1.1 INFORMATION REQUIRED IN NOTIFICATIONS CONCERNING RELEASES OF GENETICALLY MODIFIED ORGANISMS OTHER THAN HIGHER PLANTS

1.1.1 . General information

1.1.1. A. Name and address of the notifier

Intervet International B.V.
Wim de Körverstraat 35
NL - 5831 AN Boxmeer

1.1.1.B Name, qualifications and experience of the responsible scientist(s)

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1.1.2 Information relating to the GMO

1.1.2.A Characteristic of the recipient or (when appropriate) parental organism

1.1.2.A.1. Scientific name

Rhodococcus equi

1.1.2.A.2. Taxonomy

Family: Nocardiaceae
Genus: *Rhodococcus*
Species: *Rhodococcus equi*

1.1.2.A.3. Other names (usual name, strain, name, etc.)

Rhodococcus equi strain RE1

1.1.2.A.4. Phenotypic and genotypic markers

Characteristics:
- Growth on blood agar plates: small, smooth, shiny and non-haemolytic colonies after 24 hours' incubation.
- Growth at 30°C (±30-35 hours)
- Gram stain: positive rods

Catalase-, urease- and nitrate-positive, acetamidase- and nicotinamidase-negative

Growth on sole carbon sources (% $^{w}/_{v}$)
- Maltose (1.0): negative
- Mannitol (1.0): negative
- Sodium lactate (0.1): positive
- Glycerol (1.0): negative
- Sucrose (1.0): positive
- Trehalose (1.0): positive

Genotypic Markers
Positive by TRAVAP-PCR (Ocampo-Sosa *et al.* 2007, Annex 2) for *vapA* and traA and negative for *vapB*. The strain belongs to the TRAVAP category $tra^{+}/vapA^{+}B^{-}$.

1.1.2.A.5. Degree of relation between donor and recipient or between parental organisms

Not applicable.

1.1.2.A.6. Description of identification and detection techniques

After reisolation on selective agar (40 g/L blood agar base no. 2, supplemented with 6% defibrinated sheep blood, 3.125 µg/ml trimethoprim, 3.125 µg/ml cefoperazone sodium salt, 6.25 µg/ml polymyxin B sulphate, 50 µg/ml naladixic acid sodium salt and 1.5 µg/ml fungizone) *R. equi* can be identified by the phenotypic markers as described under 1.1.2.A.4. Single colonies can be analyzed by TRAVAP-PCR (Ocampo-Sosa *et al.* 2007, Annex 2).

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

Reisolation on selective agar as described in 1.1.2.A.6 is the most sensitive method. The reliability and sensitivity is dependent on the type of sample. The detection limit in faeces was found to be approximately 1.5 CFU/mg of faeces **INFORMATION DELETED FOR CONFIDENTIALITY PURPOSES.** The sensitivity in soil was not tested but since 1 mg soil contains much less bacteria compared to faeces, it can be assumed that sensitivity of the test for detection of *Rhodococcus equi* in soil is <1.5 CFU/mg.
The identity of isolated colonies (wild type or vaccine strain) can be confirmed by PCR as described in chapter 1.1.2.C.1.(f).

1.1.2.A.8. Description of the geographic distribution and of the natural habitat of the organism including information on symbionts and hosts;

The bacterium *R. equi* is a well-recognised pathogen in foals that represents a consistent and serious risk worldwide (Muscatello *et. al*. 2007, Annex 1). *R. equi* is a facultative pathogenic soil saprophyte and is therefore found in soil, especially where domesticated livestock graze. The habit of foals to consume fresh faeces may play an important role in the spreading of the pathogen. The manure of horses and other animals is the source of soil contamination. In addition to the presence of manure, temperature and pH have an influence on the multiplication of *R. equi* in soil.

R. equi can also infect non-equine species. *R. equi* infections have been described in humans, pigs, cattle, sheep goats, cats, and dogs. In humans the bacterium may cause cavitary pneumonia but this is predominantly in immunocompromised individuals.
Virulence and host tropism rely on a plasmid containing the genes for Virulence Associated Proteins (Vap), like VapA and VapB. VapA and VapB are mutually exclusive i.e. they do not occur in the same isolate, and are an important determinant of species specific virulence of certain strains (Ocampo-Sosa *et al* 2007, Annex 2). *R. equi* virulence plasmids can be classified into three general types: $VapA^{+}$, $VapB^{+}$ and $VapAB^{-}$. Each of these plasmid types is almost exclusively associated with specific non-human animal hosts, horse, pig and cattle,

respectively (Ocampo-Sosa *et al* 2007, Annex 2). By contrast all three plasmid types could be found in *R. equi* strains from humans, a host in which the infection is opportunistic and associated with immunosuppression. Additionally, strains devoid of virulence plasmids are regarded as non-pathogenic for foals and mice but have been isolated in immunocompromised humans. Immunocompetent humans are rarely affected by *R. equi*, while a compromised cell mediated immunity predisposes one to *R. equi* infection. The development of *R. equi* as an opportunistic pathogen was accelerated with the spread of the AIDS pandemic. As with other immunocompromised individuals, infection mostly results in pneumonia with fever, cough, and chest pain, but can also spread to other organs and cause bacteraemia. The fact that human isolates from pathological conditions have all types of plasmid categories, including strains without virulence plasmid, indicates that the immunocompromised human host is susceptible to a variety of *R. equi* strains and emphasises the opportunistic nature of *R. equi* in this host. It is likely that *R. equi* infections of humans are determined by the basal and chromosomally determined pathogenic potential of *R. equi* and the immunological status of the patient, rather than the presence or absence of virulence plasmid. A significantly reduced immune system is likely to allow infection with relatively avirulent organisms (Topino *et al*. 2009, Annex 3; Hondalus 1997, Annex 4).

1.1.2.A.9. Potential for genetic transfer and exchange with other organisms;

Conjugative transfer of the Vap genes containing plasmid may occur between *R. equi* strains. Although very little work is done on the transfer of the *R. equi* plasmids it has been established that they seem to belong to an ancient family of replicon that was widespread among the actinobacterial ancestors and diversified during the speciation process. A distinctive feature of this actinobacterial plasmid family is the presence of a highly conserved syntenic gene cluster which always includes conserved conjugal transfer TraG/TraD protein-coding genes. (Letek *et al*, 2008, Annex 22). For a large number of these actinobacterial plasmids the conjugative frequencies were at approximately 1×10^{-4} event per recipient cell (Yang *et al*. 2006, Annex 23).
Until today only *R. equi* strains have been identified that harbor a $vapA^{+}$, $vapB^{+}$or $vapAB^{-}$ plasmid. No evidence has been found that some *R. equi* strains might contain more than one type of virulence plasmid. The virulence plasmid might be transferred to *R. equi* that do not harbor a virulence plasmid. However the vast majority of *R. equi* strains isolated on horse farms already contain a virulence plasmid (pers. communications J. Prescott). The chance on transfer of chromosomal DNA is considered extremely low. Analysis of the available literature does not provide any reason to assume that transduction and transformation play an important role in the natural environment of *R. equi*. Furthermore natural competence has not been reported for any *Rhodococci*.

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

As described in section 1.1.2.A.9 above, genetic transfer is limited to exchange of plasmids. The main factor for this is close contact between different *R. equi* organisms and the receiving organism should not already contain a $VapA^{+}$ plasmid. Analysis of the available literature does not provide any reason to assume that transduction and transformation play an important role in the natural environment of *R. equi*. Furthermore natural competence has not been reported for any *Rhodococci*.

1.1.2.A.11. Pathological, ecological and physiological traits:

(a) Classification of hazard according to existing Community rules concerning the protection of human health and the environment

According to EU Directive 2000/54/EC *R. equi* is considered as a group 2 biological agent.

(b) Generation time in natural ecosystems, reproductive cycle

R. equi is a soil organism with simple nutrition requirements. In addition to soil, the bacterium may be recovered from intestines of herbivores and carnivores.
The organism has a long survival period in manure and soil. Survival and multiplication are strongly dependent on temperature, concentration of manure, and soil pH. Neutral to moderate alkaline soil at 30°C enriched with horse manure appears to be the optimal environment for *R. equi* growth with the organism thriving on the volatile fatty acids found in the horse manure (Hughes and Sulaiman 1987, Annex 24). *R. equi* is inactive at temperatures below 10°C.
Horses are infected by the organism while grazing or by inhalation of soil dust during dry periods. The concentration of *R. equi* in manure of adult horses may vary but is relatively low. Young foals, however, may shed high numbers of virulent *R. equi*. These observations form the basis of the hypothesis that the faeces of foals, especially foals with *R. equi* pneumonia, are the most important source of virulent *R. equi* contamination on farms. In case of a *Rhodococcus equi* pneumonia most bacteria remain in the lung abscesses, but low level shedding does occur by coughing, sneezing and/or exhalation

(c) Information on survival, including seasonality and the ability to form survival structures, e.g. spores

The organism does not produce specific survival structures such as spores but nevertheless can survive for long period of time in manure and soil. **INFORMATION DELETED FOR CONFIDENTIALITY PURPOSES.** In this context it should be noted that the vaccine strain is attenuated for survival in macrophages but outside the host it is expected to behave similarly to the wild-type parent strain.
The organism multiplies best at temperatures around 30°C and is inactive at temperatures below 10°C. The highest risk for infection of horses is during dry summer periods.

R. equi does not need the foal to survive in nature. It can cause pneumonia and enteritis but it is a saprophytic soil bacterium like most Rhodococci. In some cases it colonizes (temporarily) subclinically the gut of foals/horses. *R. equi* is a normal commensal of the intestine and faecal excretion is the main source of infection of the environment. In case of a *Rhodococcus equi* pneumonia most bacteria remain in the lung abscesses, but low level shedding does occur by coughing, sneezing and/or exhalation.

(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 host range, virulence and host tropism of *R. equi* are described in 1.1.2.A.8. The pathogenicity, disease and tropism of *R. equi* were recently reviewed by Von Bargen and Haas 2009, (provided in Annex 9). Another recent review gives an excellent overview of *R.*

equi infection in AIDS patients (Topino *et al*., 2010; Annex 3). Below is a short summary of the two papers.

The parent organism is a facultative pathogenic soil saprophyte. The soil actinomycete *R. equi* is a pulmonary pathogen of young horses and AIDS patients. *R. equi* strains are also isolated from pigs, where they can cause tuberculosis-like lesions, but can also be found in submandibular lymph nodes and tonsils of healthy animals. *R. equi* also cause tuberculosis-like lesions in lymph nodes of cattle and in the livers of young goats. Other animals occasionally infected by *R. equi* are sheep, llama, cats and dogs.

Of all species, disease caused by *R. equi* infection in foals is by far the most devastating and therefore, this has been in the centre of host–pathogen interaction studies for years. From this work it is known that *R. equi*, which normally is found in various environments from soil and ground water, infects the foal by inhalation of aerosolized dust contaminated with these bacteria, invades, survives and multiplies in alveolar macrophages by arresting the normal pathway of phagosome maturation. In the early stage of the disease, pulmonary lesions develop and alveoli fill with neutrophils, macrophages and giant cells. As disease progresses, the lung parenchyma becomes necrotic and bronchial and mesenteric lymph nodes are affected. Neutrophilic leucocytosis and hyperfibrinogenaemia are common findings, associated with abscessation and pulmonary changes.

R. equi infections of foals occur worldwide. Increased incidences of *R. equi* pneumonia is associated with large farm size, high density and population size of foals, high numbers of airborne virulent *R. equi*, low soil moisture, high temperatures and a poor pasture grass cover. Farms with endemic *R. equi* pneumonia are heavily contaminated with virulent *R. equi*. However avirulent *R. equi* are frequently found in environment and faeces on every farm. Yet, the actual proportion of virulent strains in the environment is no indication for the prevalence of *R. equi* pneumonia, as the relative proportion of virulent *R. equi* in dams' faeces is also not indicative of the development of *R. equi* pneumonia in their foals.

In the first weeks of a foal's life, ingestion of *R. equi* often leads to colonization of the intestines. Foals shed large quantities of *R. equi* as compared with adults, but the number of bacteria in faeces declines after 7 weeks of age. Ingestion of *R. equi* does not usually result in disease, but in immunization. As a result of this process, older foals and adult animals have antibodies against *R. equi* and rarely get infected.

Virulence of *R. equi* is associated with the possession of Virulence Associated Proteins (VAPs) that are encoded by the virulence plasmid. These plasmids may exist in multiple copies and are lost after repeated passages of the bacteria in broth culture at 38^{o}C. The plasmid is essential for multiplication in macrophages, prolonged inhibition of phagosome maturation and it enhances cytotoxicity. Isogenic strains from which the plasmid has been removed are avirulent in foals and mice and do not multiply in macrophages.

