addendum 1

Schering-Plough Animal Health Corp.

Project No. 00011
Report No. B01-065-01R
Date: 28Jun01
Notebook Ref. 342

Mouse LD_{50} Testing for the *Mannheimia (Pasteurella) haemolytica* Lkt(-) Deletant Strain

(APHIS Product Code _____.)

Abstract

This study was conducted to obtain data concerning the virulence of the *M. haemolytica* Lkt- Master Seed in mice and determine the 50% lethal dose (LD_{50}). For comparison, the *M. haemolytica* D153 parent strain was also tested. The only difference between the two strains is a deletion in the leukotoxin (Lkt) gene, which results in a truncated and ineffective leukotoxin. Because the Lkt is only cytolytic to ruminant leukocytes and platelets, very little or no reduction in virulence was expected to occur with the deletant strain.

Ten mice were inoculated intraperitoneally (IP) with a 0.5 mL dose of either an undiluted, 1:5 dilution, 1:25 dilution, or 1:125 dilution of a culture that was one passage from the *M. haemolytica* Lkt- Master Seed or parent working seed. The titers of the undiluted material for the parent and deletant strains were 5.7×10^9 cfu/dose and 4.7×10^9 cfu/dose, respectively. Mice were observed for seven days with the number of dead mice recorded each day. Of the total number of mice that died, 91% (32/35) did so within the first 24 hr. These mice most likely died from endotoxic shock. The LD_{50} for the parent strain was 7.6×10^8, and the LD_{50} for the deletant strain was 4.1×10^9. It can be concluded from this study that the *M. haemolytica* Lkt- deletant strain is less virulent than the parent strain.

addendum 2

Schering-Plough Animal Health Corp.

Project No. 00011
Report No. B00-150-01R1
Date: 20Jul01
Notebook Reference 342

Calf Safety Study of a *Mannheimia (Pasteurella) haemolytica* D153 Lkt- Avirulent Live Culture

(APHIS Product Code _____.)

Abstract

The objectives of this study were as follows: 1.) to determine whether the *Mannheimia haemolytica* leukotoxin gene deleted strain (Lkt-) is safe for use in cattle; 2.) to investigate the ability of the vaccine to be shed by inoculated calves and spread to control calves; 3.) to determine whether the deletant strain can be recovered from tissues after vaccination; and 4.) to determine if the *M. haemolytica* Lkt- vaccine given either subcutaneously or intranasally can protect calves from a *M. haemolytica* respiratory challenge. The results from the first three objectives are presented in this report (B00-150-01R1), and the results from the fourth objective are presented in report B00-150-01R2. The experimental vaccine was an avirulent live culture of *M. haemolytica* D153 Lkt- that was one passage from the Master Seed. Calves in Group A were inoculated subcutaneously with 5.0×10^9 cfu/dose, and calves in Group B were inoculated intranasally with the same dose. Two additional calves in Groups A and B served as sentinel controls, and calves in Group C were not inoculated. Calves were observed for 22 days post-inoculation for injection site reactions and any clinical signs attributable to the vaccine. In addition, nasal swabs were collected as an indicator of shed and spread, and whole blood was collected for culture. On study days 3 and 7, a calf from each treatment group was necropsied, and systemic tissue samples were collected to determine whether the vaccine strain could be re-isolated after inoculation.

Results indicate that the *M. haemolytica* Lkt- strain is safe at a dose of 5.0×10^9 cfu given either subcutaneously or intranasally. No serious clinical signs of respiratory disease were observed during the 22-day observation period. Following vaccination, 64% of the calves in Group A had transient fevers that were most likely due to the vaccine being administered subcutaneously. This percentage was significantly higher than Group B (0%; intranasal) and Group C (14%; not-inoculated). A transient swelling at the injection site occurred in less than 50% of the calves that were inoculated subcutaneously (Group A). Although these reactions were larger than expected, they are not a significant concern because of the low frequency of occurrence, the relatively short duration, and because they had no effect on the calves themselves. No signs of infection or disease were observed in the calves that were necropsied on study days 3 and 7. All the calves necropsied early had lung lesion scores of 0%, and *M. haemolytica* was not cultured from any of the tissues collected from these calves.

