* KOPIE *

Intervet
Schering-Plough Animal Health

Intervet International bv
Wim de Körverstraat 35
P.O. Box 31, 5830 AA Boxmeer
The Netherlands
T +31 485 58 7887
www.intervet.com

RIVM/SEC/Bureau GGO
Postbus 1
3720 BA BILTHOVEN

Boxmeer, 3 maart 2010

Beste Mevrouw van Esschoten,

In antwoord op uw verzoek om aanvullende informatie van 08 februari j.l. deel ik u het volgende mede:

Betreffende shedding, ophoping en/of uitdoving van het GGO in het milieu:

- Hoeveel ggo scheidt een gevaccineerd veulen uit (in verhouding tot de vaccinatie dosis)?
De verwachting is dat de vaccinatiedoses na verloop van tijd via de faeces zal wordt uitgescheiden. Groei (vermeerdering) in de darm is niet uitgesloten, evenals vermindering door afsterving. Dit is waarschijnlijk mede afhankelijk van de darmflora van het dier.
Uitscheiding is bij ongeveer 25% van de dieren 3 weken na vaccinatie nog aangetoond. Dit zijn enkele kolonies (+/- 3) op een rectale swab. Uitgaande dat via een swab 10 µg–100 µg feces op de kweekplaat wordt uitgesmeerd betekent dit dat er tussen $3x10^4$ en $3x10^5$ bacteriën per gram feces zit. De vaccinatie dosis ligt in de orde van 10^8-10^{10} CFU per dosis.

- Hoe lang blijft het ggo in de darm aanwezig?
De vaccinstam is 3 weken na vaccinatie in ongeveer 25% van de gevaccineerde dieren nog aantoonbaar in de feces (rectale swab). Het eindpunt van de uitscheiding is niet bekend. De dagen na vaccinatie waarop uitscheiding plaatsvindt, is van dier tot dier verschillend.

- Hoeveel dagen zal een gevaccineerd dier het ggo uitscheiden?
Zie beide voorgaande vragen

- Hoe staat de uitgescheiden hoeveelheid ggo in verhouding tot een "infectieuze" dosis?
Een challengedosis van 10^6 CFU wildtype *R. equi*, intratracheaal toegediend in een volume van 100 ml, is in staat om ziekte te veroorzaken bij veulens. Dezelfde vaccindosis (met de vaccinstam) bleek volkomen veilig en heeft geen ziekte of andere klinische verschijnselen tot gevolg.
Ongeveer 25% van de dieren scheidt 3 weken na gastro-intestinale vaccinatie nog aantoonbaar uit. Dit zijn enkele kolonies per rectale swab. Uitgaande van 10-100µg feces op een swab betekent dat er tussen de $3x10^4$ tot $3x10^5$ bacteriën per gram feces aanwezig zijn. Als een veulen meer dan 1 gram feces zou inademen, (wat niet erg waarschijnlijk is) dan is de ingesnoven dosis nog steeds aanmerkelijk lager dan een eventuele infectieuze dosis van het wildtype.

Intervet
Schering-Plough Animal Health

- Is het mogelijk dat vermenigvuldiging van het ggo in (de darm) van contactdieren (anders dan de gevaccineerde veulens) kan plaatsvinden?
Bij veulens (meest gevoelige dier) kan de vaccinstam (net als de wildtype), de darm (tijdelijk) koloniseren en wordt de stam (tijdelijk intermitterend) uitgescheiden. Bij onbehandelde veulens die in contact stonden met de gevaccineerde dieren, kon de vaccinstam niet worden aangetoond in rectale swabs (getest gedurende 3 weken), dus spreiding tussen veulens kon niet worden aangetoond.
In de longen van veulens vindt uitdoving plaats omdat de mutant niet meer kan overleven en vermenigvuldigen in macrofagen (een eigenschap die noodzakelijk is om pneumonie te veroorzaken). Hierop is de werking en veiligheid van het vaccin gebaseerd.
In het lumen van de darm en buiten het dier in het milieu zal de bacterie naar alle waarschijnlijkheid bepaalde tijd overleven. Er is geen reden om aan te nemen dat de aangebrachte deletie hierin verandering heeft gebracht t.o.v. wildtype, en dat de mutant zich hierin anders zal gedragen dan de wildtype bacterie.