So far, three types of VAPs have been identified, two of which have been sequenced and further investigated. Whereas possession of certain VAPs seems to be specific for strains infecting foals (VapA^{+}), pigs (VapB^{+}) or cattle (VapAB^{-}). By contrast all three plasmid types could be found in *R. equi* strains from humans, a host in which the infection is opportunistic and associated with immunosuppression. Additionally, strains devoid of virulence plasmids are regarded as non-pathogenic for foals and mice have also been isolated in immunocompromised humans. Immunocompetent humans are rarely affected by *R. equi*, while a compromised cell mediated immunity predisposes one to *R. equi* infection. The development of *R. equi* as an opportunistic pathogen was accelerated with the spread of the AIDS pandemic. As with other immunocompromised individuals, infection mostly results in pneumonia with fever, cough, and chest pain, but can also spread to other organs and cause bacteraemia. The fact that human isolates from pathological conditions have all types of

plasmid categories, including plasmid less strains, indicates that the immunocompromised human host is susceptible to a variety of *R. equi* strains and emphasises the opportunistic nature of *R. equi* in this host. It is likely that *R. equi* infections of humans are not determined by particular plasmids but by the basal and chromosomally determined pathogenic potential of *R. equi* and the immunological status of the patient, rather than the presence or absence of virulence plasmid, is the major factor in determining whether an infection with *R. equi* occurs. A significantly reduced immune system is likely to allow infection with relatively avirulent organisms (Topino *et al*. 2009, Annex 3; Hondalus 1997, Annex 4).
The minimum infective dose under natural conditions is not known for any species, including humans because it never has been determined. In the artificial intratracheal challenge model, doses from 10^4 CFU and higher appear infectious (Wada *et. al.* 1997, Annex 26). In an aerosol challenge model, pneumonia was induced by inhalation of $1.2x10^{10}$ CFU on 5 different days i.e. day 0, 1, 2, 6 and 7 implying a total dose of $6x10^{10}$ CFU (Chirino-Trejo *et. al.* 1987, Annex 27). In another artificial intravenous mouse challenge model the LD50 appeared $2.6x10^6$ CFU (Takai *et. al.* 1999, Annex 28).

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

The parent strain is resistant to chloramphenicol, trimethoprim-sulpha, polymixin, penicillin, oxacillin, lincomycin, flumequine, kanamycin, furazolidone, tylosin, sulphanomides, and tiamulin. A large part of the identified resistances are intrinsic features of *R. equi*. For example *R. equi*, has a complex hydrophobic cell wall which is characterized by mycolic acid-containing lipids and lipoglycans and has a thick and lamellar polysaccharide capsule which together interfere with the passage of certain antibiotics into the cell.

The parent strain is sensitive to ampicillin, erythromycin, rifampicin, ceftiofur, gentamycin, streptomycin, neomycin, amoxicillin, spectinomycin, enrofloxacin, spiramycin, and doxycycline.

The best therapy in practice seems to be a combination of a macrolide antibiotic (e.g. erythromycin) and rifampicin (Hillidge 1987, Annex 29 and Sweeney *et. al.* 1987, Annex 30) or tulathromycin (Venner et. al. 2007, Annex 31).

Both erythromycin and rifampicin are used in the treatment of human and animal diseases. Tulathromycin is used in the treatment of animal diseases only.

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

R. equi belongs to a genus of aerobic, non-sporulating, non-motile gram-positive bacteria closely related to Mycobacteria and Corynebacteria that have been found to thrive in a broad range of environments, including soil, manure and water. *Rhodococcus* is an important genus due to their ability to catabolize a wide range of compounds and produce bioactive steroids, acrylamide and acrylic acid and their involvement in fossil fuel biodesulfurization. However, as it stands now, *Rhodococcus* is not well characterized, but there are strong indications that these systems have evolved from pathways to catabolise complex plant materials like plant steroids and lignins (McLeod *et. al.*, 2006, Annex 32)

However the specific adaptation of *R. equi* to grazing animals is likely due to the predilection of these bacteria for volatile fatty acids, which are abundant in herbivore manure.

1.1.2.A.12. Nature of indigenous vectors:

(a) Sequence

Analysis of the genomic sequencing data of both the vaccine strain RG2837 and its parent RE1 demonstrated that both strains have a virulence plasmid that is identical to the published plasmid sequence of *R. equi* strain 103S which was isolated from a sick horse in 1979. The DNA sequence of the plasmid from strain 103S was thoroughly analyzed. The DNA sequence of the plasmid of strain 103 was published in 2000 (Takai *et al.*, 2000b; Annex 33, Accession no. AF116907). This analysis showed that 1 ORF might encode for a bicyclomycin resistance protein (48% homology to *E. coli* protein). However this gene appears to be present in a large number of *R. equi* isolates as all virulence plasmids (both $vapA^+$ and $vapB^+$), which have been sequenced, harbor this gene.
Finally no remnants of the plasmids that were used for the construction of the vaccine strain can be found in the chromosome, with the exception of 6 nucleotides of the restriction site (GATATC) that was used to ligate the *ipdAB1* upstream and downstream regions together, and the 6 nucleotides of the restriction site (AGATCT) that was used to ligate the *ipdAB2* upstream and downstream regions together, or in the virulence plasmid. This was demonstrated by Blasting (Blast algritm) the pSelAct plasmid backbone against the sequence reads database directly for short sequences which correspond to the antibiotic resistance genes, and other regulatory sequences that were on the plasmids used to make the clean deletions. The Blast search gave no significant hit with the DNA of the plasmids that were used in the construction of RG2837 whereas all positive and negative controls were as expected.
From this observation it can be concluded that the whole construction process has not lead to the incorporation of (fragments) of antibiotic resistance genes in the strain.
Therefore, introduction of the vaccine strain into the environment does not lead to the introduction of additional antibiotic resistances.

(b) Frequency of mobilisation

DNA sequence analysis showed that a large number of genes on virulence plasmid encode for proteins that were predicted to play a role in conjugation, but in the public domain no information is available on the frequency and efficacy of the plasmid mobilisation process. The *R. equi* plasmids belong to an ancient plasmid family that is widespread among the actinobacteria. For a large number of these actinobacterial plasmids the conjugative frequencies were at approximately 1×10^{-4} event per recipient cell (Yang et al. 2006, Annex 23).
Mobilisation of the virulence plasmid is not an issue for this vaccine. The attenuation is not located on the virulence plasmid but on the chromosome. It is not expected that the virulence plasmid of the vaccine strain encodes for antibiotic resistances other than the ones already present in the field as all pastures and stables used for horses are invested with *R. equi* strains that already harbour a virulence plasmid.

(c) Specificity

Not applicable.

(d) Presence of genes which confer resistance

Based on the sequence of other virulence plasmids it is safe to assume that the virulence plasmids do not encode for any form of resistance with the exception of a possible bicyclomycin resistance gene.

1.1.2.A.13. History of previous genetic modifications.

No previous genetic modifications have occurred.

1.1.2.B. Characteristics of the vector

1.1.2.B.1. Nature and source of the vector

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1.1.2.B.2. Sequence of transposons, vectors and other non-coding genetic segments used to construct the GMO and to make the introduced vector and insert function in the GMO

The principle of the method used to construct the GMO has been published by Van der Geize *et. al.* 2008 (Annex 7). For further details see 1.1.2.C.1a.

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

Not applicable, the GMO is a deletion mutant.

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

Not applicable, the GMO is a deletion mutant.

1.1.2.C. Characteristics of the modified organism

1.1.2.C.1. Information related to the genetic modification

(a) Methods used for the modification

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Construction of plasmid suicide vector pSelAct
See 1.1.2.B.1.

Construction of plasmids for ipdAB1 and ipdAB2 gene deletion
See 1.1.2.B.1.

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

See 1.1.2.C.1a.

(c) Description of the insert and/or vector construction

Not applicable, the GMO is a deletion mutant.

(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

Not applicable, the GMO is a deletion mutant.

(e) Methods and criteria used for selection

The vaccine strain was selected by growth on 5-fluorcytosine containing agar plates. Colonies that grew on these plates were considered to be wild type or deletion mutant. A PCR was used to select a deletion mutant. The PCR proved also that except for 6 nucleotides at the restriction sites, no remnants of the vector were present. These 6 nucleotides are remnants of the restriction sites.

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1.1.2.C.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 vaccine strain *R. equi* strain RG2837 is a deletion mutant. The main deletions were made in the *ipdAB1*-genes involved in the cholesterol metabolism. Bioinformatics analysis revealed that IpdA and IpdB represent the α and β-subunit of a heterodimeric CoA-transferase. Thus, the heterodimeric CoA-transferase encoded by *ipdAB* might be involved in the removal of the propionate moiety of methylhydroindanone propionate intermediates (e.g. HIL=3α-H-4α(3'-propionicacid)-5α-hydroxy-7αβ-methylhexahydro-1-indanone-δ-lactone) by β-oxidation during steroid degradation (see also report 10R/0075 provided in Annex 8). How HIL degradation relates to the observed hampered macrophage survival is not known (discussed further below). Outside the macrophage (i.e. in the gut or environment) the strain is expected to behave similar to the wildtype parent strain as there are no macrophages. This was confirmed by the observation that the vaccine strain survived in the environment in a similar manner as the wildtype parent strain **INFORMATION DELETED FOR CONFIDENTIALITY PURPOSES** *R. equi* can utilize a broad range of carbon sources from simple lactate molecules to complex lignins. The carbon source in the macrophage is unknown, as is the carbon source in the gut but in the latter is likely to be plant derived molecules. Information on the vaccine strain and other steroid catabolic pathway mutants was recently published (van der Geize et al., 2011, Annex 61).

As a result of the *ipdAB* deletions, the bacteria are less able to survive in macrophages, which is a requirement for pathogenicity in the target animal. This characteristic makes the strain suitable as live bacterial vaccine strain.
The other deletion, Δ*ipdAB2*, was made because *ipdAB2* showed 50% similarity with *ipdAB1*. To avoid that *ipdAB2* would take over the function of *ipdAB1*, these genes were deleted as well. Cholesterol metabolism as such is not essential for macrophage survival since mutants that are unable to take up cholesterol (i.e. *supAB* deletion mutant) are not attenuated for macrophage survival (van der Geize *et al*, 2008 Annex 7, **INFORMATION DELETED FOR CONFIDENTIALITY PURPOSES** and 10R/0075, Annex 8). However, enzymes that are part of the cholesterol degradation chain, in particular those involved in HIL degradation (i.e. *ipdAB*1 and *Fad30*), appeared to be essential for macrophage survival because deletion mutants of the respective genes were shown to be hampered in macrophage survival (see report 10R/0075, Annex 8).
The precise mechanism for the survival of *R. equi* in macrophages has yet to be elucidated., but it is evident from our studies that either the enzymes of the cholesterol degradation chain pathway appear to play a part (the enzyme pathway may serve a different function during macrophage residency) or that the buildup of the intermediates of cholesterol degradation, at the point of HIL may hamper the survival of the bacteria in macrophages. Except for a hampered macrophage survival no differences in survivability between vaccine strain and wildtype have been observed, so it is not clear whether the deletions have any negative competitive effect in certain environments (e.g. environments enriched with steroids). The most negative assumption is that outside the host there is no difference in survivability between vaccine strain and wildtype. Given the nature of the vaccine strain (unmarked deletion mutant, with no additional genes introduced into the environment), additional risks for humans, horses and environment are nearly zero.

Using genomic sequencing of both the vaccine strain RG2837 and its parent RE1 we have demonstrated that both strains have an identical virulence plasmid to the published plasmid sequence of R. equi strain 103S which was isolated from a sick horse in 1979. The DNA sequence of the plasmid from strain 103S was thoroughly analyzed. This analysis showed that 1 ORF might encode for a bicyclomycin resistance protein (48% homology to *E. coli* protein).

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

Except for 12 nucleotides at the restriction sites, no remnants of the vector are present in the final construct **(INFORMATION DELETED FOR CONFIDENTIALITY PURPOSES**

(c) Stability of the organism in terms of genetic traits;

As described under 1.1.2.C.2a, the vaccine strain *R. equi* strain RG2837 is a deletion mutant

Theoretically, the vaccine strain may encounter other bacteria and then, if able to do so, take up, integrate and express exogenous DNA encoding functional genes, thereby repairing the deletion and restoring its virulence. The chances that this process will take place will be extremely rare as the genes are not a part of a mobile element like plasmids, transposons or phages.
Furthermore natural competence (uptake of random DNA) has not been described for *R. equi* and integration will only take place if the nucleotide sequences of the genes and its flanking

regions are (almost) identical to that of *R. equi*. Analysis of the available literature does not provide any reason to assume that transduction and transformation play an important role in the natural environment of *R. equi*. Furthermore natural competence has not been reported for any Rhodococci.

An additional aspect of reversion to virulence by hypothetical DNA uptake and thereby repair of the genes, is that it results in wild type *R. equi*, already present in the fields and sites that it is used on: stables and pastures with horses.

(d) Rate and level of expression of the new genetic material. Method and sensitivity of measurement;

Not applicable, the GMO is a deletion mutant.

(e) Activity of the expressed proteins;

Not applicable, the GMO is a deletion mutant.

