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26 February 2013

Request for further information under Article 19 (1) of S. I. No. 500 of 2003 with regard to notification for consent to release GMOs into the environment for purposes other than placing on the market (GMO Register G0493-01, Reference B/IE/12/02) – 1st request

Dear Dr Cowley

I refer to the above mentioned notification, and request the following information which is required in order to assist the Agency in their review of the application.

Antibiotic resistance

1. Section 1.1.2.A.11(e) states "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*".
   Section 1.1.2.A.12(d) states "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". Yet the previous section 1.1.2.A.12 (b) states "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".
   a. Please clarify precisely the antibiotic resistances that are carried by the GM strain of *R. equi* and indicate whether they are plasmid or chromosomal borne.
   b. Similarly please clarify what is meant by "antibiotic resistances other than the ones already present in the field"
   c. Does this include some or all of the range of "identified resistances which are intrinsic features of *R. equi*"?
   d. What is the significance / role of the bicyclomycin resistance gene?

2. The deletion mutant is described as an "unmarked deletion mutant" under section 1.1.2.C.2(a). What is meant by 'unmarked' in this context? Section 1.1.2.A.12(d) refers to the possible presence of a bicyclomycin resistance gene.

Pathogenicity

1. Presence of virulence plasmids in *R. equi* is necessary to its survival and reproduction in alveolar macrophages all of which is central to the pathogenicity of *R. equi.*

   That said, R. equi vaccine strain RG2837 is unable to survive and reproduce in alveolar macrophages and so will not cause pneumonia in horses despite carrying the VapA+ virulence plasmid. Please clarify the role played by the virulence plasmid in this instance?

2. Sections 1.2.C.3.(b) 1.1.4.B.12, and ERA 3.1.2.1 state "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. Please provide the basis on which the claim that "it is expected to be unable to cause disease in (immunocompromised) humans" is made given that no direct correlation has been shown between survival in macrophages and human pathogenicity. Report 10R/0075 (which is referenced) only concludes that single and double deletion mutants are significantly attenuated for macrophage survival compared to the wild type.

Gene deletion

1. The application states that four genes have been deleted from R. equi strain RE1 and other than to say that the genes involved were ipdAB1 and ipdAB2, the four genes in themselves are not identified. Please identify the four genes that were deleted (including the 2 genes involved in pathogenicity) as well as their function.
2. Section 1.1.2.C.2(c) What is the possibility/probability that the vaccine strain of R.equi may take up and integrate DNA encoding functional genes (i.e. the deleted genes) from virulent R. equi strains in the foal's gut or in the environment.
3. The main deletions were ipdAB1 and ipdAB2 genes. As a result of these chromosomal deletions the GM strain of *R. equi* is less well able to survive in the lung macrophages which is necessary for pathogenicity in the target animal. Given that the attenuation is located on the chromosome and not on the plasmid, is the presence of the virulence plasmid $VapA^+$ (essential for reproduction in macrophages) significant? or is the transfer of the virulence plasmid $VapA^+$ between 2 *R. equi* strains significant? What is the role of the virulence plasmid $VapA^+$ (if any) in the vaccine strain of *R. equi*?

ERA

Section 3.1.2.1 'Hazard Identification' under 'Assessment of risks to humans' states that "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"
Please provide a reference to substantiate the underlined statement above.

Post vaccination and shedding

It is stated in the application that low level intermittent shedding could continue for 'four weeks' or for 'several weeks' after last vaccination '(but probably longer)'.

Please clarify the length of time over which low level intermittent shedding will continue?

Monitoring

1. With regard to monitoring, according to the application, the animals will be monitored daily for 14 days post vaccination for clinical signs.
    a. Is post-vaccination post each vaccination or post last vaccination?
    b. Who will carry out the monitoring?
    c. In the event that a foal presents with clinical signs of disease, what further investigations will be carried out in order to confirm that the clinical signs identified are owing to *R. equi* infection.

2. From approximately day 29, the health of the foals will be monitored and the presence of pulmonary abscesses in particular will be checked for every 2 – 4 weeks.
    a. The 'health' of the foal is quite general, what signs in particular will be monitored for?
    b. How will the presence of pulmonary abscesses be checked for? Will it require that the foals be euthanised in which case how will foal remains be disposed of?
    c. Once the foals are moved from the release site, what monitoring will be performed?

3. According to the application the environment will be monitored every 14 days.
    a. What area of environment will be monitored?
    b. What will the environment be monitored for and the techniques used for monitoring purposes and who will perform the monitoring?
    c. In the event of anything untoward being found, what further investigations will be taken?

Treatment of waste

1. According to the application all straw and litter from the stables where the foals are kept will be removed into closed containers and inactivated by heat treatment by SRCL Ltd. At one stage a value of 1000m^3 is given as the expected amount.
    a. Is this the expected amount of waste to be produced per foaling seson?
    b. Have SRCL agreed to the removal and inactivation of faeces from the pony garden together with litter and straw from the stables?
    c. Please provide details of SRCL's procedure for the inactivation (including time temperature and pressure where applicable) of the abovementioned waste material.
    d. Please provide details of SRCL's procedure for the heat treatment and disposal of empty vaccine vials and used syringes left over after vaccination
    e.

Contained use trials

Please provide details and supporting data relating to all contained use trials performed including the location where these trials were carried out, the number of foals (vaccinates + controls) involved and the results of those trials.

SNIF
Please provide responses to sections B.9.(a) and C.4.(a).

S.I. No 500 of 2003
In accordance with article 14.2.(d) please provide "the conclusions … … in accordance with Part D of the Second Schedule, together with any bibliographic references and detail so the methods used.

Please note that in accordance with Article 8(1) of the GMO (Deliberate Release) Regulations, S.I. No 500 of 2003, "a period of time during which the Agency is awaiting any further information on a notification, which it may have requested from the notifier, shall not be taken into account", i.e., the clock is stopped until such time as all the requested information is received by the Agency.

Yours sincerely

Bernie Murray
Inspector
Office of Climate, Licensing and Resource Use.

Antibiotic resistance

1. Section 1.1.2.A.11(e) states "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*".
Section 1.1.2.A.12(d) states "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". Yet the previous section 1.1.2.A.12 (b) states "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".

a. Please clarify precisely the antibiotic resistances that are carried by the GM strain of *R. equi* and indicate whether they are plasmid or chromosomal borne.

None of the antibiotic resistances mentioned in Section 1.1.2.A.11(e) are plasmid borne. The antibiotic resistances, mentioned in Section 1.1.2.A.11(e) are either chromosomal borne or intrinsic features of *R. equi*. For example *R. equi* and all other bacteria from the same suborder (Corynebacterineae) have a complex hydrophobic cell wall which is characterized by mycolic acid-containing lipids and lipoglycans and on top of that a thick and lamellar polysaccharide capsule which together interfere with the passage of certain antibiotics into the cell. Another example is polymyxin. After binding to lipopolysaccharide (LPS) in the outer membrane of Gram-negative bacteria, polymyxin disrupts both the outer and inner membranes. The hydrophobic tail is important in causing membrane damage, suggesting a detergent-like mode of action. *R. equi* does not have LPS and polymyxin cannot bind and cell wall is not disrupted. So for the majority of the antibiotic resistances mentioned in Section 1.1.2.A.11(e) there is no gene or set of genes involved in the resistance but is a consequence of overall structure and biochemistry of the bacterium. The introduction of the vaccine strain into the field does not impose an additional risk from the point of the above mentioned antibiotic resistances as all wild type *R. equi* strains harbor the same or comparable antibiotic resistances.

b. Similarly please clarify what is meant by "antibiotic resistances other than the ones already present in the field"

Analysis of the DNA sequence of the virulence plasmid of the vaccine strain RG2837 showed that it contained 1 ORF that might encode for a bicyclomycin resistance protein. This is however a prediction which is based on the fact that it has 48% homology to *E. coli* protein. There is no experimental evidence that demonstrates its function.
The introduction of the vaccine strain into the field does not impose an additional risk from the point of the predicted bicyclomycin resistance. Analysis of other virulence plasmids (both $vapA^+$ and $vapB^+$) showed that they all contain an identical gene to the one found in the vaccine strain. So by introducing the vaccine strain into the field we do not introduce other antibiotic resistances than the ones already present in the field, i.e. present in the highly abundant wild type *R. equi* strains.

c. Does this include some or all of the range of "identified resistances which are intrinsic features of *R. equi*"?

