



Notification 6786-01-0213

Summary of the risk assessment of the genetically modified bacterial strain *Rhodococcus equi* RG2837 carried out by the German Competent Authority within the framework of a proposed deliberate release

Berlin, 4 June 2012

Explanatory note to this document:

The following text reflects the summary of the risk assessment of (a) genetically modified organism(s) to be used for experimental field trials (deliberate releases) in Germany. The text forms part of the official authorisation regarding applications for the permit of deliberate releases (field trials) of genetically modified organisms in Germany under the legal framework of Directive 2001/18/EC and the German Gene Technology Act (Gentechnikgesetz, GenTG). The authorisation is issued by the Bundesamt für Verbraucherschutz und Lebensmittelsicherheit, BVL [*Federal Office of Consumer Protection and Food Safety*], as the German Competent Authority. It comprises the chapters

- I. Consent [to the application]
- II. Provisions [to be respected in execution of the field trials]
- III. Justification
 - III.1. Requirements for approval according to section 16 GenTG [German Gene Technology Act]
 - III.1.1. Requirements for approval according to section 16 (1) Nr. 1 GenTG
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 - III.1.4. Formal requirements according to section 16 (4, 5) GenTG
 - III.2 Appraisal of and reply to objections
- IV. Costs
- V. Legal instruction

Only the original German document is legally binding. The following passage is a courtesy translation of the chapter III.1.2. and was prepared for the Biosafety Clearing-House.

III.1.3. Authorisation requirements according to § 16 (1) No. 3 GenTG

After hearing the *Zentrale Kommission für die Biologische Sicherheit*, ZKBS [Central Commission for Biological Safety] and following the participation of the *Bundesamt für Naturschutz*, BfN [Federal Office for Nature Conservation], the *Bundesinstitut für Risikobewertung*, BfR [Federal Institute for Risk Assessment] and the Robert Koch Institute (RKI) within the scope of consultations as well as having assessed the opinions of the Julius Kühn Institute (JKI) and the Friedrich Loeffler Institute (FLI) under § 16 (4) No. 1 GenTG, the BVL has come to the conclusion that, according to the current state of science and technology, no harmful effects on the legal interests defined under § 1 No. 1 GenTG are to be expected, as will be justified in III.1.3.1. to III.1.3.4.

The purpose of the GenTG according to § 1 No. 1 is,

giving due regard to ethical values, to protect human life and health, the environment with its interacting systems, animals, plants and material assets from harmful effects of the techniques and products of genetic engineering, and to take precautions against the occurrence of such hazards.

With this wording the legislator aimed to ensure that, in addition to guarding against hazards, the “greatest possible precaution is taken against existing or suspected hazards resulting from the techniques or products of genetic engineering” (official justification for § 1 GenTG, publication of the German Bundestag 11/5622, p. 22). The yardstick for the authorisation of a deliberate release is therefore whether gene technology-specific hazards could occur, see also § 22 (2) GenTG. The assumption of a hazard depends largely on the probability of occurrence of an adverse event and on the nature and extent of the potential damage.

According to the case law of the Federal Administrative Court (*Bundesverwaltungsgericht*, BVerwG), risk prevention must also consider “possible risks which cannot be ruled out because the current state of knowledge can as yet neither confirm nor deny certain causal connections and to that extent, therefore, no hazard exists” (BVerwGE 72, 300, 315). Here the legislator exercises a special duty of care, which was also emphasised by the Federal Constitutional Court (*Bundesverfassungsgericht*, BVerfG, judgement of 24/11/2010, file no. 1 BVF 2/05, point 135, quoted after Juris).

However, the exclusion of any adverse effects whatsoever cannot be demanded, as is also pointed out in the justification for the law (see official justification for § 16 GenTG, German Bundestag publication 11/5622, p. 29). According to regulation § 16 (1) No. 3 GenTG the decisive factor is that no unacceptable adverse effects are anticipated according to the current state of science and technology in relation to the purpose of the deliberate release. According to the justification of the GenTG an overall assessment of the expected effects is to be

undertaken giving due consideration to the intended or tolerated adverse effects and the benefit of the proposed deliberate release.

Giving due regard to these legal provisions and taking the precautionary safety measures (see chapter III.1.2.) into account, it has been determined according to the current state of science and technology that no adverse effects on the protected assets defined under § 1 No.1 GenTG are to be expected to result from the proposed release, as will be justified in the following. At the same time, it has been determined that no unacceptable risks exist.