(f) Description of identification and detection techniques including techniques for the identification and detection of the inserted sequence and the vector;

The vaccine strain can be isolated from environmental samples on selective agar which contains 4 antibiotics that reduce non-specific bacterial growth (see **INFORMATION DELETED FOR CONFIDENTIALITY PURPOSES** section 1.1.2.A.6). After isolation the vaccine strain can be identified (and differentiated from wild type) by a specific PCR (see section 1.1.2.C.1.f).

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

Reisolation on selective agar as described in 1.1.2.A.6 is the most sensitive method. The reliability is dependent on the type of sample. However, it can be stated that the detection limit in faeces is 1.5 CFU/mg faeces.

(h) History of previous releases or uses of the GMO;

In 2011 and 2012 two studies were performed in 80 foals in the Netherlands under license number PorM/RB IM 09-004. No vaccine related abnormalities were observed during these studies .

1.1.2.C.3. Considerations for human health and animal health, as well as plant health

(a) Toxic or allergenic effects of the non-viable GMOs and/or their metabolic products

The vaccine strain is a gram-positive bacterium. Therefore, no toxic substances like endotoxins are present. There are no literature references indicating that exotoxins or allergenics are produced by *R. equi*.

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

The vaccine strain was derived from a pathogenic field strain that possessed a VapA encoding virulence plasmid and that was able to induce typical *R. equi* clinical signs of respiratory disease (pneumonia) when used to infect foals **INFORMATION DELETED FOR CONFIDENTIALITY PURPOSES).** The vaccine strain is unable to cause disease when administered according to the manufacturers recommendations, when it enters the horse by the natural route **INFORMATION DELETED FOR CONFIDENTIALITY PURPOSES)** or when it
is administered by the method used in the artificial challenge model used **(INFORMATION DELETED FOR CONFIDENTIALITY PURPOSES**. Using genomic sequencing of both the vaccine strain RG2837 and its parent RE1 we have demonstrated that both strains have an identical virulence plasmid to the published plasmid sequence of *R. equi* strain 103S which was isolated from a sick horse in 1979. The DNA sequence of the plasmid from strain 103S was thoroughly analyzed. Thus, the vaccine strain, which still contains a VapA encoding virulence plasmid, is attenuated and safe for foals. Since the virulence plasmid determines host tropism and since the different plasmid types A and B are mutually exclusive the vaccine strain is expected to be unable to cause disease in other animal species (Ocampo-Sosa *et al.* 2007, Annex 2). Safety in chickens, mice, rats, calves and pigs have been confirmed **(INFORMATION DELETED FOR CONFIDENTIALITY PURPOSES)**. The ability of the vaccine strain to infect wild animals has not been investigated (e.g. wild birds and pigs). Since domestic and wild pigs are of the same species and genetically very similar, it is expected that their susceptibility to *Rhodococcus* is similar. Wild boars are known to be susceptible to *R. equi* but none of the strains isolated (82 isolates examined) contained the $vapA^+$ virulence plasmid, 26% were $vapB^+$ and 74% did not contain vap plasmids (Makrai *et.al.* 2008, Annex 57). The predominant plasmid found in pigs is $vapB^+$ (70% of the 30 isolates examined) (Ocampo-Sosa *et al.* 2007, Annex 2). As the vaccine strain is $vapA^+$, the data generated on the safety of the vaccine strain in pigs is likely to be equally applicable to wild pigs, with infection being unlikely. The data on poultry most probably can be transferred to free range chickens but it is not known whether it could be transferred to other wild birds. To our knowledge problems in birds (caused by *R. equi*) have not been reported.
Since it has been demonstrated that the vaccine/mutant strain is less able to survive in human macrophages (in contrast to the parent strain), it is expected to be unable to cause disease in (immunocompromised) humans, although a direct correlation between survival in macrophages and human pathogenicity has never been tested or demonstrated.
Strains lacking the virulence plasmid (that like the vaccine strain are attenuated for macrophage survival) have been isolated from immunocompromised persons. However, since the vaccine strain was shown to be less able to survive in human macrophages it can be expected that the risk for immunocompromised persons is at most equal but most probably less compared to the wildtype *Rhodococcus equi*. In this context it is relevant to note that the vaccine strain will be used in environments where the wildtype *Rhodococcus* is (massively) present and had not caused disease in humans.

(c) Capacity for colonisation

The vaccine strain is attenuated in its ability to survive in macrophages. We have no evidence that any other aspect of its life cycle is attenuated. Wild type *R. equi* is known to infect young foals and colonise the intestines, resulting in the shedding of large numbers of bacteria (von Bargen and Haas, 2009 provided in Annex 9). After oral and/or rectal inoculation most foals have shown a transient colonisation after which the vaccine strain is not detectable anymore. However, a few foals appeared to shed the vaccine strain intermittently up to 4 weeks after

vaccination when the experiment was ended **INFORMATION DELETED FOR CONFIDENTIALITY PURPOSES**. Thus shedding can occur for at least 4 weeks but probably longer. The vaccine strain is attenuated for macrophage survival and therefore unable to colonise the lungs and to cause pneumonia. However, in the gut where *R. equi* mostly lives as a commensal (as well as outside the host) the vaccine strain is expected to behave no differently from the wild type parent strain as macrophages are not present, and as such, long term colonisation of the gut is theoretically possible. If colonisation occurs, it poses no additional risk for humans, animals or environment (compared to the wildtype that is already present in animals and the environment) because of the nature of the vaccine strain (unmarked deletion mutant, with no additional genes introduced into the environment).

(d) 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

R. equi infections in immunocompetent humans are extremely rare. The majority of reports on human infections have been associated with immune system dysfunction, in particular AIDS.
R. equi virulence plasmids can be classified into three general types: $VapA^{+}$, $VapB^{+}$ and $VapAB^{-}$. Each of these plasmid types was almost exclusively associated with a specific non-human animal host, i.e. horse, pig and cattle, respectively (Ocampo-Sosa *et al* 2007, Annex 2). By contrast all three plasmid types were found in *R. equi* strains from humans and as such the VapA possessing wildtype parent strain could be pathogenic for immunocompromised humans, a host in which the infection is opportunistic and associated with immunosuppression. Additionally, strains devoid of virulence plasmids are regarded as non-pathogenic for foals and mice have also been isolated in immunocompromised humans. Immunocompetent humans are rarely affected by *R. equi*, while a compromised cell mediated immunity predisposes one to *R. equi* infection. The development of *R. equi* as an opportunistic pathogen was accelerated with the spread of the AIDS pandemic. As with other immunocompromised individuals, infection mostly results in pneumonia with fever, cough, and chest pain, but can also spread to other organs and cause bacteraemia. The fact that human isolates from pathological conditions have all types of plasmid categories, including plasmid less strains, indicates that the immunocompromised human host is susceptible to a variety of *R. equi* strains and emphasises the opportunistic nature of *R. equi* in this host. It is likely that *R. equi* infections of humans are determined by the basal and chromosomally determined pathogenic potential of *R. equi* and the immunological status of the patient, rather than the presence or absence of virulence plasmid. A significantly reduced immune system is likely to allow infection with relatively avirulent organisms (Topino *et al*. 2009, Annex 3; Hondalus 1997, Annex 4).

However, since the mutant strain is less able to survive in human macrophages (in contrast to the parent strain) it is expected to be less able to cause disease in immunocompromised humans. The attenuation was proven in the most sensitive host, the foal.
The vaccine strain is sensitive to ampicillin, erythromycin, rifampicin, ceftiofur, gentamycin, streptomycin, neomycin, amoxicillin, spectinomycin, enrofloxacin, spiramycin, and doxycycline.

(e) Other product hazards

Pathogenicity of *R. equi* is characterised by its capability to survive in macrophages. Considering that the vaccine strain has a reduced capability to survive in macrophages, the consequence of a hazard occurring is negligible.
The risk of horizontal gene transfer of antibiotic resistance genes appears also to be very low. First of all there is little indication that the virulence plasmid contains antibiotic resistance genes that are not readily available in the environment. It must also be considered that the vaccine will be used on farm with a high infection level of *R. equi* strains that contain a VapA encoding virulence plasmid.

1.1.3 . Information Relating to the Conditions of Release and the Receiving Environment

1.1.3.A Information on the release

1.1.3.A.1 Description of the proposed deliberate release, including its purpose.

The primary purpose of the proposed deliberate release is confirmation of existing laboratory efficacy data for a licensing procedure according to EU Directive 2001/82/EC.

The proposed indication for the product is:
For immunisation of horses against *R. equi*, to reduce mortality and pyogranulomatous pneumonia caused by *R. equi*.

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

On the intended study farm, the foals are born between March and September. The applicant is seeking permission to perform the study over three foaling seasons, 2013, 2014 and 2015. This is to ensure that the required number of foals can be recruited into the study. Up to 150 foals will be recruited. The number of foals to be vaccinated in the whole study is 75 (the additional 75 foals will be used as controls). The vaccinates will receive a maximum of 4 doses of vaccine.
In one foaling season, approximately 25 foals will receive vaccine and 25 foals will remain unvaccinated. It is expected that the target of 150 vaccinated foals will be achieved in three foaling seasons. Foals will be vaccinated via the rectal route. Two doses will be given on two consecutive days at admission and another two doses on two consecutive days 14 days later. The vaccination schedule should be completed before the foals are 1 month of age. The foals will be monitored for clinical signs caused by *R. equi* infection until they are 6 months of age.

Efficacy will be demonstrated by clinical observation and serology. **INFORMATION DELETED FOR CONFIDENTIALITY PURPOSES**

1.1.3.A.3. Preparation of the site previous to release.

No special preparations are proposed, the current situation is considered adequate.

1.1.3.A.4. Size of the site

The release site where the foals are vaccinated is a medium sized and well organised horse farm located in Belmont, Co. Offaly. A plan of the site and aerial photographs are provided in Annex 53..
The site is completely surrounded by a stud rail fence and the gate is closed with an electronic lock. The release approval is requested for all buildings and associated paddocks except the stallion boxes and sporthorses yard. The fenced plot has a size of approximately 18.3 hectares. These paddocks are denoted S14901062, S14901063 and S14901074 on the aerial map. Release of the vaccine will take place on area S14901063. The foals will stay in this closed area for at least one week after each vaccination. Until at least four weeks after vaccination, the foals will be allowed in S14901062. After this period of time, foals are allowed in S14901074, as well.

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

Rectal vaccination of young foals.

1.1.3.A.6. Quantities of GMOs to be released.

Each foal will receive four doses of vaccine, each containing between 5 x 10^9 and 1x 10^{11} CFU of *R. equi* strain RG2837. A total of up to 75 foals will be vaccinated over -3 foaling seasons.

If all foals in the vaccine group receive 4 doses of vaccine containing the maximum release level of 1x 10^{11} the total amount of vaccine strain that will be released in the study will be 3x10^{13} CFU over 2-3 foaling seasons. In any one year, the maximum release level (assuming 25 foals receive 4 vaccinations) is 1 x10^{13} CFU.
Following vaccination, the amount shed by the vaccinated animals will vary from foal to foal. Peak shedding is on day 1 and 2 after vaccination; after which time some foals stop shedding while others show an intermittent low level shedding up to several weeks after vaccination (and probably longer). Theoretically the minimum number of bacteria that is shed will be equal to the inoculum but depending on multiplication in the animal and how long the foals are shedding this value might be increased by 1, 2 or even 3 logs.

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

None; the horse will be kept as usual.

1.1.3.A.8. Worker protection measures taken during release.

There are no specific risks for workers. The attenuated vaccine strain is not considered as a risk for immunocompetent people, since the attenuation was confirmed in a human

macrophage cell line and the workers are already in frequent contact with wild type *R. equi*, which is endemic at the farm. Signs of human infection or disease have never been observed at the site. As a precaution measure, immunocompromised staff will not be allowed to handle the study animals until at least 4 weeks after last vaccination.

None of the other substances included in the vaccine pose a risk for the people handling the vaccine.

1.1.3.A.9. Post-release treatment of the site.

Until 4 weeks after last vaccination, all straw and litter of the stables where the foals are kept will be removed into closed containers and inactivated by heat treatment by a specialised and approved company (SRCL, 430 Beech Road, Western Industrial Est., Dublin 12). The faeces in the foal paddock (Pony Garden), where the foals are kept until at least 1 week after each vaccination, will also be removed regularly and inactivated together with the litter and straw from the stables.
At the end the study an extra cleaning of the barns and stables that were used for vaccinated animals will be carried out as follows. All remaining straw and litter will be removed mechanically into closed containers. After the complete removal of the straw and litter, the stables, the total concrete floor before the stable, and all equipment used will be cleaned with the standard concentration of Steri-7 which is active against R. equi (see validation report, Annex 55). All straw and litter will be heat-inactivated by a specialised and approved company (SRCL, 430 Beech Road, Western Industrial Est., Dublin 12). A full hygiene plan is provided in the study protocol **(INFORMATION DELETED FOR CONFIDENTIALITY PURPOSES).**

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

Vaccination equipment and empty vials will put in a container with Steri-7 solution to effectively inactivate any R. equi on and in the equipment. The container will be closed and disposed of at Intervet International in Boxmeer.
At the end of the experiment, the site will be cleaned as follows. All remaining straw and litter in barns and stables used for the vaccinated animals will be removed mechanically into closed containers. After the complete removal the straw and litter, the stable, the total concrete floor of the barns/stables, and all equipment used will be disinfected with the standard concentration of Steri-7 which is active against *R. equi* (see validation report, Annex 55). All straw and litter will be heat-inactivated by a specialised and approved company (SRCL, 430 Beech Road, Western Industrial Est., Dublin 12). A full hygiene plan is provided in the study protocol **(INFORMATION DELETED FOR CONFIDENTIALITY PURPOSES)**.

