GMO Deliberate Release Notification

Part 1A – Technical dossier

April 2021

"Phase I, single-centre, randomized, double blind, placebo-controlled study to assess safety, tolerability and immunogenicity of the hRVFV-4s vaccine in healthy subjects."

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List of Abbreviations

ALAT	Alanine aminotransferase
ALP	Alkaline phosphatase
BSL	Biosafety level
CEPI	Coalition for Epidemic Preparedness Innovations
COGEM	Netherlands Commission on Genetic Modification
CRO	Contract Research Organization
CTA	Clinical Trial Application
CTU	Clinical Trial Unit
D	Day
DIVA	Differentiation between Infected and Vaccinated Animals
DNA	Deoxyribonucleic acid
DSMB	Data safety monitoring board
DSP	Downstream processing
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-Linked Immunosorbent Assay
FAO	Food and Agriculture Organization
FIH	First in human
GMO	Genetically modified organism
GMP	Good manufacturing practice
hr	Hour
hRVFV-4s	Four-segmented Rift Valley fever virus for human application
HEP	Humane end-point
IFN	Interferon
IM	Intramuscular
IMP	Investigational Medicinal Product
IMPM	Investigational Medicinal Product Manual
IN	Intranasal
IP	Intraperitoneal
IPMA	Immunoperoxidase monolayer assay
ml	Millilitre
MSV	Master seed virus
MVSB	Master virus seed bank
NHP	Nonhuman primate
PBMC	Peripheral blood mononuclear cells
PCR	Polymerase chain reaction
PI	Principle investigator
RNA	Ribonucleic acid
RT-qPCR	Real-time quantitative PCR
RVF	Rift Valley fever
RVFV	Rift Valley fever virus
RVFV-4s	Four-segmented Rift Valley fever virus

SAE	Serious adverse event
SC	Subcutaneous
$TCID_{50}$	50% tissue culture infective dose
UN	United Nations
VNT	Virus neutralization test
vRVFV-4s	Four-segmented Rift Valley fever virus for veterinary application
WBVR	Wageningen Bioveterinary Research
WCB	Working cell bank
WHO	World Health Organization
OIE	Office International des Epizooties
ZKBS	Zentrale Kommission für die Biologische Sicherheit

Introduction

Rift Valley fever (RVF) is a disease that is caused by the mosquito-borne Rift Valley fever virus (RVFV). The virus is pathogenic to wild- and domesticated ruminants, with sheep being most susceptible. Outbreaks in sheep herds manifest with abortion storms and high neonatal mortality rates. RVFV infects humans via contact with animal products and via mosquito bites. Infected humans generally develop a self-limiting febrile illness. However, up to 2% of patients develop severe complications, including ocular disease with temporal or permanent loss of vision, encephalitis with occasionally severe neurological sequelae, and haemorrhagic icterus with a high case-fatality rate[1]. RVF is currently endemic to Africa and the Arabian Peninsula and the global distribution of mosquito vectors is likely to facilitate future outbreaks in currently unaffected areas[1]. Due to its ability to cause severe epidemics in the absence of effective countermeasures, RVFV is included on the World Health Organization (WHO) Blueprint list[2]. Whereas no human vaccines are available, two types of veterinary vaccines are commercially available in some African countries:

- Formalin-inactivated vaccines, formulated with aluminium hydroxide gel, can be applied safely in animals of all ages, including pregnant animals. However, multiple vaccinations and yearly re-vaccinations are required for optimal efficacy. Additionally, these vaccines are expensive to produce and the relatively slow onset of immunity renders these vaccines not optimal for rapid outbreak response.

- Conventional live-attenuated vaccines have also been developed for use in animals, such as the Smithburn vaccine, a virus that was attenuated by intracerebral passage in mice, and the attenuated Clone 13 strain, which is a naturally attenuated plaque-purified clone of strain 74HB59, isolated from a human case. These live-attenuated vaccines are highly efficacious in ruminants and elicit protective immunity after a single vaccination. However, both vaccines were shown to cross the ovine placenta, resulting in stillbirths and congenital malformations[3,4]. Thus, a veterinary vaccine that optimally combines efficacy with safety that can also be applied safely in pregnant animals, is urgently needed.

To control future epidemics effectively, a safe and effective vaccine for use in humans is also needed. Until today, two candidate vaccines have been evaluated in Phase 1 and Phase 2 clinical trials: an inactivated vaccine named “TSI-GSD 200” and a live-attenuated vaccine named “MP-12”[5-8].

- The TSI-GSD 200 vaccine requires a basic scheme with three vaccinations and subsequent annual booster vaccinations and even then not all subjects seroconvert[5]. The live-attenuated MP-12 vaccine seems more efficacious, inducing neutralizing antibody responses after a single vaccination[6]. However, this vaccine is surrounded by safety concerns: the molecular basis of MP-12 attenuation is unclear, hampering evaluation of the risk for reversion to virulence. This risk is non-negligible, as MP-12 induces viremia in humans[6,7]. Furthermore, MP-12 was shown to transmit vertically in sheep, resulting in fetal demise and congenital defects[9]. Although the risk of vertical transmission in humans is unclear, RVFV infections were previously associated with miscarriage[10] and recent *in vitro* studies have shown that RVFV can infect human placental cells[11]. These findings have resulted in concerns about the potential consequences of a RVFV infected during pregnancy[12].

Wageningen Bioveterinary Research (WBVR) has used a novel technology to develop candidate live-attenuated RVF vaccines for veterinary and human use. Both candidate vaccines were shown to be completely avirulent and not to be shed to the environment in experiments with animal models. The current document is the technical dossier of the human candidate vaccine, although also some relevant information is provided about the veterinary vaccine, underscoring the efficacy and safety of the technology applied.

The RVFV genome is divided into three segments named after their size: L (large), M (medium) and S (small) (Figure 1). The L segment encodes the viral RNA polymerase, or L protein. The S segment encodes a nucleocapsid (N) protein and a non-structural protein, named NSs. The NSs protein is the major virulence factor of RVFV, by counteracting host innate immune responses[13]. The M segment encodes a polyprotein that is co-translationally cleaved into two structural glycoproteins, Gn and Gc, involved in attachment to target cells and entry. Both glycoproteins are targets of virus neutralizing antibodies, of which Gn is immunodominant[14]. The M segment additionally encodes a non-structural anti-apoptotic protein, NSm[15], and a 78-kDa NSm-Gn fusion protein, referred as large glycoprotein (LGp), or P78, which plays a role in dissemination in mosquitoes[16].

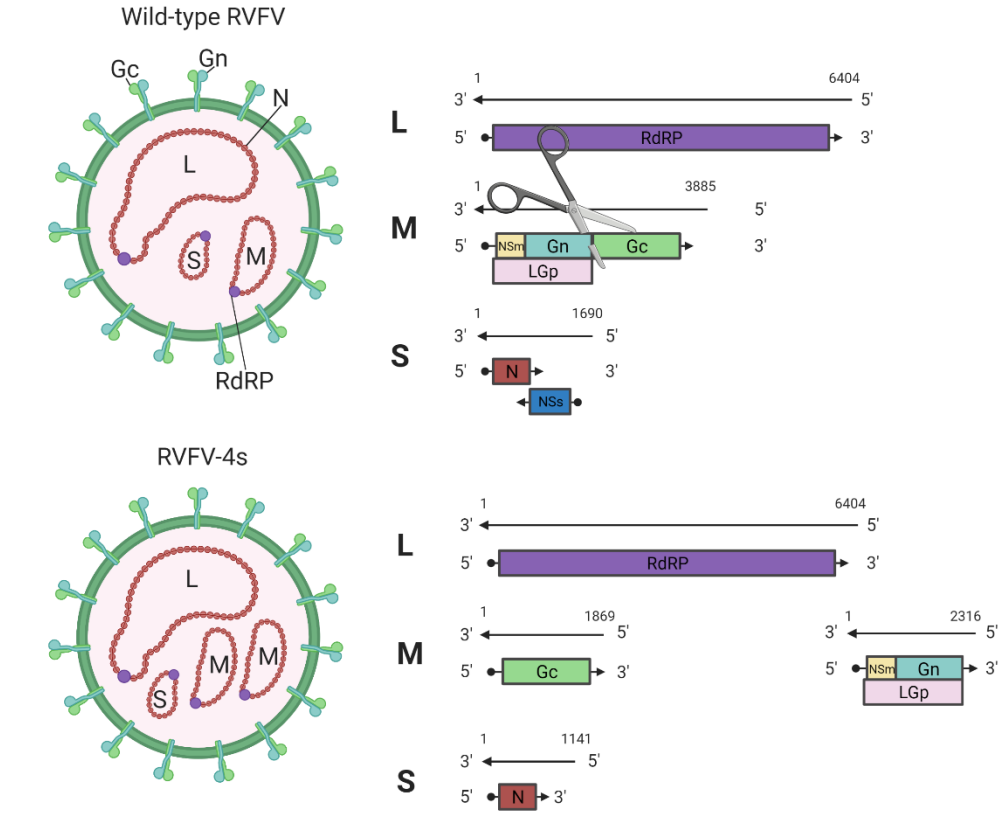

Figure 1: Schematic representation of wild-type RVFV and RVFV-4s (with deleted NSs expression and split M segment).

RVFV-4s vaccine viruses are developed by splitting the M genome segment into two M-type segments, one M-type segment encoding Gn (and accessory proteins NSm and LGp) and one M-type segment encoding Gc (Figure 1). Due to the further segmentation of the genome, assembly of infectious particles requires packaging of four, instead of three genome segments[17]. *In vitro* studies have demonstrated that packaging of four segments slows down the viral replication cycle, allowing the host's innate immune response to control dissemination effectively. To optimize vaccine safety, RVFV-4s viruses do not express the major virulence determinant NSs. The attenuating mutations (split M genome segment and deletion NSs) were shown to be stably maintained upon repeated passage *in vitro*.

