LARiSSA

GMO Deliberate Release Notification

Part 3 – Environmental Risk Assessment

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

BHK	Baby-hamster kidney
cDNA	Copy DNA
CEVAC-CTU	Centre for Vaccinology – Clinical Trial Unit
CTFG	Clinical Trial Facilitation Group
DSP	Downstream processing
EDTA	ethylenediaminetetraacetic acid
ERA	Environmental Risk Assessment
FIH	first in human
GLP	Good Laboratory Practice
GMO	genetically modified organism
GMP	Good manufacturing practice
hRVFV-4s	Four-segmented Rift Valley fever virus for human use
IFN	Interferon
IM	Intramuscular
IMP	Investigational Medicinal Product
IMPM	Investigational Medicinal Product Manual
LGp	Large glycoprotein
MSV	Master seed virus
N	nucleocapsid
NHP	Nonhuman primate
PBMC	Peripheral blood mononuclear cells
PCR	Polymerase chain reaction
RdRp	ribonucleic acid (RNA)-dependent RNA polymerase
RNA	ribonucleic acid
RDT	Repeated dose toxicity study
RT-qPCR	Real-time quantitative PCR
RVF	Rift Valley fever
RVFV	Rift Valley fever virus
RVFV-4s	Four-segmented Rift Valley fever virus
SD	Sprague Dawley
$TCID_{50}$	50% tissue culture infective dose
UN	United Nations
vRVFV-4s	Four-segmented Rift Valley fever virus for veterinary use
WBVR	Wageningen Bioveterinary Research
WOCB	Women of childbearing potential
WHO	World Health Organization

Objective of the Environmental Risk Assessment

In the context of performing a clinical trial in the European Union with the hRVFV-4s candidate vaccine, this Environmental Risk Assessment (ERA) has been conducted in accordance with Annex IIA of European Directive 2001/18/EC and Commission Decision 2002/623/EC to identify and evaluate potential adverse effects of the candidate vaccine, either direct or indirect, immediate or delayed, on human health and the environment which the conduct of a clinical trial with this genetically modified organism (GMO) may exert. It is conducted with a view of identifying if there is a need for risk management and if so, to ensure that the most appropriate methods are used to mitigate this risk.

General Principles of the Environmental Risk Assessment

This ERA is performed according to the methodology laid out in Annex IIC of directive 2001/18/EC, supplemented by the guidance notes in Commission Decision 2002/623/EC and taking into account the precautionary principle:

- Characteristics of the GMO and its use, if any, which have the potential to cause adverse effects are identified and compared to those presented by the non-modified organism from which they are derived and its use under corresponding situations;
- The ERA is carried out in a scientifically sound and transparent manner based on the available scientific and technical data;
- The ERA is carried out on a case by case basis, taking into account the GMO concerned, its intended use and the potential receiving environment.

I. Characteristics of the GMO(s) and the release

A. The recipient organism

In the strict sense, there is no “recipient” organism, as no foreign gene sequences are present in the hRVFV-4s genome. The “parent” (which is referred here as “recipient”) is Clone 13, a plaque-purified clone of Rift Valley fever virus (RVFV) that was shown to be highly attenuated in animals naturally affected by wild-type RVFV[1-4]. For completeness, also the strain from which Clone 13 was derived, named 74HB59[2], is described below.

Wild-type RVFV

Wild-type RVFV affects livestock such as sheep, goats, cattle and camelids, causing a clinical illness termed Rift Valley fever (RVF), manifesting with fever, listlessness, and anorexia. Disease results from extensive virus replication in the liver, resulting in hepatic necrosis. RVF outbreaks are characterized by extremely high rates (>90%) of neonatal mortality and abortions in sheep flocks. Infection of other wild- and domesticated ruminants, may result in fatalities and abortions, although this is much more common in sheep[5]. In humans, RVF presents as a self-limiting febrile illness that progresses in a small percentage of cases (1-2%) to encephalitis or life-threatening haemorrhagic diatheses with high case fatality rates among hospitalized individuals (>30%)[6]. RVFV can infect human placental explants *in vitro*[7], which may underlie the observed association of RVFV infection with spontaneous abortions and stillbirths in pregnant women[8,9].

Infection of ruminants with wild-type RVFV results in viremia within 2-6 days[5]. During this period the virus can be transmitted to mosquitoes during blood feeding. When infected mosquitoes take a second blood meal, the virus can be transmitted to naive animals or humans. Importantly, humans are considered dead-end hosts, as viremia is believed to be not high enough to facilitate transmission via mosquitoes. Human to human transmission, including nosocomial transmission, was never reported.

