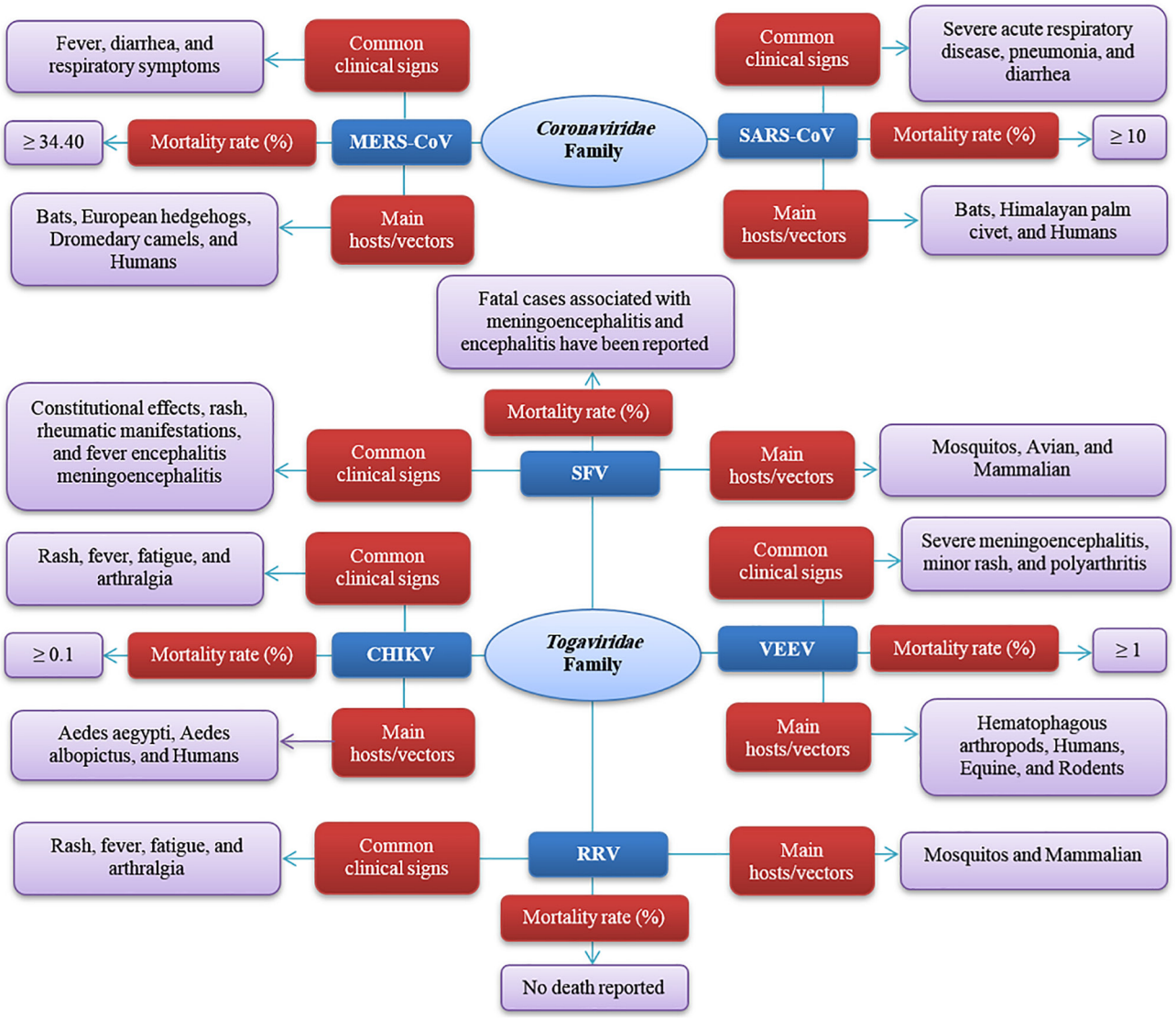

Figure 1. Summary of common clinical signs, mortality rate, and main hosts/vectors of MERS-CoV, SARS-CoV, CHIKV, Venezuelan equine encephalitis, RRV and SFV. MERS-CoV, Middle East respiratory syndrome coronavirus; SARS-CoV, severe acute respiratory syndrome coronavirus; CHIKV, Chikungunya virus; RRV, Ross River virus; SFV, Semliki Forest virus; VEEV, Venezuelan equine encephalitis virus.

Protein-based vaccines are capable of promoting immune reaction. In this group, vaccines based on the RBD (i.e., CTD), have comprehensively investigated (39), and those based on S1 and full-length S proteins are under investigation in animal models and/or non-human primates (1,40-42). An RBD fragment is a critical domain that consists of residues 377-588 and RBD proteins, including the mentioned fragment attached to either the Fc of human IgG (RBD-Fc) or the Foldon (Fd) trimeric motif (RBD-Fd) attached to the DPP4 receptor; in mice and/or rabbits, these fusion proteins evoke strong responses against various strains of MERS-CoV infections (43-45). Furthermore, a constant CHO-expressed RBD-Fc protein promoted the survival of hDPP4-Tg mice with MERS-CoV, with no immune toxicity or eosinophilic immune increment (44). MF59 is an effective adjuvant that intensely enhances the ability of the RBD protein to evoke potent reactions (46). Other fragments of the MERS-CoV S protein, including NTD and S2, are capable of being elective vaccine goals. The NTD protein can induce defined humoral and cellular immune responses, which have been analyzed in Ad5-hDPP4-transduced mice (41).

Investigations on virus-like particle (VLP)- and bacterium-like particle (BLP)-based vaccine development have also been performed. There are some MERS vaccines available based on VLPs and BLPs, some of which express full-length S protein or RBD and some others are constructed using the S, E and M structural proteins. Additionally, they have been assessed in animal models (11,47). A chimeric form of VLP, which was produced by fusing the MERS-CoV S protein with the matrix protein 1 protein of the H5N1 influenza virus, was shown to elicit particular antibodies in mice against MERS-CoV (34). The other form of a chimeric, spherical VLP was established by fusing the canine parvovirus VP2 structural protein gene to the MERS-CoV RBD (11). Moreover, a VLP expressing the MERS-CoV S, E and M proteins conjugated with an aluminum adjuvant has been studied in non-human primates (47). In the BLP-based MERS-CoV vaccine group, RLP3-GEM has been produced

Table I. MERS, SARS, Chikungunya virus, Venezuelan equine encephalitis, Ross River virus and Semliki Forest virus clinically tested vaccine candidates.

Middle East respiratory syndrome

Author (year)	Vaccine type	Candidate vaccine		Clinical trial phase	Schedule	Protective efficacy of vaccine	(Refs.)
Modjarrd *et al* (2019)	A DNA plasmid vaccine	GLS-5300	The vaccine expressed the MERS CoV spike (S) glycoprotein and consisted of 6 mg/ml of plasmid pGX9101 in sterile water.	Phase I (completed)	The vaccination program began with 0.67, 2, or 6 mg of the GLS-5300 at weeks 4 and 12 followed by co-localized intramuscular electroporation.	-	(56)
Advantages: No severe adverse events, rapid manufacture, avoidance of potential toxicities, immunogenic, induction of seroconversion and T-cell responses, durable immune responses, polyfunctional $CD8^+$ T-cell response, no laboratory abnormalities of grade 3 or higher.				Disadvantages: Solicited symptoms (mild): Injection-site reactions, headache, malaise or fatigue, administration site pain, and tenderness. Unsolicited symptoms: Infections.			
Koch *et al* (2020)	Modified vaccinia virus Ankara (MVA)	MVA-MERS-S	Modified vaccinia virus Ankara (MVA) vector that expressed the MERS-CoV spike glycoprotein (S)	Phase I	Two doses were administered as follows: 10^7 or 10^8 plaque-forming units (PFU) of MVA-MERS-S on days 0 and 28 and a booster dose of 10^8 PFU MVA-MERS-S 12 months (±4 months) after the first immunization.	-	(58)
Advantages: No severe adverse events, favorable safety, persisting T-cell responses, rapid induction of immunity, induction of both humoral and cellular immune responses.				Disadvantages: Solicited local reactions, pain, induration, swelling, headaches, and fatigue or malaise.			
Bosaeed *et al* (2022) Folegatti *et al* (2020)	Simian adenovirus-vectored vaccine	ChAdOx1	Contains the MERS Spike protein antigen.	Phase I, Phase Ib	The vaccine was administered to the participants with the following schedule: The low-dose group ($5x10^9$), the intermediate-dose group ($2.5x10^{10}$) and the high-dose group ($5x10^{10}$) viral particles.	-	(60,61)

Table I. Continued.

Middle East respiratory syndrome							
Author (year)	Vaccine type	Candidate vaccine		Clinical trial phase	Schedule	Protective efficacy of vaccine	(Refs.)
Advantages: Safe and well-tolerated, immunogenic, no serious adverse reactions induction of both humoral and cellular immune responses, inducing seroconversion and T-cell responses, good durability.				Disadvantages: Short-lived fever, Injection site pain, fatigue, headache and malaise.			