The results of this study also demonstrated the ability of the *M. haemolytica* Lkt- deletant strain to be shed by calves inoculated intranasally and spread to other calves. During the 22-day observation period, the vaccine strain was isolated from nasal swabs of calves in all three treatment groups. Shedding was not however accompanied by copious amounts of nasal discharge as only one calf from Group B was observed to have purulent nasal discharge for a single day. This suggests that nasal colonization by the *M. haemolytica* Lkt-deletant strain is relatively innocuous. Early isolation of the vaccine strain from calves in Groups A and C was most likely due to spread by the animal caretakers. Later isolations may have been due to a breach of isolation between groups B and C that occurred on study day 13. Clinically, these calves appeared no different than calves inoculated intranasally, subcutaneously, or calves that were *M. haemolytica*-free throughout the study. Regardless of how the vaccine strain was spread, no adverse reactions occurred in calves that subsequently became exposed to the vaccine strain.

addendum 3

Schering-Plough Animal Health Corp.

Project No. 00011
Report No. B05-165-01R
Date: 27 Apr 06

Safety of a Leukotoxin Deletant Strain (Lkt-) of *Mannheimia haemolytica* Following Intranasal Administration to Sheep
(APHIS Product Code No. 1861.R0)

Summary

Schering Plough Animal Health Corporation (SPAHC) is developing a modified live *Mannheimia haemolytica / Pasteurella multocida* vaccine to aid in the prevention of pneumonic pasteurellosis caused by *M. haemolytica* and *P. multocida*. Both of these strains have been genetically modified, and therefore, registration of the vaccine requires a complete Risk Assessment. One part of the Risk Assessment is the non-target animal safety studies. These studies are conducted to assess the effects of the vaccine strains on animals other than the intended host. The objective of this study was to evaluate the safety of the *M. haemolytica* leukotoxin gene deleted (Lkt-) vaccine strain in sheep. On study day 0, sheep in Group A (n=5) were inoculated intranasally with a 2.0 mL dose (1.0 mL/nare) of *M. haemolytica* Lkt- containing 4.8×10^9 colony forming units (cfu). Sheep in Treatment Group B (n=2) were inoculated with sterile tryptic soy broth + 1% yeast extract (TSBY) and served as controls. Clinical observations and rectal temperatures were monitored for 14 days following inoculation. No sheep (inoculates or controls) displayed any clinical signs of respiratory disease or elevated rectal temperatures throughout the 14-day observation period.

All seven sheep were euthanized and necropsied on study day 14. Examination revealed cranioventral lung congestion in one control and two inoculated sheep, although the lesions were not characteristic of a *M. haemolytica* infection. No lesions were observed on any major organs from the remaining sheep in either treatment group. Lung tissue samples from the three sheep with lesions and nasal swabs from all sheep in both treatment groups were collected at necropsy with no isolation of the *M. haemolytica* Lkt- deletant strain from any of the samples. These results indicate the inability of the *M. haemolytica* Lkt- strain to cause disease or persistent infection (14 days post-inoculation) in sheep when inoculated intranasally.

The conclusions of the study are as follows:

1) The *M. haemolytica* Lkt- strain, when administered intranasally to 7 month old sheep at approximately 20X the expected field dose, does not pose health or safety concerns in sheep.

2) There is low risk associated with the propagation of the *M. haemolytica* Lkt- vaccine strain in the environment via sheep.

This study was conducted in compliance with Organisations of Economic Cooperation and Development (OECD) Principles of Good Laboratory Practices (GLP).

addendum 4

Schering-Plough Animal Health Corp.

Project No. 00011
Report No. B04-175-01R
Date 07Sep06
SB B04-175-01

Reversion to Virulence Study for the *Mannheimia haemolytica* Leukotoxin Gene Deleted (Lkt-) Strain
(APHIS Product Code No. 1861.R0)

Summary

Schering Plough Animal Health Corporation (SPAHC) is developing a modified live *Mannheimia haemolytica / Pasteurella multocida* vaccine to aid in the prevention of pneumonic pasteurellosis caused by *M. haemolytica* and *P. multocida*. The *M. haemolytica* Lkt- deletant strain is attenuated by virtue of a 1,035 bp deletion within the *Lkt A* gene, which is the first gene in the leukotoxin operon that encodes the leukotoxin itself. As a result, the deletant strain is attenuated for virulence, but not growth. A reversion to virulence study was conducted to evaluate the genetic stability and potential reversion to virulence of the *M. haemolytica* D153 Lkt- Master Seed. Five passages were conducted using 3-10, sero-negative, 7-8 week old calves. For each passage, 2 calves served as un-inoculated controls. Calves in Passage 1 were inoculated intranasally with a 2 mL dose (1.2×10^9 cfu) of an avirulent live culture of *M. haemolytica* Lkt- that was one passage from the Master Seed (MSB+1). Calves in the subsequent passages were inoculated intranasally with a 2 mL dose of highest titered nasal swab material collected from the previous passage. Transient episodes of mild to severe muco-purulent discharge were observed in many of the inoculated calves in each passage, including Passage 4 despite the absence of the deletant strain in the nasal cavity. Therefore, the nasal discharge may be due to irritation of the nasal cavity from administering a large dose of Gram-negative bacteria and daily nasal swab collection rather than the pathogenicity of the deletant strain. The mean clinical score for inoculated calves in the five passages was 1.4 ± 1.7, 0 ± 0, 1.0 ± 1.7, 0.3 ± 0.6, 1.5 ± 1.5, and 2.2 ± 1.6, respectively.