According to the information provided by the applicant, the purpose of the proposed deliberate release is to confirm existing laboratory data on the efficacy of the vaccine under field conditions. The deliberate release forms part of a study aimed at collecting data which the applicant intends to submit to the *Europäische Arzneimittelagentur* [European Medicines Agency] to support an application for approval of the vaccine according EU Directive 2001/82/EC. Due to the lack of indication of risks, the stated purpose is not to be assessed and accordingly no risk-benefit analysis shall be performed.

III.1.3.1. Assessment of the genetic modification

R. equi RG2387 is a marker-free deletion mutant ($\Delta ipdAB \Delta ipdAB2$) which differs from the parent strain *R. equi* RE1 in that large parts of the encoding regions of the chromosomal genes *ipdAB* and *ipdAB2* are absent.

By analyzing the genome sequence of the vaccine strain the applicant has demonstrated that the chromosome of the deletion mutant does not contain any vector components, apart from the restriction sites (six nucleotides each) required for cloning the flanking regions, and that the recombination events have not led to any novel genomic combinations. Furthermore, a sequence analysis of the virulence plasmid contained in the vaccine strain showed that it corresponds with the plasmid of the parent strain *R. equi* RE1 and that no components of the used plasmid vectors pSelAct-ipd1 and pSelAct- $\Delta ipdAB2$ were integrated here either.

The applicant was able to demonstrate in animal trials that the loss of the cholesterol catabolic genes resulted in *R. equi* RG2837 is apathogenic for foals which were given the vaccine orally twice with a two-week interval. In a second study, the aim of which was to investigate whether oral or rectal vaccination is more effective, administration of the parent vaccine up to four times did not lead to disease in the foals either. This applies to both orally and rectally treated foals. Likewise, intratracheal administration of the strain *R. equi* RG1341, which exhibits the $\Delta ipdAB$ deletion responsible for attenuation, did not lead to disease in the foals. In contrast, animals treated with the wild-type strain *R. equi* RE1 did show symptoms of disease.

In animal trials, chickens, pigs, calves, mice and rats proved to be non-susceptible to oral and/or oronasal infection, both with the parent strain *R. equi* RE1 and with the genetically modified (GM) strain RG2837. Moreover, within the scope of these experiments no colonisation of the animals under investigation with the GM bacterial strain was detected.

R. equi belongs to the Nocardiaceae family, which includes different pathogenic agents which, like *R. equi*, can survive in macrophages from which they primarily gain their virulence. For this reason, the applicant investigated whether *R. equi* RG2837's ability to establish in human macrophages is reduced in comparison with *R. equi* RE1. To do so, cell culture experiments were carried out to determine bacterial replication in human macrophages 76 h after infection. The results of the cell culture experiments revealed that as a result of the genetic modification the vaccine strain exhibits a significantly reduced ability to establish *in vitro* in human macrophages.

Since the ability of *R. equi* to survive in macrophages is largely responsible for the virulence of the bacterium, it can be assumed that as a result of the genetic modification the vaccine strain underwent a general reduction in virulence, which would apply not only to the most susceptible host, the foal, but possibly also to all animals that are susceptible to the parent strain.

However, the experiment allows only limited conclusions about the possible pathogenicity of the vaccines for immuno-compromised humans. This is mainly because it is not known whether *R. equi* strain 103⁻, which was used as a negative control and which cannot cause disease in foals due to the absence of the VapA plasmid, is also apathogenic for immuno-compromised humans. Even if the available data cannot ultimately rule out the possibility that the vaccine strain could infect immuno-compromised humans, the reduced ability of *R. equi* RG2837 to establish in human macrophages *in vitro* indicates that compared with the parent strain RE1 the vaccine strain possesses reduced pathogenic potential for humans.

The genetic modification itself does not lead to the generation of a bacterium which has adverse effects on human health as a result of the performed gene deletion. The deleted genes do not play a role in host specificity. Moreover, since it is more difficult for the GM vaccine strain to survive in human macrophages than for the parent strain, it can be assumed that in comparison with the parent strain the vaccine strain does not demonstrate any increased pathogenic potential.

