PART 1 (COUNCIL DECISION 2002/813/EC)

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	Germany
(b)	Notification number	B/DE/10/213
(c)	Date of acknowledgement of notification	22/12/2010
(d)	Title of the project	A non-pathogenic *Rhodococcus equi* strain as vaccine in horses
(e)	Proposed period of release	From March 2012 until December 2014

2. Notifier

Name of institution or company: Intervet International bv, 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)
Deletion mutant of *Rhodococcus equi* strain RG2837

(c) Genetic stability – according to Annex IIIa, II, A(10)
It is a stable unmarked deletion mutant of *Rhodococcus equi*

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, permit PorM/RB IM 09-004

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
- Notification number B/NL/09/004, permit PorM/RB IM 09-004

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.

The 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 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 then 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 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

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	…
(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 environments it colonizes the gut and nasal cavities. 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) *R. equi* is present at practically all horse farms, but is not used as a vaccine yet

(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	colonizes the gut and nasal cavities of animals, especially herbivores

(b) If the organism is an animal: natural habitat or usual agroecosystem:
…

5. (a) Detection techniques
Isolation on selective agar and PCR

(b) Identification techniques
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)	mostly in immunocompromised humans, rarely in healthy people
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 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 submandibulary 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.

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 minutes to days.

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

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

(c) Factors affecting reproduction: temperature, nutrients

9. Survivability

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

(i)	endospores	(.)
(ii)	cysts	(.)
(iii)	sclerotia	(.)
(iv)	asexual spores (fungi)	(.)
(v)	sexual spores (funghi)	(.)
(vi)	eggs	(.)
(vii)	pupae	(.)
(viii)	larvae	(.)
(ix)	other, specify …	

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

10. (a) Ways of dissemination
Wind (attached to dust particle), animals (nasal or gut colonization), manure.

(b) Factors affecting dissemination
Presence of herbivores, husbandry methods 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
Attenuation by reduction of the ability to survive in macrophages

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 (.) No (x)

Only 2x6 nucleotides at the two ligation sites have been inserted.

If no, go straight to question 5.

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

(a) Type of vector

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

(b) Identity of the vector

…

(c) Host range of the vector

…

(d) Presence in the vector of sequences giving a selectable or identifiable phenotype

Yes (.) No (.)

antibiotic resistance	(.)
other, specify	…

Indication of which antibiotic resistance gene is inserted

…

(e) Constituent fragments of the vector

…

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

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

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

(i)	transformation	(x)
(ii)	microinjection	(.)

(iii) microencapsulation (.)
(iv) macroinjection (.)
(v) other, specify …

6. Composition of the insert:
Not applicable (deletion mutant), only 2x6 nucleotides (GATATC) and (AGATCT) at the two ligation sites were not part of the genome of strain RE1.

(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
Not applicable (deletion mutant)

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 macrophages
** No: there appears 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 reproduce in macrophages
** No: there appears 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: the GMO is less able to survive in macrophages. As growth in macrophages is essential for the development of pneumonia, the dissemination in the body will be significantly reduced.
** No: there appears no difference in growth in the gut and rectal excretion will, therefore, 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 in macrophages. As growth in macrophages is essential for the development of pneumonia, the GMO is non pathogenic for foals.

2. Genetic stability of the genetically modified organism
The strain is a stable deletion mutant.

3. Is the GMO significantly pathogenic or harmful in any way (including its extracellular products), either living or dead?
Yes (.) No (.) Unknown (x)

(a) to which of the following organisms?

humans (x) It can not ultimately be excluded that the attenuated vaccine strain could cause infections in immunocompromised humans. 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 more likely less compared to the wildtype *R. equi*.
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. First the samples must be cultured on selective agar, followed bacteriological identification of suspected colonies and GMO specific PCR testing.

(b) Techniques used to identify the GMO
Selective agar and PCRs 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 and in-use safety 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):
Flurstück 33/2, Flur 20, Germarkung 131049/Grabow, Gemeinde 19300 Grabow/Ortsteil Heidehof, Landkreis Ludwigslust, Mecklenburg-Vorpommern.

(b) Size of the site (m^2):

(i)	actual release site (m^2):	stable with a size of 1781 m^2 (49.2 m x 36.2 m)
(ii)	wider release site (m^2):	The piece of land where the stable (= actual release site) is located has a fenced plot with a length and width of approximately 110 m x 180 m.