No, this remark was only for the predicted bicyclomycin resistance. As the majority of the identified antibiotic resistances are intrinsic features of all *R. equi* we do not introduce resistance genes or set of genes, that could be transferred to other micro-organisms, into the environment by

the introduction of the vaccine strain. So the remark has only significance for the predicted bicyclomycin resistance.

d. What is the significance / role of the bicyclomycin resistance gene?

The significance of the predicted bicyclomycin resistance gene for *R. equi* is unknown. Bicyclomycin is naturally produced by Streptomyces sapporonensis. It is a broad-spectrum antibiotic for Gram-negative bacteria but is largely ineffective against Gram-positive bacteria. Bicyclomycin's site of action in is transcription termination factor Rho. Resistance can occur by single amino acid changes in the Rho protein, M219K, S266A and G337S, or by the acquisition of a bicyclomycin resistance gene that encodes a trans membrane protein that is involved in the transport of bicyclomycin from one side of a membrane.
The possibility that the protein is involved in the transport of bicyclomycin over the cell membrane is speculative. It could play a role in the transport of volatile fatty acid or other toxic compounds that are present in the soil and manure, the natural habitat of most Rhodococci.

2. The deletion mutant is described as an "unmarked deletion mutant" under section 1.1.2.C.2(a). What is meant by 'unmarked' in this context? Section 1.1.2.A.12(d) refers to the possible presence of a bicyclomycin resistance gene.

Unmarked" means that during the deletion process no foreign functional DNA is left in the vaccine strain (that could act as a marker). All genes including the possible presence of a bicyclomycin resistance were already naturally present in wildtype *Rhodococcus equi*. Occasionally researchers use attenuation methods that leave foreign genes i.e. antibiotic encoding genes, in the bacterium. However, in our case methods were used that result in a "clean" deletion mutant.

Pathogenicity

1. Presence of virulence plasmids in *R. equi* is necessary to its survival and reproduction in alveolar macrophages all of which is central to the pathogenicity of *R. equi.*
   That said, R. equi vaccine strain RG2837 is unable to survive and reproduce in alveolar macrophages and so will not cause pneumonia in horses despite carrying the VapA+ virulence plasmid. **Please clarify the role played by the virulence plasmid in this instance?**

The role of the virulence plasmid in the vaccine strain is that it is required for protection. The virulence plasmid encodes for VapA which is an important virulence factor implicated in protective immunity. Plasmid-less strains did not induce protection in mice in contrast to sub-lethal doses of wild type *R. equi*. Thus an immune response against this VapA antigen is assumed to be important for protection. For that reason the ipdAB deletion mutant was chosen as vaccine strain rather than an apathogenic plasmid-less strain.

2. Sections 1.2.C.3.(b) 1.1.4.B.12, and ERA 3.1.2.1 state "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. **Please provide the basis on which the claim that "it is expected to be unable to cause disease in (immunocompromised) humans" is made given that no direct correlation has been shown between survival in macrophages and human pathogenicity. Report 10R/0075 (which is referenced)**

only concludes that single and double deletion mutants are significantly attenuated for macrophage survival compared to the wild type.

Since we cannot perform experiments in (immunocompromised) humans we only can theorize about the relative pathogenicity of the vaccine strain in humans.
Because the vaccine strain is attenuated in foals (the most sensitive host) combined with the fact that the vaccine strain is less able to survive in human macrophages lead to the statement "it is expected to be unable to cause disease in (immunocompromised) humans". May be "expected to be less able to cause disease" or "expected to be at most equal to wild type R. equi which is ubiquitously present in areas where the vaccine is used " would be more appropriate.

Gene deletion

1. The application states that four genes have been deleted from R. equi strain RE1 and other than to say that the genes involved were ipdAB1 and ipdAB2, the four genes in themselves are not identified. **Please identify the four genes that were deleted (including the 2 genes involved in pathogenicity) as well as their function.**

The four genes that have been deleted are *ipdA1*, *ipdB1*, *ipdA2* and *ipdB2*. The genes *ipdA1* and *ipdB1* are located next to each other and are expressed and regulated as a single unit (an operon) and are therefore designated as *ipdAB1*. The same is applicable for *ipdA2* and *ipdB2* and are indicated by *ipdAB2*. In section 1.1.2.C.2. "Information on the final GMO" there is information about the function. In short, the *ipdA1* and *ipdB1*-genes are involved in the cholesterol metabolism. Bioinformatics analysis revealed that IpdA and IpdB proteins represent the α and β-subunit of a heterodimeric CoA-transferase. The heterodimeric CoA-transferase encoded by *ipdAB1* appears to 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.
The other deletion, removal *ipdA2 and ipdB2,* was made because *ipdAB2* showed 50% similarity with *ipdAB1* and probably encode for another heterodimeric CoA-transferase. To avoid that *ipdAB2* would take over the function of *ipdAB1,* these genes were deleted as well.

2. Section 1.1.2.C.2(c) **What is the possibility/probability that the vaccine strain of R.equi may take up and integrate DNA encoding functional genes (i.e. the deleted genes) from virulent R. equi strains in the foal's gut or in the environment.**

The possibility/probability that the vaccine strain of *R. equi* may takes up and integrates DNA encoding functional genes (i.e. the deleted genes) from virulent *R. equi* strains in the foal's gut or in the environment is very, very small. But even if this happens there is no risk for the foal or the environment and can only occur if wild type Rhodococcus equi is already present to act as gene donor. The result of the vaccine strain taking up all four genes and integrates them in the right place and orientation, which is theoretical almost impossible, is that one gets a wild type strain with a similar virulence as the wild type *R. equi* strains that are already present in high abundance in the environment and in the gut.

3. The main deletions were ipdAB1 and ipdAB2 genes. As a result of these chromosomal deletions the GM strain of *R. equi* is less well able to survive in the lung macrophages which is necessary for pathogenicity in the target animal. **Given that the attenuation is located on the chromosome and not on the plasmid, is the**

presence of the virulence plasmid VapA$^+$ (essential for reproduction in macrophages) significant? or is the transfer of the virulence plasmid VapA$^+$ between 2 *R. equi* strains significant? What is the role of the virulence plasmid VapA$^+$ (if any) in the vaccine strain of *R. equi*?

The presence of the Vap$^+$ virulence plasmid is indeed essential for reproduction in macrophages in wild type R. equi strains. However it can be concluded from data presented in the application that it is not the only requirement for the reproduction in macrophages, since *ipdAB1*genes also were shown to be essential for macrophage survival.
It has been demonstrated that VapA protein plays an important role in protective immune response. From this observation it was concluded that the Vap$^+$ virulence plasmid not only encodes for proteins that are involved in the reproduction in macrophages but also encodes proteins that, if recognized by the immune system, will confer protection.
The virulence plasmid is therefore an integral part of the protective ability of the current vaccine strain.

ERA

Section 3.1.2.1 'Hazard Identification' under 'Assessment of risks to humans' states that "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"
Please provide a reference to substantiate the underlined statement above.

This statement was not a literal citate but was based on and is consistent with the article of Ocampo-Sosa et al 2007, Journal of Infectious Diseases, 196:763-769 (Appendix 2 of the application).

Post vaccination and shedding

It is stated in the application that low level intermittent shedding could continue for 'four weeks' or for 'several weeks' after last vaccination '(but probably longer)'.

Please clarify the length of time over which low level intermittent shedding will continue?