Na oronasale toediening aan muizen, ratten en kippen (bijlage 3-I, 3-II en 3-III) werd geen rectale uitscheiding van de mutant of wildtype waargenomen (getest op selectieve agar, zie bijlage 3-IV). Dus in deze dieren treed wel uitdoving op. Dit wordt momenteel ook getest voor varkens en kalveren.

- Is het mogelijk dat na eventuele verspreiding van het ggo in het milieu verdere vermenigvuldiging kan plaatsvinden (bv andere landbouw huisdieren)?
Zie voorgaande antwoord.

- Is er sprake van een uitdovend effect indien bij derden een ggo besmetting van de darm optreedt?
Alleen in veulens is uitscheiding van de vaccinstam na vaccinatie aangetoond. Spreiding naar contactdieren is niet aangetoond. In muizen, ratten of kippen kon geen uitscheiding van de mutant of wildtype worden aangetoond na gerichte orale toediening. In deze dieren treed dus wel uitdoving op. Varkens en kalveren worden momenteel getest.

<u>Betreffende de farmroutines:</u>
- Wat gebeurt er met de (potentieel met ggo besmette) mest?
De mest kan gewoon op het land uitgereden worden.

- Wanneer verlaten de dieren de proef en wat gebeurt er <u>exact</u> met de paarden indien ze de proef verlaten (bijvoorbeeld, wordt getest op afwezigheid van ggo, worden de dieren geeuthanaseerd, worden de dieren met antibiotica behandeld?
We willen de werkzaamheid in het veld verder testen. De dieren zullen worden gehuisvest op de proefboerderij van Intervet. Afhankelijk van het experiment blijven de dieren 1 tot 6 maanden in de proef. Na afloop kunnen de dieren de proef verlaten, zonder extra maatregelen. Het zijn dan gevaccineerde dieren. Het gaat hier om een vaccin waarvan het risico van introductie in het milieu, gezien de aard van het GGO, als nihil wordt geschat.

- Zijn de dieren vrij van ggo indien deze de proef levend verlaten? Zo ja hoe wordt dit bewerkstelligd?
Zie voorgaande vragen.

* KOPIE *

Intervet
Schering-Plough Animal Health

Betreffende het aantal proefdieren:
In uw aanvraag geeft u aan maximaal 2000 dieren te gebruiken voor het experiment. U heeft telefonisch aangegeven dat mogelijk de proefdieren afkomstig uit de experimenten uitgevoerd onder IG99-123 zullen worden toegevoegd. U wordt verzocht van deze dieren te beschrijven welke behandeling met ggo's ze hebben ondergaan en om hoeveel dieren het gaat.
Hiermee bedoelden we dat als ten tijde van de vergunningverlening (voor introductie milieu) proeven in veulens lopen onder IG 99-123 lid 7 (handelingen met veulens in aanwezigheid van merries met de vaccin stam-RG2837) dat we deze dieren uit de DM-I inperking mogen halen en op Intervet terrein mogen huisvesten tot het einde van de proef. De gevaccineerde veulens kunnen dan gespaard worden en levend de proef verlaten, zoals ook aangevraagd voor IM09-004. Om hoeveel dieren het gaat hangt af van het tijdstip waarop de vergunning verleend wordt. De dieren zullen een of meerdere malen gevaccineerd zijn met $10^8 - 10^{10}$ CFU van de vaccinstam. De behandeling is hetzelfde als aangevraagd voor de dieren die zullen worden behandeld onder deze aanvraag IM09-004.

Er zijn 4 bijlagen bijgesloten die e.e.a. ondersteunen.