During each passage, nasal swabs were collected daily for 6 days, and a viability count performed. The *M. haemolytica* Lkt- deletant strain was recovered from the nasal cavity of at least 1 calf in every passage except Passage 4. DNA fragments that were amplified from representative isolates were the same size as the DNA fragment amplified from the *M. haemolytica* Lkt- X+1 material used for the first passage.

Calves were euthanized and necropsied at the end of each observation period. The lungs were examined for signs of congestion/consolidation and cultured for isolation of the *M. haemolytica* Lkt- strain. The mean lung lesion score for inoculated calves in the five passages was 0%, 0%, $1.7 \pm 2.9\%$, 0%, $0.8 \pm 1.6\%$, and $1.2 \pm 2.1\%$, respectively. The *M. haemolytica* Lkt- deletant strain was isolated from the lung of only one of the three inoculated calves in Passage 3.

The results of this study demonstrate that the *M. haemolytica* Lkt- deletant strain is phenotypically (remained non-hemolytic) and genetically (retained the deletion in the *Lkt A* gene) stable when passaged in the host animal. There was no significant increase in clinical signs of disease from Passage 1 to Passage 5 ($p = 0.3310$), and there was no significant increase in lung lesion scores from Passage 1 to Passage 5 ($p = 0.1228$). The collective data indicate that the *M. haemolytica* Lkt- deletant strain will not revert to a virulent strain when passaged in the host animal and is therefore safe for use in young calves.

addendum 5

Schering Plough Animal Health Corp.

Project No. 00011
Report No. B04-125-01R
Date 08Dec05

Dissemination in Host and Shed/Spread Analysis Following Administration of an Avirulent Live Culture of a *Mannheimia haemolytica* Leukotoxin Gene Deleted (Lkt-) Strain

(APHIS Product Code No. 1861.R0)

Abstract

Schering Plough Animal Health Corporation (SPAHC) is developing a modified live *Mannheimia haemolytica / Pasteurella multocida* vaccine to aid in the prevention of pneumonic pasteurellosis caused by *M. haemolytica* and *P. multocida*. A dissemination in host and shed/spread study was conducted to determine the tissue tropism of the *M. haemolytica* leukotoxin gene deleted strain (Lkt-) and determine whether it can be shed from vaccinated calves and spread to non-vaccinated control calves. On study day 0, calves in Group A (n = 14) were vaccinated subcutaneously with a 2 mL dose (7.4×10^9 cfu) of an avirulent live culture of *M. haemolytica* Lkt- that was one passage from the Master Seed (X+1). Calves in Group B (n = 5) served as contact controls for shed/spread analysis. Clinical observations and rectal temperatures were recorded daily for 21 days. Calves in both treatment groups had sporadic episodes of lethargy, nasal discharge, and increased respiratory rate during the post-vaccination observation period. Although clinical signs may be expected following a 10X dose of a live Gram-negative bacteria, the sporadic nature of the post-vaccination clinical observations among vaccinated and non-vaccinated calves throughout the 21-day observation period would indicate a role for other stressors such as other viral/bacterial infections and/or changes in environmental conditions.

Two calves from Treatment Group A were euthanized and necropsied on study days 1, 2, 3, 7, 10, 14, and 21. The following samples were collected and cultured for isolation of the *M. haemolytica* Lkt- deletant strain: injection site, tonsil, pre-scapular lymph nodes, mediastinal lymph nodes, submandibular lymph nodes, lung, liver, spleen, thymus, kidneys, fecal swab, nasal swab, tracheal swab, and whole blood. *M. haemolytica* Lkt- was only isolated from the injection site of 3 calves euthanized on study days 2 and 7. The lungs from both treatment groups were examined, the percentage of lung consolidation estimated for each lobe, and a lung lesion score calculated. Results of lung scores indicated that there was no evidence to suggest that the deletant strain was able to migrate to the lung and cause an infection that eventually would progress into pneumonic lesions.