In laboratory trials the applicant found that vaccinated foals can excrete large amounts of *R. equi* RG2837 in faeces in the first few days after vaccination. Intermittent excretion of small amounts of the vaccine strain could be demonstrated in some of the trial foals up to four weeks after vaccination. Because the trial ended after four weeks, excretion beyond the four-

week trial period cannot be ruled out. In connection with this, the applicant states that long-term intestinal colonisation of the vaccinated horses is theoretically possible. *R. equi* lives in the intestinal lumen as a harmless commensal. As a result of the genetic modification the vaccine strain's ability to establish in macrophages was weakened, which accounts for the reduced virulence of the bacterium. Due to the absence of macrophages in the intestinal lumen it can be assumed that the vaccine strain does not behave any differently to the parent strain. A macrophage-associated infection of the respiratory tract, on the other hand, turns out to be a dead end for the vaccine strain. In a study in which mother-mares and untreated contact foals came into contact with faeces from trial foals treated with the vaccine strain, the applicant was also able to demonstrate that no colonisation of these animals with the vaccine strain took place.

There are no known survival structures (spores) for *R. equi*. In survival experiments in soil as well as in pond water and fresh water with subsequent storage at 4°C, 22°C or 37°C the applicant was able to demonstrate that within a few days the number of bacteria had fallen by more than 99%. However, small amounts of the vaccine strain were able to survive for a long period of time, so that it was possible to isolate the bacterium even after more than a year. In doing so, no difference between the vaccine strain and the parent strain could be detected. Accordingly, it can be stated that the genetically modified strain *R. equi* RG2837 does not persist in the environment any longer than the wild-type strain RE1.

The genetic modification itself does not lead to the generation of a bacterium with persistent or invasive characteristics nor are there any recognisable selection advantages in the environment as a result of the performed gene deletion. Since the deleted genes are also found in related bacteria, their presence is possibly associated with an advantage. An evolutionary selection advantage resulting from the deletion of these genes in *R. equi* is however not evident.

Overall it can be stated that the genetic modification led to an attenuation of the vaccine strain so that, in contrast to the parent strain, this strain does not cause any disease in foals. In addition, there is no reason to assume that the genetic modification has led to an amplification of the pathogenicity, a change in the host spectrum or a change in environmental characteristics of the vaccine strain.

III.1.3.2. Assessment of the deliberate release trial

R. equi is a globally widespread soil bacterium found ubiquitously in soil.

Studies have shown the presence of ca. 4×10^3 to 1×10^4 cfu/g soil in the vicinity of horse farms, whereby the proportion of *R. equi* isolates containing the VapA antigen was as high as

28% at farms with numerous *R. equi* infections compared to 5% at farms without *R. equi* infections.

One study conducted at the main stud revealed that *R. equi* is widespread there. Of 64 soil samples taken in spring 2003, 87% tested positive for *R. equi*, whereby 21% of the *R. equi* isolates contained the VapA plasmid. A repeat study at the main stud in summer 2003 showed that *R. equi* could be isolated from 68% of the soil samples taken. Of these isolates as much as 81% proved to be VapA positive. Consequently, it can be assumed that staff employed at the stud come into frequent contact with *R. equi* wild-type strains. The building intended for use as stables for the proposed deliberate release is located on a fully fenced-in site at a distance of twelve kilometres from the main stud site of on which horses are kept occasionally.

All in all, as a bacterium ubiquitously distributed in soil, also in the surroundings of the deliberate release site on which horses are periodically kept, it can be assumed that *R. equi* is common.

Despite the global distribution of *R. equi* and its clustered occurrence on horse farms, the total number of detected cases of infection in immuno-compromised humans is low at approx. 34 (status in March 2012), whereby only about one third of those affected had contact with horses or pigs. Infections respond well to treatment with combinations of antibiotics such as erythromycin, rifampin or ciprofloxacin.

According to the information provided by the applicant, to date there have been no signs of any *R. equi*-induced infection or disease in humans on site. So even where there is a high degree of infestation the risk of infection for humans is still low, even at farms with a high number of infections in foals, as is the case at the Lewitz stud farm. Birds are considered to be non-susceptible to *R. equi* infection, and disease in pigs is triggered exclusively by isolates with the VapB plasmid or without the virulence plasmid.