Hoogachtend,

Dr Ir Ellen Joosten (MVF)
Intervet / Schering-Plough Animal Health

Cc Dhr. J. van Raaij (directie),
Dr. P. Vermeij

BIJLAGE 3-I

INTERVET - BOXMEER
R & D Laboratories

SUBJECT REPORT

Release no. : 10R/0014
Name of project : Rhodococcus Equi Vaccine **(REV)**
Date : 14-01-10
Pages : 1 –14

TITLE: SAFETY OF EQUILIS RHODE IN MICE AFTER ORAL ADMINISTRATION

SUMMARY

The objective of this experiment was to investigate the safety of Equilis RhodE vaccine (vaccine strain RG2837) in mice after oral administration.
Two groups of five mice each were orally treated with $6.6x10^8$ CFU of either *Rhodococcus equi* strain RG2837 or with the wild parent type strain RE1. At T=0 and then twice weekly during three weeks rectal swabs were sampled and examined for re-isolation of *Rhodococcus equi* on selective agar. In addition the mice were daily observed for general health and/or clinical abnormalities. At T= 3 weeks after vaccination, the mice were killed and a full necropsy was performed.
One day after inoculation with strain RG2837, the mice in this group were less active. The next day these mice were normal again and further no abnormalities in either group were observed. During 3 weeks after inoculation with Equilis RhodE or with the wild type strain RE1, no abnormalities in the consistency of the faeces were observed and *Rhodococcus equi* was not re-isolated from rectal swabs. During the necropsy one mouse inoculated with Equilis RhodE had an enlarged mesenterial lymph node and an enlarged spleen. Both tissues appeared sterile upon culture. Further no abnormalities of internal organs were detected in either group.
From the results it can be concluded that ingestion of *Rhodococcus equi* wild type strain RE1 or vaccine strain RG2837 by mice poses no risk to this species. It does not cause clinical signs, does not cause an infection and is not shed into the environment via mice after oral administration to mice.

BIJLAGE 3-II

INTERVET - BOXMEER
R & D Laboratories

SUBJECT REPORT

Release no. : 10R/0013
Name of project : Rhodococcus Equi Vaccine **(REV)**
Date : 14-01-10
Pages : 1 –14

TITLE: SAFETY OF EQUILIS RHODE IN RATS AFTER ORAL ADMINISTRATION

SUMMARY

The objective of this experiment was to investigate the safety of Equilis RhodE vaccine (vaccine strain RG2837) in rats after oral administration.
Two groups of five rats each were orally treated with $1.3x10^9$ CFU of either *Rhodococcus equi* strain RG2837 or with the wild type parent strain RE1. At T=0 and then twice weekly during three weeks rectal swabs were sampled and examined for re-isolation of *Rhodococcus equi* on selective agar. In addition the rats were daily observed for general health and/or clinical abnormalities. At T= 3 weeks after vaccination, the rats were killed and a full necropsy was performed.
During 3 weeks after inoculation no systemic reactions were observed in either group. Likewise, during 3 weeks after inoculation with Equilis RhodE or with the wildtype strain RE1, no abnormalities in the consistency of the faeces were observed and *Rhodococcus equi* was not re-isolated from rectal swabs. Moreover, during the necropsy no abnormalities of internal organs were observed.
From the results it can be concluded that ingestion of *Rhodococcus equi* wild type strain RE1 or vaccine strain RG2837 by rats poses no risk to this species. It does not cause clinical signs, does not cause an infection and is not shed into the environment after oral administration to rats.

BIJLAGE 3-III

INTERVET - BOXMEER
R & D Laboratories

SUBJECT REPORT

Release no. : 10R/0012
Name of project : Rhodococcus Equi Vaccine **(REV)**
Date : 14-01-10
Pages : 1 –14