Although RVF is considered a non-contagious disease, horizontal transmission from sheep to sheep was anecdotally reported in literature[10,11]. To address the possibility of horizontal transmission, a trial was performed in which young lambs were inoculated with a highly virulent strain and placed together with contact lambs. To facilitate detection of any potential horizontal transmission, contact lambs were immunosuppressed with dexamethasone treatment[12]. Contact lambs did not develop viremia and did not seroconvert for RVFV antibodies. This experiment confirmed that RVFV is not transmitted directly from animal to animal without involvement of vectors.

Wild-type RVFV is endemic to most African countries and the Arabian Peninsula. It was isolated from over 50 mosquito species collected in the field, and laboratory experiments have suggested that a similar number of mosquito species may be capable of transmitting the virus[13]. 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.

RVFV is an enveloped virus containing a three-segmented negative-sense RNA genome that 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 (RdRp), or L protein.
- The S segment encodes a nucleocapsid (N) protein and a non-structural protein, named NSs, which is the major virulence factor of RVFV (see below).
- The M segment encodes a polyprotein that is co-translationally cleaved into two structural glycoproteins, Gn and Gc. This segment additionally encodes a 14-kDa non-structural, anti-apoptotic protein, named NSm[14], and a 78-kDa NSm-Gn fusion protein, also called large glycoprotein (LGp), which plays a role in dissemination in mosquitoes[15].

Clone 13

Clone 13 is a naturally attenuated plaque-purified clone of strain 74HB59, which was obtained from a non-fatal human case during the 1974 RVF outbreak in Bangui, Central African Republic[2]. Clone 13 is unique in that it contains a 69% (549 nucleotides) deletion in the NSs gene, encoding the major virulence factor of RVFV. Clone 13 grows well in interferon (IFN)-incompetent (Vero) cells but is avirulent *in vivo*. It has no pathogenicity for mice[16], lambs[1], goats or cattle[4]. In addition, Clone 13 is highly immunogenic, leading to long-lasting immunity[1,3,4].

Importantly, as Clone 13 does not induce viremia in natural target species (sheep, goat, cattle), it cannot be transmitted via mosquitoes. Clone 13 is marketed by Onderstepoort Biological Products as a vaccine for veterinary use in South Africa and surrounding countries that recognize South African vaccine registration. With the objective to register the Clone 13 vaccine in Europe, safety trials were performed according to the requirements described in the European *Pharmacopeia*. These trials confirmed the safety of the vaccine for young lambs, even after repeated a dose or an overdose[17]. However, a trial in which an overdose was applied in pregnant ewes demonstrated that Clone 13 can cross the ovine placenta, resulting in stillbirths and congenital malformations[17]. The technology used to further attenuate the Clone 13 strain has resulted in a vaccine virus, hRVFV-4s, that was shown to be completely avirulent in pregnant ewes[18]. Please note that the “h” in hRVFV-4s was not yet used when the aforementioned manuscript was published.

B. The genetic modification(s)

For the construction of the candidate vaccine, the cDNA sequences of the S, M and L segments of Clone 13, flanked by a T7 promoter sequence at the 5’ ends and a T7 terminator sequence and hepatitis delta virus ribozyme at the 3’ ends, were introduced *in silico* into pUC57 plasmids. The L and S segments remained unchanged, whereas the M genome segment was split into two M-type genome segments, one encoding NSm/Gn and one encoding the Gc structural glycoprotein as described[19]. The resulting *in silico* designed plasmids were synthesized by the GenScript Corporation (Piscataway, NJ). Schematic representations of the genomes of wild-type RVFV, Clone 13 and hRVFV-4s are presented in Figure 1.

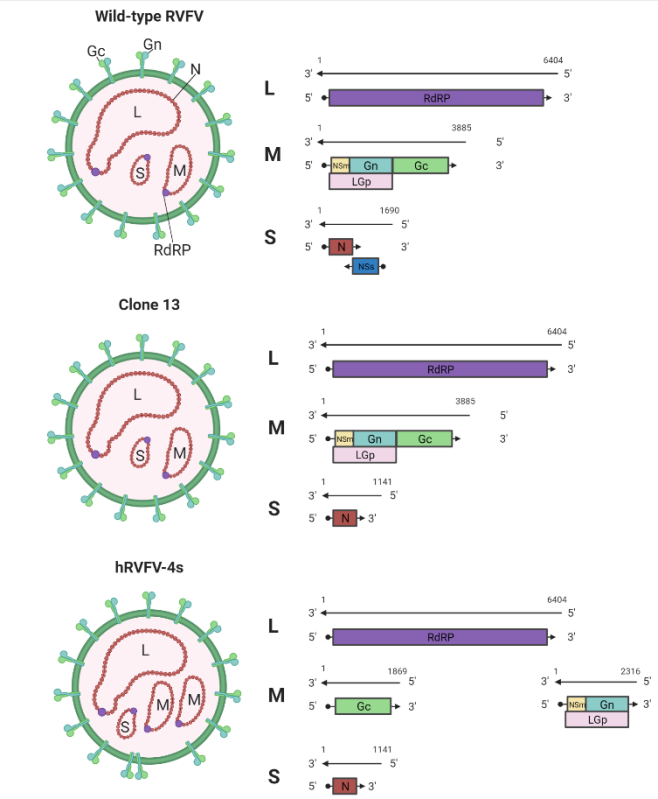

Figure 1: Schematic representation of wild-type RVFV, Clone 13 and hRVFV-4s (characterized by a 549 nucleotide deletion in the NSs gene and split M segment).