Nasal swabs for culture were collected from the 5 non-vaccinated control calves and any remaining vaccinated calves on study days 1, 2, 3, 7, 10, 14, and 21. The *M. haemolytica* Lkt- deletant strain was not isolated from the nasal swabs of any of the non-vaccinated control calves. In addition, all of the control calves remained sero-negative for antibodies against leukotoxin. These data indicate an inability of the *M. haemolytica* Lkt- deletant strain to be shed from calves vaccinated subcutaneously and spread to other susceptible calves.

The conclusions of this study are as follows:

1.) The tissue tropism of the *M. haemolytica* Lkt- deletant strain, when administered subcutaneously to 7-9 week old calves at > 10X the expected field dose, is isolated to the site of injection, with no evidence of migration to the lung.

2.) The *M. haemolytica* Lkt- deletant strain, when administered subcutaneously to 7-9 week old calves at > 10X the expected field dose, is not shed from vaccinated animals.

addendum 6

Schering-Plough Animal Health Corp.

Project No. 01005
Report No. B03-001-01R
Date: 06Aug03
Notebook Ref. 424

Mouse LD_{50} Testing for the *Pasteurella multocida* ΔhyaE Master Seed (Lot No. 90113-000)

(APHIS Product Code No.)

Abstract

This study was conducted to obtain data concerning the virulence of the *Pasteurella multocida* ΔhyaE Master Seed in mice and determine the 50% lethal dose (LD_{50}). For comparison, the *P. multocida* 1062 Parent strain was also tested. The only difference between the two strains is a 369 bp in-frame deletion in the hyaE gene, which is the first gene in the capsule biosynthetic operon that encodes for the *P. multocida* capsule. This is a repeat of study B02-187-01 in which the *P. multocida* hyaE deletant strain was found to be less virulent than the parent strain. However, in that study, the LD_{50} endpoint was not achieved.

Ten mice were inoculated intraperitoneally (IP) with a 0.5 mL dose of either an undiluted, 1:10 dilution, 1:100 dilution, 1:1,000 dilution, or 1:10,000 dilution of a culture that was one passage from the *P. multocida* ΔhyaE Master Seed (Lot No. 90113-000) or parent working seed. The titers of the undiluted material for the ΔhyaE and the parent were 2.2×10^7 cfu/dose and 6.0×10^5 cfu/dose, respectively. The mice were observed for seven days with the number of dead mice recorded each day. At the two lowest concentrations of parent strain, 95% of the mice died (18/19), compared to 55% (11/20) at the two lowest concentrations of the deletant strain. As a result, the LD_{50} for the *P. multocida* 1062 parent strain was <60, and the LD_{50} for the *P. multocida* ΔhyaE deletant strain as calculated by Reed and Muench was 2.4×10^4. These data support the conclusion that the deletion in the hyaE gene has attenuated the *P. multocida* 1062 strain and made it 400 times less virulent in mice.

addendum 7

Schering-Plough Animal Health Corp.

Project No. 01005
Report No. B05-064-01R
Date: 07Apr06

Evaluation of Safety of an Acapsular Mutant Strain (ΔhyaE) of *Pasteurella multocida* Following Intranasal Administration to Rabbits

(APHIS Product Code No. 1861.R0)

Summary

Schering Plough Animal Health Corporation (SPAHC) is developing a modified live *Mannheimia haemolytica/Pasteurella multocida* vaccine to aid in the prevention of pneumonic pasteurellosis caused by *M. haemolytica* and *P. multocida*. A complete Risk Assessment is required for the vaccine registration due to the genetic modification of these two strains, to include non-target animal safety studies. These studies are conducted to assess the effects of the vaccine strains on animals other than the intended host. The objective of this study was to evaluate the safety of the *P. multocida* ΔhyaE gene deleted vaccine strain in rabbits. On study day 0, rabbits in Group A (n=5) were inoculated intranasally with a 0.5 mL dose (0.25 mL/nare) of *P. multocida* ΔhyaE containing 1.1 x 10^9 colony forming units (cfu). Rabbits in Treatment Group B (n=2) were inoculated with sterile phosphate buffered saline (PBS) and served as controls. Clinical observations and rectal temperatures were monitored for 14 days following inoculation. No rabbits (inoculates or controls) displayed any clinical signs of respiratory distress or elevated rectal temperatures throughout the 14-day observation period.

All seven rabbits were euthanized and necropsied on study day 14. Examination revealed no gross lesions on any major organs from rabbits in either treatment group. Nasal swabs for culture were also collected at necropsy. The *P. multocida* deletant strain was not isolated from swabs of any of the inoculated or control rabbits. These results indicate the inability of the *P. multocida* ΔhyaE strain to cause disease in rabbits when inoculated intranasally and does not cause persistent infection (14 days post-inoculation).