Severe acute respiratory syndrome coronavirus							
Author (year)		Candidate vaccine	Components	Clinical trial phase	Schedule	Protective efficacy of vaccine	(Refs.)
		Advantages			Disadvantages		
Lin *et al* (2007)	Inactivated virus	ISCV (inactivated SARS-CoV vaccine)	The vaccine was obtained from a clinical strain (Sino 3) and inactivated by β-propiolactone. The syringes in which the ISCV was adsorbed to aluminum hydroxide include sterile saline without preservatives.	Phase I, completed	Doses of 16 and 32 SU/ml were administered to participants.	-	(105)
Advantages: Safe, well tolerated, induction of SARS-CoV-specific neutralizing antibodies.					-		
Martin *et al* (2008)	DNA vaccine	VRC-SRSDNA015-00-VP	The vaccine contains a single closed circular plasmid DNA macromolecule (VRC-8318). The DNA vaccine was generated at a 4 mg/ml concentration in phosphate-buffered saline (PBS). The SARS recombinant plasmid DNA vaccine encodes the SARS spike glycoprotein.	Phase I, completed	A 4 mg dose was administered on days 0, 28 and 56.	-	(104)
Advantages: Safe, well tolerated, immunogenic SARS-CoV-specific antibody, neutralizing antibody, SARS-CoV-specific CD4+ T-cell responses, $CD8^+$ T-cell responses, and rapid manufacturing.				Disadvantages: Mild injection site symptom: Pain/tenderness, swelling and redness. Mild systemic symptoms: Myalgia, malaise, headache, chills and fever.			

Table I. Continued.

Chikungunya virus

Author (year)	Vaccine type	Candidate vaccine/ manufacturer	Components	Clinical trial phase	Schedule	Protective efficacy of vaccine	(Refs.)
Harrison *et al* (1967)	Live attenuated virus (LAV)	CHIKV TSI-GSD-218	Whole attenuated virus	Phase II	1 dose	Forty seroconverted by day 14 and 98% (57 vaccinees) seroconverted by day 28	(109)
Advantages: Safe and well-tolerated.				Disadvantages: No CHIK volunteer developed clinically important reactions to the vaccine.			(117)
Reisinger *et al* (2019)	Measles viral vectored vaccine (VVV)	MV-CHIK	Capsid E3 E2 6K E1	Phase II	2 (28 days)	Results indicated that the candidate vaccine successfully displayed safety and tolerability	(120)
Advantages: Safe, well tolerated, and highly immunogenic.				Disadvantages: Injection site, tenderness injection site pain, and injection site induration.			
Chen *et al* (2019)	Virus-like particle (VLP)	VRC-CHKVLP059-00-VP	Capsid E3 E2 6K E1	Phase II	3 doses (0, 4, and 20 weeks)	There was no significant difference between effective concentration (EC50) and placebo group (still being further assessed)	(138)
Advantages: Similar immune response as native virus, safe, well-tolerated, highly immunogenic, given without adjuvant, durable vaccine induced antibodies.				Disadvantages: Mild transient alanine aminotransferase increases, transient neutropenia.			(121)

Venezuelan equine encephalitis

Author (year)	Vaccine type	Candidate vaccine/ manufacturer	Components	Clinical trial phase	Schedule	Protective efficacy of vaccine	(Refs.)
Edelman *et al* (1979)	Inactivated vaccine	C-84	Formalin-inactivated vaccine for VEE	N/A	2 Doses	The vaccine increased preexisting serum neutralizing antibody titers against the VEE	(184)

Table I. Continued.

Venezuelan equine encephalitis							
Author (year)	Vaccine type	Candidate vaccine/ manufacturer	Components	Clinical trial phase	Schedule	Protective efficacy of vaccine	(Refs.)
						virus in seropositive TC-83 vaccine recipients and elicited high neutralizing antibody titers in non-immune subjects after a primary vaccine and two-dose vaccination	
Advantages: No febrile reactions. No wheal and-flare reactions with pseudopod formation. No systemic anaphylactic reactions.					-		
Hannaman *et al* (2016)	DNA	- pWRG/VEE/ Althea Technologies, Inc., San Diego, CA	pWRG/VEEV gens	Phase I	3 Doses	All participants indicated neutralized ntibody after third dose	(186)
Advantages: Robust antibody response with a high IgG2a/IgG1 rate.					-		
Johnson (2020)	Live, attenuated	Dried TC-83	Live, attenuated vaccine for VEE	Phase II	-	In progression	(185)
		-			-		

Ross River virus							
Author (year)	Vaccine type	Candidate vaccine/ manufacturer	Components	Clinical trial phase	Schedule	Protective efficacy of vaccine	(Refs.)
Aichinger *et al* (2011)	Inactivated whole-virus	Ology Bioservices	Inactivated whole-virus Vero cell-derived	Phase I and II	0, 21 Days; 6 months later	Highly immunogenic in RRV-naïve adults	(193)
Advantages: Safe, well-tolerated, no serious adverse events, no cases of arthritis associated with RRV, and low rates of fever, no increase in adverse events after the 2nd or 3rd dose, protects ~99% of individuals who completed the vaccination.					-		(232)

Table I. Continued.

Semliki Forest virus							
Author (year)	Vaccine type	Candidate vaccine/ manufacturer	Components	Clinical trial phase	Schedule	Protective efficacy of vaccine	(Refs.)
Komdeur *et al* (2021)	rSFV-based therapeutic cancer vaccine	Vvax001/University Medical Center Groningen Groningen, Netherlands Collaborators: Dutch Cancer Society ViciniVax B. V	The Vvax001 vaccine consists of a replication-deficient Semliki Forest virus (SFV) vector which codes HPV derived tumor-antigens	A phase I clinical trial	Patients received three sequential doses, with a gap of 3 weeks.	-	(225)
Advantages: Safe, well tolerated, and induced strong HPV16 E6- and E7-specific immune responses. Capable of inducing HPV16-specific, IFN-g-producing T cells. No vaccine related grade 3 or 4 adverse events. Elicited both $CD4^+$ and $CD8^+$ $E6^-$ and E7-specific T-cell responses.				Disadvantages: The presence of either pre-existing antibodies against the virus or vaccine-induced responses that may impede booster responses against the transgenes.			

MERS, Middle East respiratory syndrome; SARS, severe acute respiratory syndrome; CHIKV, Chikungunya virus; RRV, Ross River virus; SFV, Semliki Forest virus; VEEV, Venezuelan equine encephalitis virus. The dash (-) indicates no available information.

as an alternate form to VLP-based MERS vaccines and the former anchors an RBD linked through three protein harbors (RLP3) and utilizes Gram-positive booster matrix (GEM) as a substrate (20). With the GEL01 adjuvant, the immune induction of this vaccine was tested in mice (20).

Efforts made with the design of vaccines based on nanoparticles. Multiple attempts at designing nanoparticle-based vaccines expressing the MERS-CoV full-length S protein have been carried out and conducted in insect cells, which were consequently evaluated for efficacy in mice (48,49). To achieve nanoparticle vaccines, the nano surfactant treatment and mechanical disjunction of insect cells, which express S protein was performed in order to improve nanovesicle formation and consequently achieve an optimal generation of nanoparticle vaccines (50). For immune response improvement, nanoparticle vaccines can be merged into other types of MERS vaccines, for instance, heterologous priming with rAd5 coding full-length S protein (Ad5/MERS), followed by promoting with full-length S protein nanoparticles, triggering both Th1 and Th2 immune responses that have a protective effect in mice (51).

Preclinical vaccines based on the non-S structural proteins of MERS-CoV. As aforementioned, the S protein is the most substantial one among the other structural proteins in the vaccine design approach. Apart from the S protein, the N protein may be another vaccine target; hence, it is conserved among various strains of the virus. Various N protein-based vaccines have exhibited potent immunity in immunized mice. Previous studies have tested MVA or MV vector-based recombinant vaccines presenting the MERS-CoV N protein (MVA-MERS-N; MVvac2-MERS-N), which causes MERS-CoV N-specific T-cell induction (including $CD8^+$ T-cells) in mice (24,52).

Preclinical vaccines based on the inactivated virus. The inactivated MERS-CoV virus, as another vaccine candidate, has been designed and evaluated at preclinical assessment. Agrawal *et al* (53) assessed the immunization of inactivated MERS-CoV vaccine candidates in mice, which revealed that it may increase the risk of a hypersensitive-type lung pathology from MERS-CoV infection (53). Another analysis of inactivated whole MERS-CoV in mice illustrated enhanced protection (54).

Preclinical vaccines based on live-attenuated viruses lacking structural, non-structural, or accessory proteins. MERS-CoV has several protein types; for example, ORF 3-5 as an accessory protein, the E structural protein, and the nsp16 non-structural protein (nsp), which are ascribed to pathogenicity. Nevertheless, a recombinant MERS-CoV has been tested, which lacks ORF 3-5 and was shown to lead to a reduction in viral titers in cell culture (55). Other research has suggested the possibility that rapidly generated live-attenuated MERS-CoV vaccines may have diminished virulence (40).