Plasmids were transfected into BSR-T7 cells[20]. These cells are derived from baby-hamster kidney (BHK) cells and constitutively express bacteriophage T7 polymerase, required for transcription from the T7 promoter. Introduction of the four plasmids, named pUC57-Clone13-S, pUC57-Clone13-M-Gn, pUC57-Clone13-M-Gc and pUC57-Clone13-L, resulted in the formation of the four-segmented RVFV[19]. The correct sequence of the rescued virus was confirmed by full genome sequencing.

C. The GMO

As detailed above, the hRVFV-4s vaccine contains the S and L segments and a split M segment, derived from the naturally attenuated Clone 13 strain. The hRVFV-4s candidate vaccine does not contain any foreign genetic material.

The hRVFV-4s vaccine was created by splitting the M genome segment into two M-type segments, one M segment encoding (NSm)Gn and one M segment encoding Gc (**Fout! Verwijzingsbron niet gevonden.**). Due to the further segmentation of the genome, assembly of infectious particles requires packaging of four, instead of three genome segments. *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[19]. The hRVFV-4s vaccine was shown to be completely avirulent and to be highly immunogenic in (nude) mice (Annex 1), rats (Annex 2), marmosets (Annex 3), lambs (Annex 4), and pregnant ewes [18].

D. The intended release

This “Phase I, single-centre, randomized, double blind, placebo-controlled study to assess safety, tolerability and immunogenicity of the hRVFV-4s vaccine in healthy subjects” is the first-in-human (FIH) trial in which hRVFV-4s will be assessed in 75 healthy volunteers aged between 18 (included) and 45 (included) years.

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 (Figure 2). In each cohort 20 participants will receive active substance and 5 will be given placebo via an intramuscular (IM) 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. After D180, the optimal dose, based on safety and immunogenicity, will be determined.

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. The capped vials will be shipped to the clinical trial centre, Centre for Vaccinology – Clinical Trial Unit (CEVAC-CTU), Ghent University Hospital.

The candidate vaccine is provided to CEVAC-CTU, in vials filled in 0.7 ml aliquots (0.5 ml extractable volume), sufficient for 1 dose 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 expected to replicate in infected cells but is not expected to disseminate in the host. No specific preparation of the clinical trial centre is foreseen. Care will be taken to avoid any loss or deterioration of the doses that are provided. 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 prepared 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.

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 assessed. If no clinically significant changes are observed, the participant can leave the centre and will enter the ambulatory visit period.

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.

Participants will visit the CEVAC-CTU for safety monitoring on D0 (day of vaccine administration), and Days 1, 3, 7, 14 and 28 after vaccine administration. 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 subject and the second subject. Only following a review of the safety data obtained after at least 24 hours by the PI, and in absence of any significant safety concerns, judged by the investigator, the second subject will be dosed. An additional interval of at least 24 hours will be respected between the administration of the second subject and subsequent subjects also allowing review of the safety data before dosing of the subsequent subjects. Laboratory safety parameters (haematology, biochemistry) will also be examined on all designated timepoints. CEVAC-LAB will prepare the serum, plasma, blood, saliva, urine, and semen samples and peripheral blood mononuclear cells (PBMC), cryopreserve and ship these samples to the laboratories that will execute the specific testing.

While the location of the CEVAC-CTU is known, the identity and coordinates of the participants will not be known to the notifier.