TITLE: SAFETY OF EQUILIS RHODE IN CHICKENS AFTER ORAL ADMINISTRATION

SUMMARY

The objective of this experiment was to investigate the safety of Equilis RhodE vaccine (vaccine strain RG2837) in chickens after oral administration.
Two groups of five chickens each were orally treated with $1.3x10^9$ CFU of either *Rhodococcus equi* strain RG2837 or the wild type parent strain RE1. At T=0 and then twice weekly during three weeks rectal swabs were sampled and examined for re-isolation of *Rhodococcus equi* on selective agar. In addition the chickens were daily observed for general health and/or clinical abnormalities. At T= 3 weeks after vaccination, the chickens were killed and a full necropsy was performed.
At day 14 post-inoculation one wild type treated bird had a small wound with crust on the left side of the head. This, most probably (obviously), is not related to the inoculation. At day 7 after inoculation one vaccine strain treated bird was less active. The next day this bird was normal again and further no clinical abnormalities were observed in either group, during the 3-week observation period. Likewise, during 3 weeks after inoculation with Equilis RhodE or with the wild type strain RE1, no abnormalities in the consistency of the faeces were observed and *Rhodococcus equi* was not re-isolated from rectal swabs. Moreover, during the necropsy no abnormalities of internal organs were observed.
From the results it can be concluded that ingestion of *Rhodococcus equi* wild type strain RE1 or vaccine strain RG2837 by chickens poses no risk to this species. It does not cause clinical signs, does not cause an infection and is not shed into the environment after oral administration to chickens.

BIJLAGE 3-IV

INTERVET - BOXMEER
R & D Laboratories

SUBJECT REPORT

Release no. : 09R/0098
Name of project : *Rhodococcus equi vaccine* **(REV)**
Date : 15-04-2009
Pages : 1 - 7

TITLE: DEVELOPMENT AND VALIDATION OF A SELECTIVE AGAR FOR RHODOCOCCUS EQUI STRAIN RG2837

SUMMARY

To test for shedding of the *Rhodococcus equi* vaccine strain RG2837 it is necessary to have a sensitive test to detect low numbers in animal excretions. In this study a selective agar was developed and validated. Its sensitivity was compared to PCR detection.
From the results it can be concluded that the selective agar is significantly more sensitive than the PCR methods to detect *R. equi* strain RG2837 in faeces samples. If 0.003% of the faecal flora is this strain it can still be detected. Since the selective agar cannot discriminate between strain RG2837 and *R. equi* field strains, it is necessary to determine the identity by PCR in a second step. For the ease of testing, rectal swabs can be vortexed in 1 ml physiological salt solution and then 10-fold diluted or directly inoculated on the selective agar and incubated for 3 days.

CONTENTS **Page**

1 INTRODUCTION

To test for shedding of the *Rhodococcus equi* vaccine strain RG2837 it is necessary to have a sensitive test to detect low numbers in animal excretions. In this study a selective agar was developed and validated and its sensitivity was compared to PCR detection.

2 MATERIALS AND METHODS

2.1 Test articles

Faeces suspension
20 grams of horse faeces were suspended in 90 ml isotonic phosphate buffered saline (PBS) 0.04M. One milliliter of this suspension contain the bacteria of approximately 200 milligram of faeces. This suspension was used in all experiments including growth experiments that were spiked with *Rhodococcus equi* strain RG2837.

Rhodococcus equi
Rhodococcus equi strain RG2837, working seed batch 28K08 was inoculated in RhoCD medium and incubated for 24 hours at 37 ± 2.5°C at 100 rpm. This culture was concentrated by centrifugation and used immediately after preparation.

Blood agar and selective agar
Two agars were used: blood agar (item no. 059599) and selective agar. The selective agar is composed of 40 g/L bloodagar base no. 2 (item no. 074485), supplemented with 6% defibrinated sheep blood (item no. 074324) and five antibiotics: 3.125 µg/ml trimethoprim (Sigma, T7883), 3.125 µg/ml cefoperazone sodium salt (Sigma, C4292), 6.25 µg/ml polymyxin B sulphate (Fluka, 81334), 50 µg/ml naladixic acid sodium salt (Sigma, N4382) and 1.5 µg/ml fungizone (item no. 009545).