The first RVFV-4s vaccine that was developed is based on RVFV strain 35/74, which was isolated from the liver of a sheep in 1974[18]. This strain was selected for the development of a veterinary vaccine, as the 35/74 strain originates from sheep, the primary target species of RVFV. The second vaccine that was developed

is based on Clone 13, a human isolate that naturally lacks 69% of the NSs gene[19]. This strain was selected for the development of a human vaccine as this strain replicates most efficiently in cells used for human vaccine manufacturing (Vero cells). Reverse genetics systems of both strains were used to split the M segment and, in the case of the 35/74-based RVFV-4s vaccine, to delete the NSs gene (69% of the NSs gene is already naturally absent in Clone 13).

To distinguish between the two vaccines, the veterinary vaccine is here referred to as **v**RVFV-4s and the human vaccine as **h**RVFV-4s. The vRVFV-4s vaccine was shown to be safe and immunogenic in mice[17], lambs[20], pregnant ewes[21], goats and cattle[20]. Although the vRVFV-4s vaccine will not be evaluated for human safety, the efficacy and safety data resulting from animal experiments with this vaccine strain underscore the safety and efficacy profile of hRVFV-4s. The candidate human vaccine, to be evaluated in the Phase I clinical trial, was shown to be avirulent in (nude) mice (Annex 1), rats (Annex 2), marmosets (Annex 3) and also in the most susceptible RVFV target species, being young lambs (Annex 4) and pregnant ewes[22]. This extensive non-clinical research has suggested that the vaccine viruses optimally combine safety with efficacy, holding great promise as both veterinary and human live-attenuated vaccines. RVFV-4s vaccines cannot revert to virulence and cannot disseminate, be shed or spread, and therefore hold no environmental risk of reassortment with wild-type RVFV as detailed below.

hRVFV-4s vaccination of the aforementioned range of animal models resulted in strong humoral responses, measured by a commercial ELISA detecting N antibodies and by the virus neutralization test (VNT). Importantly, the VNT is considered the 'gold standard' diagnostic serological test, having unprecedented specificity and sensitivity. The potency of the hRVFV-4s vaccine is exemplified by the minimal protective dose in lambs, the most susceptible target species. Lambs were fully protected upon a single IM vaccination with 10^5 tissue culture infective dose 50% ($TCID_{50}$) [20,23](Annex 4). Importantly, production of hRVFV-4s without additional concentration yields titres of 10^7 $TCID_{50}$/ml, heralding its excellent large-scale production potential. This means that 1 ml of the 'crude' vaccine before downstream processing (DSP) would equal the induction of complete protection of 100 individuals of the most susceptible target species.

After appropriate non-clinical evaluation, this first-in-human (FIH) trial hRVFV-4s aims to assess safety, tolerability and immunogenicity of a single dose of hRVFV-4s vaccine in 75 healthy volunteers aged ≥18 and ≤45 years. Although no untoward events, and no shedding to the environment occurred in experiments with animal models, results obtained with such models can never be extrapolated directly to humans. The safety for humans and the environment will therefore be addressed in this contained use trial.

I. GENERAL INFORMATION

A. NAME AND ADDRESS OF THE NOTIFIER

Wageningen Bioveterinary Research
Houtribweg 39
8221 RA Lelystad
the Netherlands

B. NAME, QUALIFICATIONS AND EXPERIENCE OF THE RESPONSIBLE SCIENTIST(S)

Prof. Dr. Jeroen Kortekaas
Department of Virology
Wageningen Bioveterinary Research

Prof. Kortekaas received his PhD from the University of Utrecht after completing his thesis on the Vaccine potential of iron-regulated outer membrane receptors of *Neisseria meningitidis*. In 2006, he moved to Wageningen Bioveterinary Research (formerly named Central Veterinary Institute). His main responsibility at WBVR is to develop vaccines for the control of notifiable transboundary veterinary, and zoonotic viral diseases. The first vaccine that was developed under his supervision can be applied for the control of Classical swine fever virus (*Flaviviridae*, genus Pestivirus) and enables the Differentiation between Infected and Vaccinated Animals (DIVA).

After this vaccine was transferred to industry, his focus shifted to bunyaviruses. From 2007 until today, the research of his team focuses on RVFV and other members of the order Bunyavirales. This work has resulted in the development of the vRVFV-4s and hRVFV-4s candidate vaccines. The vRVFV-4s vaccine is being further developed in collaboration with Ceva Animal Health, whereas the hRVFV-4s vaccine is being evaluated for human use in the LARISSA project financed by the Coalition for Epidemic Preparedness Innovations (CEPI).

Apart from his involvement in vaccine development and fundamental studies on arboviruses, he is an *ad hoc* member of several RVF expert panels of *e.g.* the World Organisation for Animal Health (Office International des Epizooties, OIE) and the Food and Agriculture Organization (FAO) of the United Nations. He is also member of the RVF Task Force, installed by CEPI. In November 2017, he was appointed Professor of Veterinary Arbovirology, affiliated with the Laboratory of Virology of Wageningen University. He is also member of the Netherlands Commission on Genetic Modification (COGEM) and member of the board of the Virology Division of the Royal Netherlands Society for Microbiology.

C. TITLE OF THE PROJECT

“Phase I, single-centre, randomized, double blind, placebo-controlled study to assess safety, tolerability and immunogenicity of the hRVFV-4s vaccine in healthy subjects.”

II. INFORMATION RELATING TO THE GMO

RVFV Clone 13, a human isolate that naturally lacks 69% of the NSs gene, is considered the “parent” or “recipient organism of the GMO, *i.e.* the candidate human vaccine hRVFV-4s.

A. CHARACTERISTICS OF THE RECIPIENT ORGANISM

1. Scientific name

Rift Valley fever virus (RVFV) Clone 13

2. Taxonomy

This is the taxonomy of RVFV, from which the candidate vaccine was derived

Phylum:	*Negarnaviricota*
Subphylum:	*Polyploviricotina*
Class:	*Ellioviricetes*
Order	*Bunyavirales*
Family	*Phenuiviridae* (formerly *Bunyaviridae*)
Genus	*Phlebovirus*
Species	*Rift Valley fever virus*
Strain	*Clone 13*

3. Other names

None

4. Phenotypic and genetic markers

The "recipient" organism is a negative-sense single-strand RNA virus, with an outer lipid envelope containing two glycoproteins, Gn and Gc, required for attachment to target cells and cell entry. The RVFV genome is divided into three segments named after their size: L (large), M (medium) and S (small):

- The L segment encodes the ribonucleic acid (RNA)-dependent RNA polymerase, or L protein.
- The S segment encodes a nucleocapsid (N) protein and a non-structural protein, named NSs. The NSs protein is the major virulence factor of RVFV. Clone 13 naturally lacks 69% (549 nucleotides) of the NSs gene.
- The M segment encodes a polyprotein that is co-translationally cleaved into two structural glycoproteins, Gn and Gc. The M segment additionally encodes a non-structural anti-apoptotic protein, NSm[15], and a 78-kDa NSm-Gn fusion protein, which plays a role in dissemination in mosquitoes[16].

5. Degree of relatedness between donor and recipient or between parental organisms

In the strict sense, there is no 'donor' or 'recipient' organism, as no (foreign) gene sequences were introduced into the hRVFV-4s genome. The virus differs from the parental Clone 13 strain in having a split M genome segment. Clone 13 is therefore considered the "parental" or "recipient" organism. The parent organism of Clone 13 is strain 74HB59, isolated from a human case in the Central African Republic[23].

6. Description of identification and detection techniques

RVFV (Clone 13, hRVFV-4s) can be identified by conventional reverse-transcriptase quantitative PCR (RT-qPCR) using RVFV-specific primers and probe and by Sanger- or next-generation sequencing. The virus can also be detected using antibodies against the N, Gn or Gc protein in Western blots, immunoperoxidase monolayer assays (IPMA) or virus neutralization test (VNT).

7. Sensitivity, reliability (in quantitative terms) and specificity of detection and identification techniques

Using IPMA, a single infectious particle can be detected after replication in a susceptible cell. Importantly, the ratio of infectious particles/viral RNA, as determined by our RT-qPCR of wild-type

RVFV, and our most sensitive virus isolation method, is about 1:1,000. This means that the RT-qPCR is highly suitable for showing absence of viral RNA and thereby both non-infectious and infectious virus. The RT-qPCR has been shown to be a reliable detection technique by testing thousands of samples, including serum, plasma and organ samples, from mice, sheep, goats, cattle and marmosets. Examples of the detection of RVFV RNA by RT-qPCR and isolation of the virus from different species are presented in Annexes 1-4)

8. Description of the geographic distribution and of the natural habitat of the organism including information on natural predators, preys, parasites and competitors, symbionts and hosts

Wild-type RVFV is endemic to most African countries and the Arabian Peninsula. The virus is pathogenic to wild- and domesticated ruminants among which it is transmitted exclusively via mosquitoes. Humans may become infected via contact with animal products, predominantly fluids released during the slaughtering of diseased animals, or via mosquito bite. Direct human to human transmission, including nosocomial transmission, was never reported. Humans are therefore considered as “dead-end” hosts.

RVFV was isolated from over 50 mosquito species collected in the field and laboratory experiments have suggested that a similar number may be capable of transmitting the virus under natural conditions[24]. Several of these mosquito species are prevalent outside the current habitat of the virus, suggesting that an introduction into a currently unaffected area may result in transmission and establishment.

The “parent” or “recipient” organism, Clone 13, is not pathogenic to any natural target species, with the exception of the ovine fetus[3]. Clone 13 does not cause (detectable levels of) viremia in even the most susceptible target species, and can therefore not be transmitted between humans, animals and mosquitoes. The hRVFV-4s is even further attenuated and was shown to be safe even when applied at a very high dose during ovine gestation[22]. Hence, Clone 13 and hRVFV-4s cannot survive in the environment.

9. Organisms with which transfer of genetic material is known to occur under natural conditions

Neither RVFV, nor any other member of the order Bunyavirales, is known to exchange genetic material with other organisms. A recombination event was never reported in literature.