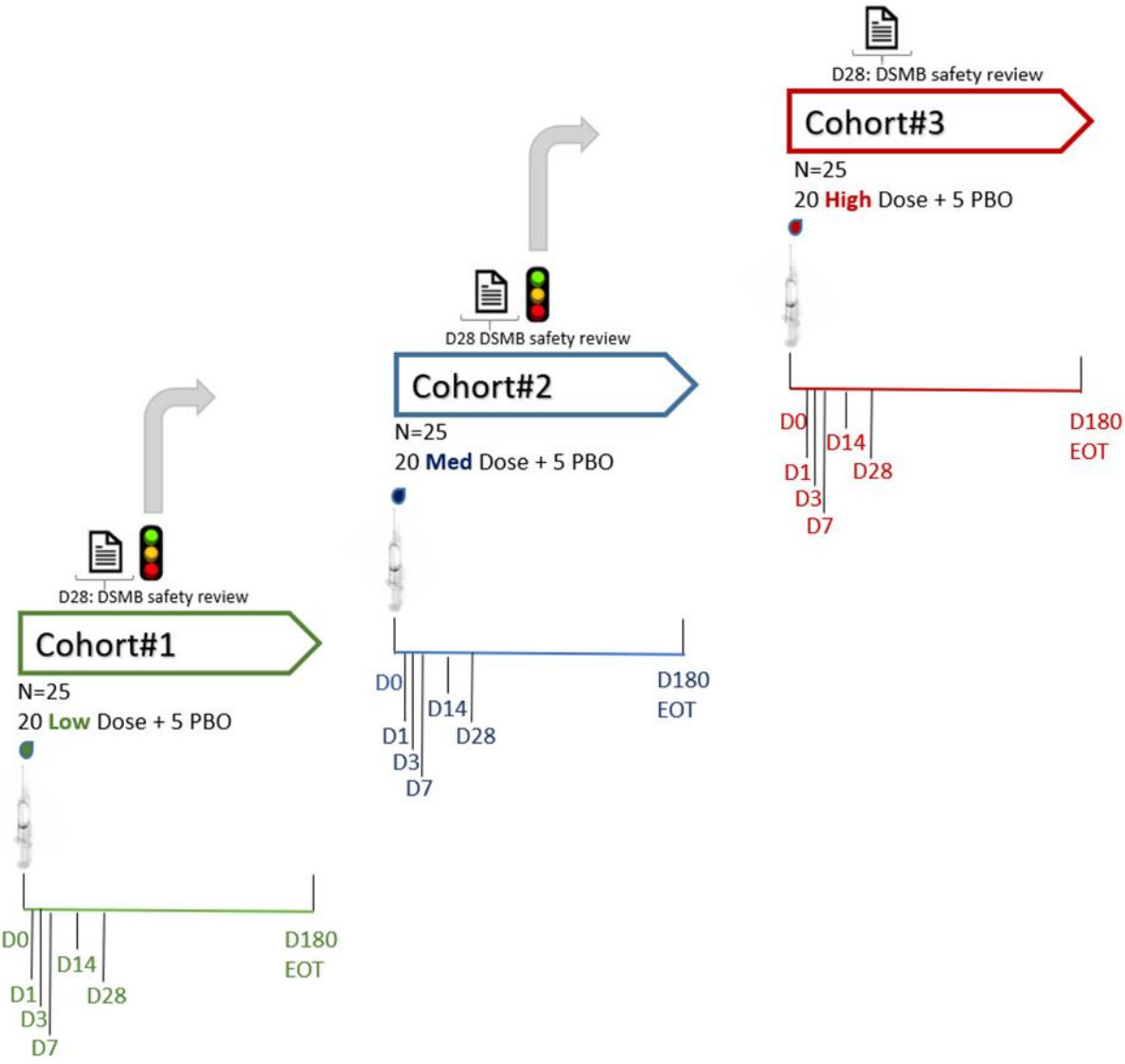

Figure 2: Schematic representation of the study outline.

E. The potential receiving environment

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

1) Handling the containers and bottles of candidate vaccine
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.

After injection of the vaccine, the injection site is covered with a wound dressing to prevent dissemination from the injection site. When the subject is allowed to return home, the wound dressing is disposed as hospital (GMO) waste and the injection site cleaned once more. Healthcare personnel administrating the vaccine will wear protective gloves. Also, the GMO could be introduced into an ecosystem via a spill. However, the Phase 1 clinical trial will be performed in a clinical trial centre and spill cleaning procedures are adopted. Therefore, introduction into an ecosystem outside the clinical trial centre setting is highly unlikely.

Exposure during contact with participants 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. Nevertheless, to investigate if indeed there is no shedding of the vaccine from humans, blood samples, saliva, urine and semen will be tested for the presence of vaccine virus. Specifically, blood, saliva and urine samples will be collected on days 0, 1, 3, 7, 14, 28 and 180, and evaluated for the presence of hRVFV-4s RNA and live virus. Semen will be investigated by analyses of semen samples collected on days -28, day 3 and day 14.

F. Interactions

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 (*e.g.* Vero cells). Preclinical data confirmed that no detectable replication in target organs of even the most susceptible species is observed. Likewise, shedding was not observed.

G. Information from releases of similar organisms and organisms with similar traits and their interaction with similar environments

The hRVFV-4s candidate vaccine is an attenuated form of the veterinary Clone 13 vaccine. Clone 13 was marketed by Onderstepoort Biological Products (South Africa) in 2010 and more than 19 million doses have been used in the field. Although this vaccine is only used for vaccination of animals, no reports of untoward events with respect to human health were ever reported.