2.2 Inoculation of test products and controls

To determine the ability of the selective agar to reduce growth of faecal flora, the faeces suspension (as described under 2.1) was serially ten-fold diluted and 100 µl of the dilutions 10^0 till 10^{-6} were plated in duplo on blood agar as well as on selective agar and incubated at 37°C for 24 hours (blood agar) or 72 hours (selective agar). Furthermore, to test for any effect of the selective agar on the sensitivity of the *Rhodococcus* detection also serial ten-fold dilutions of strain RG2837 were plated on blood agar and selective agar and incubated as described above.
To test for the suitability of the selective agar to detect strain RG2837 in faecal samples, 1 ml aliquots of faeces suspension were spiked with 100 µl of *Rhodococcus equi* strain RG2837 dilutions (prepared as described above). From every spiked sample, 100 µl was plated in duplo on blood agar as well as on selective agar. The plates were incubated as described above. Identical series of spiked faeces samples were prepared and used for PCR testing. After 24 and 72 hours *Rhodococcus equi* suspected colonies on the agar plates were counted and enumerated as CFU/ml. From the highest spike dilutions *Rhodococcus equi* suspected colonies were identified by API/Phoenix and confirmed as strain RG2837 by PCR (*ABAB2* double deletion).

2.3 PCR

The presence of the vaccine strain in the spiked faeces samples and the identity of the *AB* and/or *AB2* genotype of *Rhodococcus equi* suspected colonies on the selective (and blood) agar was tested in three different real time PCR's: *vapA*, *AB* and *AB2*.
From the spiked aliquots chromosomal DNA was isolated using Qiagen stool isolation kit and subsequently tested. From the agar plates colonie material was used for testing.
The PCR mixture of the *AB* and *AB2* PCR contained 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 $MgCl_2$, 200 nM primers and 100 nM probes. Primers 5626-OLI-3159, -3158 and -3128 and probes 5626-OLI-3130 (TXR) and 5626-OLI-3160 (FAM) from the Intervet oligo collection were used for the *AB* Q-PCR. Primers 5626-OLI-3131, -3165 and -3166 and probes 5626-OLI-3134 (FAM) and 5626-OLI-3135 (TXR) from the Intervet oligo collection were used for the *AB2* Q-PCR. The PCR program was 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 wild type strain will be positive with the FAM probe 5626-OLI-3160 and TexasRed probes 5626-OLI-3130 and -3135. An *ABAB2* mutant will only be positive with the FAM probes 5626-OLI-3160 (*AB*) and -3134 (*AB2*).
The PCR mixture of the *vapA* PCR contained 20 U/ml Taq DNA polymerase (HT Biotechnology Ltd, Cambridge, UK), Icycler buffer containing 0.2 mM dNTPs, 4 mM MgCl2, 200 nM primers and 100 nM Taqman probe. Probe 5626-OLI-3123 and primers 5626-OLI-3124 and -3125 from the Intervet oligo collection were used. The PCR program was 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 55°C.

3 RESULTS AND DISCUSSION

3.1 Development of the selective agar.

Based on literature and the antibiogram of *R. equi* strain RG2837, several antibiotics were selected for which *R. equi* strain RG2837 is resistant. These antibiotics were tested alone or in combination for their ability to inhibit growth of faecal flora. The resulting selective agar is described under 2.1.
Plating of *R. equi* strain RG2837 on blood agar and selective agar yielded $3.6x10^9$ CFU/ml and $3.3x10^9$ CFU/ml, respectively, indicating that the sensitivity of *Rhodococcus equi* detection is not influenced by the added antibiotics.
Plating faeces on blood agar and selective agar yielded $9.4x10^6$ CFU/ml and $2.0x10^3$ CFU/ml, respectively, demonstrating that the selective agar diminished growth of faeces flora by approximately 4700-fold.
Rectal swabs sampled from two different horses, vortexed in 1 ml physiological salt solution, yielded $8.4x10^5$ CFU/ml and $3.0x10^6$ CFU/ml, respectively, indicating that rectal swabs vortexed in 1 ml physiological salt gives similar counts then the faeces preparation used in this study i.e. 200 mg faeces per ml. Thus all results generated in this study are also applicable for rectal swabs.