Clinical trials testing MERS vaccine platforms. Several clinical trials have been performed testing MERS vaccine platforms, and the first vaccine candidate was a DNA plasmid vaccine. Kayvon Modjarrad and colleagues conducted the first DNA vaccine candidate against the MERS-CoV. That trial is registered on ClinicalTrials.gov (NCT02670187) and has been completed (56). The vaccine candidate GLS-5300, a DNA plasmid vaccine expressing the MERS CoV spike (S) glycoprotein, was brought out in The Lancet Infectious Diseases in 2019 (Table I) (56). To this aim, 75 adults aged 18 to 50 years at the Walter Reed Army Institute for Research Clinical Trials Center (Silver Spring, MD, USA) participated in the study and were administered a dose-increment protocol as follows: A 0.67, 2, or 6 mg GLS-5300 intramuscular injection at the starting point, then immediately at weeks 4 and 12, followed by co-localized intramuscular electroporation to evaluate the safety of GLS-5300 at one of these three dose levels. The early results of the study revealed the safety of the agent. The follow-up after dose 3 was performed up to the 48th week. To take part in the other groups of high-dose-administration, a safety monitoring committee should confirm the vaccination outcome of the first five individuals in the prior low-dose group. The secondary consequence was immunogenicity (56). The ingredient of GLS-5300 is 6 mg/ml plasmid pGX9101 in sterile water for injection. Plasmid pGX9101 comprises a gene insert planned as an optimized, full-length, macro consensus of the MERS-CoV S-glycoprotein raised from publicly present clinical sequences up to August, 2015 (Table I) (57). That phase I clinical trial revealed that the tested vaccine was well-endured, and no critical adverse events were introduced. The usual adverse effects were related to the injection site reflexes, which were in line with the other released clinical trial reports of DNA vaccines or placebo co-administered through intramuscular injection and electroporation (56). The effect of GLS-5300 vaccination in cellular stimulation and antibody responses is similar to MERS in patients who have recovered from CoV infection. Since DNA vaccines and viral-vectored vaccines use recombinant technology, they can be included in rapid designing approaches in the case of emerging infectious diseases. In comparison to living viral-vectored vaccines, DNA vaccines have this superiority in rapid production and do not have the possible occurrence of toxicity (56). Since phase I clinical trials [(NCT02670187), GLS-5300 (INO-4700)] and phase I/IIa trials [(NCT03721718), GLS-5300] on MERS-CoV DNA vaccines have been performed, Inovio Pharmaceuticals generated the GLS-5300 (INO-4700) DNA vaccine.

MVA vector vaccine candidate evokes humoral and cellular immune responses to MERS-CoV S protein. The MVA vector vaccine candidate was the other candidate used in clinical trial testing. The open-label, phase I trial was conducted at the University Medical Center Hamburg-Eppendorf (Hamburg, Germany) (58). This type of vaccine was based on an rMVA vector that expresses the full-length MERS-CoV spike glycoprotein, which relies on the sequence of EMC/2012 (GenBank accession no. JX869059). The vaccine was constructed by IDT Biologika GmbH in early chicken embryo fibroblasts. The participants were healthy adults aged 18 to 55 years. The participants were injected with doses MVA-MERS-S at $1x10^7$ plaque-forming units (PFU; low-dose group) or $1x10^8$ PFU (high-dose group) intramuscularly at the first vaccination

(Table I). The amplifier immunization was administrated intramuscularly 28 days after the first vaccination. The main aims of that study were to analyze the safety and tolerability of the two dosage plans in addition to determining the reactogenicity after administration. The participants in the low-dose or high-dose groups did not exhibit any severe adverse effects. The comparison of the two-step vaccination demonstrated that the booster dose elicited humoral and cellular immune responses to the MERS-CoV spike protein (58). Among various preclinical analyses, mice vaccinated with MVA-MERS-S produce neutralizing antibodies and $CD8^+$ T-cells and a protective effect occurs in Ad-hDPP4-transduced mice infected with MERS-CoV (59). Even though research on animal models has illustrated the vital role of antibodies in protecting against MERS-CoV, information obtained on humans has not revealed the potent connection between the MERS-CoV viral load and MERS-CoV-specific antibody responses (57). T-cells cause dominant responses in survivors of MERS-CoV (16). The importance of T-cell responses has not been proven to be critical in humans; however, in mouse models, the clearance effect of T-cells has been revealed (48). In conclusion, the aforementioned trial (ClinicalTrials.gov, NCT03615911; EudraCT, 2014-003195-23) demonstrates humoral and cellular immunogenicity in humans. Since vaccination for MVA-MERS-S had no crucial adverse effects, the vaccine was considered safe. A phase Ib clinical trial (ClinicalTrials.gov, NCT04119440) on was conducted in order to scrutinize the safety, tolerability and immunogenicity of two ascending doses of the candidate vaccine MVA-MERS-S_DF-1 against MERS (MVA MERS-S). The last update for this trial was on November 8, 2022.

Adenovirus-vectored vaccine encoding the full-length spike surface glycoprotein has yielded promising results. The other phase I clinical trial is based on assessing the safety and immunogenicity of the candidate simian adenovirus-vectored vaccine encoding the full-length spike surface glycoprotein, ChAdOx1 MERS (NCT03399578 and NCT04170829), in humans was conducted at the Centre for Clinical Vaccinology and Tropical Medicine (Oxford, UK) (Table I) (60). A total of 48 healthy participants aged 18 to 50 years received the ChAdOx1 MERS in a single injection, intramuscularly. In total, three different doses were administrated as follows: The low-dose group was administered $5x10^9$ viral particles, the intermediate-dose group with $2.5x10^{10}$ viral particles, and the high-dose group with $5x10^{10}$ viral particles. ChAdOx1 MERS composition is the replication-deficient simian adenovirus vector ChAdOx1 expressing a codon-optimized coding sequence for the full-length spike protein (S1 and S2 subunits) of the MERS-CoV isolate from a camel in Qatar (GenBank Accession no. KJ650098.1), containing a 32 amino acid N-terminal tissue plasminogen activator leader sequence. In that study, the candidate ChAdOx1 MERS vaccine was evaluated, and the safety was revealed in all three dose groups, although a higher reactogenicity profile was considered in the high-dose group. In addition, no severe adverse reactions were observed (Table I) (60). For the safety evaluation of the ChAdOx1 MERS vaccine (NCT04170829), a phase Ib trial was conducted in healthy Middle Eastern adults. The vaccine dosage in this trial was similar to the clinical trial phase I, and the outcome was desirable and conformed with the clinical trial phase I (61).

According to preclinical research on the BVRS-GamVac-Combi vaccine, a heterologous prime-boost immune vaccine with recombinant adenovirus types 26 and 5, depicted high titers of specified antigen-neutralizing antibodies in mice (62); thus, phase I/II clinical trials of the vaccine (NCT04128059) and BVRS-GamVac (NCT04130594) are currently underway.

Although various tests have been performed on the MERS vaccine, no commercial vaccine has been marketed to date, and all these findings are derived from laboratory-based trials.

3. Severe acute respiratory syndrome coronavirus

CoVs are included in the family of *Coronaviridae* and comprise α-CoV, β-CoV, γ-CoV and δ-CoV genera (1). The most pathogenic human CoVs have caused considerable infections, which include SARS-CoV, MERS-CoV and the newly recognized SARS-CoV-2 [known as CoV 2019 (COVID-2019)]; all infections are associated with the genus β-CoV (63). SARS-CoV was first identified in Guangdong, China, in 2002, resulting in a worldwide outbreak in 2003, which led to an ~10% fatality (Fig. 1) (64). Several structural proteins, including nucleocapsid, membrane, envelope and S proteins, which are expressed by SARS-CoV cause severe infections (65). The target cells to be infected by this virus are lung epithelial cells and the entry to the host cell occurs by binding to ACE2 (66). SARS-CoV infection begins with flu-like signs, and subsequently leads to severe acute respiratory disease, pneumonia, diarrhea and even death (67). SARS-CoV is found in bats, which can be transferred into the Himalayan palm civet as another host that causes the amplification of the virus (68). There are two probable mechanisms for the transmission of SARS-CoV as a zoonotic virus, including animal-to-human and human-to-human (Fig. 1) (67). The evolution of the strategies regarding the SARS vaccine comprises three generations. Live attenuated and inactivated vaccines are categorized into the first-generation group. Related to the natural antigenic substance, live attenuated vaccines have always yielded significant results due to their rapid access and potent immunogenic response (69). The successful administration of these vaccines against variant diseases, such as polio, rubella, chickenpox, mumps, etc. has been previously reported (69). Based on preclinical research in which SARS-CoV mutants lacking the E gene were evaluated in hamsters challenged with SARS-CoV, preventive effects were inferred (70). Since snp16 can function as a target for the CoV vaccine, both SARS-CoV and MERS-CoV nsp16 mutant vaccine has evaluation revealed a conservative effect (71). Another target for live attenuated CoV vaccine is nsp14, which encodes exoribonuclease (ExoN). Graham *et al* assessed the effects of ExoN deletion, which demonstrated that the ExoN-deleted SARS-CoV vaccine can exert a protective effect against challenges in these mice (72). Nevertheless, none of the preclinical analyses of live attenuated vaccines have led to clinical trials for either SARS-CoV or MERS-CoV (73).