The conclusions for the study are as follows:

1) The *P. multocida* ΔhyaE strain, when administered intranasally to 8 week old rabbits at >10X the expected field dose, does not pose health or safety concerns in rabbits.

2) There is low risk associated with the propagation of the *P. multocida* ΔhyaE vaccine strain in the environment via rabbits.

This study was conducted in compliance with Organisations for Economic Cooperation and Development (OECD) Principles of Good Laboratory Practices (GLP).

addendum 8

Schering-Plough Animal Health Corp.

Project No. 01005
Report No. B06-035-01R
Date: 10May06

Safety of an Acapsular Mutant Strain (ΔhyaE) of *Pasteurella multocida* Administered to Chickens by Swab to the Nasal Cleft

(APHIS Product Code No. 1861.R0)

Summary

Schering Plough Animal Health Corporation (SPAHC) is developing a modified live *Mannheimia haemolytica/Pasteurella multocida* vaccine to aid in the prevention of pneumonic pasteurellosis caused by *M. haemolytica* and *P. multocida*. A complete Risk Assessment is required for the vaccine registration due to the genetic modification of these two strains, to include non-target animal safety studies. These studies are conducted to assess the effects of the vaccine strains on animals other than the intended host. The objective of this study was to evaluate the safety of the *P. multocida* acapsular mutant (ΔhyaE) strain in chickens. On study day 0, chickens in Group A (n=10) were administered a 0.2 mL dose of *P. multocida* ΔhyaE containing 6.2×10^8 colony forming units (cfu) by swab to the nasal cleft. Chickens in Treatment Group B (n=5) were administered sterile phosphate buffered saline (PBS) and served as controls. Clinical observations were monitored for 14 days following inoculation. No chickens (inoculates or controls) displayed any clinical signs of respiratory distress throughout the 14-day observation period.

All fifteen chickens were euthanized and necropsied on study day 14. Examination revealed no gross lesions on any major organs from chickens in either treatment group. Nasal cleft swabs for culture were also collected at necropsy. The *P. multocida* acapsular mutant strain was not isolated from swabs of any of the inoculated or control chickens. These results indicate the inability of the *P. multocida* ΔhyaE strain to cause disease or persistent infection (14 days post-inoculation) in chickens when inoculated by swab to the nasal cleft.

The conclusions of the study are as follows:

1) The *P. multocida* ΔhyaE strain, when administered by swab to the nasal cleft to 16 week old chickens at 12X the expected field dose, does not pose health or safety concerns in chickens.

2) There is low risk associated with the propagation of the *P. multocida* ΔhyaE vaccine strain in the environment via chickens.

This study was conducted in compliance with Organisations for Economic Cooperation and Development (OECD) Principles of Good Laboratory Practices (GLP).

addendum 9

Schering-Plough Animal Health Corp.

Project No. 01005
Report No. B06-037-01R
Date: 26May06

Safety of an Acapsular Mutant Strain (ΔhyaE) of *Pasteurella multocida* Following Intranasal Administration to Sheep

(APHIS Code 1861.R0)

Summary

Schering Plough Animal Health Corporation (SPAHC) is developing a modified live *Mannheimia haemolytica / Pasteurella multocida* vaccine to aid in the prevention of pneumonic pasteurellosis caused by *M. haemolytica* and *P. multocida.* Both of these strains have been genetically modified, and therefore, licensing of the vaccine requires a complete Risk Assessment. One part of the Risk Assessment is the non-target animal safety studies. These studies are conducted to assess the effects of the vaccine strains on animals other than the intended host. The objective of this study was to evaluate the safety of the *P. multocida* acapsular mutant (ΔhyaE) strain in sheep. On study day 0, sheep in Group A (n=5) were inoculated intranasally with a 2.0 mL dose (1.0 mL/nare) of *P. multocida* ΔhyaE containing 7.0×10^8 colony forming units (cfu). Sheep in Treatment Group B (n=2) were inoculated with sterile phosphate buffered saline (PBS) and served as controls. Clinical observations were monitored for 14 days following inoculation. No sheep (inoculates or controls) displayed any clinical signs of respiratory distress or elevated rectal temperatures throughout the 14-day observation period.

All seven sheep were euthanized and necropsied on study day 14. Examination revealed no gross lesions on any major organs from sheep in either treatment group. Nasal swabs for culture were also collected at necropsy. The *P. multocida* acapsular mutant strain was not isolated from swabs of any of the inoculated or control sheep. These results indicate the inability of the *P. multocida* ΔhyaE strain to cause disease or persistent infection (14 days post-inoculation) in sheep when inoculated intranasally.

The conclusions of the study are as follows:

1) The *P. multocida* ΔhyaE strain, when administered intranasally to 3 month old sheep at 14X the expected field dose, does not pose health or safety concerns in sheep.