Immuno-compromised humans are sensitive to a range of naturally occurring *R. equi* strains (see chapter I.1.1., "Description of the non-GM recipient organism"). In immuno-compromised humans *R. equi* infections are – just like many other infections in immuno-compromised individuals with bacteria which are harmless to healthy individuals - of an opportunistic nature. The current data suggest that in comparison with the wild-type strain the vaccine strain does not demonstrate any increased pathogenicity as a result of the genetic modification. Rather the reduced ability of *R. equi* RG2837 to establish in human macrophages *in vitro* suggests that in comparison with the parent strain RE1 the vaccine strain demonstrates a lower pathogenic potential for humans. The possibility of immuno-compromised humans being infected with *R. equi* RG2837 would only be conceivable if extremely large amounts of the GM bacteria were able to establish in the surroundings of the

release site, and even then, this would not be attributable specifically to the genetic modification. In any case, the deliberate release proposed here is a spatially and temporally limited trial in which the vaccine strain is to be administered to a total of 120 foals over a period of three years. Specific containment measures, which were planned by the applicant and defined as a supplement to the Provisions under point II, will counteract the establishment of large amounts of the GM bacteria outside the stable building. Compared against the widespread distribution of pathogenic *R. equi* wild-type strains, the possible discharge of small amounts of the vaccine strain, e.g. by birds or small mammals, does not represent a risk to humans, animals or the environment.

The vaccine strain is sensitive to both a tested disinfectant (Halamid®) and a range of antibiotics. Genome analyses of the vaccine strain revealed that no transfer of antibiotic resistance genes from the plasmid vectors used in the construction of *R. equi* RG2837 took place. Therefore, the antibiotics shown to be effective in the treatment of infections caused by the parent strain have the same effect on the vaccine strain.

At the age of about one week, the foals that are to participate in the study are to be transferred with the mother-mares from the main stud to stables located 12 km away. Here, within the scope of a double-blind trial, either the vaccine or a placebo will be administered to the animals. The stable building is located on a fully fenced-in area of 100 m in length and 180 m in width. In accordance with Provision II.14., the fence has to carry signs designed to forbid entry to the site by unauthorised persons.

In addition to the stable building proposed for use for the deliberate release trial, the enclosed area also contains three further stables which are not to be used during the trial period. The stable proposed for use in the deliberate release covers an area of roughly 1800 square metres and consists of several individual boxes located at the side which are only to be used in emergencies during the trial, as well as a main room which is divided by a concrete wall. The stable has a concrete floor and solid walls on three sides as well as a gate on the fourth side.

The foals are to be transported back to the main stud, at the earliest, six weeks after the last vaccination. Until such time, the horses are to be held exclusively in the stable building proposed in the application for use for the release trial, which they may not leave at any time in between. Before the foals are transferred to the main stud, it must be determined whether the vaccine has established in the intestines of the foals by rectal swab. To do so, a pool of ten *R. equi* colonies, randomly isolated from the respective swabs, is to be tested for the presence of the vaccine strain by means of PCR. According to Provision II.8, only foals shown not to excrete the vaccine strain in the course of this examination will be permitted to return to the main stud.

The applicant plans to instruct all staff who have access to the trial site on the nature of the vaccine strain in writing, by providing a product brochure. In this connection, the staff will be instructed to consult a doctor in the case of illness and to present a copy of the product brochure indicating the possibility of contact with the vaccine strain. All events relating to human health which can be traced back to the vaccine strain are to be reported immediately to the competent authorities. Since staff participating in the trial may come into contact with very large amounts of the vaccine strain, in accordance with Provision II.13., the operator has to ensure that staff are also instructed about the possible health hazard emanating from the bacterium *R. equi* in general which could thus also result from the handling of very large amounts of the GM vaccine strain. As a precaution members of staff with known immuno-compromised status and members of staff who are scheduled to receive or who are already receiving immuno-suppressant therapy should not participate directly in the trial in order to avoid contact with very large amounts of the GM vaccine strain.

Furthermore, the applicant provides that only authorised personnel may enter the stable, whereby the main entrance to the stable (a door located in one of the three solid side-walls of the building) is to be used. All staff who enter the stable must change their clothes and wear boots which are to be left in place for use at all times. Before leaving the trial animals staff must step on a disinfectant mat. A log book is to be kept in which all entry and exit movement of persons and horses are to be recorded. Entry or exit through the gated side of the building is only to be permitted when the straw needs to be replaced or when the animals are being transported to or from the building.

According to Provision II.7. the applicant is required to control the stable building and its immediate surroundings on a daily basis, as long as the horses participating in the trial are being held at the site of the release trial (stable building). This includes monitoring for abnormalities in the interactions between the GMO and the trial horses and, where applicable, other organisms which could come into contact with the GM bacteria. If necessary, risk-minimising measures are to be taken.