10. Verification of the genetic stability of the organisms and factors affecting it

With respect to genetic stability, three different mechanisms have to be distinguished: 1) mutation, 2) recombination and 3) reassortment:

- With respect to mutation, it is relevant to note that RVFV is genetically very stable, accumulating mutations at an average of 2.9×10^{-4} substitutions/site/year, resulting in 5% genetic diversity among isolates[25]. With specific reference to Clone 13 (and the hRVFV-4s vaccine virus derived thereof), it is important to stress that the 69% (549 nucleotides) of the NSs gene, given its size, cannot restore upon replication of the virus.
- Recombination was never reported for any member of the order Bunyavirales.
- Reassortment was demonstrated to be possible *in vitro* and although some indications of reassortment events were noted in literature, this seems to be a rare event[26].

11. Pathological, ecological and physiological traits

a) classification of hazard according to existing Community rules concerning the protection of human health and/or the environment

The wild-type RVFV is classified as a biosafety level-3 (BSL-3) organism in Belgium[27], in line with Directive 2000/54/EC of the European Parliament and of the Council on the protection of workers from risks related to exposure to biological agents at work. The German "Zentrale Kommission für die Biologische Sicherheit" (ZKBS) has classified Clone 13 as a risk class 2 organism[28].
Importantly, the data demonstrating safety for laboratory animals and natural target species and absence of dissemination, shedding and spreading to the environment[20] was used to request downscaling to BSL-2 in the Netherlands, which has been approved (Annex 5, in Dutch). COGEM and the Netherlands Bureau Genetically Modified Organisms (Bureau GGO) subsequently recommended vaccination of animals under BSL-2 containment and allows housing of the animals under D-I conditions (minimal containment level in closed pasture) from 24h after vaccination.

b) generation time in natural ecosystems, sexual and asexual reproductive cycle

Infection of animals or humans with wild-type RVFV results in viremia within 2-6 days. During this period the virus can be transmitted, at least theoretically, via blood-feeding mosquitoes or via contact with animal- or human blood. However, humans are considered dead-end hosts, as viremia is believed to be not high enough to facilitate transmission via mosquitoes. Importantly, Clone 13 does not induce viremia in natural target species, making clear that Clone 13 cannot be transmitted via mosquitoes[3].

c) information on survival, including seasonability and the ability to form survival structures

Wild-type RVFV may survive on contaminated surfaces for a number of hours, but data on this is not available. Cases of non-vector borne transmission have not been described, making information on the potential survival of the virus less relevant for the risk assessment.

On the other hand, RVFV survives in vector organisms (mosquitoes). Climatic conditions that favour the expansion of mosquito populations may facilitate transmission of wild-type RVFV. However, the "parent" ("recipient") organism (Clone 13) does not induce viremia in even the most susceptible target species, rendering the risk of transmission to mosquitoes negligible.

d) pathogenicity: infectivity, toxigenicity, virulence, allergenicity, carrier (vector) of pathogen, possible vectors, host range including non-target organism. Possible activation of latent viruses (proviruses). Ability to colonise other organisms

The pathogenesis of wild-type RVF is described in a comprehensive review of Ikegami and Makino [29]. Briefly, wild-type RVFV causes a self-resolving flu-like illness in most (~98-99%) of infected humans. Clinical signs and symptoms are believed to result from replication of the virus in the liver, resulting in host innate immune responses, including the production of inflammatory cytokines. Particularly type I interferons are believed to be responsible for the influenza-like illness (fever, chills, weakness, nausea) that results from RVFV infection. In most cases the disease resolves within one to two weeks.

Up to 10% of infected humans develop ocular complications, resulting in temporal or permanent reduced sight or even blindness as a consequence of retinal lesions[30]. Wild-type RVFV infections can furthermore result in encephalitis which, when given supportive treatment, is generally not fatal. However, patients may suffer from life-long neurological sequelae. Patients that develop haemorrhagic fever have the poorest prognosis, with high fatality rate[29].

Importantly, the parent ("recipient") organism (Clone 13) was never associated with disease in humans.

e) antibiotic resistance, and potential use of these antibiotics in humans and domestic organisms for prophylaxis and therapy

Clone 13 is not susceptible to antibiotics and does not contain antibiotic resistance genes.

f) involvement in environmental processes: primary production, nutrient turnover, decomposition of organic matter, respiration, etc.

Clone 13 is not involved in any environmental process.

12. Nature of indigenous vectors

RVFV was isolated from over 50 mosquito species collected in the field and laboratory experiments have suggested that a similar number may be capable of transmitting the virus under natural conditions[24]. However, as Clone 13 does not induce viremia in natural target species, the risk of transmission via mosquitoes is considered negligible.

13. History of previous genetic modifications.

There have been no previous genetic modifications of Clone 13. As noted above, Clone 13 is a human isolate that naturally lacks 69% of the NSs gene[23].

B. CHARACTERISTICS OF THE VECTOR

1. Nature and source of the vector

For the construction of the candidate vaccine, the sequences of the S, M and L segments of Clone 13 were obtained from GenBank, accession numbers DQ375417.1 (L segment), DQ380213.1 (M segment), and DQ380182.1 (S segment).The cDNA sequences of the genome segments were introduced *in silico* into pUC57 plasmids, flanked by a T7 promoter sequence at the 5' end and a T7 terminator sequence and hepatitis delta virus ribozyme at the 3' ends. The L and S segments remained unchanged, whereas the M genome segment was split into two M-type genome segments, one encoding Gn, and accessory proteins NSm and LGp, and one encoding the Gc structural glycoprotein as described[17].

The resulting *in silico* designed plasmids were synthesized by the GenScript Corporation (Piscataway, NJ). Plasmid maps are given below and full genome sequences are provided as Annex 6.

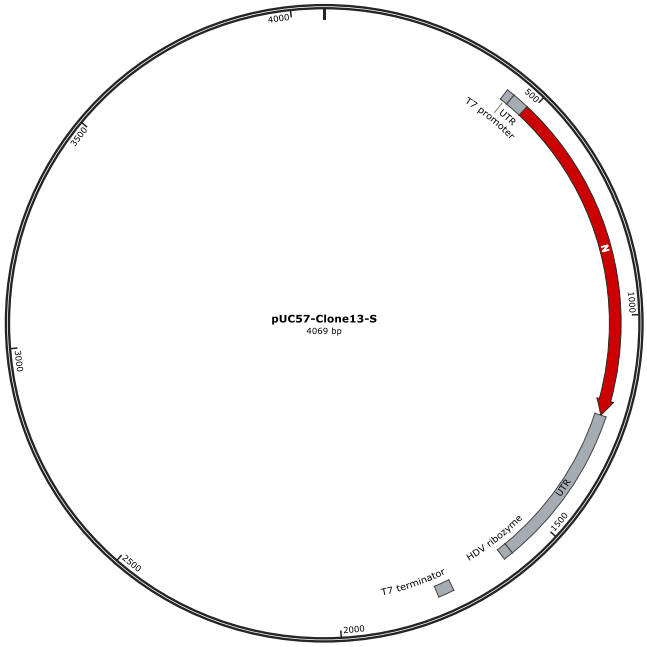

Figure 2A: pUC57 plasmid encoding the Clone 13 S segment

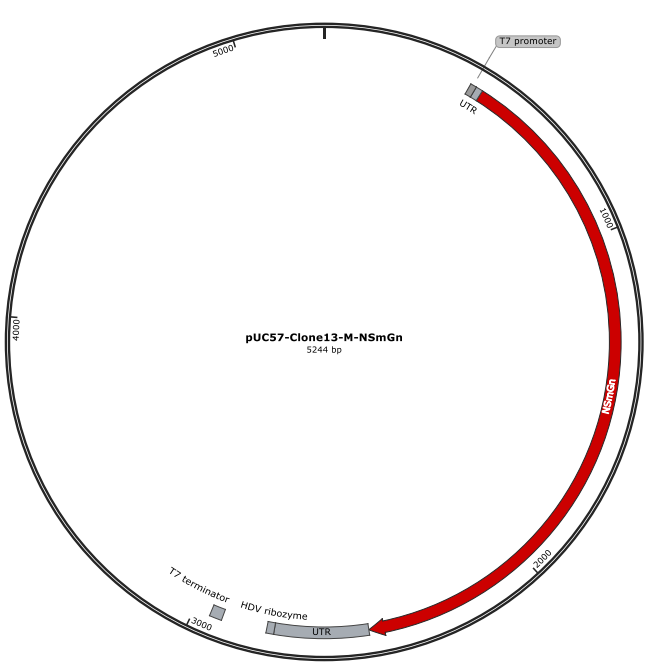

Figure 2B: pUC57 plasmid encoding the Clone 13 NSmGn sequence flanked with M segment UTRs

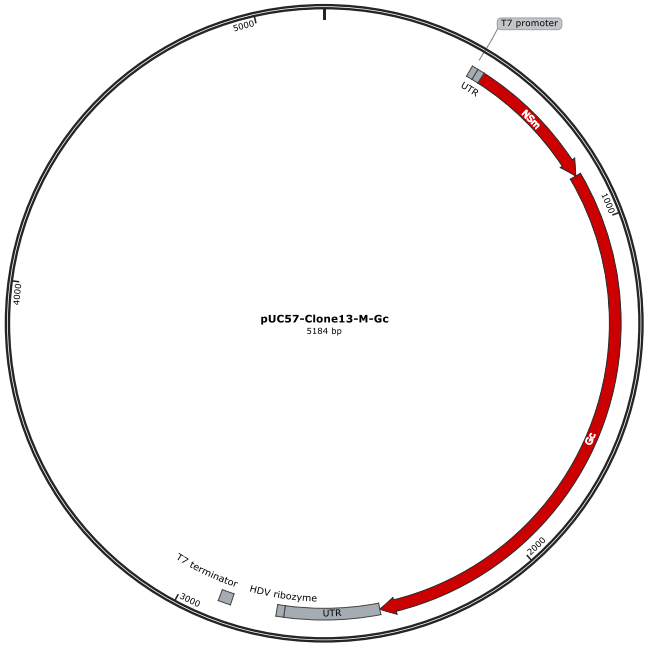

Figure 2C: pUC57 plasmid encoding the Clone 13 Gc gene flanked with M segment UTRs