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

A deliberate release license has been received from the Dutch authorities. The reference number is IM09-004.

The COGEM (the Dutch GMO board) gave a positive opinion concerning the Intervet request for deliberate release into the environment. The COGEM concluded that the risks for humans

and the environment are negligible. The licence (PorM/RB IM 09-004) was published on October 4th 2010. A copy of the licence is provided in Annex 20. Under this license two studies were performed in 80 foals in the Netherlands in 2011 and 2012. No vaccine related abnormalities were observed during these studies.

Also a license for deliberate release of the vaccine was received from the German authorities (BVL 107/2012/4). The study in Germany is planned to start in 2013 foaling season.

1.1.3.B Information on the environment (both on the site and the wider environment)

1.1.3.B.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 release site is located in Belmont, Co. Offaly (GPS Reference N53° 15.072 W007° 53.887). Plans of the site, photographs and aerial photographs are provided in Annex 53. Belmont Stud is located adjacent to Belmont village (population just in excess of 200). Other farms are located nearby and the fields adjacent to the release site are used for rearing cattle. A horse farm is located on the opposite side of Belmont village but no contact with horses will occur from adjacent farms.

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

The farm is located close to Belmont village with latest census data suggesting a population of just in excess of 200 people. The location is sparsely populated with 52 people per mile^2 . The nearest town larger than 50,000 inhabitants takes about 1:40 hours by local transportation. The closest site where livestock (cows) is kept is located beside the farm, and while contact with cattle is inhibited by boundary hedgerows, direct contact is unavoidable.

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

The farm is located in an agricultural area. The area is characterised by a clay soil covered with permanent pasture. A map of the area is provided in Annex 56. The nearest Special Area of Conservation (SAC) is Clara Bog, located 22 km from the release site and therefore not affected by the release of the vaccine strain. The release of this organism will have no impact on any local species.

Groundwater is the principle source of water in Offaly. 72% of the people of Offaly receive water from schemes with groundwater sources, compared with a national average of less than 25%. A groundwater protection scheme has been adopted for all public and group water supply sources in Offaly. Groundwater, which is the source for a large proportion of the drinking water in Co. Offaly, receives a precautionary dose of chlorine to provide disinfection. Surface water is the source for 4 drinking water supplies in Co. Offaly, namely. The River Shannon is the closest supply source for Belmont through the Banagher Regional Water Supply. A tributary of the Shannon is adjacent to the Front Field of Belmont farm and the horses use it for drinking. The closest point of entry to the Shannon is approximately 8 km from the release site. The Shannon river sources receive full physio-chemical treatment, consisting of coagulation, floculation, sedimentation, filtration, pH correction and disinfection. The release of this organism will have no impact on water quality. As *R. equi* is

widespread in the environment on all horse farms in the hinterland and the vaccine strain does not introduce any new genes or plasmids into the environment, the risk is effectively zero.

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

The mean annual precipitation in the Offaly site has not been determined. It is located at 59 metres above sea level. The average rainfall of 948 mm and average temperature of 9.7°C has been recorded in Gurteen Agricultural College, the nearest measured site, located at a distance of approximately 32 km from Belmont.

1.1.3.B.5. Geographical, geological and pedological characteristics.

The site is named Belmont Stud and has a landscape characterised by a clay soil. The land is mainly used for agriculture.

1.1.3.B.6. Flora and fauna, including crops, livestock and migratory species.

Cattle are maintained in farms adjacent to the release site. The release site is surrounded by fields where badgers, foxes and rabbits are present. A direct contact with these animals is possible though the site is completely fenced in. Smaller wild life, such as birds and rodents, can enter the release site. However, the number of rodents and birds is limited. Spring migratory birds (e.g. swallows) would potentially be exposed to the vaccine strain as the barns are not enclosed.
The area is characterised by a clay soil covered with permanent pasture.
Wild animals are not considered at risk because *R. equi* is not pathogenic for birds or wildlife, therefore the release of this organism will have no impact on protected or other important species

1.1.3.B.7. Description of target and non-target ecosystems likely to be affected.

It is not expected that the release will affect any eco-system.

1.1.3.B.8. A comparison of the natural habitat of the recipient organism with the proposed sites of release.

R. equi is a normal inhabitant of soil, especially when herbivore manure is present. Since the site is part of a horse farm, wild type *R. equi* containing $VapA^{+}$ plasmid will be present.

1.1.3.B.9. Any known planned developments or changes in land use in the region, which could influence the environmental impact of the release.

No such developments are known to the applicant.

1.1.4 . Information Relating to the Interactions between the GMOs and the Environment

1.1.4.A. Characteristics affecting survival, multiplication and dissemination

1.1.4.A.1. Biological features which affect survival, multiplication and dispersal

The vaccine strain is a deletion mutant which is less able to survive in macrophages and, as a result, unable to colonise the lungs and consequently not pathogenic for horses and other species. The other characteristics are identical to wild type *R. equi*. Thus in the gut as well as outside the host (where no macrophages exist) the vaccine strain is expected to behave similarly to the wildtype *R. equi* and thus long term colonization of the gut is theoretically possible. Except for a hampered macrophage survival no differences in survivability between vaccine strain and wildtype have been observed, so it is not clear whether the deletions have any negative competitive effect in certain environments (e.g. environments enriched with steroids). The most negative assumption is that outside the host there is no difference in survivability between vaccine strain and wildtype. Given the nature of the vaccine strain (unmarked deletion mutant, with no additional genes introduced into the environment), additional risks for humans, horses and environment are nearly zero.

1.1.4.A.2. Known or predicted environmental conditions which may affect survival, multiplication and dissemination (wind, water, soil, temperature, pH etc.)

The presence of manure, temperatures above 10°C and a neutral pH have a positive influence on the multiplication of *R. equi* in soil (see Hughes and Sulaiman, 1987, Annex 24).

1.1.4.A.3. Sensitivity to specific agents

Steri-7 can be used to disinfect materials which were in contact with the vaccine strain. A validation report demonstrating the effect of Steri-7 on *Rhodococcus equi* is provided in Annex 55.
The vaccine strain is sensitive to ampicillin, chloramphenicol, erythromycin, rifampicin, gentamycin, neomycin, amoxicillin, spectinomycin, enrofloxacin, spiramycin, and doxycycline.

1.1.4.B. Interactions with the environment

1.1.4.B.1. Predicted habitat of the GMOs

The predicted habitats of the GMO are the gut of vaccinated foals and soil.

1.1.4.B.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, animal houses etc. may also be of relevance to medicinal products;

The organism does not produce specific survival structures such as spores but nevertheless can survive for long period of time in manure and soil. **INFORMATION DELETED FOR CONFIDENTIALITY PURPOSES.** The bacterium could be isolated after more than a year of incubation under the above conditions and there was no difference in survival between the parent strain and the deletion mutant strain. In this context it should be noted that the vaccine strain is attenuated for survival in macrophages and therefore cannot induce disease (see **INFORMATION DELETED FOR CONFIDENTIALITY PURPOSES** 10R/0075, Annex 8) but in the gut and outside the host it is expected to behave similarly to the wildtype parent strain.

1.1.4.B.3. Genetic transfer capability:

(a) Post-release transfer of genetic material from GMOs into organisms in affected ecosystems

The intended vaccine strain *R. equi* RG2837 was constructed by deletion of four genes from its genome. The genome of *R. equi* strain RG2837 and the wild type parent strain RE1 were sequenced. **INFORMATION DELETED FOR CONFIDENTIALITY PURPOSES**

The gene deletion makes the vaccine strain less able to survive in macrophages and therefore unable to cause clinical disease in the lung. Intervet is not aware of any other phenotypic difference with its parent *R. equi* RE1. This also means that we do not expect the vaccine strain to differ from its parent with regard to the transfer genetic material post-release. However, the vaccine strain cannot transfer any genetic material that is not already present in the environment, as it does not contain foreign/recombinant genetic material. Until today only *R. equi* strains have been identified that harbour a $vapA^+$, $vapB^+$ or $vapAB^-$ plasmids. No evidence has been found that some *R. equi* strains might contain more than one vap plasmid. The virulence plasmid might be transferred to a *R. equi* organism that does not harbour a virulence plasmid. The vaccine strain contains $vapA^+$ plasmid and the vast majority of *R. equi* strains isolated on horse farms already contain a virulence plasmid (pers. communications J. Prescott). The predominant virulence plasmid found in horses is $vapA^+$ and therefore the balance of virulence plasmids found in the *R. equi* population present on the release site environment is unlikely to be changed (Ocampo-Sosa *et al.* 2007, Annex 2).

(b) Post-release transfer of genetic material from indigenous organisms to the GMOs

See 1.1.2.A.9.

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

The GMO is a deletion mutant without any foreign genes. Therefore, the assessment of likelihood that post-release selection may lead to the expression of unexpected and/or undesirable traits in the modified organism is negligible.

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

The GMO is a deletion mutant and therefore it does not contain any genes which are not already present in the environment. The chance that the *ipdAB1*deletion is transferred to a wild type *R. equi* strain is very limited because the deletion is present in a gene on the chromosome and not on a mobile element like a plasmid or transposon. In the unlikely event that the deletion will be transferred to a wild type *R. equi* strain, the new strain will be a deletion mutant as well, with a reduced capability to survive in macrophages. This is not considered as an evolutionary benefit.

Genetic stability can be demonstrated by sequencing, PCR or the macrophage survival assay.

1.1.4.B.6. Known routes of biological dispersal or potential modes of interaction with the disseminating agent, including inhalation, ingestion, surface contact etc.

Potential modes of interaction with the disseminating agent are ingestion and inhalation of soil and litter contact. Peak spreading occurs on day 1 after vaccination. Thereafter, only low level intermittent shedding was observed in some foals up to several weeks after vaccination. An endpoint has not been determined and therefore it cannot be excluded that the vaccine strain could result in lifelong (subdetectable) colonization. If it occurs it does not pose an additional risk since the vaccine strain is a clean deletion mutant (containing only 12 residual nucleotides from the vector) that lacks 4 genes in comparison to the wild type strain that is already present in the environment, especially on horse farms.

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

The ecosystems into which the vaccine strain can be disseminated with the litter of recently vaccinated foals are primarily paddocks and fields. At later stage, at least 4 weeks after last vaccination, some of the foals can also be moved to a field along a tributary of the river Shannon.

Since the vaccine strain contains the VapA encoding virulence plasmid which determines species specificity (in this case horses), it is not expected to colonize other animal species such as cattle, pigs or rodents. The safety and lack of colonization in other animals has been demonstrated under experimental conditions **INFORMATION DELETED FOR CONFIDENTIALITY PURPOSES.** All three plasmid types (including strains lacking VapA and VapB) were found in *R. equi* strains isolated from humans and as such the $VapA^{+}$ possessing wildtype parent strain could be pathogenic for immunocompromised humans. However, since the deletion mutant vaccine strain is less able to survive in human macrophages (in contrast to the parent strain) it is expected to be unable to cause disease in immunocompromised humans. The attenuation and safety was further proven in the most sensitive host, the foal.

To our knowledge, *Rhodococcus* has only been isolated from diseased immunocompromised people (especially AID patients) and never from healthy persons. Healthy people do not carry *Rhodococcus.* Subclinical infections with *Rhodococcus* and subsequent infection of others are unlike to occur**.**

As *R. equi* is widespread in the environment on all horse farms in the area and the vaccine strain does not introduce any new genes or plasmids into the environment, the risk is effectively zero.