During the different licensing studies foals have been swabbed rectally (during 4w after vaccination) in order to test for shedding. Peak shedding was observed 1 day after vaccination. Thereafter, in most foals the shedding strongly decreased to undetectable levels and at 4w after vaccination most animals were negative but a few animals still showed intermittent low level shedding at 4w after vaccination. Since we did not test beyond 4w we assume that (intermittent) low level shedding is still possible beyond 4w.

Monitoring

1. With regard to monitoring, according to the application, the animals will be monitored daily for 14 days post vaccination for clinical signs.
    a. **Is post-vaccination post each vaccination or post last vaccination?**

The animals will be monitored for clinical signs post each vaccination.

b. Who will carry out the monitoring?

The monitoring will be carried out by the investigator on days -1, 0, 1, 4, 7 and 14 and staff of the farm on days 2, 3, 5, 6, 8, 9, 10, 11, 12 and 13 after vaccination.

c. In the event that a foal presents with clinical signs of disease, what further investigations will be carried out in order to confirm that the clinical signs identified are owing to *R. equi* infection.

If clinical signs are observed that are possibly related to R. equi infection, then the lungs will be scanned with ultrasound equipment to determine if lung abscesses are present and what their size is.

2. From approximately day 29, the health of the foals will be monitored and the presence of pulmonary abscesses in particular will be checked for every 2 – 4 weeks.

a. The 'health' of the foal is quite general, what signs in particular will be monitored for?

Because the vaccine has not caused any disease in the contained use studies and the wild type R. equi does not show typical symptoms, the observations will not be focused on specific clinical signs. In addition to the rectal temperature and diarrhea (because of the rectal application) the monitoring will be on (absence) of clinical signs and normal demeanour. A foal is considered healthy if it is active, drinks well and no abnormalities are observed. In case a foal is diseased (i.e. the score for consistency of faeces, general health or feed intake is > 0) , the necessary examinations will be performed and usual veterinary care will be given. The foals will be scored according to the scoring systems mentioned below:

Consistency of faeces		**General health**		**Feed intake**	
Normal	0	Normal, attentive	0	Normal	0
Pulp	1	Signs of discomfort	1	Impaired	1
Watery	2	Signs of disease	2	Hardly eating	2
Bloody	3	Very ill	3	Will not eat	3

b. How will the presence of pulmonary abscesses be checked for? Will it require that the foals be euthanised in which case how will foal remains be disposed of?

The lungs will be scanned on a 2 weekly basis with ultrasound equipment to determine if lung abscesses are present. Only foals that are moribund will be euthanized and post-mortem examined.

c. Once the foals are moved from the release site, what monitoring will be performed?

Every 2 weeks the foals will be examined by ultrasound scan to determine the presence and size of lungs abscesses.

3. According to the application the environment will be monitored every 14 days.

a. What area of environment will be monitored?

The release site and the paddocks where vaccinated foals are present will be monitored.

b. What will the environment be monitored for and the techniques used for monitoring purposes and who will perform the monitoring?

During monitoring, performed by the farm personnel 1) the foals and the other horses present in the paddocks will be observed for signs of disease and other abnormalities and 2) any abnormalities in the environment, like mortality of wild animals, will be recorded.

c. In the event of anything untoward being found, what further investigations will be taken?

Horses with signs of disease will be examined by the investigator or another qualified veterinarian. If clinical signs are observed that are possibly related to R. equi infection, then the lungs will be scanned with ultrasound equipment to determine if lung abscesses are present and what their size is.
If in other animals signs of disease are observed that are possibly related to R. equi infection the inspection will immediately be notified.

Treatment of waste

1. According to the application all straw and litter from the stables where the foals are kept will be removed into closed containers and inactivated by heat treatment by SRCL Ltd. At one stage a value of 1000m^3 is given as the expected amount.

a. Is this the expected amount of waste to be produced per foaling season?

1000 m^3 of waste is an expected amount per foaling season.

b. Have SRCL agreed to the removal and inactivation of faeces from the pony garden together with litter and straw from the stables?

Farm staff will remove the faeces from the release site and straw from the stables include in this site and place in plastic liners. These will then be placed in wheelie bins for collection by SRCL staff and subsequent inactivation.

c. Please provide details of SRCL's procedure for the inactivation (including time temperature and pressure where applicable) of the abovementioned waste material.

Sterilisation (Inactivation) of waste will take place in a Tempico Rotoclave (Revolving Autoclave). The Rotoclave sterilises waste on set sterilisation parameters of a minimum 134^{o}C for 15 minutes at 45psi. The quantity of waste to be processed in an individual cycle of the Rotoclave shall not exceed 0.7 tonnes. The performance of the Rotoclave is evaluated as follows:

- by determining residence time in the Rotoclave
- by use of thermal indicator strips; and
- by performing challenge tests against spore-forming bacteria in accordance with *Schedule D: Monitoring*, of this licence.
  The parameter settings, which control residence time and temperature, shall be tamper proof.

During the operation of the autoclave chambers, is continuously monitored to ensure that operating parameters are being maintained within the established limits. The autoclave chambers are set in such a manner so as to ensure that the chambers are not opened prior to having attained the required operating parameters, unless an incident occurs.
The Rotoclave is operated such that there is a 6 log reduction of Bacillus strearothermophilus spores in challenge tests.
Should any of the samples taken for the purposes of *Schedule D: Monitoring*, of this licence indicate test failure:
a) the batch of processed waste being held shall be further processed and retested;
b) all methods of sampling and testing specified in *Schedule D: Monitoring*, of this licence shall be carried out for each batch for the next four consecutive working days;
c) should this additional sampling and testing show the continued presence of the relevant microorganisms, the acceptance and processing of health care risk waste shall cease until written notice from the EPA agreeing to the resumption is received;
d) a written report shall be submitted to the Agency within five working days of the availability of the test results referred to in (b) above

d. Please provide details of SRCL's procedure for the heat treatment and disposal of empty vaccine vials and used syringes left over after vaccination

Both waste types will be classified the same way and the same inactivation method (stated above) will apply.

Contained use trials

Please provide details and supporting data relating to all contained use trials performed including the location where these trials were carried out, the number of foals (vaccinates + controls) involved and the results of those trials.

The contained use trials were reported and these reports are part of the application. These contained use trials were performed at the MSD-AH animal farm in St. Anthonis, The Netherlands and demonstrated the safety and efficacy of the vaccine. Based on these data a "deliberate release license" was obtained. Trials under this deliberate release license were also performed at the MSD-AH animal farm in St. Anthonis, The Netherlands and further confirmed the safety and efficacy of the vaccine.

SNIF

Amended SNIF including responses to sections B.9.(a) and C.4.(a) to follow.

S.I. No 500 of 2003

Amended conclusions in accordance with Part D of the Second Schedule, together with any bibliographic references and details of the methods used to follow.

Environmental Protection Agency
An Ghníomhaireacht um Chaomhnú Comhshaoil

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South County Business Park
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13 March 2013

Request for further information under Article 19 (1) of S. I. No. 500 of 2003 with regard to notification for consent to release GMOs into the environment for purposes other than placing on the market (GMO Register G0493-01, Reference B/IE/12/02) – 2nd request

Dear Dr Cowley

I refer to the above mentioned notification, and request the following information which is required in order to assist the Agency in their review of the application.

1. Annex 8 ("Macrophage survival of different *Rhodococcus equi* deletion mutants", Release No 10R/0075) focussing on the ability of the mutant vaccine strain to survive in macrophages contains a graph (Figure 1) plotting the survival of *R. equi* mutants in macrophages. The standard deviation bars if shown, would demonstrate an overlapping standard error which in turn would suggest that there is no statistical difference between the macrophage survival rate for the wildtype and the vaccine strain. Please comment

2. The comparative ANOVA table (also contained in Annex 8) is not consistent with the significance table (Table 4) and the reasons for this need to be clarified.

3. The ANOVA table documents 196 observations instead of a maximum of 288. Please explain what this substantial difference may be attributed to and please provide reasoning.