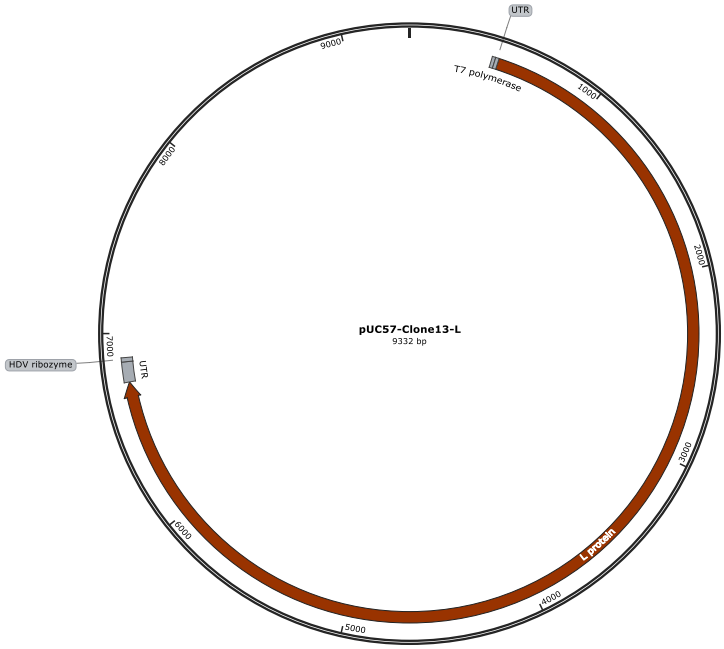

Figure 2D: pUC57 plasmid encoding the Clone 13 L segment

Plasmids were transfected into BSR-T7 cells. These cells are derived from baby-hamster kidney (BHK) cells and constitutively express bacteriophage T7 polymerase, required for transcription from the T7 promoter[31]. Introduction of the four plasmids, named pUC57-Clone13-S, pUC57-Clone13-M-Gn, pUC57-Clone13-M-Gc and pUC57-Clone13-L, resulted in the production of infectious particles in the supernatant. The correct sequence of the rescued virus was confirmed by next-generation sequencing (sequences provided as Annex 6). After rescue, the hRVFV-4s candidate vaccine was amplified in Vero cells.

2. Sequence of transposons, vectors and other non-coding genetic segments used to construct

See Section II.B.1.

3. Frequency of mobilisation of inserted vector and/or genetic transfer capabilities and methods of determination

Plasmids were used to rescue the vaccine virus. Vector (plasmid) DNA is not present in the GMO.

4. Information on the degree to which the vector is limited to the DNA required to perform the intended function.

Vectors were used to produce viral RNA sequences in Vero cells, resulting in the rescue of the vaccine virus. The plasmids have been completely characterized and sequenced, no unknown functions are present and they have been used routinely in research. Vector (plasmid) DNA is not present in the GMO.

C. CHARACTERISTICS OF THE MODIFIED ORGANISM

1. Information Relating to the Genetic Modification

a) methods used for the modification

The hRVFV-4s vaccine virus was rescued from copy DNA of the attenuated Clone 13 strain. The hRVFV-4s vaccine contains the S and L segments of Clone 13 and a split M segment. The modifications of the plasmids described in the previous section were made *in silico* and by standard cloning methods.

b) methods used to construct and introduce the insert(s) into the recipient or to delete a sequence

The modifications of the plasmids are described in the previous section, in particular Section II.B.1. The 69% deletion (549 nucleotides) of the NSs gene was already present in the RNA of the Clone 13 attenuated strain. The split M segment was created by *in silico* cloning followed by gene synthesis. The resulting plasmids were used to rescue the recombinant vaccine virus.

c) description of the insert and/or vector construction

The modifications of the plasmids are described in the previous section, in particular Section II.B.1.

d) purity of the insert from any unknown sequence and information on the degree to which the inserted sequence is limited to the DNA required to perform the intended function

The cDNA used for construction of the candidate vaccine was designed *in silico* and obtained from GenScript. No sequences of unknown source have been used.

e) methods and criteria used for selection

The modifications of the plasmids are described in the previous section. The correct sequence of the rescued virus was confirmed by full-genome next-generation sequencing and is provided as Annex 6. After rescue, the hRVFV-4s candidate vaccine was amplified in Vero cells. The Vero Master Cell Bank (MCB, continuous Vero W.H.O. derived from ECACC seed material cell line no. 88020401) cell line Lot: MCB 0020207 Passage 139) was generated at IDT Biologika and was used to generate a Working Cell Bank (WCB) suitable for production of human vaccines.
The RVFV-4s Master Virus Seed Bank (MVSB) was generated using Vero MCB or WCB derived cells. The Master Virus Seed Bank was tested for purity, content and identity.

f) sequence, functional identity and location of the altered/inserted/deleted nucleic acid segment(s) in question with particular reference to any known harmful sequence.

The candidate vaccine was rescued from cDNA, hence the product contains only the viral RNA segments, L, S, M-Gn and M-Gc. No new sequences have been introduced. The sequences of the different segments are provided in Annex 6.

2. Information on the final GMO

a) description of genetic trait(s) or phenotypic characteristics and in particular any new traits and characteristics which may be expressed or no longer expressed

The hRVFV-4s candidate vaccine differs in two major features from wild-type RVFV:

- First, as present in Clone 13, the NSs gene contains a deletion of 69% (549 nucleotides). This deletion ensures that hRVFV-4s is incapable of antagonizing the host innate immune response, including the type I IFN response.
- Second, the M segment of the virus is split into two M-type genome segments. This splitting of the M segment results in further attenuation by complicating genome packaging (the virus needs to package 4 instead of the naturally occurring 3 genome segments). Importantly, a RVFV-4s still containing an intact NSs gene was found to be completely avirulent in the highly sensitive BALB/c mouse model[17], demonstrating that the splitting of the M segment alone results in strong attenuation of the virus.

The combination of the 69% deletion in NSs and the split M genome segment resulted in a live-attenuated RVFV that is completely avirulent in all species evaluated: nude mice (Annex 1), rats (Annex 2), lambs (Annex 4), marmosets (Annex 3), and pregnant ewes[22].

b) structure and amount of any vector and/or donor nucleic acid remaining in the final construction of the modified organism

The hRVFV-4s candidate vaccine does not carry any foreign genetic material.

c) stability of the organism in terms of genetic traits

The hRVFV-4s virus was passaged >20 times in Vero cells and found to be genetically stable by next generation full-genome sequencing (Annex 6). Furthermore, using PCR, the stable maintenance of the split M segment and stable maintenance of the 69% deletion in NSs upon passage was confirmed (Figure 3).
Reconstruction of the M genome segment is theoretically possible through a recombination event. Considering that there are no overlapping sequences on the two M-type genome segments of the hRVFV-4s candidate vaccine, homologous recombination cannot occur. Although heterologous recombination can never be excluded (for any live-attenuated vaccine), it has never been observed in any bunyavirus. Furthermore, repeated passage (>20 times) of hRVFV-4s *in vitro* did not result in the restoration of the M segment. This exceeds the requirements for good manufacturing practice (GMP) and DSP, whereby genetic stability is generally demonstrated through 10 passages, to cover Master Seed Virus (MSV) through to vaccine product (as per WHO guidance on yellow fever vaccine manufacture).

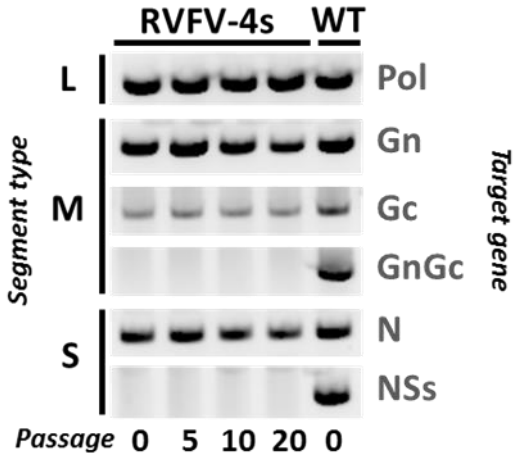

Figure 3. PCR amplification of individual open reading frames demonstrates stable maintenance of the split M genome segment and deletion of the NSs gene upon repeated passage in Vero cells.

d) rate and level of expression of the new genetic material/ method and sensitivity of measurement

There are no heterologous or new proteins expressed by hRVFV-4s as a result of the modifications. The modified sequences do not translate into modified protein sequences. The truncated NSs gene (as originally present in Clone 13) is translated, but results in an NSs fragment that is immediately degraded in the cytoplasm of infected cells[32].

e) activity of the expressed protein(s)

There are no heterologous proteins present in the vaccine. Only RVFV proteins are expressed: N protein, Gn protein, Gc protein, LGp protein, NSm, RNA dependent RNA polymerase (RdRp, or L protein). The NSs protein is not expressed (with exception of a truncated product that is rapidly degraded [32].

f) description of identification and detection techniques including techniques for the identification and detection of the inserted sequence and vector

The hRVFV-4s vaccine does not carry any foreign genetic material. The vaccine can be identified by conventional reverse-transcriptase quantitative PCR (RT-qPCR) using RVFV-specific primers and a probe. The hRVFV-4s vaccine virus can be identified by Sanger- or next-generation sequencing. The virus can also be detected using antibodies against the N, Gn or Gc protein in Western blots and IPMA.

g) sensitivity, reliability (in quantitative terms) and specificity of detection and identification techniques

See Section II.A.7

h) history of previous releases or uses of the GMO

So far, all activities with the candidate vaccine have been performed in BSL-2 containment (NB: BSL-2 containment of GMOs is named ML-II [laboratory] and DM-II [animal facilities] in the Netherlands). However, after demonstrating in these experiments that the virus does not induce viremia and is completely avirulent in all species evaluated, COGEM confirmed the environmental safety of the vaccine and has approved housing of RVFV-4s-vaccinated animals outside GMO containment facilities, 24 hrs after vaccination (Annex 5).