The other form of the vaccine in this category known as an inactivated vaccine may be achievable by inactivating the virus, using radiation method (UV-ray, X-ray org-radiation) or chemicals (such as formalin, methanol, or b-propiolactone),

in which the antigenic feature of the virus remains active, although it is not able to cause infection (74).

To date, diverse inactivated vaccines are available against various diseases, such as influenza, polio, hepatitis A, rabies pathogen, etc. (74). Various studies have been designed based on first-generation vaccines against SARS. The assessments have been tested on different animal models, such as mice (75,76), hamsters (77), African green monkey (78) and rhesus monkey (79), which were revealed to be safe candidates in animals.

Along with all these data, whole inactivated vaccines which were tested in both SARS-CoV and MERS-CoV, have depicted an eosinophil-related lung pathology as a downside (80). Nonetheless, later research has manifested that UV-inactivated SARS-CoV with Toll-like receptor agonists as adjuvants, and formaldehyde-inactivated MERS-CoV with alum and unmethylated CpG as adjuvants, have the potential of suppressing or preventing lung injury (81).

As regards second-generation vaccines, protein subunit vaccines and vector-based vaccines have been assessed. The first attempts for protein subunit vaccine generation were based on surrounding full-length S protein-based vaccines; S protein RBD-based vaccines subsequently attracted increasing attention. The formulation of a protein subunit vaccine is based on synthetic, isolated, recombinant, or derived highly antigenic protein base subunits with the short antigen part proposing a safer strategy in the vaccine project. Diverse protein subunit vaccines have been designed successfully against multiple pathogens, such as the influenza virus, hepatitis B, pneumonia and meningitis, etc. (82-84). According to previous studies, the full-length S protein, extracellular domain of the S protein and trimeric S proteins (triSpike) have an immunogenic capacity that can exert protective effects against SARS-CoV infection (84,85). According to the study by Du *et al* (86), RBD-based SARS-CoV vaccines have the potency of evoking RBD-specific IFN-γ producing cellular immune responses in mice.

Studies using various animal models, such as rabbits and mice have yielded acceptable outcomes using subunit vaccine candidates for SARS prevention (87,88). Moreover, the assessment of the immunogenicity of recombinant baculovirus-expressed SARS-CoV S protein in a mouse model, yielded positive results, demonstrating protective effects (85). Other structural proteins, N protein-based vaccines, have also been tested. Although N protein-based vaccines cannot impel neutralizing antibodies, they are more conserved across CoV species than S protein, which renders them a possible target for a T-cell inducing global CoV vaccine (89). Testing M protein-based vaccines has revealed high antibody titers, but no NAb (77). Studies on CoV E protein-based immunization are limited, and neither neutralizing antibodies nor protective immunity has been reported (90).

The evaluation of the SARS-CoV protein subunit vaccines in preclinical assessments has revealed promising results, despite the fact that these have not entered clinical trials (91). Superior immunogenic responses are concluded from vector-based vaccines (92). There are disparate viral vectors that are being used as a transfer instrument in vaccination, such as the MVA virus, adenovirus, adeno-associated viruses, retrovirus vector, lentivirus vector, Sendai virus, etc. (88). Adenovirus, as a popular viral vector vaccine, has been surveyed in order to examine the effectiveness of the adenovirus-based SARS-CoV vaccine. Based on research conducted on monkeys and rats, adenoviral vector representing the S1 fragment has the potency of inducing NAbs (88,93). A ferret model of SARS-CoV infection was previously tested; the results revealed that it could prevent pneumonia (94). Based on research on a rat model, an adenoviral-based vaccine outlined potent SARS-CoV-specific humoral immune responses (95).

The other vaccine platform evaluated in the SARS-CoV challenge was MVA. In a study on mice immunized with attenuated MVA containing a full-length S gene, a protective outcome was attained (96). Although NAbs have been observed in mice, ferrets and monkeys tested with recombinant MVA expressing SARS-CoV S protein, no protective effects were detected (97). On the other hand, some studies have provided conflicting data depicting certain adverse effects in ferrets, including inflammatory responses and focal necrosis in the liver while using the MVA vaccine expressing SARS-CoV S protein (98,99).

Deming *et al* (100) evaluated the VEEV-based SARS-CoV vaccine, and concluded that VEE VRPs expressing S protein exerted protective effects in mouse models. Further investigations have been conducted using the parainfluenza-based vaccine in hamsters and monkeys, and attenuated VSV in mice revealed promising results for SARS-CoV vaccines (78,90,101).

Analyses on viral vector-based vaccines in comparison to the first-generation vaccines have demonstrated efficacious results attributed to the presence of the live virus by recombination of the antigenic protein ingredient of a pathogenic virus into a non-virulent vector. In due course, the stimulation of cellular and humoral immunogenicity is obtained. The precise information about epidemiology, genotoxicity and virology of both viruses (pathogenic and vector virus) needs to be examined further, in order to design a proper and effective vaccine (92).

In order for this to be achieved, however, several obstacles may have to be combatted, such as a risk of mutation and unanticipated virulence potency, the delay of an actual expected immune response, and the need for precise information about the epidemiology, genotoxicity and virology of both viruses (pathogenic and vector virus), etc. (60,92).

Other vaccine development strategies have been tested, VLPs, which are self-assembled viral structural proteins that imitate the compound of native viruses without a viral genome. Based on a study in which chimeric VLPs composed of SARS-CoV S protein and mouse hepatitis virus E, M, and N proteins were analyzed, the induction of NAb responses and the reduction of SARS-CoV viral titers in mouse lungs were observed (102). Another study that utilized the same chimeric VLPs as the aforementioned study by Lokugamage *et al* (102), revealed the disadvantage of this vaccine type, which was pulmonary immunopathology (80).

Conduction of clinical trials according to the prior favorable consequences obtained from preclinical studies. DNA vaccines as a rapid and flexible vaccine development platform consist of genes encoding viral antigenic components. Among various evaluations including the S, M and N protein-based vaccines, only the S protein-based DNA

vaccine can exert a conservative effect against SARS-CoV infection. According to a previous study, DNA encoding full-length S protein can provide NAbs and exdert protective effects in mice (103). Since the preclinical results were encouraging, a phase I clinical trial based on SARS-CoV full-length S protein DNA vaccine was conducted (NCT00099463) (104). In that clinical trial, the vaccine, VRC-SRSDNA015-00-VP, comprised a single closed circular plasmid DNA macromolecule (VRC-8318). For this purpose, 10 healthy adults received a three-dose vaccine schedule and were then tested in order to evaluate the immunity and safety of the vaccine. The vaccine administration was based on three doses of 4 mg/ml on days 0, 28 and 56. The results depicted a promising outcome, demonstrating a safe and well-tolerated vaccine that can elicit NAbs and exert a protective effect (Table I) (104).

In a study conducted by Sinovac Biotech, the response to an inactivated vaccine was examined in 36 healthy adults (SARS-CoV seronegative), aged 21 to 40 years. The clinical trial was performed in a randomized manner, double-blinded and placebo-controlled in China (Table I) (105). The control group was administered saline with aluminum hydroxide as aluminum hydroxide absorbed the inactivated vaccine.

The doses were established based on preclinical assessments in mice, rats and rhesus monkeys, in which the safety and immunogenicity of the vaccine were proven. The participants received 16 SU or 32 SU of the vaccine or the placebo, via intramuscular injection (105). The results demonstrated a safe and well-tolerated vaccine candidate, evoking SARS-CoV-specific NAbs (106). Although several clinical trials have been running for a number of years, thus far, there is no licensed vaccine available for SARS-CoV.

4. Chikungunya and O'nyong-nyong virus

CHIKV belongs to the *Togaviridae* family, the Alphavirus genus. CHIKV is a positive-sense single-stranded RNA virus, that was first isolated in 1953 in Tanzania. Since then, the CHIKV epidemic or endemic infections have been reported in 106 countries and territories, including the USA and Europe (107).

CHIKV consists of three genotypes, having a single serotype behavior (107). *Aedes aegypti* and *Aedes albopictus* mosquitoes are the vectors of CHIKV (108). Four nsps, namely nsp1, nsp2, nsp3 and nsp4, and five structural genes (C-E3-E2-6K-E1) are encoded in most of the CHIKV genome, and are responsible for viral replication and transcription. CHIKV virions are spherical, enveloped particles of ~70 nm in diameter. The E1 and E2 glycoproteins form heterodimers and assemble into spikes on the surface. At the center of the virion, is the nucleocapsid core, ~35 nm in diameter, which is composed of the C protein in a complex with the viral genome (109).