1.1.4.B.8. Potential for excessive population increase in the environment

The GMO is an attenuated *R. equi* strain; two genes that are important for the pathogenicity of wild-type *R. equi* have been deleted. Therefore, the potential for excessive population increase in the environment is considered low. Outside the host the vaccine strain is expected to behave similarly to the wildtype parent strain. Spiking experiments indicate a long survival time in soil but no excessive increase **INFORMATION DELETED FOR CONFIDENTIALITY PURPOSES).**

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

The deletion responsible for attenuation is the deletion in the *ipdAB1* gene which is involved in the cholesterol metabolism. This resulted in a strain which is attenuated for macrophage

survival and therefore no longer pathogenic for the target species. Cholesterol metabolism as such is not essential for macrophage survival since mutants that are unable to take up cholesterol (i.e. SupAB deletion mutant) are not attenuated for macrophage survival (van der Geize *et al*, 2008, Annex 33, **INFORMATION DELETED FOR CONFIDENTIALITY PURPOSES** and 10R/0075, Annex 8). However, enzymes that are part of the cholesterol degradation chain, in particular those involved in HIL degradation (i.e. ipdAB and Fad30), appeared to be essential for macrophage survival because deletion mutants of the respective genes were shown to be hampered in macrophage survival (see report 10R/0075, Annex 8).
The precise mechanism for the survival of *R. equi* in macrophages has yet to be elucidated, but it is evident from our studies that either the enzymes of the cholesterol degradation chain pathway appear to play a part (the enzyme pathway may serve a different function during macrophage residency) or that the buildup of the intermediates of cholesterol degradation, at the point of HIL may hamper the survival of the bacteria in macrophages.

In the respiratory tract, the competitive advantage is therefore considered to be in favour of the parent organism. In the gut and outside the animal host there is probably no difference in competitive advantage because macrophages are not present. See also under 1.1.4.B.2 and 1.1.4.B.8.

Except for a hampered macrophage survival no differences in survivability between vaccine strain and wildtype have been observed, so it is not clear whether the deletions have any negative competitive effect in certain environments (e.g. environments enriched with steroids).
The most negative assumption is that outside the host there is no difference in survivability between vaccine strain and wildtype. Given the nature of the vaccine strain (unmarked deletion mutant, with no additional genes introduced into the environment), additional risks for humans, horses and environment are nearly zero.

1.1.4.B.10. Identification and description of the target organisms if applicable

Horses, i.e. young foals at risk of mortality and pyogranulomatous pneumonia caused by *R. equi*.

1.1.4.B.11. Anticipated mechanism and result of interaction between the released GMOs and the target organism if applicable

Active immunisation of horses against *R. equi*, to reduce mortality and pyogranulomatous pneumonia caused by *R. equi*.

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

Since the vaccine strain contains the VapA encoding virulence plasmid which determines species specificity (in this case horses), it is not expected to colonize other animals such as cattle or rodents. The safety and lack of colonization in other animals (rats, mice, chickens, pigs and calves) has been demonstrated under experimental conditions **INFORMATION DELETED FOR CONFIDENTIALITY PURPOSES.**

The vaccine/mutant strain is less able to survive in human macrophages (in contrast to the parent strain) and therefore it is expected to be unable to cause disease in (immuno-compromised) humans, although a direct correlation between survival in macrophages and human pathogenicity has never been tested or demonstrated.
Strains lacking the virulence plasmid (that, like the vaccine strain are attenuated for macrophage survival) have been isolated from immunocompromised persons. However, since the vaccine strain was shown to be less able to survive in human macrophages it can be expected that the risk for immunocompromised persons is at most equal but most probably less compared to the wildtype *Rhodococcus equi*. In this context it is relevant to note that the vaccine strain will be used in environments where the wildtype *Rhodococcus* is (massively) present and had not caused disease in humans.

To our knowledge, *Rhodococcus* has only been isolated from diseased immunocompromised people (especially AID patients) and never from healthy persons. Healthy people do not carry *Rhodococcus.* Subclinical infections with *Rhodococcus* and subsequent infection of others are unlike to occur,

The attenuation of the vaccine strain was proven in the most sensitive host, the foal.

Except for a hampered macrophage survival no differences in survivability between vaccine strain and wildtype have been observed, so it is not clear whether the deletions have any negative competitive effect in certain environments (e.g. environments enriched with steroids).
The most negative assumption is that outside the host there is no difference in survivability between vaccine strain and wildtype. Given the nature of the vaccine strain (unmarked deletion mutant, with no additional genes introduced into the environment), additional risks for humans, horses and environment are nearly zero.

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

Deletions have been made in 4 genes of which two are involved in the pathogenicity of wild type *R. equi*. Therefore, the likelihood of post-release shifts in biological interactions or in host range is therefore considered negligible.

1.1.4.B.14. Known or predicted effects on non-target organisms in the environment, impact on population levels of competitors, hosts, symbionts and pathogens

There are no known or predicted effects on non-target organisms in the environment. The GMO could not be recovered from rats, mice, calves and chickens after being inoculated with high doses of the GMO **(INFORMATION DELETED FOR CONFIDENTIALITY PURPOSES).**

1.1.4.B.15. Known or predicted involvement in biogeochemical processes

There are no known or predicted involvements in biogeochemical processes.

1.1.4.B.16. Other potentially significant interactions with the environment

None.

1.1.5. Information on Monitoring, Control, Waste Treatment and Emergency Response Plans

1.1.5.A. Monitoring Techniques

1.1.5.A.1. Methods for tracing the GMOs, and for monitoring their effects

Reisolation on selective agar plates as described in 1.1.2.A.6 is the most sensitive method for *R. equi*. Since the selective agar cannot discriminate between vaccine strain RG2837 and field (wild type) strains, it is necessary to determine the identity by PCR in a second step.

1.1.5.A.2. Specificity (to identity the GMOs, and to distinguish them from the donor, recipient or, where appropriate, the parental organisms), sensitivity and reliability of the monitoring techniques

PCR is considered as a specific and highly sensitive and reliable technique to confirm the identity of the vaccine strain by demonstrating the presence of the deletions. The selective agar used to reisolate *R. equi* has a detection limit in faeces of 1.5 CFU/mg. This means that if 0.003% of the detectable faecal flora consists of the vaccine strain, it can still be detected. **INFORMATION DELETED FOR CONFIDENTIALITY PURPOSES**.

1.1.5.A.3. Techniques for detecting transfer of the donated genetic material to other organisms.

Not applicable, the GMO is a deletion mutant

1.1.5.A.4. Duration and frequency of monitoring.

The focus of monitoring will be on possible adverse effects on animals, including wild life, and humans. The monitoring period is every year between first vaccination and last clinical examination, when the last foal reaches the age of 6 months.

- Foals will be closely monitored for clinical signs (general demeanour, faecal consistency and rectal temperatures) for up to 14 days after each vaccination Thereafter the health of the foals and especially the presence of pulmonary abscesses is checked every 2-4 weeks.
- Twice per month one of the staff members of Belmont farm will inspect the site for any unexpected mortality in wild animals.
- The owner and farm staff will be instructed to report immediately any disease in the other horses or in the personnel that could possibly related to the vaccine. For further details see the emergency plan (chapter 1.1.5.D.2).

As *R. equi* is widespread in the environment and the introduction of the vaccine strain does not introduce any new genes or plasmids into the environment the risk is effectively zero.

1.1.5.B. Control of the Release

1.1.5.B.1. Methods and procedures to avoid and/or minimise the spread of the GMOs beyond the site of the release or the designated areas of use

The foals will remain in the foal shed, stables or the foal paddock (Pony Garden) (S14901063 on the aerial map) for at least 1 week after each vaccination (peak shedding is expected the first few days after rectal vaccination). During this period, the straw, litter and faeces will be removed and inactivated by heat treatment to reduce the spread of the vaccine strain.
Thereafter, the foals and the mares can be allowed onto the field that is denoted S14901062 on the aerial map. Big Field and Mill field. Once the foals are 4 weeks after last vaccination, the possible shedding will be minimal and the animals can be moved to the other fields and paddocks.
At the end of the study, the release site will be cleaned as follows. After the complete removal the straw and litter, the stable, the total concrete floor before the stable and all equipment used will be cleaned with the standard concentration of Steri-7 (see validation report, Annex 55). The straw and litter will be heat inactivated by a specialised and approved company (SRCL, 430 Beech Road, Western Industrial Est., Dublin 12).
As the vaccine strain does not readily colonize other species **INFORMATION DELETED FOR CONFIDENTIALITY PURPOSES),** no extra measures will be taken to avoid rats and mice entering the unit.
Other mares and foals from other farms will not be allowed on the site until at least 4 weeks after last vaccination

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

The farm is surrounded by a locked fence. Only authorised personnel are allowed to enter the facilities. A logbook to register the visiting persons and horses will be maintained.

1.1.5.C. Waste treatment

1.1.5.C.1. Type of waste generated

The following types of waste are generated:
- used (disposable) vaccination equipment
- empty vials
- litter and straw

As with all livestock it is possible that a treated animal dies during the study. In such a case a post mortem investigation will be performed by the Irish Equine Centre (or another approved laboratory). The remains will be treated as contaminated waste

1.1.5.C.2. Expected amount of waste

Up to 300 vials (4 x 75 vials of the vaccine and 4 x 75 vials of the diluent to be used with the vaccine).

Up to 320 syringes with or without applicator to be used with the vaccine.

Up to 1000 m^3 of straw and litter.

1.1.5.C.3. Description of treatment envisaged

Dispose of waste material by heat inactivation or immersion in the standard concentration of Steri-7 (see validation report, Annex 55).

1.1.5.D. Emergency response plans

In case an adverse event occurs, the authority that is responsible for the inspection of the release will be informed and consulted.

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

Standard antibiotics for *R. equi* infection. The vaccine strain is sensitive to ampicillin, chloramphenicol, erythromycin, rifampicin, gentamycin, neomycin, amoxicillin, spectinomycin, enrofloxacin, spiramycin, and doxycycline.

1.1.5.D.2. Methods for decontamination of the areas, e.g. eradication of the GMOs.

At the end of the study, the stables will be cleaned and decontaminated by using Steri-7 (see validation report Annex 55).

1.1.5.D.3. Methods for disposal or sanitation of areas affected by the spread.

The straw and litter from the affected stables will be transported by an approved company to an approved centre for heat inactivation (SRCL, 430 Beech Road, Western Industrial Est., Dublin 12). The stables/barns will be cleaned and decontaminated as described above.

1.1.5.D.4. Methods for the isolation of the areas affected by the spread.

The vaccinated animals will be kept inside or on the foals paddock (Pony Garden) (S14901063) for a one week after each vaccination. During this period of peak shedding the litter is removed from the stables and the field. Thereafter the study animals are only allowed onto plot S14901062 until at least 4 weeks after last vaccination and the shedding is minimal. Considering the restrictions of a normal horse farm this is the maximum isolation that is practically possible.

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

R. equi infections in immunocompetent humans are extremely rare. To our knowledge, *Rhodococcus* has only been isolated from diseased immunocompromised people (especially AIDS patients) and never from healthy persons. Healthy people do not carry *Rhodococcus.* Subclinical infections with *Rhodococcus* and subsequent infection of others are unlike to occur.

R. equi virulence plasmids can be classified into three general types: $VapA^{+}$, $VapB^{+}$ and $VapAB^{-}$. Each of these plasmid types was almost exclusively associated with a specific non-human animal host, i.e. horse, pig and cattle, respectively (Ocampo-Sosa *et al* 2007, Annex 2). By contrast all three plasmid types were found in *R. equi* strains from humans and as such the $VapA^{+}$ plasmid possessing wild type parent strain could be pathogenic for immunocompromised humans. However, since the mutant strain is less able to survive in human macrophages (in contrast to the parent strain) it is expected to be unable to cause disease in immunocompromised humans. The attenuation and safety was further proven in the most sensitive host, the foal. Since

the vaccine strain possesses the VapA encoding virulence plasmid which determines species specificity (in this case horses) it is not expected that other animals will be colonised **INFORMATION DELETED FOR CONFIDENTIALITY PURPOSES).** Therefore, occurrence of an undesirable effect affecting the environment can be considered as effectively zero.

Despite the rationale given above, an emergency plan has been established in which 3 operating phases are implemented:

1. Alert phase

Any observation which cannot be related to normal post vaccination reactions must be reported to the investigator and to the monitor of the trial.

2. Investigation phase

Appropriate samples are collected and sent to the laboratory for isolation and identification. If present, diseased animals will be treated with antibiotics. Dead animals will be destroyed. In the unlikely case that humans are affected they also will be treated with antibiotics.

3. Action phase

The study will be cancelled and the unit is treated as described under 1.1.5.D.2 above. The animals will remain in isolation until a decision has been taken by the applicant in consultation with the responsible authorities concerning the consequences for the animals. This may, for instance, consist of antibiotic treatment, monitoring of shedding or a combination of measures.

2. SUMMARY NOTIFICATION INFORMATION FORMAT FOR THE RELEASE OF GENETICALLY MODIFIED ORGANISMS OTHER THAN HIGHER PLANTS IN ACCORDANCE WITH ARTICLE 11 OF DIRECTIVE 2001/18/EC

In order to tick one or several possibilities, please use crosses (meaning x or X) into the space provided as (.)

A. General information

1. Details of notification

(a) Member State of notification Ireland…
(b) Notification number B/IE/12/02
(c) Date of acknowledgement of notification 14/December/2012
(d) Title of the project: 'A blinded, placebo controlled, clinical and efficacy study of Equilis RhodE in horses in Ireland'.
(e) Proposed period of release From 13/03/2013 until 30/09/2016

2. Notifier

Name of institution or company: Intervet International B.V., Wim de Korverstraat 35, NL - 5831 AN Boxmeer, the Netherlands.