The applicant plans, if necessary, to treat sick animals with antibiotics. In the unlikely case of an infection in humans, they too are to receive treatment with the appropriate antibiotics. If a diseased animal dies during the trial, an autopsy is to be performed by the University of Hannover (or another authorised laboratory) and the remains are to be treated as contaminated waste.

The applicant plans to dispose of used vaccination equipment and empty ampoules by incineration or immersion in an appropriate disinfectant.

According to Provision II.9., the concrete floor in front of the stable building is to be controlled on a regular basis for the discharge of manure and faeces from the stable. Where necessary,

the discharge is to be removed and disposed of and the affected floor area is to be disinfected.

After each group of foals present has left the stable, straw, litter and manure are to be transferred mechanically to closed containers and transported off site to an authorised specialist contractor for incineration. In this context, Provision II.11. provides that the containers, in the case that they are not co-incinerated, are to be disinfected after use. According to the information provided by the applicant, if necessary, the straw can also be replaced from time to time. In this regard, Provision II.10. provides that faeces or manure produced during the peak shedding period (in the first two days after vaccination) is to be removed in the meantime and straw and litter are to be replaced. Contaminated straw, litter and manure are to be disposed of as already specified.

In addition, each year one additional cleaning operation is to take place after all trial horses have left the release site. In the process, after all straw, litter and manure has been removed, the stable, the entire concrete floor inside and in front of the building as well as all equipment used are to be first cleaned with water and subsequently disinfected with Halamid® (chloramine-T) in standard concentration. With reference to this, Provision II.12. provides that care must be taken to ensure complete disinfection of the entire affected area with Halamid®. Moreover, the applicant is to ensure that the cleaning water is disinfected prior to disposal.

Random samples of the ground around the stable should be tested for the presence of *R. equi* RG2837. This should be done 1.) on the day of vaccination, 2.) when the foals are transported off site and 3.) six weeks after the last group of foals of the respective year have left the release site, whereby samples of approx. 25 g of the topsoil should be taken a) from two places at the edge of the concrete floor in front of the stable building, b) from two places close to the gate of the release site as well as c) from one place close to the back of the stable building. The soil samples taken should be tested for the presence of the vaccine strain by typing up to five isolates per sample site. If one of the soil samples taken at the end of the respective foal crop year tests positive for the vaccine strain, the applicant plans to repeat the test at intervals of two months until the test proves negative on two consecutive occasions. Under Provision II.4, the applicant is required to include the results of the planned soil sample tests for the presence of the GM bacteria in its report on the deliberate release to be submitted to the authority.

III.1.3.3. Assessment of the possibility of a transfer of the genetic material from the genetically modified bacteria into the environment or to other organisms

Since the vaccine strain is a marker-free deletion mutant, no new genes can be introduced into the environment or transferred to other organisms within the scope of the deliberate release trial. Hence, no genetic material other than the ubiquitously distributed wild-type strain can be introduced into the environment or transferred to other organisms by the vaccine strain. This applies both to the bacterial chromosome and to the VapA plasmid, which are carried by the wild-type strain and the GM strain alike.

The proposed deliberate release is a spatially and temporally limited experiment in which the GM vaccination strain is to be administered to 120 foals over a period of three years. In any case, the establishment of large amounts of the GM bacteria outside the stable building will be counteracted with specific containment measures which are planned by the applicant and/or which have been additionally defined in the Provisions under point II. Thus, in view of the widespread distribution of wild-type *R. equi*, the overall frequency of a transfer of the genetic material, which is carried by the GM strain and the wild-type strain alike, into the environment or to other organisms as a result of the proposed deliberate release is not discernibly increased.

III.1.3.4. Evaluation of the genetic stability of the genetic modification

It is theoretically possible that the deletions undertaken in the genome of the vaccine strain could be reconstructed through an exchange of DNA with other bacteria which carry the deleted gene in their genome. Such a gene transfer would allow the GM bacteria to survive unrestrictedly in macrophages and to proliferate there, so that they would again be able to cause pulmonary infection in young foals. However, the likelihood of a repair of the gene deletion by recombination is extremely low because the deletions were not performed on mobile elements but on the bacterial chromosome. Moreover, since almost the complete operon was deleted, both the *ipdA* gene and the *ipdB* gene would have to be replaced in order to restore its function. In the highly unlikely case of such a homologous recombination event between the GM bacterium and the wild-type *R. equi*, it must again be taken into account that the resulting strain would be identical to the wild-type strain, which in any case is widespread in the environment.