The clinical symptoms of CHIKV exhibit similarities with other Alphaviruses, including the O'nyong-nyong virus and the Ross River virus (RRV) (Fig. 1). Since there is no effective antiviral treatment for CHIKV infections, several techniques have been used for the development of CHIKV vaccines, including non-infectious (110) and infectious DNA vaccines (111), VLPs and inactivated virus. Live attenuated vaccines under development include rationally attenuated Alphavirus chimeras (112) and deletion mutants (113); a vesicular stomatitis-vectored vaccine (114) and an internal ribosome entry site-modified CHIKV strain (115). For this purpose, various clinical human (116-121) and animal (122) trials have been performed.

Preclinical studies. The main portion of current available knowledge on the immune system response to CHIKV has been obtained from animal models. The first efforts of researchers date back to the attempts of the Reed Army Institute of Research (WRAIR) in Washington, D.C., USA, and are related to the formalin-inactivated virus vaccine (109). For the preparation of this candidate vaccine, chick embryo, suckling mouse-brain (SMB) and green monkey kidney cells (GMKCs) were used. Due to the weak immune response elicit, chick embryo could not pass the test, and between SMB and GMKCs, the former was selected for continuation. However, the study was suspended to limit the risks of inducing encephalitis in males. Following the success of the CHIKV 168 vaccine in eliciting homologous protection in mice, the African CHIKV strain 168; the CHIKV strain E.103 isolated from a pool of 78 *Aedes africanus* mosquitoes (123), the Asian strain BAH-306 isolated from Thai patients (124), and the Indian CHIKV strain C-266 isolated from Calcutta by K.V. Shah were selected to assess heterologous protection in rhesus macaques (125). CHIKV strain 168 was administered in 0, 7 and 21 days. After 30 days of homologous and heterologous challenges, no viremia was observed in all vaccinated subjects (Table I) (109).

Other candidate vaccines are VLPs which express the structural protein of viruses that have the ability to infect the host (original); however, the new structure does not have the ability of infection, since it is an empty shape (126). Akahata *et al* (127) at the Vaccine Research Center (VRC) at the NIH National Institute of Allergy and Infectious Diseases (NIAID) in Bethesda, MD, USA developed a VLP vaccine. The vaccine carried structural CHIKV proteins Capsid, E3, E2, 6K and E1 sequences of the CHIKV strain 37997. All macaques (n=6) were resistant to the CHIKV (strain LR2006 opy-1) challenge and NAbs were induced (127). Other CHIK-VLP vaccines were developed based on the expression of yeast-derived CHIKV-like particles. Saraswat *et al* (128) used a novel yeast expression system (*Pichia pastoris*) and evaluated this as a vaccine candidate. This elicited neutralizing activity against CHIKV in BALB/c mice. Various doses of CHIK-VLPs also succeeded in inducing humoral and cellular immune responses (128).

The MV-CHIKV vaccine is a type of recombinant live-attenuated vaccine based on inducing high titers of CHIKV antibodies in mice challenged with the measles virus. It has been demonstrate that single vaccination with this vaccine candidate protected all subjects (mice) from a lethal CHIKV challenge (122). In addition, two chimeric Alphavirus vaccine candidates for CHIKV have reached the animal model phase, as demonstrated by Wang *et al* (129). The first one was designed for Alphavirus genus members in 2007. Chimeric Alphavirus/CHIKV vaccine viruses were created employing recombinant DNA methods (129). The Alphavirus backbone includes Sindbis virus (SINV) strain AR339, the attenuated

VEEV vaccine strain TC-83 and a South American strain of eastern equine encephalitis virus (EEEV), BeAr436087, which is unable to cause disease in adult mice. The structural genes and 5'-UTR of the sub-genomic RNA were obtained from the LR strain of CHIKV. The results indicated that the CHIKV chimera versions that used the attenuated VEEV strain TC-83 or naturally attenuated EEEV backbones were consistently more immunogenic in outbred or inbred mice than the chimera with a SINV backbone, and also produced robust NAb responses (129). In the other study, in order to establish an Alphavirus vaccine, chimeric genomes encoding VEEV- or EEEV-derived non-structural and CHIKV-specific structural proteins (VEE/CHIKV and EEE/CHIKV) were used. To obtain a safer vaccine and to hint replication in mosquito cells, a novel modification was introduced. Their replication was also dependent on the function of the encephalomyocarditis virus internal ribosome entry site. Three different chimeric candidate vaccines were used, including Sham, VEE/IRES-CHIKV and VEE/IRES-C/CHIKV. Following a single immunization, mice exhibited a protective immune response against subsequent CHIKV challenge, characterized by high titers of NAbs (112).

Another type of candidate vaccine is the MVA. Three different research groups have investigated new vaccine platforms. Van Den Doel *et al* (130), Weger-Lucarelli *et al* (131) and García-Arriaza *et al* (132) used different candidate vaccine platforms and demonstrated the efficiency of vaccines separately. Furthermore, various groups have investigated different platforms of the CHIKV vaccine, such as VSV (114), adenovirus 5 (133), DNA, protein E2 (134), E1 and E2 (135), live attenuated (115,116,136) and inactivated (FIV) (109).

Clinical trials. Some candidate vaccines could pass through phase I and II clinical trials. Inactivated (FIV 15562) (109), live attenuated (137), VLP (138), MV-CHIKV (120) and ChAd0x1-CHIKV (NCT03590392) (6) were the vaccines that proceed to phase I trials.

On the other hand, some candidate vaccines proceeded to phase II trials. The MV-CHIKV vaccine is one of the vaccine candidates against CHIKV. In 2021, this vaccine's safety and immune response were completed and compared to the commercially available MMR vaccine, but no publication has been done yet (ClinicalTrials.gov Identifier: NCT03101111). In 2000 CHIKV TSI-GSD-218 entered Phase II (117). The platform was based on live attenuated and the CHIKV strain 15561 (seed) was obtained from an infected patient during the 1962 outbreak CHIKV in Thailand. The vaccine was provided by passaging 18 plaque-to-plaque passages in MRC-5 cultures (116). During the investigation, 73 healthy volunteers participated in the study. A total of 59 volunteers received the vaccine once subcutaneously and 14 were immunized with a placebo (tissue culture fluid). Consequently, the candidate vaccine was highly immunogenic. According to the results, 69% of the volunteers (n=40) seroconverted by day 14, and 98% (57 vaccinees) seroconverted by day 28; in addition, after 12 months NAb was detectable in 85% of the volunteers (117). The PXVX0317 [CHIKV-like particle vaccine (CHIKV VLP)], alum adjuvant vaccine was evaluated in a clinical to determine the safety of the vaccine in adults, and assess the induction of anti-CHIKV NAb responses following a single dose of PXVX0317 (40 μg CHIKV VLP, alum-adjuvanted) as measured 7 days (day 8), 14 days (day 15), and 21 days (day 22) and 56 days (day 57) after vaccination. Additionally, 25 volunteers participated in the trial (NCT05065983). A phase III clinical study on PXVX0317 is currently under research (ClinicalTrials.gov Identifier: NCT05072080).

In 2015, the NIAID conducted a study to investigate the safety and tolerability of a CHIKV vaccine, VRC-CHKVLP059-00-VP, in healthy adults. During this project, 400 subjects participated. From the 400 volunteers, 201 received intramuscular injections 28 days apart (20 μg) and 199 received a placebo and were followed-up for 72 weeks. After the injection, severe, and mild-to-moderate unsolicited adverse events were observed. In addition, 8 weeks after the first administration, the half maximal effective concentration geometric mean titer in the vaccine group was 2005 (95% CI, 1680-2392) vs. 43 (95% CI, 32-58; P<0 .001) in the placebo group. Finally, there was no significant difference between the treatment and the placebo group. However, further assessments and phase III trials are warranted (Table I) (138).

The immunogenicity, safety and tolerability of the measles-vectored CHIKV vaccine MV-CHIK have been investigated in double-blind, randomized, placebo-controlled and active-controlled trial. A total of 263 patients in two different groups received two different concentrations ($5x10^4$ or $5x10^5$ 50% tissue culture infectious dose) intramuscularly, with an interval of 28 (D0 and D28) or 168 (D28 and D196) days between the prime vaccine and the booster. At day 56, NAb was detectable. In addition, the results revealed that a low vaccine dose induced a PRNT50 titer of 50.16 and 12.87 (short and long intervals, respectively), while the high dose induced titers of 174.80 and 33.64 (short and long intervals, respectively). The results indicated that the vaccine candidate succeed to display safety and tolerability (Table I) (120).

A phase II, multicenter, randomized, placebo-controlled and double-blind study was conducted to evaluate the safety and immunogenicity of a two-injection vaccine regimen (days 0 and 28) with CHIKV virus-like particle vaccine (CHIKV VLP, VRC-CHKVLP059-00-VP) in healthy adults, ages 18-60 years, that resided in CHIKV endemic regions (NCT02562482); the results demonstrated the safety and tolerability of the vaccine; however, phase III trials are warranted in order to assess the clinical efficacy (139).