3. GMO characterisation

(a) Indicate whether the GMO is a:

viroid (.)

RNA virus (.)

DNA virus (.)

bacterium (X)

fungus (.)

animal

- mammals (.)
- insect (.)
- fish (.)
- other animal (.)

specify phylum, class …

(b) Identity of the GMO (genus and species)
Genus: *Rhodococcus*
Species: *Rhodococcus equi (*Deletion mutant of Rhodococcus equi strain RG2837)

(c) Genetic stability – according to Annex IIIa, II, A(10)

Genetic transfer is limited to exchange of plasmids. The main factor for this is close contact between different *R. equi* organisms and the receiving organism should not already contain a $VapA^+$ plasmid. Analysis of the available literature does not provide any reason to assume that transduction and transformation play an important role in the natural environment of *R. equi*. Furthermore natural competence has not been reported for any *Rhodococci*.

4. Is the same GMO release planned elsewhere in the Community (in conformity with Article 6(1)), by the same notifier?

Yes (X) No ()

If yes, insert the country code(s) NL and DE

5. Has the same GMO been notified for release elsewhere in the Community by the same notifier?

Yes (X) No (.)

If yes:

- Member State of notification : NL and DE
- Notification number B/NL/09/004 and BVL 107/2012/4

Please use the following country codes:

Austria AT; Belgium BE; Germany DE; Denmark DK; Spain ES; Finland FI; France FR; United Kingdom GB; Greece GR; Ireland IE; Iceland IS; Italy IT; Luxembourg LU; Netherlands NL; Norway NO; Portugal PT; Sweden SE

6. Has the same GMO been notified for release or placing on the market outside the Community by the same or other notifier?

Yes (.) No (X.)

If yes:

- Member State of notification …
- Notification number B/../../…

7. Summary of the potential environmental impact of the release of the GMOs.

Rhodococcus equi is a common soil bacterium that also can colonize the gut and nasal passages of animals, especially herbivores, and is the cause of severe pneumonia in foals. The GMO (vaccine strain) is shed in the environment with the manure of vaccinated animals during a short period following vaccination (is detectable for at least 4 weeks post vaccination). The GMO is an unmarked deletion mutant of *Rhodococcus equi*. Because of this deletion the bacterium is less able to survive in macrophages (in contrast to the wild type) and therefore safe for foals (in contrast to the wild type). The deletions do not provide any competitive benefits outside the vaccinated animals, compared to wild type *R. equi.* The attenuation (deletion of the *ipdAB1* and *ipdAB2* genes from the chromosome and therefore less able to survive in macrophages), does not play a role outside the animal. It must be assumed that the vaccine strain will be shed into the environment, where it will behave the same in soil as wild type *R. equi*. This was confirmed by spiking experiments where the vaccine strain and the parent strain both survived for more than a year in soil and water and no difference between the two strains were apparent. However, the attenuation will reduce the spreading by horses or other animals. Except for a hampered macrophage survival no differences in survivability between vaccine strain and wild type have been observed, so it is not clear whether the deletions have any negative competitive effect in certain environments (e.g. environments enriched with steroids). The most negative assumption is that outside the host there is no difference in survivability between vaccine strain and wildtype. Given the nature of the vaccine strain (unmarked deletion mutant, with no additional genes introduced into the environment), environmental impact of release of the GMO is judged effectively zero.

B. **Information relating to the recipient or parental organism from which the GMO is derived**

1. Recipient or parental organism characterisation:

(a) Indicate whether the recipient or parental organism is a:

(select one only)

viroid	(.)
RNA virus	(.)
DNA virus	(.)

bacterium (X)

fungus (.)

animal

- mammals (.)
- insect (.)
- fish (.)
- other animal (.)
  (specify phylum, class) …

other, specify …

2. Name
   (i) order and/or higher taxon (for animals) …
   (ii) genus *Rhodococcus*
   (iii) species *Rhodococcus equi*
   (iv) subspecies
   (v) strain RE1

   (vi) pathovar (biotype, ecotype, race, etc.) …
   (vii) common name …

3. Geographical distribution of the organism

(a) Indigenous to, or otherwise established in, the country where the notification is made:
Yes (X) No (.) Not known (.)

(b) Indigenous to, or otherwise established in, other EC countries:
(i) Yes (X)

If yes, indicate the type of ecosystem in which it is found: The bacterium occurs world-wide in soil and surface water, especially where herbivores graze. In these animals it colonizes nasal cavities and gut. In foals colonization of airways can lead to pneumonia.

Atlantic ..
Mediteranean ..
Boreal ..
Alpine ..
Continental ..
Macaronesian ..

(ii) No (.)
(iii) Not known (.)

(c) Is it frequently used in the country where the notification is made?
Yes (.) No (X)

(d) Is it frequently kept in the country where the notification is made?
Yes (.) No (X)

4. Natural habitat of the organism

(a) If the organism is a microorganism

water	(X)
soil, free-living	(X)
soil in association with plant-root systems	(.)
in association with plant leaf/stem systems	(.)
other, specify	…

If the organism is an animal:

5. (a+b) Detection and identification techniques

Isolation on selective agar and PCR followed by bacteriological determination and PCR

6. Is the recipient organism classified under existing Community rules relating to the protection of human health and/or the environment?
Yes (X) No (.)

If yes, specify EC class 2 organism (EC 2000/54/EG)

7. Is the recipient organism significantly pathogenic or harmful in any other way (including its extracellular products), either living or dead?
Yes (X) No (.) Not known (.)

If yes:

(a) to which of the following organisms:

humans	(X) only immunocompromised humans
animals	(X) pneumonia in foals
plants	(.)
other	(.)

(b) give the relevant information specified under Annex III A, point II. (A)(11)(d) of Directive 2001/18/EC
The parent organism is a facultatively pathogenic soil saprophyte. The live wildtype *Rhodococcus equi* can cause pneumonia in foals and can cause

infections in immunocompromised humans (e.g. AIDS patients). *R equi* rarely infects immunocompetent humans. *R. equi* strains are also isolated from pigs, where they can cause tuberculosis-like lesions, but can also be found in submandibular lymph nodes and tonsils of healthy animals. *R. equi* also cause tuberculosis-like lesions in lymph nodes of cattle and in the livers of young goats. Other animals occasionally infected by *R. equi* are sheep, llama, cats and dogs. Of all species, disease caused by *R. equi* infection in foals is by far the most devastating. *R. equi,* which normally is found in various environments from soil and ground water, infects the foal by inhalation of aerosolized dust contaminated with these bacteria, invades, survives and multiplies in alveolar macrophages by arresting the normal pathway of phagosome maturation. Neutrophilic leucocytosis and hyperfibrinogenaemia are common findings, associated with abscessation and pulmonary changes. Experimental data suggest that *R equi* is capable of inhibiting oxidative bactericidal functions of polymorphonuclear cells. Electron microscopy of *R equi* in equine macrophages demonstrates that the organisms appear to avoid being killed by interfering with phagosome-lysosome fusion. Most of the information about the pathogenesis of *R equi* infections is derived from animal isolates. However, the infection in humans seems to differ from that in foals. A 15- to 17-kd virulence-associated protein antigen (VapA), is highly associated with virulence in foals. Nearly all isolates from pigs have a 20-kd virulence-associated protein antigen (VapB). In human beings, only about 20-25% of isolates have been reported to express either VapA, or VapB. The rest does not have Vap or VapB encoding genes. There are no reports about toxigenicity, allergenicity or vectors.*R. equi* infections of foals occur worldwide. Increased incidences of *R. equi* pneumonia is associated with large farm size, high density and population size of foals, high numbers of airborne virulent *R. equi*, low soil moisture, high temperatures and a poor pasture grass cover. Farms with endemic *R. equi* pneumonia are heavily contaminated with virulent *R. equi*. However avirulent *R. equi* are frequently found in environment and faeces on every farm.

In the first weeks of a foal's life, ingestion of *R. equi* often leads to colonization of the intestines. Foals shed large quantities of *R. equi* as compared with adults, but the number of bacteria in faeces declines after 7 weeks of age. Ingestion of *R. equi* does not usually result in disease, but in immunization. As a result of this process, older foals and adult animals have antibodies against *R. equi* and rarely get infected.

Virulence of *R. equi* is associated with the possession of Virulence Associated Proteins (VAPs) that are encoded by the virulence plasmid. The plasmid is essential for multiplication in macrophages, prolonged inhibition of phagosome maturation and it enhances cytotoxicity. Isogenic strains from

which the plasmid has been removed are avirulent in foals and mice and do not multiply in macrophages.

So far, three types of VAPs have been identified, two of which have been sequenced and further investigated. Whereas possession of certain VAPs seems to be specific for strains infecting foals (VapA+), pigs (VapB+) or cattle (VapAB-). By contrast all three plasmid types could be found in *R. equi* strains from humans, a host in which the infection is opportunistic and associated with immunosuppression. Additionally, strains devoid of virulence plasmids are regarded as non-pathogenic for foals and mice have also been isolated in immunocompromised humans. Immunocompetent humans are rarely affected by *R. equi*, while a compromised cell mediated immunity predisposes one to *R. equi* infection. As with other immunocompromised individuals, infection mostly results in pneumonia with fever, cough, and chest pain, but can also spread to other organs and cause bacteraemia. The fact that human isolates from pathological conditions have all types of plasmid categories, including plasmid less strains, indicates that the immunocompromised human host is susceptible to a variety of *R. equi* strains and emphasises the opportunistic nature of *R. equi* in this host.

The minimum infective dose under natural conditions is not known for any species, including humans because it never has been determined. In the artificial intratracheal challenge model, doses from 10^4 CFU and higher appear infectious.

8. Information concerning reproduction

(a) Generation time in natural ecosystems:
Depending on conditions 30 min to days

(b) Generation time in the ecosystem where the release will take place:
See previous

(c) Way of reproduction: ~~Sexual~~ ..
Asexual: cell division

(c) Factors affecting reproduction:
temperature, nutrients

9. Survivability

(a) ability to form structures enhancing survival or dormancy:

(i)	endospores	(.)
(ii)	cysts	(.)
(iii)	sclerotia	(.)
(iv)	asexual spores (fungi)	(.)
(v)	sexual spores (funghi)	(.)
(vi)	eggs	(.)
(vii)	pupae	(.)

(viii) larvae (.)
(ix) other, specify …

(b) relevant factors affecting survivability:
temperature, pH, availability of nutrients

10. (a) Ways of dissemination

Wind (attached to dust particles), animals (nasal and or gut colonization)

(b) Factors affecting dissemination

Presence of herbivores, housing practices and weather conditions

11. Previous genetic modifications of the recipient or parental organism already notified for release in the country where the notification is made (give notification numbers)
Not applicable

C. **Information relating to the genetic modification**

1. Type of the genetic modification

(i) insertion of genetic material (.)
(ii) deletion of genetic material (X)
(iii) base substitution (.)
(iv) cell fusion (.)
(v) others, specify …

2. Intended outcome of the genetic modification
Unable to survive in macrophages (in contrast to wildtype)

3. (a) Has a vector been used in the process of modification?
Yes (X) No (.)

If no, go straight to question 5.

(b) If yes, is the vector wholly or partially present in the modified organism?
Yes (X.) No ()

Only 2x6 nucleotides (GATATC) and (AGATCT) remain at the two ligation sites. These nucleotides are remnants of the plasmid used for gene deletion.

.

If no, go straight to question 5.

4. If the answer to 3(b) is yes, supply the following information

(a) Type of vector

plasmid (.)
bacteriophage (.)

virus (.)
cosmid (.)
transposable element (.)
other, specify …

(b) Identity of the vector
…
(c) Host range of the vector
…

(d) Presence in the vector of sequences giving a selectable or identifiable phenotype
Yes (.) No (.)

antibiotic resistance (.)
other, specify …

Indication of which antibiotic resistance gene is inserted
…

(e) Constituent fragments of the vector
…

(f) Method for introducing the vector into the recipient organism

(i) transformation (.)
(ii) electroporation (.)
(iii) macroinjection (.)
(iv) microinjection (.)
(v) infection (.)
(vi) other, specify …

5. If the answer to question B.3(a) and (b) is no, what was the method used in the process of modification?

(i) transformation (X)
(ii) microinjection (.)
(iii) microencapsulation (.)
(iv) macroinjection (.)
(v) other, specify …

6. Composition of the insert

Not applicable (deletion mutant). (a) Composition of the insert
…

(b) Source of each constituent part of the insert
…

(c) Intended function of each constituent part of the insert in the GMO

…

(d) Location of the insert in the host organism

- on a free plasmid (.)
- integrated in the chromosome (.)
  - other, specify …

(e) Does the insert contain parts whose product or function are not known?
Yes (.) No (.)

If yes, specify …

D. Information on the organism(s) from which the insert is derived

1. Indicate whether it is a:

viroid (.)