2) There is low risk associated with the propagation of the *P. multocida* ΔhyaE vaccine strain in the environment via sheep.

This study was conducted in compliance with Organisations of Economic Cooperation and Development (OECD) Principles of Good Laboratory Practices (GLP).

addendum 10

Schering-Plough Animal Health Corp.

Project No. 01005
Report No. B03-013-01R
Date: 29May03
Notebook Ref. 424

Calf Safety Study of a *Pasteurella multocida* ΔhyaE Avirulent Live Culture

Summary

The objectives of this study were as follows: 1.) to determine whether the *Pasteurella multocida* hyaE deletant Master Seed given at 10X the expected typical field dose is safe for use in 3 month old calves; 2.) to determine whether the deletant strain can be recovered from nasal passages and tissues after subcutaneous vaccination; and 3.) to investigate the ability of the deletant strain to be shed by inoculated calves and spread to control calves. Four calves were enrolled in the study: 2 vaccinates (Treatment Group A) and 2 sentinel controls (Treatment Group B). One calf from each treatment group was housed together in an isolation room to maximize nose-to-nose contact. On study day 0, the 2 calves in Treatment Group A were vaccinated subcutaneously with a 2 mL dose (5.0×10^9 cfu/dose) of a live *P. multocida* ΔhyaE culture that was one passage from the Master Seed. The calves were monitored daily (21 days) for clinical signs and adverse reactions following vaccination. Nasal swabs were collected on study days -3, 1, 3, 7, 14, and 21. Whole blood for culture was collected on study days 0-4. After 21 days, calves were necropsied, the lungs scored for consolidation, and tissue samples collected for isolation of the vaccine strain.

At a titer of 5.0×10^9 cfu/dose, the *P. multocida* ΔhyaE deletant strain caused some undesirable adverse reactions such as fever, lethargy, rapid breathing, and large injection site reactions. The calves quickly recovered from the clinical manifestations of the vaccine without any treatment, but the injection site reactions were still evident 21 days post-vaccination. Reactions for calf #5551 ranged from 8x9x2 cm on study day 3 to 9x4x2 cm on study day 21. Reactions for calf #5553 ranged from 30x20x3 cm on study day 3 to 4.5x3.5x1 cm on study day 21. Since the two sentinel control calves didn't show any signs of *P. multocida* ΔhyaE infection (no clinical signs and no isolation from nasal swabs), it was decided to vaccinate these calves with a 10-fold lower dose and evaluate the safety. On study day 14, calves in Treatment Group B were vaccinated with 5.0×10^8 cfu *P. multocida* ΔhyaE. The lower dose caused a slight increase in rectal temperature in only one calf (#5552; 103.3°F and 103.1°F on study days 15 and 16 respectively) and injection site reactions that were much smaller than those induced by the higher dose. Reactions for calf #5552 ranged from 9x5x1.5 cm on study day 15 to 5x2.5x1 on study day 21. Reactions for calf #5554 ranged from 5x3x1 cm on study day 15 to 3.5x2x1 cm on study day 21.

After 21 days, a pure culture of *P. multocida* ΔhyaE was isolated from the injection site (1/2 calves in Group A and 1/2 calves in Group B) and pre-scapular lymph nodes (1/2 calves in Group A and 1/2 calves in Group B). The deletant strain could not be isolated from the blood 1-4 days post-vaccination and did not appear to migrate to and colonize in the nasal passages or lung. The ability of this strain to reside in the injection site of a calf and elicit a constant immune response supports the potential for long-term duration of immunity. A longer duration of immunity claim would be a clear advantage over currently licensed products.

Results obtained from this study indicate that the *P. multocida* ΔhyaE acapsular mutant is safe for use in 3 month old calves at a dose of 5.0×10^8 cfu. At a 10-fold higher dose, the deletant strain produces fever, lethargy, rapid breathing, and large injection site reactions. In addition, there is no evidence that the *P. multocida* ΔhyaE deletant strain can be shed from calves vaccinated subcutaneously and spread to non-vaccinated animals or the environment.

addendum II

Schering Plough Animal Health Corp.