The live recombinant measles-virus-based CHIKV vaccine exhibited immunogenicity. It was also safe in the presence of anti-vector immunity and had an acceptable tolerability profile. This vaccine is the first promising measles-virus-based candidate vaccine for use in human beings (119).

A phase II study to evaluate the safety and immunogenicity of the CHIKV vaccine (MV-CHIK-202) was completed in 2021 (NCT02861586). In September 2022, a clinical study was completed as a randomized double-blind interventional. That study evaluated the safety and immunogenicity of the investigational V184 live recombinant measles-vectored CHIKV vaccine delivered in two doses, 28 days apart compared with a saline placebo. After providing informed consent, individuals were monitored for eligibility, including verification of previous exposure to the CHIKV virus. However, the related study has not yet been published (NCT03807843). In July

2022, a phase III clinical study was conducted on ~4,060 male and female subjects aged ≥18 years evaluating the final dose of VLA1553 (NCT04546724). Nevertheless, the results have not yet been published, at least to the best of our knowledge.

Although O'nyong-nyong virus infection does not have any effective antiviral treatment or vaccine, the CHIKV-IRES (V1/V2) vaccine for CHIKV has been found to elicit a potent cross-NAb response and to confer protection against O'nyong-nyong virus challenge in an A129 mouse model (140).

5. Venezuelan equine encephalitis virus

VEEV, of the Alphavirus genus in the *Togaviridae* family, is a zoonotic pathogen that is transmitted via hematophagous arthropods, through mosquitoes. It is an enveloped virus with a non-segmented, positive-sense RNA genome. The genus comprises VEEV, EEEV and western equine encephalitis virus (WEEV) (141). The VEEV species incorporates six antigenic subtypes, specifically IA/B, IC, ID and IE (I-VI) (142). Alphavirus causes acute infections characterized by high-titer viremia and since vertebrate hosts are infected, induces a variety of diseases from severe meningoencephalitis to minor rash and polyarthritis (Fig. 1) (143). The *Togaviridae* family induces human disease outbreaks and equine epizootics in the American continent, including South, Central, and North America, particularly outbreaks in Texas in 1971 (144). Two live-attenuated strains of VEEV, specifically TC-83 and V3526, can be securely taken care of at biosafety level 2 control (145). The virus glycopeptide forms the icosahedral shape with T=4 symmetry. The virus RNA is surrounded by 240 copies of the viral capsid protein-linked N-terminus of the protein. Moreover, the capsid is bound to the E2 glycoprotein at the C terminus (146).

Preclinical studies. To date, to the best of our knowledge, there is no approved licensed vaccine against VEEV. Reportedly there are several vaccine candidates against VEEV, which are still in progress at different stages of development. This system of classification includes live attenuated viruses, inactivated viruses, recombinant subunit or chimeric viruses, VLPs, or passive immunization. The type and features of VEEV candidate vaccines have been reviewed in detail by Sharm a and Knollmann-Ritschel (142). Live attenuated vaccines are obtained by a mutation in VEEV strains through serial passage in cell culture or manipulation in the viral genome via mutation. According to Berge *et al* (147), initially, TC-83 (a live-attenuated strain of VEEV) obtained by 83 passages of the Trinidad donkey (TrD) strain of VEEV in guinea pig heart cells was used for vaccination in humans. Mexico and Colombia are using live TC-83 as a vaccine for immunizing equines; however, it is not currently marketed in the USA (148).

Inactivated candidate vaccines may be suggested as an alternative candidate, despite the risk of the live virus escaping. One of the candidates for VEEV vaccines for development was the formalin-inactivated TrD strain of VEEV (149). Formerly, it was used to vaccinate equine endemic areas; however, due to the risk of escaped live viral particles, its administration was restricted. By 1970, the incomplete inactivated VEEV vaccine was known as the major cause of outbreaks of VEEV in endemic areas (150). Currently, formalin-inactivated TC-83 is available for the immunization of horses against VEEV and WEEV in the USA. During mutations in the 50 non-coding regions, nsp3, E2, E1 and 30 non-coding regions, and serial passage of the virulent TrD strain through guinea pig heart cell cultures, TC-83 was developed (147,151). Several side-effects and adverse effects related to TC-83 have been reported. It can be transmitted by mosquitos and causes adverse effects in ~20% of recipients; on the other hand, it has a high rate (almost 18%) of serological non-response and is likely to cause pancreatic disease (152,153). Another inactivated VEEV was obtained by using the chemical, 5-iodonaphthyl-1-azide (INA). INA is a type of hydrophobic alkylating agent, divided into biological membranes and accumulating hydrophobic domain of the lipid bilayer; following short-term exposure to UV (long wavelength) it selectively binds to transmembrane proteins in the viral envelope and completely inactivates V3000, a full-length infectious clone of the wild-type TrD strain of VEEV (154,155). INA-inactivated V3526 was shown to be able to induce immunization in mice against an aerosol challenge with TrD (156). Another procedure carried out to inactivate V3526 and generate the VEEV vaccine was by ionizing gamma radiation; V3526 was exposed to a 50 kGy dose of gamma radiation, which led to a 30-50% loss in epitope integrity. However, gamma-irradiated V3526 failed to protect mice against aerosol challenge with virulent TrD and the fatality rate of underlying mice was almost 60% (157,158). The novel material is known as manganese-decapeptide-inorganic phosphate complex derived from gamma radiation-resistant bacteria *Deinococcus radiodurans*, was used to protect VEEV epitopes, while the virus genome degraded completely. Hence, it protected 90% of mice from an aerosol challenge with TrD (159,160).

Recombinant live attenuated vaccines have been dedicated to novel techniques to approach vaccines based on an Alphavirus, which led to a practicable, safe, immunogenic and effective vaccine against encephalitis Alphaviruses. The genome of SINV, which is a non-pathogenic member of Alphavirus in humans, is being used as a vector to design chimeric SIN/VEE virus(es) to express all the structural proteins of the virulent Alphavirus (129,161). SIN-83, SAAR/TrD, SIN/TrD and SIN/ZPC, four different types of SINV vaccines were developed against VEEV (162). The SIN-83 vaccine-induced immune profile was lower than that of TC-83 vaccination, although it caused negligible disease in mice. SIN-83 vaccination successfully induced immunization against the intranasal and subcutaneous challenges; however, the result against intracerebral challenge with heterologous VEEV strain ZPC 738 was not completely acceptable (161). In addition, Paessler *et al* (162) evaluated the immunization potential of three vaccines in mice and hamsters challenged with the VEEV strain ZPC 738, which demonstrated 100% protection. Nevertheless, safety is a priority and live vaccines attract concern. In a 6-day-old mouse model of VEEV central nervous system infection, the immune efficacy of three different vaccines, TC-83, AAR/TRD, SIN/TRD, or SIN/ZPC, and SIN-83 was examined in mice. Accordingly, the lethal rate was 100% for TC-83; in addition, all mice vaccinated with SIN-83 survived, which indicated complete success; the mice administered the other vaccines demonstrated a moderate rate of survival of 60-80% (162).

Another chimeric vaccine candidate is EILV/TC83, in which structural genes of EILV (C-E3-E2-6k-E1) were replaced with those of the TC-83 strain. It induced immunization against the virulent VEEV 3908 strain in mice (163). MVA was manipulated to express E3-E2-6k-E1 proteins of the TrD strain of VEEV tested as a vaccine candidate. MVA-Bavarian Nordic (MVA-BN) was employed as a vector under the control of a synthetic PrHyb promoter to clone enveloped proteins of VEEV (164). A single dose of the MVA-BN-VEEV chimera did not infect mice; however, after 2 weeks, the immune systems of all animals excreted nAb titers after the booster dose and the animals survived against the challenge with virulent TrD (164).

Subunit vaccine candidates introduce novel, safe and high-tech methods for vaccine production. pWRG7077 was utilized as a vector to express structural genes of the TrD strain of VEEV (C-E3-E2-6K-E1) in mammals using the gene gun immunization of the epidermis. In addition, compared with the wild-type plasmid, the VEEV aerosol challenge protected mice (165) and macaques (166). However, the response of nAbs was low, and one macaque exhibited low viremia after infection and exhibited non-sterile immunity (166). DNA vaccination regularly conveys a DNA plasmid encoding at least one antigen to incite an immune reaction (167). Another study demonstrated that a DNA E2 recombinant plasmid vaccine which contains E2 gene sequences from VEEV IA/B and IE, WEEV and EEEV, and Mucambo virus and E1 glycoprotein sequences induced a cross-reactive antibody response against all viruses following intradermal administration (168).