4. Annex 44 (confidential information- Figure 3.E.6.36, Determined DNA sequence of the ipdAB1 region and the ipdAB2 region of *R. equi* strain RG2837) shows schematics of the deleted sequences however they are not aligned with a wild type control sequence as is standard practice. Please provide a schematic aligning the deleted sequences with the wild type control sequence for comparison.

5. Annexes 47 – 51 addressing the safety of Equilis RhodE vaccine in chickens, rats, mice, calves and pigs respectively. Each of these studies typically tests 5 animals which is a very small sample size and precludes the observation of any but the most serious rapidly developing and immediately obvious adverse effect. Small sample sizes also limits statistical analysis. Please comment providing justification for the small number of animals used in the safety experiments.

6. In a number of cases there appears to be no or inadequate follow-up on an animal's wellbeing e.g. one chicken and all of the mice were noted to be 'less active' following vaccination. A mouse developed an enlarged mesenterial lymph node and an enlarged spleen, mucus was observed in the faeces of a calf. These abnormalities do not appear to have been further investigated therefore on what basis was it decided that they were not vaccine related?

7. Please provide information on the detection limits of the microbiology tests reported with particular reference to Annexes 13 (Macrophage survival of *R. equi* vaccine strain RG2837 before and after animal passage) and 25 (survival of *R. equi* vaccine strain RG2837 and wildtype parent strain RE1 in different environments)

8. No testing was performed in immune compromised animals despite the increased vulnerability of immune-compromised persons to infection by Rhodococcus. Please comment.

Please note that in accordance with Article 8(1) of the GMO (Deliberate Release) Regulations, S.I. No 500 of 2003, "a period of time during which the Agency is awaiting any further information on a notification, which it may have requested from the notifier, shall not be taken into account", i.e., the clock is stopped until such time as all the requested information is received by the Agency.

Yours sincerely

B Murray

Bernie Murray

Inspector
Office of Climate, Licensing and Resource Use.

1. Annex 8 ("Macrophage survival of different *Rhodococcus equi* deletion mutants", Release No 1 OR/007 5) focussing on the ability of the mutant vaccine strain to survive in macrophages contains a graph (Figure 1) plotting the survival of *R. equi* mutants in macrophages. The standard deviation bars if shown, would demonstrate an overlapping standard error which in turn would suggest that there is no statistical difference between the macrophage survival rate for the wildtype and the vaccine strain. Please comment

Report 10R/0075 describes macrophage survival tests done with 9 different (mutant) strains. Adding the standard error bars would not improve the readability of the graph with 9 strains. Therefore, also a statistical analysis was done on the results (ANOVA using linear mixed model). Not all mutants tested were statistically significantly different from the wild type parent strain. However, the vaccine strain (Re1-ipdABipdAB2 deletion mutant) demonstrated a clear significantly lower macrophage survival compared to the parent strain (p=0.0065) as shown in Table 4 of the report. Besides the tests described in the report, many more macrophage survival tests have been performed in the past (some can be found in the other annexes of the application). These tests were not statistically analyzed but always showed the same pattern of reduced survival for the vaccine strain compared to the wild type parent strain.

Moreover, by analyzing the data in a repeated measurement setting resulted in reasonable small standard errors (e.g. wildtype RE1 (code A), lsmeans 4.72 with standard error 0.27; vaccine strain (code D) lsmeans 3.22 with standard error 0.25, Addendum 1 report 10R/0075). Hence, a higher discriminative power was achieved.

2. The comparative ANOVA table (also contained in Annex 8) is not consistent with the significance table (Table 4) and the reasons for this need to be clarified.

Summary Table 4 in report 10R/0075 was made for an easier interpretation of the results of the ANOVA. Comparisons of interest were mutants (coded B-H) vs. the wildtype REI (code A), where by default in the used software, the comparisons are made in alphabetic order i.e. difference in lsmeans A - D where D - A is the research question.

Double checking Table 4 against the ANOVA output in Addendum 1of the report still revealed three minor inconsistencies 1: C vs. A diff = +1.00 instead of -1.00; 2: H vs. A 95%CI interval of difference [-3.69 ; -0.38] instead of [-2.84 ; -0.34]; 3: I vs. A lower limit 95% CI -2.84 instead of -2.74. These inconsistencies had no impact on the conclusions of the analysis. A corrected table 4 is attached.

3. The ANOVA table documents 196 observations instead of a maximum of 288. Please explain what this substantial difference may be attributed to and please provide reasoning.

Data presented in Table 2 of report 10R/0075, were statistically analyzed by ANOVA. Per time point 49 observations (i.e. measurements) were available. Data at time zero were included as covariate in the model to account for any differences in the starting value. Thus, leaving 4 time points each with 49 observations giving a total of 196 observations.

4. Annex 44 (confidential information- Figure 3.E.6.36, Determined DNA sequence of the ipdAB1 region and the ipdAB2 region of *R. equi* strain RG2837) shows schematics of the deleted sequences however they are not aligned with a wild type control sequence as is standard practice. Please provide a schematic aligning the deleted sequences with the wild type control sequence for comparison.

See attachment 44A+B.

5. Annexes 47 - 51 addressing the safety of Equilis RhodE vaccine in chickens, rats, mice, calves and pigs respectively. Each of these studies typically tests 5 animals which is a very small sample size and precludes the observation of any but the most serious rapidly developing and immediately obvious adverse effect. Small sample sizes also limits statistical analysis. Please comment providing justification for the small number of animals used in the safety experiments.

We agree that a group of 5 animals is a minimal group size but when doing animals trials we always need to balance between the number of animals and the question to be answered taking into account the expected discomfort for the animals, the expected frequency of the phenomena to be observed etc. If we challenge 5 foals with wild type Rhodococcus equi, all 5 will develop pneumonia. Thus for clear serious rapidly developing effects, a group of 5 animals seems to be sufficient. For rare effects much larger groups are needed (e.g. > 100) and it would be difficult if not impossible to obtain permission from the Dutch ethical committee for such numbers of animals for this purpose. In addition, the bacterium is safe for the foal which is the most sensitive host making it even more unlikely to observe adverse reactions in other species. In other species even the wild type is only very rarely implicated in clinical abnormalities and did not cause disease in our trials. Because it is a deletion mutant (no new genes or characteristics are added) it is highly unlikely that the vaccine strain would cause problems in other species. We used groups of 5 animals to confirm this.

6. In a number of cases there appears to be no or inadequate follow-up on an animal's wellbeing e.g. one chicken and all of the mice were noted to be 'less active' following vaccination. A mouse developed an enlarged mesenterial lymph node and an enlarged spleen, mucus was observed in the faeces of a calf. These abnormalities do not appear to have been further investigated therefore on what basis was it decided that they were not vaccine related?

The cause of the “less active” qualification of an animal immediately after vaccination (one chicken, all mice) is not known but is most probably not related to an infectious process since the incubation period for *Rhodocccus equi* signs is normally approximately two weeks even when administered at a high dose. Since the signs had disappeared on the next day they probably were related to the handling and large amount of bacteria (proteins etc) that were administered to the animal rather than being of infectious nature.

Mucus was observed in the faeces of a calf at 7 days after inoculation. This was an observation but is not necessarily related to a pathological phenomenon and can be observed in healthy calves as well. In addition, at post-mortem no *Rhodococcus* related abnormalities were found in the gut of this animal.

The enlarged mesenterial lymph node (of a mouse) and the enlarged spleen (of a mouse) were all sterile upon culture and thus were not infected by the vaccine strain. In addition all known pathological phenomena associated with *Rhodococcus equi* (in all species) are typically suppurative, purulent abscessations; such abnormalities were not observed.