Project No. 01005
Report No. B04-057-01R
Date 09Nov05

Dissemination in Host and Shed/Spread Analysis Following Administration of an Avirulent Live Culture of a *Pasteurella multocida* Acapsular Mutant Strain (ΔhyaE)

(APHIS Product Code No. 1861.R0)

Abstract

Schering Plough Animal Health Corporation (SPAHC) is developing a modified live *Mannheimia haemolytica* / *Pasteurella multocida* vaccine to aid in the prevention of pneumonic pasteurellosis caused by *M. haemolytica* and *P. multocida*. A dissemination in host and shed/spread study was conducted to determine the tissue tropism of the *P. multocida* ΔhyaE acapsular mutant strain and determine whether it can be shed from vaccinated calves and spread to non-vaccinated control calves. On study day 0, calves in Group A (n = 14) were vaccinated subcutaneously with a 2 mL dose (1.3×10^9 cfu) of an avirulent live culture of *P. multocida* ΔhyaE that was one passage from the Master Seed (X+1). Calves in Group B (n = 6) served as contact controls for shed/spread analysis. Clinical observations and rectal temperatures were recorded daily for 21 days. The non-vaccinated control calves were normal throughout the study. However, calves in Group A (vaccinates) were lethargic at 24 hours post-vaccination, but returned to normal by 48 hours post-vaccination. Elevated rectal temperatures were recorded for 1/6 of the non-vaccinated control calves and 2/14 of the vaccinated calves at 24 hours post-vaccination. No other clinical observations or elevated rectal temperatures were observed during the remainder of the study.

Two calves from Treatment Group A were euthanized and necropsied on study days 1, 2, 3, 7, 10, 14, and 21. The following samples were collected and cultured for isolation of the *P. multocida* ΔhyaE acapsular mutant strain: injection site, tonsil, pre-scapular lymph nodes, mediastinal lymph nodes, submandibular lymph nodes, lung, liver, spleen, thymus, kidneys, fecal swab, nasal swab, tracheal swab, and whole blood. The *P. multocida* ΔhyaE deletant strain was isolated from the pre-scapular lymph node of 1 calf and the injection site of 8 calves. The lungs from both treatment groups were examined, the percentage of lung consolidation estimated for each lobe, and a lung lesion score calculated. None of the non-vaccinated control calves developed lung lesions; however, 2 vaccinated calves had lung lesion scores of 6.6% and 4.4%, but without any isolation of the deletant strain.

Nasal swabs for culture were collected from the 6 non-vaccinated control calves and any remaining vaccinated calves on study days 1, 2, 3, 7, 10, 14, and 21. The *P. multocida* ΔhyaE acapsular mutant strain was not isolated from the nasal swabs of any of the non-vaccinated control calves. In addition, all of the control calves remained sero-negative for antibodies against *P. multocida* OMPs. These data indicate an inability of the *P. multocida* ΔhyaE deletant strain to be shed from calves vaccinated subcutaneously and spread to other susceptible calves.

The conclusions of this study are as follows:

1.) The tissue tropism of the *P. multocida* ΔhyaE acapsular mutant strain, when administered subcutaneously to 7-8 week old calves at > 10X the expected field dose, includes the site of injection and adjacent lymph nodes, with no evidence of migration to the lung.

2.) The *P. multocida* ΔhyaE acapsular mutant strain, when administered subcutaneously to 7-8 week old calves at > 10X the expected field dose, is not shed from vaccinated animals.

addendum 12

Schering-Plough Animal Health Corp.

Project No. 01005
Report No. B05-006-01R
Date 05Sep06
SB B06-006-01

Reversion to Virulence Study for the *Pasteurella multocida* Acapsular Mutant Strain (ΔhyaE)
(APHIS Product Code No. 1861.R0)

Summary

Schering Plough Animal Health Corporation (SPAHC) is developing a modified live *Mannheimia haemolytica* / *Pasteurella multocida* vaccine to aid in the prevention of pneumonic pasteurellosis caused by *M. haemolytica* and *P. multocida*. The *P. multocida* ΔhyaE deletant strain is attenuated by virtue of a 366 bp deletion within the *hyaE* gene, which is the first gene in the capsule biosynthetic operon that encodes for the *P. multocida* capsule. As a result, the deletant strain is attenuated for virulence, but not growth. A reversion to virulence study was conducted to evaluate the genetic stability and potential reversion to virulence of the *P. multocida* 1062 ΔhyaE Master Seed. Five passages were conducted using 3-9, sero-negative, 7-8 week old calves for each passage. In addition, 2 calves in each passage served as un-inoculated controls. Passage 5 was repeated due to a concurrent *Salmonella* infection that affected the health of the calves enrolled in the first Passage 5. Calves in Passage 1 were inoculated intranasally with a 2 mL dose (1.1×10^8 colony forming units) of Master Seed. Calves in the subsequent passages were inoculated intranasally with a 2 mL dose of highest titered nasal swab material collected from the previous passage. Transient episodes of mild to severe muco-purulent discharge were observed in many of the inoculated and control calves in each passage, indicating a possible cause to be irritation of the nasal cavity from frequent nasal swab collection rather than the pathogenicity of the deletant strain. The mean clinical score for inoculated calves in the five passages was 1.2 ± 1.9, 2.0 ± 1.7, 0.3 ± 0.6, 1.0 ± 1.7, and 0.6 ± 0.9, respectively.