(c) Proximity to internationally recognised biotopes or protected areas (including drinking water reservoirs), which could be affected:
The European bird sanctuary ‘Ludwigsluster-Grabower Heide’ (DE 2635-401) is within 50 metre distance from the actual release site. Within this reserve and at a distance of approximately 400 meter to the site is located the Natura 2000 reserve ‘Ludwigsluster-Grabower heide, Weisses Moor und Griemoor’ (DE2635-303). The area is characterised by a poor sandy soil covered with pine forest and moors.

(d) Flora and fauna including crops, livestock and migratory species which may potentially interact with the GMO
The natural reserve(s) indicated above house a protected plant (Schwimmendes Froschkraut) and number of protected bird species, which are not at risk because *R. equi* is not pathogenic for plants or birds. The nearest livestock (cattle) is on a farm at a distance of 930 meter from the release site.

4. Method and amount of release

(a) Quantities of GMOs to be released:

The vaccine contains between $5x10^9$ and $1x10^{11}$CFU (Colony Forming Units) of *R. equi* strain RG2837 per dose. .
The number of foals to be vaccinated in the whole study is 120. The vaccinates will receive a maximum of 4 doses of vaccine. If all 120 foals receive 4 doses of vaccine containing the maximum release level, the total amount of vaccine strain that will be released in the study will be 4.8 $x10^{13}$ CFU over 2-3 foaling seasons.

(b) Duration of the operation:
Up to three foaling seasons: 2012 - 2014

(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, 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 will be removed mechanically into closed containers after each group of foals has left the stable. The straw and litter will be incinerated by a specialised and approved company. Each year when the last study animals have left, the release site will be cleaned and disinfected.

5. Short description of average environmental conditions (weather, temperature, etc.)
The environmental conditions are typical for Mecklenburg Vorpommern, a mean annual rain fall of approximately 600 mm and an average temperature of 8-9°C with significant seasonal differences.

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.
In 2011, a study was performed in 40 foals in the Netherlands under license number PorM/RB IM 09-004. No vaccine related abnormalities were observed during this study.

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)	Vertebrates, mammals
(ii)	family name for plants	Equidae
(iii)	genus	*Equus*
(iv)	species	*ferus*
(v)	subspecies	*caballus*
(vi)	strain	…
(vii)	cultivar/breeding line	warmblood horses
(viii)	pathovar	…
(ix)	common name	…

2. Anticipated mechanism and result of interaction between the released GMOs and the target organism (if applicable)
The vaccine strain will transiently be present in the body (mainly intestine) and interact with the local lymph nodes and thereby induce 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
Not applicable, non-pathogenic vaccine strain.

(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

(c) likely consequences of gene transfer:
It is unlikely that gene transfer will have any consequences. The situation for the GMO (= unmarked deletion mutant, with no additional genes introduced into the environment) is the same as for the wildtype *R. equi* that are already present in the environment.

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 of *R. equi* on selected agar and identification by PCR.

2. Methods for monitoring ecosystem effects
The foals in the study will be monitored until they are at least 6 months old. No specific monitoring of ecosystem effects is foreseen. However, the ground around the stable will be monitored at random for the presence of *R. equi* RG2837 in the soil.

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)
The actual release site has a surface of 1781 m^2

5. Duration of the monitoring
The foals will be monitored until they are at least 6 months old. The release site will be monitored for the presence of the GMO in the soil until to 6 weeks after the foals have left. In case one or more of the soil samples are positive for the vaccine strain, then the monitoring will continue at 2-monthly intervals until they are negative on 2 consecutive occasions.

6. Frequency of the monitoring
Shortly after vaccination the foals will be monitored daily, later this will be every two weeks.

I. Information on post-release and waste treatment

1. Post-release treatment of the site
Each year when the last study animals have left, 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.

2. Post-release treatment of the GMOs
See above

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

3. (b) Treatment of waste
By incineration 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
In case of adverse events caused by the GMO, standard antibiotic treatment for *R. equi* infection can be administered. The vaccine strain is sensitive to ampicillin, chloramphenicol,

erythromycin, rifampicin, gentamycin, neomycin, amoxicillin, spectinomycin, enrofloxacin, spiramycin, and doxycycline.

2. Methods for removal of the GMO(s) of the areas potentially affected
The GMO is sensitive for most disinfectants, including Halamid ®

3. Methods for disposal or sanitation of plants, animals, soils, etc. that could be exposed during or after the spread
Not foreseen because the GMO is a deletion mutant and wildtype *R. equi* is a normal soil saprophyte that is found at most farms where herbivores are kept.

4. Plans for protecting human health and the environment in the event of an undesirable effect
No undesirable effects expected. In case an adverse event occurs, the following action plan applies:
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 cleaned and disinfected (if possible). 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.