7. Please provide information on the detection limits of the microbiology tests reported with particular reference to Annexes 13 (Macrophage survival of *R. equi* vaccine strain RG2837 before and after animal passage) and 25 (survival of *R. equi* vaccine strain RG2837 and wildtype parent strain RE 1 in different environments)

When using the selective agar *R. equi* can still be detected if it constitutes 0.003% of the intestinal bacterial flora (see report annex 13). Such calculation was not made for soil isolation but it can be theorized that the sensitivity in soil is higher than for faeces because the density of bacteria in soil is much less compared to faeces. Moreover, the *Rhodococcus* bacteria are cultured at 37°C whereas the optimum temperature for most soil bacteria is lower. Although we do not know the exact sensitivity of the selective agar for soil, it apparently is sufficiently sensitive for its purpose because we were able to culture *Rhodococcus equi* even after a period of more than a year (after soil inoculation).

8. No testing was performed in immune compromised animals despite the increased vulnerability of immune-compromised persons to infection by Rhodococcus. Please comment.

Since we are not allowed to perform experiments in (immunocompromised) humans we only can theorize about the relative pathogenicity of the vaccine strain in humans. Because the vaccine strain was shown to be attenuated for foals (the most sensitive host) combined with the fact that the vaccine strain is less able to survive in human macrophages leads to the assumption that the vaccine strain is less able to cause disease in immunocompromised humans compared to wild type *R. equi*, which is ubiquitously present in the area where the study will be performed. A similar theorization can be made for immunocompromised animals.

In addition, immunocompromised animals do not survive wildlife conditions. So the chance that those animals come in contact with the vaccine strain is very low. It is more likely that they will encounter wild type *Rhodococcus equi* which is abundantly present in the area where the study will be performed and which is more virulent compared to the vaccine strain. Even if an immunocompromised animal would be infected by the vaccine strain, there is no risk that the gene deletions will be repaired and an equal or more virulent strain (compared to wild type *R. equi*) will become present in the field.

Table 4 (corrected): Summary results statistical analysis on difference between mutants

		Mutant code							
Mutant code		**A** **RE1**	**B** **RE1 ΔipdAB**	**C** **RE1 ΔipdAB2**	**D** **RE1 ΔipdABipdAB2**	**E** **RE1 ΔsupAB**	**F** **RE1 ΔfadA6**	**G** **RE1 ΔfadE28**	**H** **RE1 ΔfadE30**
B **RE1 ΔipdAB**	diff.[a] 95%CI diff p-value	-1.46 -2.71; -0.21 **0.0118**							
C **RE1 ΔipdAB2**	diff. 95%CI diff p-value	-0.46 -1.71 ; 0.79 0.9523	1.00 -0.25 ; 2.26 0.2040						
D **RE1 ΔipdABipdAB2**	diff. 95%CI diff p-value	-1.50 -2.71 ; -0.28 **0.0065**	-0.035 -1.25 ; 1.18 1.00	-1.04 -2.25 ; 0.17 0.1414					
E **RE1 ΔsupAB**	diff. 95%CI diff p-value	-0.076 -1.69 ; 1.54 1.00	1.39 -0.23 ; 3.00 0.1423	0.38 -1.23 ; 1.99 0.9970	1.43 -0.16 ; 3.00 0.1092				
F **RE1 ΔfadA6**	diff. 95%CI diff p-value	-0.40 -2.01 ; 1.22 0.9959	1.06 -0.55 ; 2.68 0.4516	0.058 -1.56 ; 1.67 1.00	1.10 -0.48 ; 2.68 0.3789	-0.32 -2.23 ; 1.59 0.9997			
G **RE1 ΔfadE28**	diff. 95%CI diff p-value	-0.067 -1.54 ; 1.41 1.00	1.39 -0.079 ; 2.87 0.0760	0.39 -1.09 ; 1.87 0.9938	1.43 -0.026 ; 2.89 0.0577	0.009 -1.79 ; 1.80 1.00	0.33 -1.48 ; 2.14 0.9995		
H **RE1 ΔfadE30**	diff. 95%CI diff p-value	-2.04 -3.69 ; -0.38 **0.0068**	-0.58 -2.24 ; 1.09 0.9638	-1.58 -3.23 ; 0.066 0.0683	-0.54 -2.15 ; 1.07 0.9698	-1.96 -3.91 ; -0.018 **0.0464**	-1.64 -3.57 ; 0.29 0.1505	-1.97 -3.85 ; -0.098 **0.0326**	
I **103-** **(neg. control)**	diff. 95%CI diff p-value-	-1.59 -2.84 ; -0.34 **0.0046**	-0.13 -1.38 ; 1.12 1.00	-1.13 -2.38 ; 0.12 0.1017	-0.093 -1.31 ; 1.12 1.00	-1.51 -3.13 ; 0.099 0.0804	-1.19 -2.81 ; 0.43 0.3029	-1.52 -3.00 ; -0.049 **0.0382**	0.45 -1.21 ; 2.11 0.9924

[a] Difference in between mutants in row and column 95% confidence interval of the difference and p-value, using Tukey-Kramer post-hoc test
e.g difference B - A = -1.46 with 95%CI [-2.71 ; - 0.21] P=0.0118

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)

Dr. A.A.C. Jacobs
The CV and copies of the diplomas of the responsible scientist (project leader) are given in Annex 14.

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 **(Report 09R/0098, provided in Annex 21).** 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. **Spiking experiments of soil, pond water or tap water that were subsequently kept at 4°C, 22°C or 37°C demonstrated long survival times of the bacterium. After the initial spiking, bacterial numbers decreased significantly in all environments by more than 99%. However, 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 (report 11R/0038 provided in Annex 25).** 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

Construction of suicide vector pSelAct
To enable the generation of unmarked in-frame gene deletions in *R. equi* a non-replicative suicide vector designated pSelAct was developed using CD-UPRT based 5-fluorocytosine (5-FC) conditional lethality (see Van der Geize *et. al*. 2008, Annex 7). Cytosine deaminase (CD, EC 3.5.4.5) and uracil phosphoribosyltransferase (UPRT, EC 2.4.2.9) are enzymes involved in the pyrimidine salvage pathway, converting cytosine via uracil into dUMP. The genes encoding these activities in *Escherichia coli, codA* and *upp*, respectively, have been cloned and characterized (Andersen *et al*. 1989, Annex 34; Danielsen *et al*., 1992, Annex 35). Interestingly, microorganisms expressing CD convert the innocuous cytosine analog 5-fluorocytosine (5-FC) into 5-fluorouracil (5-FU), a highly toxic compound lethal to living cells. The cytotoxicity is largely exerted following UPRT mediated conversion of 5-FU into 5-fluoro-dUMP, which irreversibly inactivates thymidylate synthase inhibiting both RNA and DNA synthesis (Heidelberger *et al.*, 1983, Annex 36).
Plasmid pSelAct was constructed in three steps. First plasmid pORF-Pkan-codAupp was constructed by cloning of the amplified *aphII* promoter from pRESQ (primers Pkan-F and Pkan-E5-R; 367bps) into *EcoRV* digested pBluescript(II)KS (Stratagene), giving pBs-Pkan. A *SalI*/*NotI* restriction released a 431 bp fragment comprising the *aphII* promoter which was then cloned into *SalI*/*NotI* digested pORF-codA::upp (InvivoGen, San Diego, USA), yielding plasmid pORF-Pkan-codAupp. A second plasmid pBs-Apra-ori was constructed from pBluescript(II)KS in which the *bla* cassette was removed with *BspHI*, followed by Klenow treatment and replaced by an apramycin-oriT cassette obtained as a 1.3 kb *XbaI* fragment (Klenow treated) from pIJ773 (Gust *et al*., 2003, Annex 37). Finally a 2.4 kb Klenow-treated *EcoRI*/*NheI*

fragment from pORF-Pkan-codAupp was ligated into *SspI* digested pBs-Apra-ori that was first dephosphorylated with alkaline phosphatase.
Vector pSelAct is a small (5.6 kb) conjugative plasmid based on pBlueScript(II)KS, harboring the *aac(3)IV* cassette for apramycin resistance that can be used both in *E. coli* and *R. equi*. The vector contains a MCS with several unique restriction sites for cloning flanking regions of the gene of interest using lacZ dependent blue-white screening in *E. coli*.