Non-clinical studies performed with the vaccine virus are summarized in Table 1.

Table 1: Overview of non-clinical experiments performed with hRVFV-4s n: number of animals included in the trial; intramuscular(IM), intranasal (IN) and/or subcutaneous (SC) route)

Species	n	10Log ($TCID_{50}$)	# of vaccinations	Route	Type of trial	Reference
Pregnant ewes	10	7	1	IM/SC	Safety	[22]
Mice	10	4	1	IN	Safety	[22]
Rats	10	4.8	1	IM	Immunogenicity	Annex 2
Rats	10	5.8	1	IM	Immunogenicity	Annex 2
Rats	10	6.8	1	IM	Immunogenicity	Annex 2
Lambs	8	5	1	IM	Efficacy	Annex 4
Lambs	8	6	2	IM	Efficacy	Annex 4
Lambs	8	7	1	IM	Efficacy	Annex 4
Nude mice	10	5	1	IM	Safety	Annex 1
Nude mice	10	6	1	IM	Safety	Annex 1
Nude mice	10	7	1	IM	Safety	Annex 1
Marmosets	6	5	1	IM	Safety	Annex 3
Marmosets	6	6	1	IM	Safety	Annex 3
Marmosets	6	7	1	IM	Safety	Annex 3

Safety and efficacy of hRVFV-4s in mice

Inoculation of BALB/c mice with wild-type RVFV via the intraperitoneal (IP) route generally results in 100% mortality within 3-4 days. Mortality results from widespread liver necrosis and is either acute or per-acute. Inoculation of BALB/c mice via the intranasal (IN) route results in fatal encephalitis, with massive virus replication in all areas of the brain. Mice that are not solidly protected by either passive or active immunization either succumb to liver necrosis with a slightly delayed onset of clinical signs or succumb from encephalitis after 8-14 days. Thus, mice develop both hepatic and encephalitic forms of the disease, which makes these animals particularly relevant for the first safety and efficacy evaluation of (human) vaccine candidates[29].

To establish the attenuating effect of splitting the M genome segment, BALB/c mice were inoculated with either wild-type RVFV or with the corresponding virus containing a split M segment and an intact NSs gene. The results of this experiment demonstrate that splitting of the M segment only is sufficient to completely abolish virulence in the mouse model[17]. No other experimental live-attenuated RVFV vaccine virus exist with the same level of attenuation. Importantly, the ability of RVFV to cause encephalitis in the mouse model does not depend on the NSs protein. Accordingly, IN inoculation of BALB/c mice with Clone 13 resulted in lethal encephalitis within 11 days, and is associated with high viral RNA levels in the brain[22]. In contrast, mice inoculated with hRVFV-4s via the IN route maintained normal bodyweights and none of these mice developed any clinical signs[22]. These results demonstrate that RVFV-4s is innocuous, even in the most susceptible (immunocompetent) mouse model.

Safety of the hRVFV-4s vaccine in nude mice

To gain further support for the safety of the hRVFV-4s vaccine, nude mice (mice lacking T cells) were inoculated via IM route with a dose of either $10^{4.4}$, $10^{5.4}$ or $10^{6.4}$ TCID50 (n=10/group). None of these mice developed clinical signs of disease. Importantly, with the exception of low viral RNA levels in 3/10 spleen samples in the high-dose group, most likely representing virus taken up by phagocytic cells (DCs and macrophages), all other organ samples, including testes, were negative for viral RNA on the day of necropsy (14 days post inoculation). Urine samples were also negative (Annex 1).

Safety and efficacy of hRVFV-4s in young lambs

To demonstrate hRVFV-4s immunogenicity and vaccine efficacy, lambs were vaccinated once via the IM route with escalating doses of the vaccine (10^5, 10^6 or 10^7 $TCID_{50}$) and challenged three weeks later with wild-type RVFV. All vaccinated lambs were fully protected, whereas all mock-vaccinated lambs developed high viremia and clinical signs (Annex 4). Importantly, no vaccine virus RNA was detected in daily collected blood samples, demonstrating that hRVFV-4s does not cause viremia. This makes the risk of dissemination, shedding and spreading, including transmission via vectors negligible.

Safety of hRVFV-4s in pregnant ewes

The most characteristic feature of RVF outbreaks are abortion storms, during which virtually all pregnant ewes abort their foetuses. The high susceptibility of the ovine foetus is also revealed by teratogenic effects associated with classical live-attenuated vaccines, like those based on the Smithburn strain[4] and Clone 13[3]. Experimental live-attenuated vaccines, including recombinant viruses lacking NSs expression, also were shown to cross the ovine placenta[33]. Vertical transmission may result in abortion, stillbirths, and severe teratogenic effects, such as hydranencephaly and arthrogryposis.

To study the safety of hRVFV-4s for the ovine foetus, pregnant ewes in the beginning of the second trimester were inoculated with a high dose (10^7 $TCID_{50}$) of hRVFV-4s via both the IM and subcutaneous (SC) routes. No hRVFV-4s viremia was detected by PCR and no clinical signs or other untoward effects were observed. Four weeks after inoculation, all ewes were euthanized and foetuses inspected for abnormalities. No pathology was noted in either ewes or foetuses. Moreover, no hRVFV-4s RNA was detected in organ samples from ewes or foetuses at necropsy[22]. Whereas the vaccine was completely safe for pregnant ewes and their foetuses, all ewes developed neutralizing antibodies within one week after vaccination[22].

Safety and immunogenicity of hRVFV-4s in the common marmoset (*Callithrix jacchus*)

The common marmoset is the most susceptible nonhuman primate (NHP) model for RVFV[34]. To assess the safety of the hRVFV-4s vaccine in this model, 3 groups of 6 marmosets were inoculated with escalating doses of hRVFV-4s: 10^5, 10^6, or 10^7 $TCID_{50}$ via IM route. A group of equal size was inoculated with a high dose (10^7 $TCID_{50}$) of the parent strain of Clone 13 and of hRVFV-4s, named 79HB59. Body temperatures were measured continuously with an implanted probe (Anipill) and activity with an Actiwatch mini. Body weights were measured daily. Blood samples were collected regularly for haematology, clinical chemistry and for measuring viremia. Animals were euthanized when a humane end-point (HEP) was reached or between days 21-24 followed by full necropsy and collection of organ samples. The results of this experiment are provided in Annex 3.

Briefly, significant changes in body weights were noted only in three animals inoculated with wild-type RVFV, two of which reached a HEP and were euthanized on days 13 and 14. Temperature reactions were observed in all animals, although the kinetics were strikingly different. In the group that received the wild-type virus, body temperatures started to increase on day 2, whereas this was observed in the groups that received the hRVFV-4s vaccine 24 hrs earlier. This increase in body temperature correlated with a peak in neutrophil levels in marmosets that had received the hRVFV-4s vaccine, suggesting that an innate immune response (primarily type I IFN) caused this temperature reaction. This peak in neutrophil levels was not observed in marmosets that had received the wild-type virus. We postulate that the NSs protein, as a demonstrated strong antagonist of innate immune responses, delays the type I IFN response in wild-type RVFV-inoculated marmosets. Importantly, the strong innate immune response detected in hRVFV-4s-inoculated marmosets could positively stimulate adaptive immunity.

Blood biochemistry demonstrated increased alanine aminotransferase (ALAT) and alkaline phosphatase (ALP) levels in two, respectively four wild-type RVFV-inoculated marmosets, suggestive of impaired liver function. Whereas one marmoset in the low dose group had an elevated ALAT level on one day, no further changes in blood biochemistry were noted in the hRVFV-4s-inoculated marmosets. From this, we conclude that a high dose of hRVFV-4s was well tolerated by the marmosets.

The marmoset study was also used to investigate potential persistence, dissemination, shedding and spreading of the vaccine virus. First of all, blood samples were analysed for the presence of viral

RNA. In marmosets inoculated with wild-type RVFV, viral RNA levels increased from day one, and peaked on day 3. Viral RNA levels in this group subsequently declined to undetectable levels on day 11. In marmosets inoculated with hRVFV-4s, viral RNA was measured on day 1, steadily declining to undetectable levels on day 6 or 7. Importantly, viral RNA levels never increased in these animals, hence, no evidence of virus replication was detected. Of note, considering the RNA genome copies that were injected (10^7 $TCID_{50}$ equals 10^{10} RNA copies) and the total blood volume of a marmoset (30 ml in a marmoset of 400 grams), a theoretical copy number of $10^{8.8}$ RNA copies/ml would be detected if all vaccine virus RNA would be released into the circulation. The average peak level $10^{6.4}$ RNA copies/ml measured, supports our hypothesis that replication of the hRVFV-4s vaccine virus is very limited. Furthermore, whereas infectious virus was isolated from marmosets inoculated with wild-type RVFV, no live virus was isolated from hRVFV-4s inoculated marmosets.
Analysis of organ samples, collected at necropsy, revealed viral RNA in different target organs of marmosets inoculated with wild-type RVFV, whereas only low levels of viral RNA were detected in lymphoid organs of hRVFV-4s inoculated marmosets, likely representing vaccine virus that was taken up by phagocytic cells (macrophages and DCs). Analysis of oral and rectal swabs revealed viral RNA in swabs of marmosets inoculated with wild-type RVFV, but not in hRVFV-4s-inoculated marmosets. This makes clear that hRVFV-4s is not shed from vaccinated marmosets.
Analysis of antibody responses by the commercial species-independent RVFV ELISA demonstrated that all marmosets seroconverted, and the VNT demonstrated that all marmosets developed neutralizing antibodies within 14 days post vaccination, with marmosets in the medium- and high dose groups developing these antibodies within 7 days.
From this, we can conclude that the hRVFV-4s vaccine can be applied safely in the most susceptible NHP model, with only manifestation temporal pyrexia. The vaccine virus does not disseminate to RVFV target organs and is not shed or spread to the environment from these animals. A single vaccination resulted in neutralizing antibody responses, suggesting that the vaccine would be protective in this species. A complete report of the marmoset trial is provided as Annex 3.

i) considerations for human health and animal health, as well as plant health

(i) toxic or allergenic effects of the GMOs and/or their metabolic products

There is no indication that the GMO is toxic or allergenic. However, to demonstrate absence of toxicity, a repeated dose toxicity study with rats will be performed before start of the Phase 1 clinical trial.