Another experimental vaccine for human use, named MP-12, was previously evaluated in the US. MP-12 is an attenuated strain of RVFV that was developed by passage of a wild-type RVFV in cell culture in the presence of the mutagen 5-fluorouracil[21]. This resulted in the accumulation of mutations in all three genome segments[22]. Although the MP-12 vaccine is clearly attenuated in animals, the virus still induces viremia and can still cross the ovine placenta and induce congenital defects. Nevertheless, MP-12 was evaluated in human Phase 1 and Phase 2 clinical trials[23,24]. From these trials, it was concluded that MP-12 is safe and immunogenic in humans. Nevertheless, the residual virulence of MP-12, at least for sheep, as well as its ability to induce viremia in humans explains the remaining concerns about transmission to the environment, transmission to the foetus and reversion to virulence. In contrast, the hRVFV-4s vaccine does not induce any viremia in even the most susceptible target species and cannot revert to virulence due to the 69% deletion (549 nucleotides) of the NSs gene and the stably split M genome segment.

II. Environmental Risk Assessment

In this section, the characteristics of the GMO linked to the genetic modification that may result in adverse effects on human health or the environment are reviewed. They are based on a generic series of potential adverse effects of GMOs and some that are not applicable for the specific release were discarded at this first step of the risk assessment, *i.e.* the "identification of characteristics which may cause adverse effects". For others, the potential impact as well as the likelihood is further analysed, leading to an estimation of the risk for human health or the environment posed by each identified characteristic of the GMO.

A. Disease to humans, including allergenic or toxic effects

1. Identification of characteristics which may cause adverse effects

There are no characteristics of hRVFV-4s known that are expected to cause adverse effects.

Wild-type RVFV infection of humans results generally (98-99%) in a febrile, self-resolving illness. However, up to 10% of infected persons develop transient or permanent loss of vision resulting from retinal lesions, regardless of disease severity[25]. In a minority of cases, estimated at 1-2%, encephalitis or haemorrhagic fever may develop. Encephalitis may result in neurological sequelae and haemorrhagic fever has a high case fatality[6]. Importantly, the hRVFV-4s candidate vaccine is based on the Clone 13 strain, which lacks 69% (549 nucleotides) of the only known virulence factor of RVFV, the NSs protein[26].

The Clone 13 strain is non-pathogenic for laboratory animals (mice, rats, hamsters) as well as for target animals[1,3,4]. Whereas Clone 13 was reported to be safe also for pregnant animals[1], a study in which an overdose of Clone 13 was applied in pregnant animals resulted in vertical transmission, stillbirths and congenital malformations[17]. These elaborate studies have shown that Clone 13 is completely avirulent in all species evaluated, except pregnant ewes. Clone 13 was marketed by Onderstepoort Biological Products in South Africa in 2010, and more than 19 million doses have been used in the field. Due to its strong attenuation, The German "Zentrale Kommission für die Biologische Sicherheit" (ZKBS) classified Clone 13 as a risk class 2 organism[27]. Importantly, Clone 13 does not induce viremia in natural target species, making clear that Clone 13 cannot be transmitted via mosquitoes. Although Clone 13 is not used as a human vaccine, untoward events in humans were never reported.

Clone 13 has been modified with the aim to attenuate the virus even further. The candidate hRVFV-4s vaccine was created by splitting the M genome segment into two M-type segments, one M segment encoding Gn, and the accessory proteins NSm and 78-kDa protein (LGp), and one M segment encoding Gc. Due to the further segmentation of the genome, assembly of infectious particles requires packaging of four, instead of three genome segments. *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[19]. This strong attenuation is further confirmed by data showing that RVFV-4s still containing an intact NSs gene was found to be completely avirulent in the highly sensitive BALB/c mouse model[19].

The candidate hRVFV-4s vaccine thus combines 2 independent and complementary attenuating features providing a safe, highly attenuated vaccine:

- as present in Clone 13, the substantial deletion of 69% (549 nucleotides) of the NSs gene ensures that hRVFV-4s is incapable of antagonizing the host innate immune response, including the type I IFN response. Consequently, the hRVFV-4s vaccine does not, or very poorly, replicate in type I IFN competent cells. Importantly, if low-level replication would occur, the infection is expected to result in very low titres, prohibiting passage in cell culture (abortive infection).

- 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)[19].

Importantly, the hRVFV-4s vaccine was shown to be completely avirulent and to be highly immunogenic in (nude) mice (Annex 1), rats (Annex 2), marmosets (Annex 3), lambs (Annex 4), and pregnant ewes[18]. The candidate hRVFV-4s vaccine has no residual pathogenicity in all animal species evaluated. The risk of untoward effects in humans is therefore considered negligible.

hRVFV-4s does not cause viremia in even the most susceptible target species (young lambs, pregnant ewes and marmosets). 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. Nevertheless, although dissemination/shedding and spreading does not occur in animals and the fact that humans are less susceptible to the wild-type virus than ruminants, it is still important to confirm absence of shedding from humans. To this end, blood, saliva, urine and semen will be collected in the Phase I study and inspected for the presence of vaccine virus.