Construction of plasmids for ipdAB1 and ipdAB2 gene deletion
Plasmid pSelAct-ipd1 (schematic provided in Annex 38), for the generation of an unmarked gene deletion of the *ipdAB1* operon in *R. equi* RE1, was constructed. as follows. The upstream (1,368 bp; primers ipdAB1equiUP-F and ipdAB1equiUP-R) and downstream (1,398 bp; primers ipdAB1equiDOWN-F and ipdAB1equiDOWN-R) flanking regions of the *ipdAB1* genes were amplified by PCR oligonucleotide sequences of primers provided in Annex 39). The obtained amplicons were ligated into *Eco*RV digested pBluescript(II)KS, rendering plasmids pEqui14 and pEqui16 for the upstream and downstream region, respectively. A 1.4 kb *Spe*I/*Eco*RV fragment of pEqui14 was ligated into *Spe*I/*Eco*RV digested pEqui16, generating pEqui18. A 2.9 kb *Eco*RI/*Hind*III fragment of pEqui18, harboring the *ipdAB1* gene deletion and its flanking regions, was treated with Klenow fragment and ligated into *Sma*I digested pSelAct suicide vector (*ipdAB1* region shown in Fig. 1 of Annex 40). The resulting plasmid was designated pSelAct-ipd1 for the construction of *ipdAB* gene deletion mutant *R. equi* ΔipdAB1 (RG1341).
Double gene deletion mutant *R. equi* ΔipdAB1ΔipdAB2 (RG2837) was made by unmarked gene deletion of the *ipdAB2* operon in *R. equi* ΔipdAB1 mutant strain using plasmid pSelAct-ΔipdAB2 (schematic provided in Annex 38). Plasmid pSelAct-ΔipdAB2 was constructed as follows. The upstream (1,444 bp; primers ipdAB2equiUP-F and ipdAB2equiUP-R) and downstream (1,387 bp; ipdAB2equiDOWN-F, ipdAB2equiDOWN-R) regions of *ipdAB2* were amplified by PCR using genomic DNA as template (oligonucleotide sequences of primers provided in Annex 39).
The amplicons were ligated into *Sma*I digested pSelAct, resulting in plasmids pSelAct-ipdAB2equiUP and pSelAct-ipdAB2equiDOWN, respectively. Following digestion with *Bgl*II/*Spe*I of both plasmids, a 1,381 bp fragment of pSelAct-ipdAB2equiDOWN was ligated into pSelAct-ipdAB2equiUP, resulting in pSelAct-ΔipdAB2 used for the construction of a Δ*ipdAB2* gene deletion.

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

Culture media and growth conditions for R. equi strain RE1
***R. equi* strain RE1 was grown at 30°C in Luria-Bertani (LB) medium. For growth on solid media Bacto-agar (15 g/l) was added.**

Cloning, PCR and genomic DNA isolation
***E. coli* DH5α was used as host for all cloning procedures. Restriction enzymes were obtained from Fermentas GmbH (St.Leon-Rot, Ireland). PCR was performed in a reaction mixture (25 µl) consisting of Tris-HCl (10 mM, pH 8), 10x High-Fidelity polymerase buffer (Fermentas), dNTPs (0.2 mM), DMSO (2%), PCR primers (10 ng/µl each, oligonucleotide sequences of primers provided in Annex 39) and High-Fidelity polymerase enzyme (1-2 U, Fermentas). For colony PCR, cell material was mixed with 100 µl of chloroform and 100 µl of 10 mM Tris-HCl pH 8, vortexed vigorously and centrifuged (2 min, 14,000 x g). A sample of the upper water phase (1 µl) was subsequently used as template for PCR. Chromosomal DNA of *R. equi* cell cultures was isolated using the GenElute Bacterial Gnomic DNA Kit (Sigma-Aldrich) according to the instructions of the manufacturer.**

Electrotransformation of R. equi strain RE1
Cells of *R. equi* strains were transformed by electroporation essentially as described (Navas *et al.*, 2001 provided in Annex 41). Briefly, cell cultures were grown in 50 ml LB at 30^0C until OD_{600} reached 0.8-1.0. The cells were pelleted (20 min at 4,500 x g) and washed twice with 10% ice-cold glycerol. Pelleted cells were re-suspended in 0.5-1 ml ice-cold 10% glycerol and 200 µl aliquots were put on ice. MilliQ-eluted plasmid DNA (5-10 µl; GenElute Plasmid Miniprep Kit, Sigma-Aldrich) was added to 200 µl cells in 2 mm gapped cuvettes. Electroporation was performed with a single pulse of 12.5 kV/cm, 1000Ω and 25 µF. Electroporated cells were gently mixed with 1 ml LB medium and allowed to recover for 2 h at 37^0C and 200 rpm. Aliquots (200 µl) were plated onto LB agar medium containing apramycin (50 µg/ml). Transformants appeared after 2-3 days of incubation at 30^0C.

Unmarked gene deletion in R. equi strains using 5-fluorocytosine (5-FC) selection
***R. equi* transformants obtained from electroporation of wild type or mutant cells were streaked onto LB agar medium supplemented with apramycin to confirm apramycin resistance ($Apra^R$). Four $Apra^R$ transformants per transformation were grown overnight (20-24 h) at 30^0C and 200 rpm in 25 ml LB medium, and plated in 10^{-1}-10^{-3} fold dilutions in MM-Ac medium onto MM-Ac agar plates supplemented with 5-FC (100 µg/ml) in 100 µl aliquots. 5-FC resistant colonies, appearing after 3 days of incubation at 30^0C, were replica streaked onto LB agar and LB agar supplemented with apramycin (50 µg/ml) to select for apramycin sensitive ($Apra^S$) and 5-FC resistant ($5\text{-}FC^R$) colonies. $Apra^S/5\text{-}FC^R$ colonies were checked for the presence of the desired gene deletion by colony PCR using primers amplifying the locus of the gene deletion (see**

oligonucleotide sequences of primers provided in Annex 39). Genomic DNA was isolated from potential gene deletion mutants and used to confirm the gene deletion using primers amplifying the *ipdAB1* or *ipdAB2* gene locus, as well as the upstream and downstream regions of these loci with primers as described in Annex 38.

Identification of ipdA and ipdB in R. equi strain 103S
A BLAST search with the *ipdA* and *ipdB* genes of *Rhodococcus erythropolis* SQ1, involved in the degradation of methylhexahydroindanedione propionate (HIP; 3a*α*-H-4*α*(3'-propionic acid)-7aß-methylhexahydro-1,5-indanedione), revealed that these genes and their apparent operonic organization were conserved in the *R. equi* 103S genome (http://www.sanger.ac.uk/Projects/R_equi/). Genome analysis furthermore revealed that *R. equi* 103S harbors additional paralogous genes of *ipdA* and *ipdB*, designated *ipdA2* and *ipdB2*, respectively.

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.

Construction of mutant strains R. equi ΔipdAB1 (RG1341) and R. equi ΔipdAB1ΔipdAB2 (RG2837)

***R. equi* unmarked gene deletion mutants of *ipdAB1* (RG1341, in literature referred to as *ipdAB*) and *ipdAB1ipdAB2* (RG2837) were constructed using a two-step homologous recombination strategy with 5-fluorocytosine counter-selection developed for *R. equi* (Van der Geize *et al*., 2008, Annex 7 and see for general overview of the procedure provided in Annex 42, diagram A).**
For construction of the *ΔipdAB1* mutant *R. equi* strain RG1341, the non-replicative plasmid pSelAct-ipd1 (see plasmid diagrams provided in Annex 38) was mobilized to *R. equi* strain RE1 by electrotransformation. Four $Apra^R$ transformants, resulting from homologous recombination between plasmid pSelAct-ipd1 and the RE1 genome, were subsequently subjected to 5-FC selection in order to select for the occurrence of the second rare homologous recombination event resulting in gene deletion. Eighteen randomly picked $Apra^S/5FC^R$ colonies were subjected to colony PCR and three $FC^R/Apra^S$ colonies gave an amplicon of the expected size (296 bp, oligonucleotide sequences of primers provided in Annex 39). Genomic DNA was isolated from these putative *ΔipdAB* mutants and subjected to PCR analysis of the *ipdAB1* locus and its up- and downstream flanking regions (oligonucleotide sequences of primers provided in Annex 39). This analysis confirmed the presence of a genuine *ipdAB1* gene deletion in two out of three cases and revealed no aberrant genomic rearrangements at the *ipdAB1* locus. The presence of *vapA* as a marker for the presence of the virulence plasmid was confirmed by PCR (oligonucleotide sequences of primers provided in Annex 39). One *ipdAB1* mutant strain was chosen, designated *R. equi* RG1341, and was used for further work.