(ii) comparison of the modified organism to the donor, recipient or (where appropriate) parental organism regarding pathogenicity

Of note, the hRVFV-4s vaccine is compared with wild-type RVFV below, although the parent of the hRVFV-4s is actually the Clone 13 strain of RVFV. Clone 13 is avirulent in non-pregnant animals and thus a strongly attenuated strain of RVFV, attributed to the 69% deletion in the NSs gene.

Wild-type RVFV causes a self-limiting febrile (flu-like) illness in most (98-99%) infected humans. However, a significant number of infected humans may develop temporal or permanent blindness resulting from retinal damage. A small percentage (1-2%) of infected humans develop encephalitis or haemorrhagic fever, the latter with high fatality ratio.
Wild-type RVFV induces fatal liver disease after intraperitoneal inoculation of BALB/c mice (and several other inbred mouse strains) and fatal encephalitis when applied intranasally. The virus is also highly pathogenic to young lambs and pregnant ewes, which succumb to the infection and abort, respectively. Severe disease is associated with high viremia levels. A review on the pathogenesis of RVF is provided[29].
The “parent” or “recipient” organism of hRVFV-4s, is the Clone 13 strain. Clone 13 is used as a veterinary vaccine in South Africa and several surrounding countries recognizing South African vaccine registration. Clone 13 is avirulent in all three target species (sheep, goats, cattle)[35-37], although a high dose was shown to result in vertical transmission in pregnant ewes[3].

In strong contrast to wild-type RVFV, the hRVFV-4s vaccine is completely avirulent in all evaluated species: nude mice (Annex 1), rats (Annex 2), marmosets (Annex 3), lambs (Annex 4) and pregnant ewes[22]. No viremia was detected in any of these species, as determined by RT-qPCR and virus isolation.

(iii) capacity for colonisation

Colonization was never described for any bunyavirus and is therefore not expected to occur in humans. As noted in Section II.A.8, there is a limited host range and this would also apply to the candidate vaccine.

(iv) if the organism is pathogenic to humans who are immunocompetent:

- diseases caused and mechanism of pathogenicity including invasiveness and virulence,
- communicability,
- infective dose,
- host range, possibility of alteration,
- possibility of survival outside of human host,
- presence of vectors or means of dissemination,
- biological stability,
- antibiotic resistance patterns,
- allergenicity,
- availability of appropriate therapies.

-The virus is completely avirulent in all animal models evaluated and we therefore do not expect hRVFV-4s to be pathogenic to humans.
-The virus does not cause viremia in all animal models evaluated and we therefore do not expect that the vaccine can be transmitted from human to human (or animals) via mosquitoes.
-The infective dose is low in animal models (seroconversion in all vaccinated lambs after a single vaccination with 10^5 $TCID_{50}$), but unknown in humans.
-The host range of the vaccine is believed to be similar as that of the wild-type virus (ruminants and humans), although the tissue tropism is expected to be very restricted due to the strong attenuation.
-The vaccine virus may survive for a number of hours on surfaces (although this could never be confirmed), but cannot survive outside the host for longer periods.
-Whereas potential mosquito vectors of RVFV are present in Europe, the hRVFV-4s vaccine is not expected to cause viremia in humans. The risk of transmission via mosquitoes is therefore considered negligible.
-The vaccine virus (without formulation/freeze drying) can remain viable for months when stored at 4°C and many years at <-70°C. However, at ambient temperature, the virus in solution loses infectivity within days to weeks.
-The vaccine is not sensitive to antibiotics and the vaccine does not contain any antibiotics.
-The vaccine was not yet evaluated in humans, therefore no data on allergenicity is available. However, viruses have evolved to not induce any allergenic response as this would not be beneficial for replication.
-The vaccine is not expected to cause disease in humans, but no therapies are available.

(v) other product hazards

No other product hazards are known or anticipated.

III. INFORMATION RELATING TO THE CONDITIONS OF RELEASE AND THE RECEIVING ENVIRONMENT

A. INFORMATION ON THE RELEASE

The information on the intended release is based on the planning of the notifier and might be subject to possible modifications arising from indications before and during the clinical trial. Whenever such modification would arise, the GMO competent authorities will be informed, in particular if the modification might affect the GMO risk assessment.

1. Description of the proposed deliberate release, including the purpose(s) and foreseen products

After the hRVFV-4s vaccine is produced in GMP-compliant facilities, and the vaccine has successfully passed a repeated dose toxicity study, the proposed deliberate release marks the start of the Clinical Trial Application procedure to perform the clinical phase with a FIH, Phase I, single-centre, randomized, double blind, placebo-controlled study to assess safety, tolerability and immunogenicity of the hRVFV-4s vaccine in 75 healthy subjects aged ≥18 and ≤45 years.

A schematic overview of the Phase 1 study is provided as Figure .
Three study cohorts consisting of 25 subjects per cohort will be created to examine safety, tolerability and immunogenicity of increasing (low, intermediate and high) doses of the hRVFV-4s vaccine. In each cohort 20 participants will receive active substance and 5 will be given placebo via an intramuscular injection on Day 0. An interim analysis evaluating the safety profiles and magnitude of the antibody responses of the three vaccine doses will allow us to select the optimal dose.

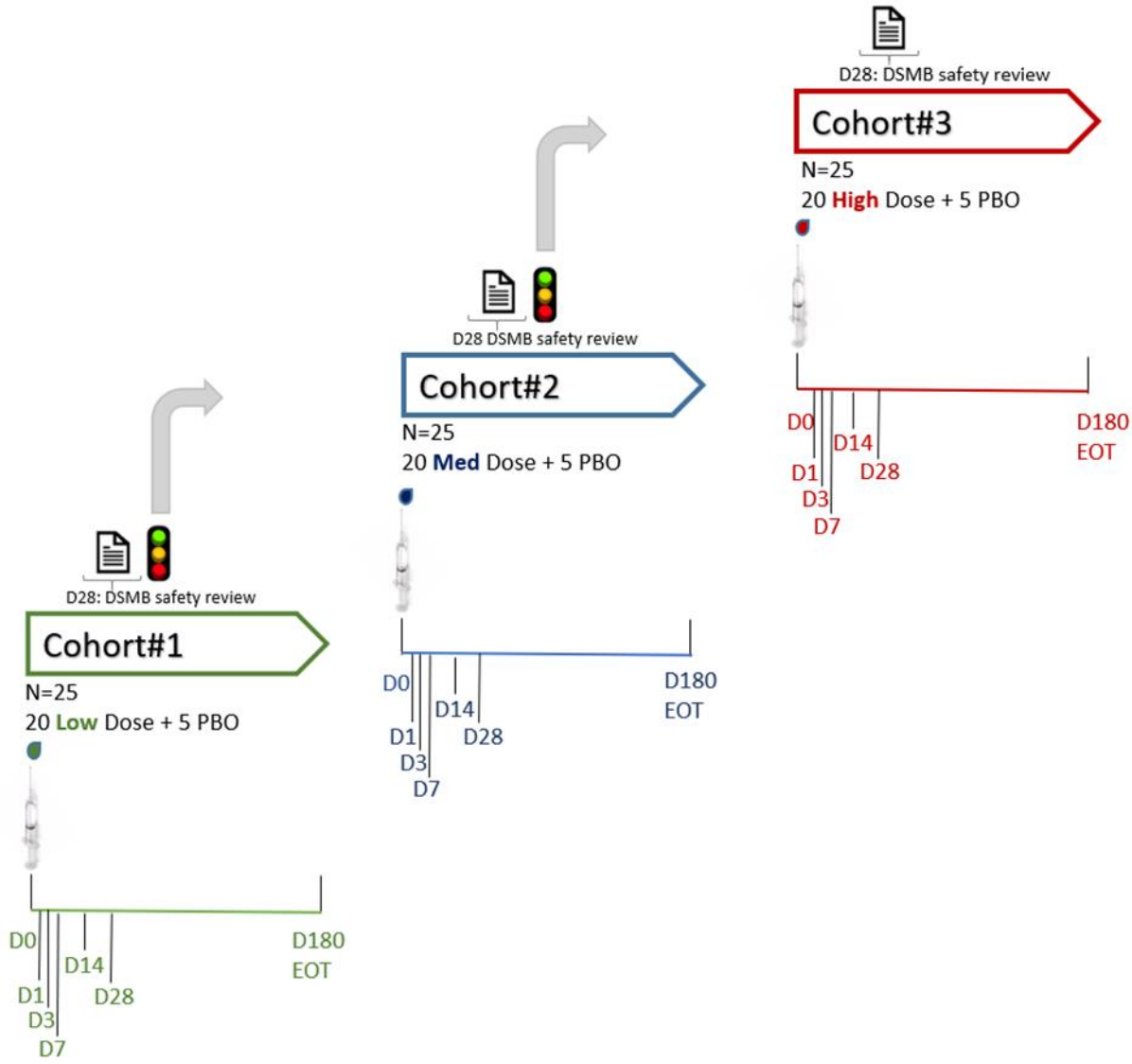

Figure 4: Schematic representation of the Phase 1 study

The study will consist of two phases:

- Active treatment phase from D0 to D28 after first vaccine administration, with unblinding and primary analysis by the DSMB of safety (incl. tolerability) and immunogenicity data collected up to D28 aimed at finding the optimal dose. The study team will remain blinded.
- Follow-up phase from D28+1 day to D180 post-vaccine administration to further evaluate safety and investigate immunogenicity.