The common marmoset is the most susceptible nonhuman primate (NHP) model for RVFV[28]. 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.
The results show 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. A single vaccination resulted in neutralizing antibody responses, suggesting that the vaccine would be protective in this species.

There is no indication that the hRVFV-4s is toxic or allergenic. However, to evaluate potential toxicity of the vaccine, a GLP repeated dose toxicity (RDT) study will be performed with Sprague Dawley (SD) rats. The SD rat was selected as the model, as these rats were previously demonstrated to be highly susceptible to wild-type RVFV[29]. Immunogenicity of the hRVFV-4s vaccine in the same rat breed was confirmed before start of the RDT study (Annex 2).

2. Evaluation of the potential consequences of the adverse effect, if it occurs

No adverse effects are foreseen. The virus is not shed or spread to the environment even after inoculation of the most susceptible NHP species with the highest dose to be applied in the Phase I trial. The vaccine virus also does not cause viremia in the most susceptible target species, suggesting that the risk of transmission from vaccinated individuals via mosquitoes is negligible. If humans are exposed to hRVFV-4s, either during administration of the vaccine (needlestick incident) or after an incidental spill, no adverse effects are foreseen, apart from a potential immune response.
As noted above, the vaccine cannot revert to full virulence, as this would require a reconstruction of 69% (549 nucleotides) of the NSs gene, which is not possible. Additionally, the M segment cannot restore through homologous recombination, as homologous sequences are not present in the vaccine. The worst possible outcome following accidental exposure is seroconversion.

3. Evaluation of the likelihood of the occurrence of each identified potential adverse effect

- Shedding from vaccinated individuals is extremely unlikely, since the vaccine is not shed and does not spread from even the most susceptible animals.

- Transmission via mosquitoes is also extremely unlikely, since the vaccine virus does not cause viremia in even the most susceptible target species.

- Transmission via urine or semen is considered unlikely, as no vaccine virus/viral RNA was detected in urine or semen samples collected from marmosets inoculated with a high dose (Annex 3).

- Mutation: 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[30]. The hRVFV-4s virus was passaged >20 times in Vero cells and found to be genetically stable by next generation full-genome sequencing. Furthermore, using PCR, the stable maintenance of the split M segment and stable maintenance of the 69% deletion in NSs upon passage was confirmed.

- Reassortment: There are several arguments why genome segment reassortment would not occur, and even when this would occur, that it would not pose a risk to humans or the environment: First, whereas the occurrence of reassortment was anecdotally reported in literature, this was never confirmed to occur in nature[31]. Second, the hRVFV-4s vaccine is unlikely to cause viremia in humans, and without viremia, reassortment cannot occur. Third, for reassortment to occur, the wild-type RVFV should also be present in the individual. Considering that there is only one pathotype (no differences in virulence of strains) and one serotype of RVFV, such an event is unlikely to result in a strain that has an altered tropism and/or is more virulent than the wild-type virus. Fourth, as RVFV is not present in Belgium, the risk that a vaccinated person will be infected with wild-type RVFV is negligible. Participants of the Phase I study are not allowed to travel to countries where RVFV is endemic during the course of the study.

- Recombination was never reported for any member of the order Bunyavirales. Neither RVFV, nor any other member of the order Bunyavirales, is known to exchange genetic material with other organisms. 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 (See Technical dossier). This exceeds the requirements for good manufacturing practice (GMP) and downstream processing (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).

4. Estimation of the risk posed by each identified characteristic of the GMO(s)

Like wild-type RVFV, hRVFV-4s can theoretically infect liver cells, monocytes (DCs and macrophages), placental cells, and several additional cell types that are more sporadically found to become infected, at least in animals. Retinal cells and cells of the peripheral and central nervous system can potentially also be infected. However, considering that hRVFV-4s replication is very limited or absent in cells that have an intact interferon system (most cells of the human body, including the cells listed above), the hRVFV-4s vaccine is not expected to replicate to significant levels in cells of the vaccinated person. In contrast, inoculation in the muscle most likely results in uptake of the vaccine virus by dendritic cells, followed by transport to the local lymph node(s). In these lymph nodes, vaccine-derived antigens are presented to T and B cells. This hypothesis is supported by preclinical studies with hRVFV-4s in several species, including marmosets, in which the vaccine virus was exclusively detected in lymphoid organs, predominantly lymph nodes (Annex 3).

5. Application of management strategies for risks from the deliberate release or marketing of GMO(s)

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.