RNA virus (.)

DNA virus (.)

bacterium (.)

fungus (.)

animal

- mammals (.)
- insect (.)
- fish (.)
- other animal (.)
  (specify phylum, class) …

other, specify …

2. Complete name

(i) order and/or higher taxon (for animals) …
(ii) family name for plants …
(iii) genus …
(iv) species …
(v) subspecies …
(vi) strain …
(vii) cultivar/breeding line …
(viii) pathovar …
(ix) common name …

3. Is the organism significantly pathogenic or harmful in any other way (including its extracellular products), either living or dead?

Yes (.) No (.) Not known (.)
If yes, specify the following:

(b) to which of the following organisms:

humans (.)
animals (.)
plants (.)
other ..

(b) are the donated sequences involved in any way to the pathogenic or harmful properties of the organism
Yes (.) No (.) Not known (.)

If yes, give the relevant information under Annex III A, point II(A)(11)(d):
…

4. Is the donor organism classified under existing Community rules relating to the protection of human health and the environment, such as Directive 90/679/EEC on the protection of workers from risks to exposure to biological agents at work?
Yes (.) No (.)

If yes, specify …

5. Do the donor and recipient organism exchange genetic material naturally?
Yes (.) No (.) Not known (.)

E. Information relating to the genetically modified organism

1. Genetic traits and phenotypic characteristics of the recipient or parental organism which have been changed as a result of the genetic modification

(a) is the GMO different from the recipient as far as survivability is concerned?
Yes (X)* No (X)** Not known (.)
Specify
*Yes: the GMO is less able to survive in macrophage. Invasion and growth of the

alveolar macrophages is essential for the development of pneumonia.

**No: there appears to be no difference in survival in the environment.

(b) is the GMO in any way different from the recipient as far as mode and/or rate of reproduction is concerned?
Yes (X)* No (X)** Unknown (.)
Specify
*Yes: the GMO is less able to survive and reproduce in macrophage. Invasion and growth of the alveolar macrophages is essential for the development of pneumonia.

**No: there appears to be no difference in growth in the environment.

(c) is the GMO in any way different from the recipient as far as dissemination is concerned?

Yes (X)* No (X)** Not known (.)

Specify

*Yes: as the GMO is less able to grow in the lungs there will be less dissemination.

**No: there appears to be no difference in growth in the gut and therefore rectal

excretion will be similar.

(d) is the GMO in any way different from the recipient as far as pathogenicity is concerned?

Yes (X) No (.) Not known (.)

Specify

The GMO is less able to survive and grow in alveolar macrophage, the site of infection that is central in the development of R. equi pneumonia in foals.

2. Genetic stability of the genetically modified organism
See section A.3.c

3. Is the GMO significantly pathogenic or harmful in any way (including its extracellular products), either living or dead?

Yes (.) No (X) Unknown (.)

(a) to which of the following organisms?

humans (.)
animals (.)
plants (.)
other …

(b) give the relevant information specified under Annex III A, point II(A)(11)(d) and II(C)(2)(i)

…

4. Description of identification and detection methods

(a) Techniques used to detect the GMO in the environment

The GMO cannot be monitored directly in the environment. Indirect monitoring can be done by taking samples and plate them out on selective agar. Positive

identification will follow from R. equi and GMO specific PCR's.

(b) Techniques used to identify the GMO

Selective agar and PCR's based on the genome region that has been modified.

F. Information relating to the release

1. Purpose of the release (including any significant potential environmental benefits that may be expected)
   Study the efficacy of the vaccine under field conditions

2. Is the site of the release different from the natural habitat or from the ecosystem in which the recipient or parental organism is regularly used, kept or found?
   Yes (.) No (X)

   If yes, specify …

3. Information concerning the release and the surrounding area

   (a) Geographical location (administrative region and where appropriate grid reference):
   The vaccine will be tested in foals on Belmont Stud Farm, Belmont, Co. Offaly
   Grid reference: N53° 15.072 W007°53.887

   (b) Size of the site (m^2): 52.4 Ha.
   (i) actual release site (m^2): 1.11 Ha.
   (ii) wider release site (m^2): 52.4 Ha.

   (c) Proximity to internationally recognised biotopes or protected areas (including drinking water reservoirs), which could be affected:

   The farm is located in an agricultural area. The area is characterised by a clay soil covered with permanent pasture. The nearest Special Area of Conservation (SAC) is Clara Bog, located 22 km from the release site and therefore not affected by the release of the vaccine strain.

   Groundwater is the principle source of water in Offaly. 72% of the people of Offaly receive water from schemes with groundwater sources, compared with a national average of less than 25%. A groundwater protection scheme has been adopted for all public and group water supply sources in Offaly. Groundwater, which is the source for a large proportion of the drinking water in Co. Offaly, receives a precautionary dose of chlorine to provide disinfection. Surface water is the source for 4 drinking water supplies in Co. Offaly, namely. The River Shannon is the closest supply source for Belmont through the Banagher Regional Water Supply. A tributary of the Shannon is adjacent to the Front Field of Belmont farm and the horses use it for drinking. The closest point of entry to the Shannon is approximately 8 km form the release site. The Shannon river sources receive full physio-chemical treatment, consisting of coagulation, flocculation, sedimentation, filtration, pH correction and disinfection. The release of this organism will have no impact on water quality. As *R. equi* is widespread in the environment on all horse farms in the hinterland and the vaccine strain does not introduce any new genes or plasmids into the environment, the risk is effectively zero.

(d) Flora and fauna including crops, livestock and migratory species which may potentially interact with the GMO

Rodents and birds can come into contact with pasture. Neighbouring farms contain cattle.

4. Method and amount of release

(a) Quantities of GMOs to be released:

Vaccine dose consists of approximately 0.5 -9 x 10^{10} CFU per dose. Up to 300 doses will be used.

(b) Duration of the operation:

Field trial on one farm; duration per location approximately 12-24 months.

(c) Methods and procedures to avoid and/or minimise the spread of the GMOs beyond the site of the release

The foals will be physically contained on the release site, in stables and/or (fenced) pasture, and remain there for at least 6 weeks after last vaccination (peak shedding is expected the first few days after rectal vaccination). The straw and litter of the foals during the first week after each vaccination, the period of peak shedding, will be removed mechanically into closed containers. The straw and litter will be heat-inactivated by a specialised and approved company. Each year the stables on the release site will be cleaned and disinfected.

5. Short description of average environmental conditions (weather, temperature, etc.)

The weather conditions are as usually found in Ireland

6. Relevant data regarding previous releases carried out with the same GMO, if any, specially related to the potential environmental and human health impacts from the release.

None available

G. Interactions of the GMO with the environment and potential impact on the environment, if significantly different from the recipient or parent organism

1. Name of target organism (if applicable)

(i)	order and/or higher taxon (for animals)	Animals / Vertebrates / Mammals / Equidae
(ii)	family name for plants	
(iii)	genus	Equus
(iv)	species	ferus
(v)	subspecies	caballus
(vi)	strain	…
(vii)	cultivar/breeding line	all breeds
(viii)	pathovar	…
(ix)	common name	…

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

Vaccine strain will be present transiently in the intestine and interact with the local lymph nodes and thereby inducing a protective immune response

3. Any other potentially significant interactions with other organisms in the environment
Outside the animal host the vaccine strain will behave similar to the wild type.

4. Is post-release selection such as increased competitiveness, increased invasiveness for the GMO likely to occur?

Yes (.) No (X) Not known (.)

Give details

…

5. Types of ecosystems to which the GMO could be disseminated from the site of release and in which it could become established
In the soil (pasture) where the horses graze.

6. Complete name of non-target organisms which (taking into account the nature of the receiving environment) may be unintentionally significantly harmed by the release of the GMO
None known

(i) order and/or higher taxon (for animals) …
(ii) family name for plants …
(iii) genus …
(iv) species …
(v) subspecies …
(vi) strain …
(vii) cultivar/breeding line …
(viii) pathovar …
(ix) common name …

7. Likelihood of genetic exchange in vivo

(a) from the GMO to other organisms in the release ecosystem:
Unlikely

(b) from other organisms to the GMO:
Unlikely

(a) likely consequences of gene transfer:
Consequences of genes transfer will be unlikely. Occurrence of gene transfer is not more likely than for wildtype R. equi.

8. Give references to relevant results (if available) from studies of the behaviour and characteristics of the GMO and its ecological impact carried out in stimulated natural environments (e.g. microcosms, etc.):
Not available

9. Possible environmentally significant interactions with biogeochemical processes (if

different from the recipient or parental organism)
None

H. Information relating to monitoring

1. Methods for monitoring the GMOs
Isolation on selective agar and further identification by PCR.

2. Methods for monitoring ecosystem effects
The protocol contains a description of the monitoring and data system concerning the animal and its immediate environment.

3. Methods for detecting transfer of the donated genetic material from the GMO to other organisms
Not applicable. It is a deletion mutant.

4. Size of the monitoring area (m^2)
1.11 Ha.
This corresponds to the area of all buildings where vaccine administration will take place and the pastures where the foals will be held for up to one week after vaccine administration. Peak shedding is expected to occur during this period.

5. Duration of the monitoring
Up to three consecutive foaling seasons: from 1st vaccination until the last vaccinated foals reaches the age of 6 months.

6. Frequency of the monitoring
During the 14 days after each vaccination the animals will be monitored daily. Later the foals will be examined every 2 weeks and also the environment will be monitored with 14 day intervals.

I. Information on post-release and waste treatment

1. Post-release treatment of the site

Each year after the last study animals have been vacinated, the release site will be cleaned as follows. After the complete removal the straw and litter, the stable, the total concrete floor before the stable and all equipment used will be cleaned with water and disinfected. No post-release treatment of the paddocks is necessary; it is an attenuated deletion mutant.

2. Post-release treatment of the GMOs
See above.

3. (a) Type and amount of waste generated
Vials, syringes, applicators and up to 1000 m^3 of straw and litter

3. (b) Treatment of waste
By heat inactivation by an approved company or immersion in an appropriate disinfectant

J. Information on emergency response plans

1. Methods and procedures for controlling the dissemination of the GMO(s) in case of unexpected spread

   Controlling spread is not necessary since it is an attenuated deletion mutant, there is not more risk than the already present wildtype Rhodococcus equi. See section J4

2. Methods for removal of the GMO(s) of the areas potentially affected
   Not applicable (see above).

3. Methods for disposal or sanitation of plants, animals, soils, etc. that could be exposed during or after the spread
   Not applicable (see above).

4. Plans for protecting human health and the environment in the event of an undesirable effect

   Despite the negligible risk related to the use of the vaccine strain RG2837, an emergency

   plan has been established in which three operating phases are implemented.

   1. Alert phase

   Any observation which cannot be related to normal post vaccination reactions must be reported to the investigator and to the monitor of the trial.

   2. Investigation phase

   Appropriate samples are collected and sent to the laboratory for isolation and identification. If present, diseased animals will be treated with antibiotics. Dead animals will be destroyed. In the unlikely case that humans are affected they also will be treated with antibiotics.

   3. Action phase

   The study will be cancelled and the unit will be cleaned and decontaminated by using an approved disinfectant. The animals will remain in isolation until a decision has been taken by the applicant in consultation with the responsible authorities concerning the consequences for the animals. This may, for instance, consist of antibiotic treatment, monitoring of shedding or a combination of measures.

3.1 THE ENVIRONMENTAL RISK ASSESSMENT CONDUCTED UNDER THE REQUIREMENTS OF SECTIONS B AND C OF THE SECOND SCHEDULE OF S.I. 500 OF 2003

An assessment of risk according to the guideline for conduct of the environmental risk assessment for veterinary medicinal products which contain or consist of genetically modified organisms is given below.

3.1.1 SUMMARY

This environmental risk assessment concerns the vaccine Equilis RhodE containing live deletion mutant *R. equi* strain RG2837 as active ingredient.

R. equi is a soil bacterium primarily causing infections in grazing animals, mainly foals. *R. equi* is zoonotic although *R. equi* rarely infects immunocompetent humans, it is considered as a pathogen for immunocompromised people. The ability of *R. equi* to persist in macrophages is believed to be the basis of its pathogenesis in all species. This has been best studied in horses (other species have not been studied in that detail). The virulence associated protein VapA appears essential for *R. equi* survival and growth in macrophages and arrest of phagosome maturation (Von Bargen *et al* 2009, Annex 10).

Due to the *ipdAB1* deletion (and despite the presence of $VapA^+$ plasmid) the vaccine strain is less able to survive in (human) macrophages and therefore considered safe for target and non-target species, including humans. Attenuation for foals **(INFORMATION DELETED FOR CONFIDENTIALITY PURPOSES)** and safety in mice, rats, chickens, calves and pigs **(INFORMATION DELETED FOR CONFIDENTIALITY PURPOSES)** has been demonstrated.