Safety considerations for dosing and dose-escalation:
The study is designed to determine safety, tolerability and immunogenicity of hRVFV-4s, investigated at three different dose levels in 75 healthy adults in Belgium.
For safety reasons, an interval of at least 24 hours will be respected between the administration of the first dose of any dosage level to the first participant and the second participant. An additional interval of at least 24 hours will be respected between the administration of the second participant and subsequent participants. Subsequent subjects will be vaccinated with an interval of at least 60 minutes (1hr) to assess for immediate hypersensitivity reaction (within 60 minutes after vaccination). Each participant will be examined by a study physician 60 minutes after vaccine administration. Local and general adverse events and vital signs (pulse rate, blood pressure and oral body temperature) will be examined. If no clinically significant changes are observed, the participant can leave the center and will enter the ambulatory visit period (see schedule of assessments). Serious adverse events (SAEs) will be recorded throughout the duration of study. Subjects will visit the clinical site for safety monitoring on D0 (day of vaccine administration), and Days 1, 3, 7, and 28 after vaccine administration. Laboratory safety parameters will be examined using samples collected on these days.

The medical monitor and sponsor representative will perform a blinded safety assessment of data obtained until day 7 after the immunization of all subjects of the low/intermediate/high dose and advise whether administration of the next higher dose is justified.

The criteria (*i.e.* halting criteria) for considering a contraindication or preventive action to the start of the treatment of the 2nd or 3rd cohort (increasing dosage) or administration of test article to subsequent participants in the current cohort will be:

- the occurrence of one (1) SAE considered at least possibly related to the Investigational Medicinal Product (IMP) administration in one subject that belongs to the vaccine group;
- the occurrence of severe (grade 3) non-serious adverse events considered as at least possibly related to the IMP administration in two subjects belonging to the vaccine group in the same cohort, independent of within or not within the same system-organ-class. Both clinical and laboratory abnormalities are considered.

To ensure optimal safety for the subjects enrolled in this FIH trial the study will be conducted at the CEVAC-CTU. Together with CR2O (an experienced early stage infectious disease clinical CRO), CEVAC-CTU will be collectively responsible for assessing the safety, tolerability and immunogenicity of the hRVFV-4s vaccine in this study. CEVAC-LAB will prepare the serum samples and peripheral blood mononuclear cells (PBMC), cryopreserve and ship these samples to the laboratories that will execute the specific testing. CEVAC-CTU will be responsible for and acting as the clinical trial unit, whereas CR2O will be responsible for all CRO activities. Prior to study start, CR2O will have completed a site-readiness procedure ensuring all involved site staff are trained on respective legislation and guidelines, protocol specific procedures (including but not limited to subject recruitment and retention strategies).

2. Foreseen dates of the release and time planning of the experiment including frequency and duration of releases

Clinical trial is planned to initiate in November 2021. Completion will depend on availability of participants fulfilling all selection criteria. It is estimated to last at maximum until October 2022.

3. Preparation of the site previous to the release

The purified vaccine will be formulated in an optimized buffer containing a suitable stabilizer. The product will be filled in 2 R glass vials using a qualified container closure system. The presentation of the clinical trial material for the Phase 1 study will be liquid frozen. After filling, the vials will be capped, inspected for defects and stored until labelling and packaging takes place.

No specific preparation of the investigational site (clinical trial centre) is foreseen. According to the protocol, the material will be logged upon arrival and stored separately.

The primary release is the moment when the vaccine is prepared and administered to the participant. The preparation of the vaccine dose required for each cohort and placebo will be performed as instructed in the Investigational Medicinal Product Manual (IMPM) and/or trial protocol and documented within the IMP Preparation Logs at the hospital pharmacy by an unblinded pharmacist, independent of the (blinded) clinical staff. After vaccine preparation and dosing, all medical hazardous waste will be removed in UN-approved yellow polyethylene containers, collected by an approved transporter of medical hazardous waste and transported directly to one of the approved incinerators in Belgium.

While the location of the clinical trial centre will be known, the identity and coordinates of the participants will not be known to the notifier.

4. Size of the site

Not relevant.

5. Method(s) to be used for the release

The vaccine will be administered via IM injection.

6. Quantities of GMOs to be released

The candidate vaccine is provided to the clinical trial centre in 2R (2 ml) vials filled in 0.7 ml aliquots, sufficient for 1 dose (0.5 mL) per vial, and presented as solution for IM injection.
The GMO will be administered at a maximum dose of 10^7 $TCID_{50}$, present in a volume of 500µl.
Following administration, the hRVFV-4s candidate vaccine is not expected to multiply in the host (based on absence of viremia in non-clinical studies with nude mice, rats, lambs, pregnant ewes, and marmosets).

7. Disturbance on the site (type and method of cultivation, mining, irrigation, or other activities)

Not relevant.

8. Worker protection measures taken during the release

There are 2 manipulations during which the involved clinical staff may be exposed to the candidate vaccine:

1) handling the prepared doses of candidate vaccine from reception to administration (no direct contact given packaging)
2) administration of the candidate vaccine

Although still limited, the most important likelihood of exposure is in step 2, given that the vaccine is viable and present in high concentration.
Staff will wear a lab coat, disposable gloves, safety glasses and a face mask (FFP2). Disposable wipes will be used when handling samples. All waste material will be handled as hazardous medical waste.
Exposure during contact with participant in follow-up visits or when handling of samples collected from the participant and preparing for shipment is not expected, as no multiplication or shedding is anticipated. Irrespective, standard clinical practice will be implemented as a general workers protection measure.

9. Post-release treatment of the site

Type of site	Treatment
Clinical trial centre Preparation & administration	• Standard handling of all clinical material. • Collection of vaccine vials as hazardous medical waste • After the last visit of the last subject in the study, any used and unused vaccine will be returned to the Sponsor, or destroyed at the clinical trial site with the Sponsor's written permission.

10. Techniques foreseen for elimination or inactivation of the GMOs at the end of the experiment

For any handling in the clinical trial centre during preparation, administration or follow-up, either chemical inactivation or collection as hazardous medical waste for heat inactivation/incineration will be used. Chemical decontamination of surfaces or equipment contaminated with hRVFV-4s and chemical inactivation of the GMO will be done with hypochlorite (Javel) at 0.1% active chlorine. To this end, 1 part of Javel (5.5% chlorine) will be diluted approximately 50-fold in water. Minimal decontamination time will be 60 minutes.

The elimination will also take into account GCP requirements related to data-logging/reconciliation of vials and residuals of clinical trial material.

11. Information on, and results of, previous releases of the GMOs, especially at different scales and in different ecosystems

See previous sections, in particular Section II.C.2.

B. INFORMATION ON THE ENVIRONMENT (BOTH ON THE SITE AND IN THE WIDER ENVIRONMENT):

1. Geographical location and grid reference of the site(s) (in case of notifications under part C the site(s) of release will be the foreseen areas of use of the product)

The clinical trial centre is the Centre for Vaccinology (CEVAC), Ghent University Hospital
At this location, exposure and release into the environment will be limited (storage & administration, follow-up of participants and handling of samples).
The actual release will occur at the moment of the application to the participant. No subsequent shedding is expected.

2. Physical or biological proximity to humans and other significant biota

Obviously, the participants will be directly exposed to the candidate vaccine during vaccination. Viremia is not expected to occur (based on results from non-clinical studies: see Annexes) and therefore transmission to other humans or other RVFV-susceptible species (ruminants) is not expected.

3. Proximity to significant biotopes, protected areas, or drinking water supplies

Administration will only occur at the clinical trial centre.

4. Climatic characteristics of the region(s) likely to be affected

Not applicable.

5. Geographical, geological and pedological characteristics
Not applicable.

6. Flora and fauna, including crops, livestock and migratory species
Not applicable.

7. Description of target and non-target ecosystems likely to be affected
Not applicable.

8. Comparison of the natural habitat of the recipient organism with the proposed site(s) of release
Not applicable.

9. Any known planned developments or changes in land use in the region which could influence the environmental impact of the release
Not applicable.

IV. INFORMATION RELATING TO THE INTERACTIONS BETWEEN THE GMOs AND THE ENVIRONMENT

A. CHARACTERISTICS AFFECTING SURVIVAL, MULTIPLICATION AND DISSEMINATION

1. Biological features which affect survival, multiplication and dispersal
Wild-type RVFV only survives in the environment in either mosquito vectors or susceptible mammals. However, considering that the risk of viremia in vaccinated individuals is considered negligible, the risk of survival, multiplication and dispersal is also considered negligible.

The hRVFV-4s vaccine can only multiply in cells with a defective type I interferon pathway (Vero cells). Preclinical data confirmed that no detectable replication in target organs of even the most susceptible species occurs. Likewise, shedding was not observed.

2. Known or predicted environmental conditions which may affect survival, multiplication and dissemination (wind, water, soil, temperature, pH, etc.)
Considering that wild-type RVFV only survives in infected mosquitoes or susceptible mammals, no specific environmental conditions affect survival, multiplication and dissemination.

3. Sensitivity to specific agents
Wild-type RVFV and, hence, the hRVFV-4s vaccine virus is susceptible to low pH, lipid solvents and detergents, ether, chloroform and solutions of sodium or calcium hypochlorite with a residual chlorine content greater than 5,000 ppm.

B. INTERACTIONS WITH THE ENVIRONMENT

1. Predicted habitat of the GMOs

There is no natural habitat of hRVFV-4s and the very limited or even absent replication of the vaccine strain in inoculated animals, as expected in humans, renders the survival of the vaccine virus in the environment negligible.

2. Studies of the behaviour and characteristics of the GMOs and their ecological impact carried out in simulated natural environments, such as microcosms, growth rooms, greenhouses

Not applicable.