Another vaccine was developed using novel iDNA vaccine technology, which is based on infectious DNA. This type of vaccine possesses the advantages of DNA and live attenuated vaccines. To develop such a VEEV-DNA vaccine, the full-length genomic RNA of the TC-83 live attenuated virus was used under the control of a CMV promoter in a pcDNA3.1-derived plasmid vector. *In vivo* developed viral RNA initiated the limited replication of the vaccine virus. Consequently, to evaluate its efficacy, a single dose of the pTC-83 iDNA vaccine was administered to BALB/c mice. Immediately after vaccination, all mice were seroconverted with no adverse reactions and after 4 weeks, all animals were challenged with the lethal epidemic strain of VEEV. All vaccinated animals survived, while the unvaccinated control groups succumbed to the infection and thus did not survive (169). Rico *et al* (170) designed an alternate strategy to manage vaccine advancement, which involved using E1 glycoproteins as the antigen for immunization advancement (170). Lipid-antigen-nucleic acid-complexes containing VEEV E1 and WEEV E1 antigens were formed through the integration of the purified E1 glycoprotein of the TrD strain of VEEV and WEEV in cationic liposomes.

Since the major conditions for the replication of the Alphavirus genome are non-structural proteins and *cis*-acting RNA sequences, the genes of structural proteins can be altered and foreign antigens are highly expressed (171,172). VRPs, which can only replicate during one cycle due to the lack of structural genes in the virus offspring, have been shown to exert protective effects against VEEV infection when administered as early as 6 h prior to viral infection. The VRP was generated using the V3000 backbone, which contains a mature VRP263 mRNA transcription initiation site nucleotide downstream, a V3000 5'UTR, and a genome containing non-structural genes 1-4. The VRP envelope contains E3 and E1 glycoproteins, derived from V3000 (173). This corresponding non-specific defense mechanism is not yet fully understood; however, the activation of the innate immune response and the inclusion of the antiviral state, possibly through the release of endogenous type I IFN, may mediate defense (174). Human adenovirus type 5 (Rad/VEEV)-based replication-deficient VEEV vaccine expressing the E2 glycoprotein VEEV serogroups IA/B has also been improved. For this, the structural genes of TC-83 (E3-E2-6K) were cloned into PMV100 plasmids and site-directed mutagenesis was used to convert the TC-2E2 glycoprotein into TDE2 glycoprotein. The modified VEEV structural gene sequence was cloned into a pMV60 plasmid to create pMV60/VEEV. Subsequently, the homologous recombination of pMV60/VEEV and pJM17 (containing the entire genome of Ad5) plasmids in 293 cells was utilized to generate replication-deficient human adenovirus type 5 (Ad5) containing VEEV structural proteins (Rad/VEEV) (175). BALB/c mice, 6 to 8 weeks old immunized intranasally with 10^7 PFU of recombinant adenovirus (Rad/VEEV) at various intervals (0 to 21) were protected from low-to-intermediate doses of infectious VEEV; however, this immunization was not successful against high doses of infectious VEEV (175).

nAbs provide a protective shield against peripheral inoculation or natural Alphavirus infection (175,176). The E2 and E3 glycoproteins of VEEV can induce monoclonal antibodies which have been shown to protect mice from challenge with infectious VEEV, whereas E1 glycoproteins provide only weak protection against the infectious viral challenge (177). In the study by O'Brien *et al* (178), they distinguished an extensively responsive monoclonal immunizer, CUF37-2a, from animals that were first vaccinated with TC-83 followed by an introduction to six distinctive serotypes of VEEV (subtypes I, II, III, IV, V and VI). CUF37-2a was discovered to be explicit to the E2 glycoprotein of VEEV and recognized all the VEEV subtypes, aside from the subtype VI, with which it demonstrated a more fragile reactivity. The antibodies protected the mice from subcutaneous presentation to the wild-type TrD strain of VEEV (178).

Clinical trials. Currently, multiple groups are performing clinical trials that aim to study and assess the safety and immunogenicity of the TC-83 obtained vaccine in adult healthy volunteers. TC-83 has certain disadvantages; thus, it is highly probable that it will not be affirmed for mass vaccination in the human population. TC-83 is capable of causing an immunological reaction. Vaccines lead to 23-37% spontaneous flu-like symptoms, such as rash, headache, fever, chills, nausea, diarrhea and myalgia (148,152,179). TC-83 has been isolated from mosquitoes in the southern states of the USA and poses a significant environmental risk of spreading following immunization (180). It has a poor response rate that is suggested to depend on the HLA typing f the host. Among responders, during the first year, the antibody titer decreases following immunization, requiring booster immunizations to maintain the protective antibody titer. There is also a possibility of reversion to virulence (152,181). Site-directed mutagenesis has been employed to generate mutant strains of VEEV that exhibit differential replication and/or tissue tropism in mice compared

to parent full-length V3000 clones (182,183). Davis *et al* (183) evaluated V3526, one the live-attenuated strains of VEEV as a potential vaccine. They obtained V3526 through the sequence of clonal isolates (J9-1a and J9-1b) of the mutant V3022 strain. V3526 was produced by site-specific mutagenesis that removes the furin-like cleavage site of V3022's E2 (PE2) precursor protein (183). In phase I clinical trials, the V3526 vaccine demonstrated adverse reactions, such as myalgia, lymphopenia, pyrexia and tachycardia in the volunteers. Since nasal and throat samples were positive for V3526 and emerging febrile reactions co-occurred with this, the development of the vaccine was terminated. On the other hand, the safety profile provided by this vaccine was found to be excellent in animal models (141).

Inactivated vaccines for VEEV can elicit high nAb titers. In the 1970s, a new, formalin-inactivated vaccine for VEE (C-84) was developed by the US Army based on the TC-83 live attenuated vaccine. Incidental, mild, local and systemic reactions were only observed in 28 volunteers; there were no significant changes in clinical laboratory parameters. The vaccine increased preexisting serum-neutralizing antibody titers against the VEE virus in seropositive TC-83 vaccine recipients and elicited high nAb titers in non-immune subjects after a primary vaccine and two-dose vaccination (Table I) (184).

Live attenuated vaccines may be a potential VEE vaccine. Another research group commenced an investigation on the VEEV candidate vaccine on February 9, 2017. The study sponsored by the US Army Medical Research and Development Command was conducted in phase II and aimed to evaluate the safety and immunogenicity of the live attenuated vaccine for VEE, dried TC-83, in 500 healthy volunteers. 18-65 years of age. The volunteers were administered 0.50 ml of the VEE vaccine subcutaneously in the upper outer aspect of the triceps region. The estimated study completion date is on April 1, 2023 (ClinicalTrials.gov Identifier: NCT03051386) (Table I) (185).

DNA vaccines are safer than inactivated vaccines. On December, 2013, Hannaman *et al* designed a study to investigate the DNA vaccine candidate, which expressed the E3-E2-6K-E1 genes of VEE (pWRG/VEEV) and performed a phase I clinical study to assess the safety, reactogenicity, tolerability and immunogenicity of the vaccine. Participants were administered intramuscular or intradermal electroporation. In the intramuscular electroporation group, members received 0.50 mg, 2.00 mg of pWRG/VEE, or the saline placebo in a 1.0 ml injection, respectively. Participants in the other group (intradermal electroporation) received 0.08 or 0.30 mg of DNA or saline placebo in a 0.15 ml injection. The safety of each administration dose was assessed on days 0, 28 and 56. After two doses, all subjects exhibited measurable levels of nAbs. In addition, nAbs were detectable in samples from all subjects after the third vaccination (Table I) (186).

6. Ross River virus

Ross River virus (RRV) which belongs to the *Togaviridae* family of the Alphavirus genus and is a mosquito-transmitted virus that has a specific molecular characterization. This zoonotic, positive-strand RNA virus causes a rash, fever, fatigue and most prominently, arthralgia, which may persist for months to even years (Fig. 1) (187). This virus causes the most widespread vector-borne disease in Australia with >5.000 cases annually (188).

RRV has a complex ecology that has been isolated from >40 species of mosquitos and also infects/amplifies in at least 18 animal host species (Fig. 1) (189). There are currently no approved treatments or vaccines available against the virus. nAbs may be the only effective path to discovering new treatments and vaccine designs. There are human monoclonal antibodies designed for distantly related Alphavirus that binds to the Mxra8 Alphavirus receptor. With a cryo-electron microscope, the attachment of these antibodies with RRV reveals a conserved footprint of neutralizing monoclonal antibody RRV-12 in a region of the virus surface protein (187).

There are >80 copies of a trimer of heterodimeric glycoproteins on the surface of the virus. These heterodimers consist of two glycoproteins (E1 and E2), which are the target of nAbs (190).

The E2 glycoprotein is exclusively divided into three domains (A, B and C). The A domain, which is exposed at the surface, connects domains B and C. The B domain shields the fusion loop of the E1 protein and the C domain of the E2 glycoprotein. This B domain of E2 glycoprotein is the target site of monoclonal nAb in the RRV (191).

RRV-12 monoclonal antibody and its defined epitopes neutralize and prevent virus entrance to cells in a mouse model. Accordingly, this monoclonal antibody binds to the B domain of E2 glycoprotein; thus, it is a good candidate for further research on vaccine developments (187). CHK-265 cross-reactive murine antibody also inhibits and neutralizes the RRV, but this inhibition occurs more potently for CHIK and MAYV viruses (187).