B. Disease to animals and plants including toxic, and where appropriate, allergenic effects

1. Identification of characteristics which may cause adverse effects

Other hosts may be wild- and livestock ruminants (cattle, sheep, goats, camels), as well as some rodent species and primates.

The candidate hRVFV-4s vaccine was evaluated in (nude)mice, rats, lambs, pregnant ewes, and marmosets. None of these non-clinical studies indicated any negative effect on the animals, even on the most susceptible target species (lambs and pregnant ewes). Non-clinical studies with natural target species showed that hRVFV-4s does not cause viremia. This makes the risk of dissemination, shedding and spreading, including transmission via vectors negligible.

Since no characteristics related to disease to animals and plants including toxic effects, and where appropriate, allergenic effects, were identified, this risk was not further evaluated.

C. Effects on the dynamics of populations of species in the receiving environment and the genetic diversity of each of these populations

Colonization was never described for any bunyavirus and is therefore not expected as a consequence of the trial. There is a limited host range and this would also apply to the candidate vaccine.

Since no characteristics related to effects on the dynamics of populations of species in the receiving environment and the genetic diversity of each of these populations were identified, the risk was not further evaluated.

D. Altered susceptibility to pathogens facilitating the dissemination of infectious diseases and/or creating new reservoirs or vectors

1. Identification of characteristics which may cause adverse effects

Infection of humans with wild-type RVFV results in a self-limiting febrile illness that progresses in a small percentage of cases (1-2%) to encephalitis or life-threatening haemorrhagic diatheses with high case fatality rates among hospitalized individuals (>30%)[6]. RVFV can infect human placental explants *in vitro*[7], which may underlie the observed association of RVFV infection with spontaneous abortions and stillbirths in pregnant women[8]. Although the pathology that results from wild-type RVFV

infection may predispose to other infections, no information about this is available. However, considering the strong attenuation of the Clone 13 virus and the demonstrated further attenuation of hRVFV-4s, no predisposition to any other infection is expected to result from vaccination with hRVFV-4s. Therefore, this risk was not further evaluated.

E. Compromising prophylactic or therapeutic medical, veterinary, or plant protection treatments

The hRVFV-4s vaccine virus does not contain any antibiotic resistance genes. Since no characteristics related to compromising prophylactic or therapeutic medical, veterinary, or plant protection treatments were identified, the risk was not further evaluated.

F. Effects on biogeochemistry(biogeochemical cycles)

Since no characteristics related to effects on biogeochemistry were identified, the risk was not further evaluated.

G. Determination of the overall risk of the GMO(s)

Based on the extensive non-clinical studies performed with the hRVFV-4s candidate vaccine in (nude) mice, lambs, pregnant ewes and marmosets, no untoward effects are expected in humans. Additionally no viremia, shedding or spreading to the environment is foreseen, either directly or via mosquitoes. The hRVFV-4s candidate vaccine is genetically stable and as detailed above, there is no environmental risk of reversion to virulence through recombination or reassortment.

Although we consider the risk of vertical transmission extremely small, the following precautions are made to exclude any untoward effects during pregnancy: Study subjects should not be pregnant and should prevent becoming pregnant until at least 6 months after administration of hRVSV-4s. Study subjects should commit to use adequate and effective contraception in accordance with the Clinical Trial Facilitation Group (CTFG) criteria, as detailed in the Clinical Study Protocol.
Women of childbearing potential (WOCBP) must use adequate and effective contraception means (CTFG criteria) for at least 60 days prior to the first hRVFV-4 administration. Male subjects should refrain from donating sperm, abstain from intercourse with a WOCBP or use a male condom and advise partner to use a highly effective contraceptive method until at least 6 months after hRVFV-4 administration.

Wild-type RVFV was previously isolated from urine of a patient[32]. Additionally, wild-type RVFV was previously detected in semen of an immunocompromised patient[33]. Although hRVFV-4s is not expected to cause viremia and not expected to disseminate in vaccinated study subjects based on non-clinical data (Annexes), the presence of hRVFV-4s in urine, semen and saliva will be investigated. To this end, urine samples will be collected on days 0, 1, 3, 7, 14, 28 and 180, and evaluated for the presence of hRVFV-4s RNA and live virus. Semen will be investigated by analyses of semen samples collected on days -28, day 3 and day 14. Finally, whereas live RVFV was never reported to be present in saliva samples, saliva samples will be collected on days 0, 1, 3, 7, 14, 28 and 180 and assayed for the presence of viral RNA and, when viral RNA is detected, for the presence of live virus.