3. Genetic transfer capability

a) postrelease transfer of genetic material from GMOs into organisms in affected ecosystems

Recombination was never shown for any bunyavirus.

Genome segment reassortment is a theoretical possibility. However, as RVFV is not present in Belgium, the risk that a vaccinated person will be infected with wild-type RVFV is negligible. The finding that hRVFV-4s does not induce viremia in all species evaluated is another argument that the risk of reassortment is negligible. Importantly, even if a vaccinated person would be exposed to wild-type RVFV, the chance that the vaccine and wild-type viruses infect the same cell is negligible. Furthermore, even if reassortment would occur, this would pose no environmental risk: For reassortment to occur, the vaccinated person would have to be exposed to the wild-type virus (or *vice versa*). There would only be added risk of such an event, if exchange of a genome segment could result in novel tropism and/or virulence. However, as there is only one serotype and one pathotype of RVFV, a reassortment event would not result in a novel strain with enhanced tropism and/or virulence[26].

b) postrelease transfer of genetic material from indigenous organisms to the GMOs

The chance of this is negligible. See previous argumentation.

4. Likelihood of post-release selection leading to the expression of unexpected and/or undesirable traits in the modified organism

The genetic make-up of the hRVFV-4s vaccine has resulted in strong attenuation and thereby does not provide a selective advantage. The hRVFV-4s vaccine virus was found to be stable upon repeated passage in cells in which the virus replicates efficiently. Considering that vaccine virus is unlikely to replicate to significant levels in vaccinated individuals, the risk of post release selection leading to the expression of unexpected and/or undesirable traits is considered negligible.

5. Measures employed to ensure and to verify genetic stability. Description of genetic traits which may prevent or minimise dispersal of genetic material. Methods to verify genetic stability.

The genetic stability of the hRVFV-4s vaccine was demonstrated after repeated passage (>20 times) *in vitro*, using PCR of target genes (Figure 3). Whereas L, Gn and Gc genes were found intact, the gene encoding the Gn/Gc precursor polyprotein and the gene encoding NSs were not detected in hRVFV-4s preparations. Also, the full genome sequence of the hRVFV-4s vaccine virus was determined upon passage of the virus and was found to be stably maintained (Annex 6).

6. Routes of biological dispersal, known or potential modes of interaction with the disseminating agent, including inhalation, ingestion, surface contact, burrowing, etc

Considering that wild-type RVFV is likely to be infectious via aerosol exposure (although not experimentally demonstrated), the hRVFV-4s vaccine virus is also likely to be infectious via this route. Nevertheless, the very limited or even absent replication in even the most susceptible target species suggests that the risk of dissemination is negligible (the virus is not contagious).

7. Description of ecosystems to which the GMOs could be disseminated

After vaccine administration the injection site will be inspected for any leakage and covered with a wound dressing to prevent dissemination of the vaccine virus. Health care personnel administering the vaccine will wear personal protective material, including a facial shield and protective gloves. In order to avoid any introduction of the GMO in the environment, possible spills of the investigational material will be cleaned by trained personnel of the Phase 1 unit following the standard operation procedures that are in place. Therefore, introduction into an ecosystem outside the hospital setting is negligible.

8. Potential for excessive population increase in the environment

Viruses depend on host cells for survival and replication in the environment. Considering that the hRVFV-4s vaccine does not replicate, or to very low levels, in even the most susceptible animals, the risk of population increase is considered negligible.

9. Competitive advantage of the GMOs in relation to the unmodified recipient or parental organism(s)

The candidate vaccine has no competitive advantage in comparison with the unmodified recipient organism, Clone 13. On the contrary, the candidate vaccine is strongly attenuated.

10. Identification and description of the target organisms if applicable

There is no target organism in the strict sense. The candidate vaccine is expected to induce an immunogenic reaction in the vaccinated human participants.

11. Anticipated mechanism and result of interaction between the released GMOs and the target organism(s) if applicable

If humans are considered target organisms, then the goal of this research program is to develop a novel RVFV vaccine with an improved benefit/risk profile. IM inoculation of humans will result in exposure of target cells and expression of viral proteins. This expression of viral proteins is expected to result in innate and adaptive immune responses, as observed in experiments with animal models.

12. Identification and description of non-target organisms which may be adversely affected by the release of the GMO, and the anticipated mechanisms of any identified adverse interaction

Based on non-clinical studies with animal models, which have demonstrated that the vaccine does not disseminate and is not shed or spread to the environment, we consider the risk of transmission to non-target organisms negligible.

13. Likelihood of post-release shifts in biological interactions or in host range

The genetic modifications to attenuate the virulence of RVFV and construct the hRVFV-4s vaccine, have no influence on tropism or range of susceptible cell types and species. See also answer to question B12.

14. Known or predicted interactions with non-target organisms in the environment, including competitors, preys, hosts, symbionts, predators, parasites and pathogens
No specific interactions with non-target organisms have been identified.

15. Known or predicted involvement in biogeochemical processes
Not applicable

16. Other potential interactions with the environment
No other potential interactions with the environment have been identified.

V. INFORMATION ON MONITORING, CONTROL, WASTE TREATMENT AND EMERGENCY RESPONSE PLANS

A. MONITORING TECHNIQUES

1. Methods for tracing the GMOs, and for monitoring their effects
Although hRVFV-4s is not expected to cause viremia and not expected to disseminate in vaccinated study subjects based on non-clinical data (Annexes), after vaccination, the presence of the vaccine virus in blood samples, collected on days 0, 1, 3, 7, 14, 28 and 180, will be monitored by RT-qPCR. On the same days, urine and saliva samples will be collected, and evaluated for the presence of hRVFV-4s RNA and live virus. Semen will be investigated by analyses of samples collected on days -28, day 3 and day 14.

2. Specificity (to identity the GMOs, and to distinguish them from the donor, recipient or, where appropriate, the parental organism(s), sensitivity and reliability of the monitoring techniques
This has been addressed in Section II.A.7 and Section II.C.2.g).

3. Techniques for detecting transfer of the donated genetic material to other organisms
Dissemination, shedding and spreading of the vaccine virus will be monitored by RT-qPCR of blood samples, saliva, semen and urine samples.

4. Duration and frequency of the monitoring
The participants will visit the clinical trial centre at regular times for monitoring.

B. CONTROL OF THE RELEASE

1. Methods and procedures to avoid and/or minimise the spread of the GMOs beyond the site of release or the designated area for use
At the clinical trial centres, the material is stored and handled in controlled and contained conditions. Following administration, very limited shedding may occur from the injection site. The main instructions for avoiding exposure are indicated in Section III.A.9. No additional measures are taken to avoid or minimize the spread of the candidate vaccine.

2. Methods and procedures to protect the site from intrusion by unauthorised individuals

Until administration, the material is stored under controlled conditions and restricted access at the clinical trial centres. Similarly, any study sample will be securely stored. In both cases, unauthorised individuals will not have access to the material.
Once applied, no further protection is foreseen.

3. Methods and procedures to prevent other organisms from entering the site.

A similar reasoning as provided under V.B.2 can be developed. At the clinical trial centre standard precautions are in place to avoid other organisms (pets, insects, rodents, etc.) from entering the site and the storage equipment.
Once administered to the participant, family members and visitors, pets, insects and rodents are not expected to be exposed.

C. WASTE TREATMENT

1. Type of waste generated

Waste that can be expected to carry candidate vaccine is limited to materials at the clinical trial centres that contain or have been exposed to the hRVFV-4s vaccine (*e.g.* residual doses, empty containers, equipment used during visits of and sampling of participants, ..)

2. Expected amount of waste

The amount of waste generated at the clinical trial centres is not expected to be significant and will be within the normal handling capacity.

3. Description of treatment envisaged.

According to standard practices at the clinical trial centres, all waste is collected and treated as hazardous medical waste, *i.e.* collected in dedicated and certified bins, which are hermetically sealed and transported by a certified shipper to a specialized incineration facility. Surfaces and non-disposable materials will be chemically decontaminated with hypochlorite (Javel) at 0.1% active chorine. For this 1 part of Javel (5.5% chlorine) will be diluted approximately 50-fold in water. Minimal decontamination time will be 60 minutes.

D. EMERGENCY RESPONSE PLANS

1. Methods and procedures for controlling the GMOs in case of unexpected spread

Unexpected spread would mainly be limited to accidental opening of the packaged doses. Even if all the doses are spilled, the quantity remains limited (0.7 ml per dose) and can easily be handled via a spill procedure.

2. Methods for decontamination of the areas affected, for example eradication of the GMOs

The hRVFV-4s candidate vaccine can only infect the vaccinated individual. It is not expected to survive in the environment. If needed, and depending on the affected area, chemical disinfection can be used. Chemical decontamination of surfaces or equipment contaminated with RVFV-4s and chemical inactivation of the GMO will be done with hypochlorite (Javel) at 0.1% active chorine. For this 1 part of Javel (5.5% chlorine) will be diluted approximately 50-fold in water. Minimal decontamination time will be 60 minutes.

3. Methods for disposal or sanitation of plants, animals, soils, etc., that were exposed during or after the spread

Material that has been exposed to the candidate vaccine will be either disinfected or inactivated as hazardous medical waste. No specific sanitation measures are foreseen.

4. Methods for the isolation of the area affected by the spread

Before application and when handling samples, the area where the material is handled and hence where a spill could occur, will be physically isolated at the clinical trial centre.

5. Plans for protecting human health and the environment in case of the occurrence of an undesirable effect.

In the unexpected event that hRVFV-4s vaccination results in viremia, the risk of further dissemination is considered negligible, as transmission of RVFV from humans to other humans (or animals) via mosquitoes was never demonstrated. Humans are consequently considered dead-end hosts as they are not believed to develop sufficiently high viremia to enable transmission via mosquitoes. Accordingly, most human infections are attributed to contact with tissues of diseased animals (nosocomial transmission was also never reported).

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