3.2 Comparison of sensitivity of selective agar compared to blood agar and PCR.

Aliquotes of the faeces suspension, containing $9.4x10^6$ CFU/ml bacterial flora were spiked with 10-fold dilutions of *R. equi* strain RG2837 and then plated on blood agar and selective agar. The spiked faeces suspensions were also tested in three different PCR's: *vapA*, *AB* and *AB2*.
From the results (Table 1 and 2) it is clear that the selective agar is the most sensitive method to detect strain RG2837 in faeces samples. As few as 300 bacteria of strain RG2837 in 1 ml can be detected amongst $9.4x10^6$ CFU of faecal flora in the same ml (=0.003%). Since the selective agar cannot discriminate between strain RG2837 and field strains of *R. equi*, the identity has to be confirmed by *AB* and/or *AB2* PCR in a second step.

4 CONCLUSION

From the results it can be concluded that the selective agar is significantly more sensitive than the PCR methods to detect *R. equi* strain RG2837 in faeces samples. If 0.003% of the faecal flora consists of this strain it can still be detected. Since the selective agar cannot discriminate between strain RG2837 and *R. equi* field strains, it is necessary to determine the identity by PCR in a second step. For the ease of testing, rectal swabs can be vortexed in 1 ml physiological salt solution and then 10-fold diluted or directly inoculated on the selective agar and incubated for 3 days.

Table 1 Detection of *R. equi* strain RG 2837 spikes in faeces by PCR and by plating on bloodagar

theoretic R. equi spike (CFU/ml)	equi in faeces (CFU/ml)	detection VapB PCR	detection AB2 PCR	detection VapA PCR	count/detection on blood agar at dilution 10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}
3.4×10^8	2.8×10^8	+/-	+	+	-	-	-	+	+	+	+	+
3.4×10^7	2.7×10^7	+/-	+	+	-	-	+	+	+	+	+	+
3.4×10^6	2.2×10^6	-	+	+	-	-	-	+[a]	+[a]	+[a]	+[a]	-
3.4×10^5	2.5×10^5	-	-	+	-	-	-	+[a]	+[a]	-	-	nt
3.4×10^4	nd	-	-	+	-	-	-	-	-	-	nt	nt
3.4×10^3	nd	-	-	-	-	-	-	-	-	nt	nt	nt
3.4×10^2	nd	-	-	-	-	-	-	-	nt	nt	nt	nt
3.4×10^1	nd	-	-	-	-	-	-	nt	nt	nt	nt	nt
3.4	nd	-	-	-	-	-	nt	nt	nt	nt	nt	nt

Table 2 Detection of *R. equi* strain RG 2837 spikes in faeces by PCR and by plating on selective agar

theoretic R. equi spike (CFU/ml)	equi in faeces (CFU/ml)	detection VapB PCR	detection AB2 PCR	detection VapA PCR	count/dilution on selective agar at dilution 10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}
3.4×10^8	5.6×10^8	+/-	+	+	+	+	+	+	+	+	+	+
3.4×10^7	9.0×10^7	+/-	+	+	+	+	+	+	+	+	+	+
3.4×10^6	2.6×10^6	-	+	+	+	+	+	+	+	+	+	+
3.4×10^5	3.2×10^5	-	-	+	+	+	+	+	+	+	-	nt
3.4×10^4	2.3×10^4	-	-	+	+	+	+	+	-	-	nt	nt
3.4×10^3	2.9×10^3	-	-	-	+[a]	+[a]	+[a]	+[a]	-	nt	nt	nt
3.4×10^2	3.0×10^2	-	-	-	+[a]	+[a]	+[a]	-	nt	nt	nt	nt
3.4×10^1	nd	-	-	-	-	-	-	nt	nt	nt	nt	nt
3.4	nd	-	-	-	-	-	nt	nt	nt	nt	nt	nt

[a] identity confirmed as *R. equi* strain RG2837 by PCR

nd = not detectable because of overgrowth by faeces flora

nt = not tested