The vaccine strain is shed in the environment with the manure of vaccinated animals during a short period following vaccination. The deletions do not provide any competitive benefits outside the vaccinated animals, compared to wild type *R. equi*, since the attenuation (less able to survive in macrophages) does not play a role outside the animal. It must be assumed that the vaccine strain will be shed into the environment, where it will behave the same in soil as wild type *R. equi*. This was confirmed by spiking experiments where both the vaccine strain as well as the parent strain survived for more than a year in soil and no difference between the two strains were apparent **(INFORMATION DELETED FOR CONFIDENTIALITY PURPOSES).** Except for a hampered macrophage survival no differences in survivability between vaccine strain and wildtype have been observed, so it is not clear whether the deletions have any negative competitive effect in certain environments (e.g. environments enriched with steroids). The most negative assumption is that outside the host there is no difference in survivability between vaccine strain and wildtype. Given the nature of the vaccine strain (unmarked deletion mutant, with no additional genes introduced into the environment), additional risks for humans, horses and environment are nearly zero.

The level of risk for both immunocompetent humans and the environment for Equilis RhodE is considered as effectively zero.

3.1.2. ASSESSMENT OF RISK TO HUMANS

3.1.2.1 Hazard identification

R. equi primarily causes infections in grazing animals, mainly foals. Although *R. equi* rarely infects immunocompetent humans, it is considered as a pathogen for immunocompromised people, especially those with acquired immunodeficiency syndrome (AIDS) where it acts as an opportunistic pathogen most commonly manifesting necrotising pneumonia.

The *R. equi* infection in humans seems to differ from that in foals. Makrai *et. al*. demonstrated that about 88% of the clinical isolates from foals were tested positive for a 15- to 17-kd virulence-associated protein antigen (VapA). According to the virulence classification for *R. equi*, $VapA^+$ containing strains are considered virulent (Makrai *et. al.*, 2002, Annex 57). Nearly all isolates from pigs contain a 20-kd virulence-associated protein antigen (VapB), which is considered to be of intermediate virulence. By contrast both plasmid types were found in *R. equi* strains from humans and as such the $VapA^+$ plasmid possessing wild type parent strain could be pathogenic for immunocompromised humans, a host in which the infection is opportunistic and associated with immunosuppression. Additionally, strains devoid of virulence plasmids are regarded as non-pathogenic for foals and mice have also been isolated in immunocompromised humans.

Immunocompetent humans are rarely affected by *R. equi*, while a compromised cell mediated immunity predisposes one to *R. equi* infection. As with other immunocompromised individuals, infection mostly results in pneumonia with fever, cough, and chest pain, but can also spread to other organs and cause bacteraemia. In a study performed by Takai *et al* in Thailand, about 75% of human isolates expressed VapB, and 25% were avirulent. Most of these patients were infected with HIV (Takai *et. al*., 2003, Annex 58). In other studies VapA containing *R. equi* was isolated from humans, but this was never more than 25% of the total number of human isolates (Takai *et. al*., 2003, Annex 58). The fact that human isolates from pathological conditions have all types of plasmid categories, including plasmid less strains, indicates that the immunocompromised human host is susceptible to a variety of *R. equi* strains and emphasises the opportunistic nature of *R. equi* in this host. It is likely that *R. equi* infections of immunosupressed humans are not determined by particular plasmids but by the basal and chromosomally determined pathogenic potential of *R. equi* and the immunological status of the patient, rather than the presence or absence of virulence plasmid, is the major factor in determining whether an infection with *R. equi* occurs. A significantly reduced immune system is likely to allow infection with relatively avirulent organisms (Topino *et al*. 2009, Annex 3; Hondalus 1997, Annex 4).

To our knowledge, *Rhodococcus* has only been isolated from diseased immunocompromised people (especially AIDS patients) and never from healthy persons. Healthy people do not carry *Rhodococcus*. Subclinical infections with *Rhodococcus* and subsequent infection of others are unlike to occur,

The ability of *R. equi* to persist in and destroy macrophages is the basis of its pathogenesis. Due to the deletion of the *ipdAB* genes the vaccine strain is less able to survive in macrophages. This was demonstrated *in vitro* using a human macrophage cell line (see reports **INFORMATION DELETED FOR CONFIDENTIALITY PURPOSES** and 10R/0075, Annex 8). It is therefore expected that the vaccine strain is unable to cause disease in (immuno-compromised) humans, although a direct correlation between survival in macrophages and human pathogenicity has never been tested or demonstrated

Strains lacking the virulence plasmid (that like the vaccine strain are attenuated for macrophage survival) have been isolated from immunocompromised persons. However, since the vaccine strain was shown to be less able to survive in human macrophages it can be expected that the risk for immunocompromised persons is at most equal but most probably less compared to the wildtype *Rhodococcus equi*. In this context it is relevant to note that the vaccine strain will be used in environments where the wildtype *Rhodococcus* is (massively) present and had not caused disease in humans.

It is unlikely that foreign DNA uptake thereby repairing one or both of the gene deletions will occur under field conditions since the deletions were made in the chromosomal DNA. In the case that full gene repair (both genes) would occur, the GMO would become identical to the RE1 parent strain.

3.1.2.2 Assessment of the degree of exposure and the likelihood of the hazard occurring

The vaccine is presented in well-closed containers and the full amount of reconstituted vaccine is required for vaccination. The vaccine is administered rectally and the person handling the vaccine will normally not be exposed to the vaccine strain. Since no needles are used, self-injection can be excluded.

People handling horses and performing daily husbandry at horse farms may come into contact with the vaccine strain when it is excreted with the manure. This is not different from wild type *R. equi*, which is widely spread in the environment, especially on horse farms. The clinical history shows that *R. equi* is also present on the study farm Since human disease caused by wild type *R. equi* is almost exclusively limited to immunocompromised people and the vaccine strain's ability to enter macrophages is reduced, the likelihood that a hazard will occur is considered negligible.

3.1.2.3 Assessment of level of risk.

Taking all the risk factors in consideration the assessment of level of risk for immunocompetent humans for Equilis RhodE can be considered as effectively zero.

3.1.2.4 Consequences of a hazard occurring

If a hazard would occur this is limited to an individual and will not spread to the community. The consequence would be that this person needs treatment with antibiotics.

3.1.3. ASSESSMENT OF RISK TO THE ENVIRONMENT

3.1.3.1 Hazard identification

R. equi is readily found in soil, especially where domesticated livestock graze. The host range for wild type *R. equi* are primarily in grazing animals.

The vaccine strain was prepared by deleting the *ipdAB1*and *ipdAB2* chromosomal genes of *R. equi* strain RE1. It also contains a plasmid with the gene for VapA (Virulence Associated Protein A). This VapA is species specific for horses.

The *ipdAB1* gene is known to be involved in the cholesterol metabolism of the bacterium. As a result of this deletion, the bacteria are less able to survive in macrophages, which is a requirement for pathogenicity in the target animal. The other genes (*ipdAB2*) were deleted as a precaution because they show similarity with *ipdAB1*. The deletions have no influence on the behaviour of the bacterium in soil.

Cholesterol metabolism as such is not essential for macrophage survival since mutants that are unable to take up cholesterol (i.e. SupAB deletion mutant) are not attenuated for macrophage survival (van der Geize *et al*, 2008, Annex 7; reports **INFORMATION DELETED FOR CONFIDENTIALITY PURPOSES** and 10R/0075, Annex 8). However, enzymes that are part of the cholesterol degradation chain, in particular those involved in HIL degradation (i.e. ipdAB and Fad30), appeared to be essential for macrophage survival because deletion mutants of the respective genes were shown to be hampered in macrophage survival (see report 10R/0075, Annex 8).

The precise mechanism for the survival of *Rhodococcus equi* in macrophages has yet to be elucidated, but it is evident from our studies that either the enzymes of the cholesterol degradation chain pathway appear to play a part (the enzyme pathway may serve a different function during macrophage residency) or that the build-up of the intermediates of cholesterol degradation, at the point of HIL may hamper the survival of the bacteria in macrophages. Except for a hampered macrophage survival no differences in survivability between vaccine strain and wildtype have been observed, so it is not clear whether the deletions have any negative competitive effect in certain environments (e.g. environments enriched with steroids). The most negative assumption is that outside the host there is no difference in survivability between vaccine strain and wildtype. Given the nature of the vaccine strain (unmarked deletion mutant, with no additional genes introduced into the environment), additional risks for humans, horses and environment are nearly zero.

The horse is the primary target species for $VapA^+$ containing *R. equi*. Since the ability to enter and survive in macrophages is required for pathogenicity of *R. equi* in any species, pathogenicity of the vaccine strain for other species is highly unlikely. The strain is safe for the most sensitive category of the target species being young foals when given orally **(see INFORMATION DELETED FOR CONFIDENTIALITY PURPOSES)**. A *ΔipdAB1* single deletion mutant appeared also to be safe after oral and intratracheal administration **(INFORMATION DELETED FOR CONFIDENTIALITY PURPOSES)**. In addition, the vaccine strain has been tested for safety in the non-target species of chickens, rats, mice, calves, and pigs. **(INFORMATION DELETED FOR CONFIDENTIALITY PURPOSES)** A high dose of the bacterium was orally administered and could not be reisolated from rectal swabs during 3 weeks post infection.

In the field horses are infected by contaminated soil or faeces. While grazing it may come in the gut with the grass, and, especially during dry periods, it may come into the lungs with soil dust when the horses are galloping through the pasture. When horses are not immune to the bacterium, the lung infection may lead to severe pneumonia often resulting in mortality when not treated. A deletion mutant derived from the same parent strain but having only *ipdAB1* gene deleted did not cause pneumonia in young foals at a dose of 7.1 $x10^6$ CFU administered intratracheally whereas the parent strain RE1 caused severe pneumonia in all animals at a lower dose **(INFORMATION DELETED FOR CONFIDENTIALITY PURPOSES).**

The double deletion mutant, for which this application is intended, appeared to be safe in young foals when a high dose was administered by the oral route **(INFORMATION DELETED FOR CONFIDENTIALITY PURPOSES).**

The vaccine strain is administered rectally and, as a result, is shed by vaccinated animals **INFORMATION DELETED FOR CONFIDENTIALITY PURPOSES)**. The vaccine strain may be shed for 4 weeks after vaccination or longer. The deletions do not have any competitive benefits outside the vaccinated animals compared to wild type *R. equi,* since the attenuation (less able to survive in macrophages) does not play a role in survival outside the animal. It must be assumed that in soil the vaccine strain will behave similarly to the wild type *R. equi*.

To determine the genetic stability in the target animal, a reversion to virulence study was performed, which showed that the vaccine strain remains stable during five animal passages **(INFORMATION DELETED FOR CONFIDENTIALITY PURPOSES).** Following the 5 passages the deletion was still present in the passaged bacteria and the organism had the same macrophage survival profile as the unpassaged material **(INFORMATION DELETED FOR CONFIDENTIALITY PURPOSES)**.

Repair of the gene deletions may only occur via recombination with a non-gene deleted *R equi* strain. Since the deletions have been made at the chromosomal DNA it is highly unlike that genes will be exchanged. In the highly unlikely case that this would occur, the bacterium would be similar to wild type *R. equi* that is already present in the environment.

3.1.3.2 Assessment of likelihood

Pathogenicity of *R. equi* is related to the virulence factor on a plasmid (VapA or VapB) and the ability to survive in macrophages. Conjugative transfer of the virulence factor containing plasmid may occur between *R. equi* strains. However, *R. equi* bacteria like the vaccine strain already containing a $VapA^+$ plasmid are less able to receive a $VapB^+$ plasmid from another strain. $VapA^+$ strains occur on the site and therefore no new virulence plasmid is being introduced.

The deletion mutant vaccine strain is less able to survive in macrophages, which is required for pathogenicity in any species and due to the nature of the deletion gene repair can be excluded under environmental conditions.

3.1.3.3 Assessment of level of risk

Taking all the risk factors in consideration the assessment of level of risk to the environment for Equilis RhodE can be considered as effectively zero.

3.1.3.4 Assessment of the consequence

In the theoretical case that gene recombination with a field strain would occur thereby repairing the gene deletions, the bacterium will be similar to *R. equi* strains already present in the environment. Since in this case no new type of bacterium is introduced into the environment, but only a strain similar to other strains already present in the environment, the consequence for the environment is considered as effectively zero.

3.1.4. ASSESSMENT OF THE OVERALL RISK

The product Equilis RhodE complies with all obligations under Directive 2001/18/EC on the deliberate release into the environment of genetically modified organisms.

Taking all the risk factors into consideration the assessment of level of risk for both humans and the environment can be considered as effectively zero.

4. CONCLUSION

The active ingredient of Equilis RhodE is the live deletion mutant *Rhodococcus equi* strain RG2837. The vaccine is administered to foals by the rectal route.

The genetic stability and the safety for the target animal was discussed and considered satisfactory. The vaccine strain can be traced and identified by culture on selective agar followed by PCR.

From the environmental risk assessment it is concluded that the level of risk for both humans and the environment for Equilis RhodE can be considered as effectively zero.