Double gene deletion mutant strain RG2837 was constructed from strain RG1341 using plasmid pSelAct-*ΔipdAB2* (see plasmid diagrams provided in Annex 38) essentially as described for the isolation of the *ΔipdAB* single mutant (a general overview of the procedure is provided in Annex 42, diagram B). Four $Apra^R$ transformants, obtained

from electroporation of cells of strain RG1341 with pSelAct-*ΔipdAB2*, were subjected to 5-FC selection to select for Apra^S/5-FC^R colonies. Subsequent PCR analysis of eighteen Apra^S/5-FC^R colonies confirmed that two colonies harbored a *ΔipdAB2* gene deletion, as evident from the obtained 123 bp amplicon using oligonucleotide developed to amplify the *ipdAB2* operon (oligonucleotide sequences of primers provided in Annex 39). Further analysis of the upstream and downstream regions of the *ipdAB2* locus by PCR confirmed the presence of an *ipdAB2* gene deletion and revealed no aberrant genomic rearrangements. A summary of the PCR method used to confirm the presence of the deletions in mutant strains *R. equi ΔipdAB1* (RG1341) and *R. equi ΔipdAB1ΔipdAB2* (RG2837) is provided in Annex 43. The results of the PCR are also shown. This information may be made available to the public.

Also, the presence of the VapA virulence gene was confirmed by PCR (primer sequences provided in Annex 39). One *ΔipdAB1ΔipdAB2* double deletion mutant strain was designated RG2837 and chosen for further work. The genome of *R. equi* strain RG2837 and the wild type parent strain RE1 were sequenced. Chromosomal DNA was isolated from a 100 ml overnight culture using Puregene core A kit from QiaGen according to the instructions of the manufacturer. Ten microgram DNA was sent to BaseClear (Leiden, The Netherlands) and sequencing was done using a 75 base read paired end method on a Solexa Genomic Analyzer II. Sequencing data was transferred to Intervet Innovations. Genome assembly was done using CLC-Bio software with the published genome of R. equi strain 103S as reference. The 8,289,126 reads for RG2837 and the 9,409,993 reads for RE1 were assembled (coverage >80-fold) into a genome. The CLC Genomics Workbench software identifies all SNPs (or point mutations) between an assembled genome of the investigated strains (*R. equi* RE1 or RG2837) in comparison to the reference strain *R. equi* 103S. All identified point mutations with the reference strain were manually confirmed, by examination of raw data. The two strains only differed in 13 positions of the genome with the exception of the *ipdAB1* and *ipdAB2* deletions (Annex 59). Eleven of the thirteen single-base substitutions (SNP 2 to 12) appear to have been introduced by the PCR amplification of the ipdAB1 up- and downstream regions. All these point mutation are silent and do no introduce amino acid changes in the encoded proteins. The other two single-base substitutions (SNP 1 and 13) appear to be spontaneous random mutations. If transcribed they both induce an amino acid change, changing the encoded protein. SNP 1 will introduce a conservative alanine into valine amino acid change in a protein which bears some characteristics of a transcription regulator of unknown function. If translated into protein SNP 13 will introduce a proline into a leucine amino acid change in a putative sulfatase, again of unknown function

(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.

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

The nucleotide sequence of the genes in which the deletions were made is provided in a schematic overview of the regions (Annex 40, Fig.1 and Fig.2) as determined by DNA sequencing of parent strain *R. equi* RE1. The positions of the deletions are highlighted as they were determined by DNA sequencing of the vaccine strain *R. equi* RG2837. Two highly specific PCRs were developed to positively identify both the parent and the vaccine strain.
First a quantitative PCR was designed to discriminate between wild type strain and the ipdAB deletion strain. PCR amplification can be performed on colony material, culture or purified DNA. The PCR mixture contains 20U/ml DNA polymerase (HT Biotechnology Ltd, Cambridge, UK), 1x Icycler buffer (100mM Tris-HCl and 500 mM KCl, pH 8.5), 0.2 mM dNTP's (HT Biotechnology, Ltd, Cambridge, UK), 4 mM MgCl2, 200 nM primers and 100 nM probes. Primers 5626-OLI-3159 (GCCGAGAAGGTCGATGTG), -3158 (GTCGCCGGTATGATATCGAAC) and -3128 (GTTCGCCTTGACGTCCTC) and probes 5626-OLI-3130 (CGACAAGGTCGATCCGGAGAAC; TXR) and 5626-OLI-3160 (CCCTGGAAGTCGAAGACGCCCAG; FAM) from the Intervet oligo collection are used for the ipdAB Q-PCR.
The PCR program is composed of a 5 minutes denaturation step at 95°C and 50 cycles of 10 seconds at 95°C, 15 seconds at 55°C and 30 seconds at 72°C. Data collection is performed at 72°C. A wildtype strain (e.g. strain RE1) will be positive with the FAM (5626-OLI-3160) and the TexasRed probe (5626-OLI-3130). An ipdAB mutant will only be positive with the FAM probe (5626-OLI-3160).
Secondly a quantitative PCR was designed to identify the *ipdAB2* genotype. PCR amplification is performed on colony material. The PCR mixture was identical to the ipdAB PCR described above. Primers 5626-OLI-3131 (GCTACGCCTGGTCTCCGTG), -3165 (TTGTCGGACAAGAGAATGTCGG) and -3166 (TCACGCGGGAACCTCCTTGTCG) and probes 5626-OLI-3134 (CTCCTCCGCACTAGATCTCCGGCCG; FAM) and 5626-OLI-3135 (TCCCGGACTCGGTGCCGCAGAC; TXR) from the Intervet oligo collection are used in this ipdAB2 Q-PCR.
The PCR program is composed of a 5 minutes denaturation step at 95°C and 50 cycles of 10 seconds at 95°C, 15 seconds at 55°C and 30 seconds at 72°C. Data collection is

performed at 72°C. A wildtype strain (e.g. strain RE1) will be positive with the Texas Red probe (5626-OLI-3135). An *ipdAB2* mutant will only be positive with the FAM probe (5626-OLI-3134).
Both the *ipdAB1* and *ipdAB2* regions were sequenced from the *R. equi* vaccine strain RG2837 and confirmed that the genes were deleted as designed (see sequence information provided in Annex 44).

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 **(Report 11R/0038, Annex 25).** *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, **reports 08R/0234 Annex 5** 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**(see Annex 44).**

(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 **report 09R/0098, Annex 21 and** 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 **(see report 08R/0234, Annex 5).** The vaccine strain is unable to cause disease when administered according to the manufacturers recommendations, when it enters the horse by the natural route **(see reports 09R/0227, Annex 46; 10R/0086, Annex 11 and 08R/0234, Annex 5)** or when it
is administered by the method used in the artificial challenge model used **(see report 08R/0234, Annex 5)**. 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 **(see reports 10R/0012 (chickens), 10R/0013 (rats), 10R/0014 (mice), 10R/0153 (calves) and 10R/0273 (pigs), provided in Annex 47 to Annex 51 respectively.)**. 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 **(see reports 09R/0227, Annex 46 and 10R/0086, Annex 11)**. 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. **The study protocol for the trial is provided in Annex 52, along with the consent from the owner of the trial site.**

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 **(provided in Annex 52).**

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 **(provided in Annex 52)**.