III. Conclusions on the potential environmental impact from the release or the placing on the market of GMOs

On the basis of an ERA presented in Section II the following conclusions can be drawn:

- Likelihood of the GMO to become persistent and invasive in natural habitats under the conditions of the proposed release(s).
  Natural habitats of wild-type RVFV are ruminants and mosquitoes. Apart from the expected absence of viremia (as observed in non-clinical studies) and demonstrated absence of shedding and spreading to the environment, the only, albeit purely theoretical possibility how the hRVFV-4s vaccine could be introduced into the environment is via mosquito blood feeding on a study subject that, despite previous findings, develops sufficiently high viremia levels to enable transmission. Even if this would occur, the risk that such an event would result in continued transmission is negligible as direct transmission of RVFV from human to human via mosquitoes was never demonstrated.

- Any selective advantage or disadvantage conferred to the GMO and the likelihood of this becoming realised under the conditions of the proposed release(s).
  The hRVFV-4s vaccine is an attenuated form of the already highly attenuated Clone 13 strain of RVFV. Like Clone 13, the hRVFV-4s vaccine virus lacks 69% of the gene encoding the major virulence determinant, the NSs protein. The virus was attenuated further by splitting the M genome segment into two M-type segments. In consequence, the candidate vaccine has no competitive advantage in comparison with the unmodified recipient organism, Clone 13. In contrast, the hRVFV-4s vaccine is likely to replicate less efficiently as Clone 13 due to its augmented attenuation.

- Potential for gene transfer to other species under conditions of the proposed release of the GMO and any selective advantage or disadvantage conferred to those species.
  Bunyaviruses do not exchange genetic information with other viruses or organisms and the hRVFV-4s candidate vaccine does not contain any heterologous sequences. Gene transfer from the hRVFV-4s vaccine to wild-type RVFV would only be possible when both are present in the same individual in the same cell. The chance of such an event is negligible as RVFV is not present in Belgium. Furthermore, gene transfer from the vaccine to the wild-type RVFV genome would not provide a selective advantage. In contrast, reassortment events with wild-type RVFV would result in either the wild-type virus, or viruses with attenuated genome segments, either an S segment with a deletion in the NSs gene or one of the two M-type genome segments.

- Potential immediate and/or delayed environmental impact of the direct and indirect interactions between the GMO and target organisms (if applicable).
  Not applicable. There is no target organism in the strict sense. The candidate vaccine is expected to induce an immune response in the vaccinated human participants. 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.

- Potential immediate and/or delayed environmental impact of the direct and indirect interactions between the GMO with non-target organisms, including impact on population levels of competitors, prey, hosts, symbionts, predators, parasites and pathogens.
  No specific interactions with non-target organisms are expected. Based on non-clinical studies with four animal species, 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.

- Possible immediate and/or delayed effects on human health resulting from potential direct and indirect interactions of the GMO and persons working with, coming into contact with or in the vicinity of the GMO release.
  The hRVFV-4s vaccine is designed to protect humans from RVF. The hRVFV-4s vaccine is not expected to induce viremia as this was not observed in any of the four animal species evaluated. Non-clinical studies with the hRVFV-4s were conducted with nude mice (Annex 1), rats (Annex 2), marmosets (Annex 3), young lambs (Annex 4), and pregnant ewes[18], and safety was demonstrated in all these species. Hospital workers may accidentally come in contact with the hRVFV-4s vaccine preparation (*e.g.* shedding from the injection site after administering the vaccine), but based on the characteristics of hRVFV-4s, no untoward effects are foreseen.
  The NSs gene, encoding the major virulence determinant of RVFV, contains a 69% deletion, resulting in strongly reduced virulence and the M segment is split into two M-type segments, which was shown to independently attenuate the virus. No residual pathogenicity was detected in four animal species.

  The clinical staff will use good clinical practices and will therefore be protected against inadvertent exposure, should it occur, staff is protected by 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.

- Possible immediate and/or delayed effects on animal health and consequences for the feed/food chain resulting from consumption of the GMO and any product derived from it, if it is intended to be used as animal feed.
  Not applicable. The hRVFV-4s vaccine is not intended for livestock animals. It is extremely unlikely that animals would accidentally be exposed to the hRVFV-4s. Even if this is the case, this is not expect to result in any effect.

- Possible immediate and/or delayed effects on biogeochemical processes resulting from potential direct and indirect interactions of the GMO and target and non-target organisms in the vicinity of the GMO release(s).
  Not applicable. The hRVFV-4s vaccine will be administered to healthy volunteers in a clinical setting. The risk of dissemination, shedding or spreading from humans to the environment is estimated to be negligible, as none of these effects are observed in susceptible animal species. Furthermore, RVFV or any of its derivatives is not known to affect biogeochemical processes.

- Possible immediate and/or delayed, direct and indirect environmental impacts of the specific techniques used for the management of the GMO where these are different from those used for non-GMOs.
  The techniques used for the management of the hRVFV-4s vaccine are identical to routine vaccination clinical trials. No specific impact is expected.

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