The similarity of both RRV-12 and CHK-265 binding sites within the B domain reveals the importance of vaccine design based on epitope for research, particularly when the exact residue at the binding site is shown. For example, CHK-265 binds to residues 182-189, 203-206, and 214-218 in the B domain of the E2 glycoprotein (191).

Preclinical trials. In 2011, Holzer *et al* (192) performed a study to evaluate inactivated RRV vaccine in active and passive mouse immunization. A formalin- and UV-inactivated whole virus vaccine was derived from animal protein-free cell culture. In the first group, the mean active immunization group, female CD-1 mice received a solution of 500 μl containing vaccine doses of 10, 2.5, 0.625, 0.156, 0.039, 0.01 or 0.0025 μg, on days 0 and 28. After 42 days, the mice were challenged with the mouse-virulent RRV prototype strain T48 [ATCC VR-373] in a volume of 100 μl. Another group, the active immunization group (IFN-$\alpha/\beta R^{-/-}$), mice were injected with a solution of 50 μl containing vaccine doses of 1, 0.25 and 0.063 μg on days 0 and 21. At 42 days after the first shot, the mice were challenged with $10^{2.5}$ $TCID_{50}$ of the RRV prototype strain T48 in a volume of 10 μl. The passive immunization of young mice with sera from human vaccines was carried out using 100 μl complement-inactivated human serum intraperitoneally. After

24 h, the animals were challenged with 10^4 $TCID_{50}$ of infectious RRV T48. In that study, the vaccine elicited a potent antibody response in both models (192).

Clinical trials. Another investigation was performed in the same year with an inactivated whole-virus Vero cell-derived RRV vaccine in 382 healthy adults. Volunteers received 1.25, 2.5, 5, or 10 μg aluminum hydroxide-adjuvanted or non-adjuvanted RRV vaccine. After 21 days, the second dose was administered and consequently, booster doses were injected 6 months later. To evaluate the safety and immunogenicity of the vaccine, serum IgG and nAb titers were tested. The results indicated that the optimum concentration of the vaccine formulation was the adjuvanted 2.5 μg dose. Consequently, the candidate vaccine, adjuvanted inactivated whole-virus Vero cell-derived Ross River virus vaccine, was found to be highly immunogenic in RRV-naïve adults and was well-tolerated at all dose levels (Table I) (193).

7. Semliki Forest virus

Semliki Forest virus (SFV), as a positive-stranded RNA enveloped virus, is a member of the *Togaviridae* family and the Alphavirus genus along with the Sindbis and VEE virus, which are arthropod-borne (194). The genome of wild-type SFV expresses non-structural proteins that are in control of the transcription and replication of viral RNA and structural proteins, such as the capsid protein and envelope glycoproteins. The membrane envelope possesses the E1, E2, and E3 glycoproteins, which are involved in receptor distinction and fusion (195). Animal hosts for SFV differ from mosquitos to avian and mammalian species (Fig. 1) (196). Some vaccine strategies are based on peptides or protein subunits (197,198), recombinant vaccinia virus-vectored vaccines (199,200), adenovirus, or baculovirus-derived vectors (201,202) and naked DNA-based vaccines (203) are followed (204).

In the process of vaccine development, the SFV system was applied first as a DNA or an RNA-derived vaccine, in which the structural genes were replaced with an external gene (203,205). The recombinant SFV (rSFV) particles, which have the ability to infect host cells without replication, have been studied in mice in different virus models, such as the influenza virus (206), louping ill virus (207), human immunodeficiency virus (208), and human papillomavirus (209).

The inactivation of the SFV can be achieved using different processes, such as formalin, β-propiolactone, hydroxylamine and 2-ethyl ethylenimine; formalin inactivation appears to be the optimal process. These types of vaccines have been evaluated in white mice (210).

Various analyses have been conducted to assess the efficacy of different vaccine platforms, such as testing the recombinant SFV particles expressing the hepatitis C virus non-structural protein 3 (NS3) (211), and constructing and testing the infectious but non-replicative SFV particles encoding *Brucella abortus* Cu, Zn superoxide dismutase (SOD) (212-218). Vectors based on the SFV have been broadly tested *in vitro* and *in vivo* to express heterologous genes in animal cells (219).

Some clinical trials were conducted an RNA replicon vaccine based on the SFV, in which these replication-defective SFV replicon particles demonstrated potent immune responses in animal models against a variety of viral and tumor antigens (172,206,220-222). These preclinical studies have promised to certify other clinical studies of this vector platform (223).

Preclinical and clinical trials. To achieve the rSFV vectors, the structural genes were deleted from the SFV genome and replaced by the targeted gene. This genome was packaged in a viral particle containing a nucleocapsid and a membrane envelope (195). In order to develop a therapeutic vaccine against HPV-induced cancers, an SFV-based vector platform was utilized (224). An rSFV-based therapeutic vaccine, Vvax001, encoding a fusion protein of HPV16 E6 and E7, which is the first-in-human clinical, was developed by Komdeur *et al* (225). The results of the phase I revealed the safety of the Vvax001, which can elicit potent immune responses from participants (Table I) (225). Several studies have been conducted based on using rSFV-based RNA for vaccination against a range of pathogens, as for example in a study in which mice were immunized with rSFV RNA encoding HIV-1 HXB2 gp160 protein which led to an antigen-specific humoral immune response (108,226,227,228). In another study, the intradermal electroporation with the SFV-based RNA, expressing luciferase and galactosidase proteins, exerted potent cellular and humoral responses in mice (229). These vectors have also been used as antitumor vaccines (200). In another assessment, the potency of the SFV-based self-amplifying RNA as a recombinant vaccine against HIV-1C in mice was evaluated, as it is considered an encouraging approach for preventing the transmission and eradication of HIV/AIDS. Consequently, the extraction of clear cell-mediated and humoral immune responses in mice by rSFV2 gen RNA encoding HIV-1C antigens illustrated the potency of self-amplifying rSFV2gen RNA as a promising candidate for anti-HIV vaccine development (208). In the process of searching for new respiratory syncytial virus (RSV) vaccine candidates, a strategy based on an Alphavirus vector and SFV particles, which are self-abortive vector particles expressing RSV F and G proteins from an RNA replicon, was designed (206).

The analysis of recombinant RNA based on the SFV replicon expressing the nucleoprotein of the influenza virus was tested in mice, which demonstrated that the self-replicative recombinant SFV RNA may be useful as a nucleic acid vaccine (228).

The SFV expression system was estimated as a basis for avian vaccine development due to preliminary studies revealing that 1-day-old specific pathogen-free (SPF) chicks were sensitive to infection with a strain of SFV, generating SFV-specific antibodies, but exhibiting no clinical disease. Intramuscular immunization with recombinant replication-defective SFV particles, which express the *Escherichia coli* lacZ reporter gene, led to high titers of β-gal-specific antibodies at 4 weeks p.i. after two inoculations. However, significantly lower antibody levels were observed in chicks immunized with a recombinant SFV-based DNA construct or a conventional CMV promoter-based DNA

plasmid. rSFV particles which encode the protective VP2 protein or the VP2/VP4/VP3 polyprotein of infectious bursal disease virus (IBDV) were produced and the antigens that were expressed were characterized in cell culture. Proteins were generated and found to respond against a range of IBDV-specific monoclonal antibodies. The outcome of the immunization of 1-day-old SPF chicks with rSFV particles expressing the IBDV proteins was specific antibodies evoked in all birds and the production of nAbs in some, but not all birds (230).

8. Conclusions and future perspectives

Having noted the absence of effective therapeutic drugs and the rapid worldwide spread of viral infections, the development of novel vaccine technologies, such as recombinant subunit and DNA vaccines, should not be neglected. It is a fact that finding any single nucleotide polymorphisms in the genome of viruses may reduce the cross-protective immune responses against these (231). Therefore, finding much more effective and functional antiviral vaccines needs to be prioritized before the emergence of new viral pandemics. The present review aimed to provide sufficient data on various vaccine platforms in different stages of human clinical trials against the most significant zoonotic viruses of Coronavirus and Alphavirus. Over the past decades, the massive outbreaks of these viral families, such as MERS-CoV, SARS-CoV-2 and CHIKV, illustrate the essential need for stimulating research activities with large-scale investment to design and formulate new vaccine candidates in the future. As there are other viruses belonging to these families that have the propensity to 'spill over' around the globe, further investigations that aim to contribute toward the development of new vaccine generations are warranted. Consequently, these approaches may be employed when society requires to confront the next unavoidable contagious infection outbreak.

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AT, SSB and DM designed the study. ES, DAS and DM were involved in the screening of the literature for inclusion in the review. AT was involved in quality assessment. SSB, HZ, ES, NSG, MK, ST, DAS and HS were involved in the writing and revision of the manuscript. All authors have read and approved the final manuscript. Data authentication is not applicable.

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Not applicable.

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