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. **Spiking experiments of soil, pond water or tap water that were subsequently kept at 4°C, 22°C or 37°C demonstrated long survival times of the bacterium (report 11R/0038 provided in Annex 25).** 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 **reports 09R/0227, Annex 46;10R/0086, Annex 11; 08R/0234, Annex 5; 08R/0247, Annex 45 and** 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. **Chromosomal DNA was isolated from a 100 ml overnight culture using Puregene core A kit from QiaGen according to the instructions of the manufacturer. Ten microgram DNA was sent to BaseClear (Leiden, The Netherlands) and sequencing was done using a 75 base read paired end method on a Solexa Genomic Analyzer II. Sequencing data was transferred to Intervet Innovations. Genome assembly was done using CLC-Bio software with the published genome of R. equi strain 103S as reference. After the 8,289,126 reads for RG2837 and the 9,409,993 reads for RE1 were assembled (coverage >80-fold) into a genome, the genome sequences were subjected to a SNP**

analysis. This analysis showed that the two strains only differed in 13 position of the genome with the exception of the *ipdAB1* and *ipdAB2* deletions. No evidence was found that the antibiotic resistance and any other fragment of the plasmid had incorporated in the genome during the construction process to make RG2837.

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 **(see reports 10R/0012 (chickens), 10R/0013 (rats), 10R/0014 (mice), 10R/0153 (calves) and 10R/0273 (pigs), provided in Annex 47 to Annex 51 respectively).** 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 **(report 11R/0038-01 provided in Annex 25).**

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, **reports 08R/0234 Annex 5** 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 **(see reports 10R/0012 (chickens), 10R/0013 (rats), 10R/0014 (mice), 10R/0153 (calves) and 10R/0273 (pigs), provided in Annex 47 to Annex 51 respectively).**

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 **(see reports 10R/0012 (chickens), 10R/0013 (rats), 10R/0014 (mice) 10R/0153 (calves) and 10R/0273 (pigs), provided in Annex 47 to 51 respectively).**

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. **The full report 09R/0098 is provided in Annex 21**.

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 **(see reports 10R/0012 (chickens), 10R/0013 (rats), 10R/0014 (mice), 10R/0153 (calves) and 10R/0273 (pigs), provided in Annex 47 to Annex 51 respectively),** 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 **(see**

reports 10R/0012 (chickens), 10R/0013 (rats), 10R/0014 (mice), 10R/0153 (calves) and 10R/0273 (pigs), provided in Annex 47 to Annex 51 respectively). 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	no such structures are known

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

(b) Identity of the vector
pSelAct-gene1 for the deletion of ipdAB1
pSelAct-geneAB2 for the deletion of ipdAB2

(c) Host range of the vector
Escherichia coli

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

antibiotic resistance (apramycin, kanamycin)
other, specify …cytosine deaminase, fosforybosyl transferase

Indication of which antibiotic resistance gene is inserted
None

(e) Constituent fragments of the vector
None

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

(i)	transformation	(.)
(ii)	electroporation	(X)
(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 **(see reports 09R/0227 (Annex 46) and 08R/0234 (Annex 5)** and safety in mice, rats, chickens, calves and pigs **(reports 10R/0012 (chickens), 10R/0013 (rats), 10R/0014 (mice), 10R/0153 (calves) and 10R/0273 (pigs), provided in Annex 47 to Annex 51 respectively)** 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 **(report 11R/0038, Annex 25).** 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 **08R/0234, Annex 5** 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 **08R/08234 Annex 5** 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 report 09/R0227, Annex 46)**. A *ΔipdAB1* single deletion mutant appeared also to be safe after oral and intratracheal administration **(see reports 08R/0247, Annex 45 and 08R/0234, Annex 5 respectively)**. In addition, the vaccine strain has been tested for safety in the non-target species of chickens, rats, mice, calves, and pigs. **(see reports 10R/0012 (chickens), 10R/0013 (rats), 10R/0014 (mice), 10R/0153 (calves) and 10R/0273 (pigs), provided in Annex 47 to Annex 51 respectively)** 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 **(see report 08R/0234, Annex 5).**

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 **(see report 09/R0227, Annex 46).**

The vaccine strain is administered rectally and, as a result, is shed by vaccinated animals **(see report 10R/0086, Annex 11)**. 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 **(report 11R/0028draft, Annex 12).** 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 **(report 10R/0357, Annex 13)**.

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.

3.1.3.5 Conclusions on the potential environmental impact from the release

1. Likelihood of the genetically modified organism to become persistent and invasive in natural habitats under the conditions of the proposed release(s).

In natural habitats, 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. Wild type *R. equi* is known to infect young foals and colonise the intestines, resulting in the shedding of large numbers of bacteria. 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 so 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 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).

2. Any selective advantage or disadvantage conferred to the vaccine strain and the likelihood of this becoming realised under the conditions of the proposed release(s).

Except for a hampered macrophage survival no differences in survivability between vaccine strain and wildtype have been observed. 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.

3. Potential for gene transfer to other species under conditions of the proposed release of vaccine strain and any selective advantage or disadvantage conferred to those species.

The potential for gene transfer is not considered any more likely than with wild type. 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.

4. Potential immediate and/or delayed environmental impact of the direct and indirect interactions between the vaccine strain and horses.

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. 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 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). In 2011 and 2012 two studies were performed in 80 foals in the Netherlands (the most susceptible type of target organism) under license number PorM/RB IM 09-004 (Annex 20). No vaccine related abnormalities were observed during these studies .

5. Potential immediate and/or delayed environmental impact of the direct and indirect interactions between the vaccine strain with non-target organisms, including impact on population levels of competitors, prey, hosts, symbionts, predators, parasites and pathogens.

Safety in chickens, mice, rats, calves and pigs have been confirmed **(see reports 10R/0012 (chickens), 10R/0013 (rats), 10R/0014 (mice), 10R/0153 (calves) and 10R/0273 (pigs), provided in Annex 47 to Annex 51 respectively.)**. 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. However, 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 a published study demonstrated that none of the strains isolated contained the $vapA^+$ virulence plasmid, 26% were $vapB^+$ and 74% did not contain vap plasmids. The predominant plasmid found in pigs is $vapB^+$ (70% of the 30 isolates examined). 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.

6. Possible immediate and/or delayed effects on human health resulting from potential direct and indirect interactions of the vaccine strain and persons working with, coming into contact with or in the vicinity of the vaccine strain release.

Immunocompetent humans are rarely affected by *R. equi*, while a compromised cell mediated immunity predisposes one to *R. equi* infection. 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. As the vaccine strain is a deletion mutant, it is not considered likely that any adverse effects will occur in humans that are not observed due to exposure to the wild type strain.

7. Possible immediate and/or delayed effects on animal health and consequences for the feed/food chain resulting from consumption of the vaccine strain and any product derived from it, if it is intended to be used as animal feed.

Safety in chickens, mice, rats, calves, pigs and foals have been confirmed. Regarding use of product derived from the foals as feed, the vaccine strain is not disseminated to other parts of the body than the intestine and respiratory tract and both, to the best of our knowledge, are not consumed. The vaccine strain is expected to behave no differently from the wild type parent strain should it gain access to feed as, because of the nature of the vaccine strain (unmarked deletion mutant) no additional genes are introduced into the environment.

8. Possible immediate and/or delayed effects on biogeochemical processes resulting from potential direct and indirect interactions of the vaccine strain and target and non-target organisms in the vicinity of the genetically modified organism release(s).

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*. If it persists 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.

9. Possible immediate and/or delayed, direct, and indirect environmental impacts of the specified techniques used for the management of the vaccine strain where these are different from those used for non-genetically modified organisms.

The only specified technique used for the management of the vaccine strain is heat inactivation of the manure and bedding material associated with treated animals. No immediate or delayed direct or indirect environmental impact is envisaged from this technique. Steri-7 is selected as a disinfectant for its efficacy against *R. equi*. However, the disinfection programme planned does not differ significantly from normal disinfection practices which already take place on the farm.

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.