During each passage, nasal swabs were collected at least every other day for 14 days, and a viability count performed. The *P. multocida* ΔhyaE acapsular mutant strain was recovered from the nasal cavity of at least one calf in every passage. DNA fragments that were amplified from representative isolates were the same size as the DNA fragment amplified from material that was one passage from the *P. multocida* ΔhyaE Master Seed.

Calves were euthanized and necropsied at the end of each observation period. The lungs were examined for signs of congestion/consolidation and cultured for isolation of the *P. multocida* ΔhyaE strain. The mean lung lesion score for inoculated calves in the five passages was 0.3 ± 0.8, 0 ± 0, 0 ± 0, 0 ± 0, 0.9 ± 1.2, respectively. The *P. multocida* ΔhyaE deletant strain was not isolated from the lung of any of the calves.

The results of this study demonstrate that the *P. multocida* ΔhyaE deletant strain is phenotypically (remained acapsular) and genetically (retained the deletion in the hyaE gene) stable when passaged in the host animal. There was no significant increase in clinical signs of disease from Passage 1 to Passage 5 ($p = 0.7098$), and there was no significant increase in lung lesion scores from Passage 1 to Passage 5 ($p = 0.5055$). The collective data indicate that the *P. multocida* ΔhyaE acapsular mutant strain will not revert to a virulent strain when passaged in the host animal and is therefore safe for use in young calves.

addendum 13

Schering-Plough Animal Health Corp.

Project No. 98006
Report No. B03-155-01R1
Date: 07Dec04
Notebook Ref. 457

Development of a Modified Live Bacterial Vaccine Containing a Leukotoxin Deletant Strain of *Mannheimia haemolytica* D153 and an Acapsular Mutant Strain of *Pasteurella multocida* 1062: Immunogenicity Study for *M. haemolytica*

(APHIS Product Code No. 1861.R0)

Abstract

This immunogenicity study evaluated the efficacy of a modified live bacterial vaccine containing a leukotoxin deletant strain of *Mannheimia haemolytica* D153 (Lkt-) and an acapsular mutant strain of *Pasteurella multocida* 1062 (ΔhyaE) in 9-11 week old calves challenged transtracheally with virulent *M. haemolytica*. Each deletant strain was at maximum passage from the Master Seed (MSB + 5). The vaccine was produced and tested according to the draft Outline of Production. Calves were randomized into two groups of vaccinates (Group A; n=20 and Group C; n=40) and one group of PBS-vaccinated controls (Group D; n=40). The vaccine preparation administered to calves in Group A (B03-155-01A) contained 1.8×10^8 cfu/dose *M. haemolytica* Lkt-. The vaccine preparation administered to calves in Group C (B03-155-01B) contained 6.9×10^7 cfu/dose *M. haemolytica* Lkt-. Each vaccine was administered subcutaneously as a single 2 mL dose. Fever, diarrhea, and lethargy were observed post-vaccination in all three treatment groups, suggesting an environmental or other biological stress and not the vaccine, as the cause for the clinical anomalies. Injection site reactions developed following vaccination with both vaccines; however, the reactions were smaller and less frequent in the group vaccinated with the lower dose (Group C). After 21 days, calves were challenged transtracheally with a live culture of *M. haemolytica* and then monitored for clinical signs of pneumonia including lethargy / depression, persistent coughing, nasal discharge, and increased respiratory rate. The median clinical scores for Treatment Groups A (high dose), C (low dose), and D (controls) were 6.0, 4.0, and 10.0, respectively. The scores for both vaccinate groups were significantly lower than the control group; $P = 0.0457$ and 0.0002, respectively. After 7 days, calves were euthanized and necropsied. The lungs were examined, swabbed, the percentage of lung consolidation estimated for each lobe, and a lung lesion score calculated. The median lung lesion score for Groups A, C, and D was 6.7%, 1.9%, and 18.3%, respectively. The scores for both vaccinate groups were significantly lower than the control group; $P = 0.0273$ and 0.0001, respectively.

This study successfully met the test validity and acceptability criteria outlined in the study protocol to demonstrate that a *M. haemolytica* Lkt- dose of 6.9×10^7 cfu/dose was 89.6% effective at protecting calves from pneumonia and clinical disease due to virulent *M. haemolytica*.

addendum 14

Schering-Plough Animal Health Corp.

Project No. 98006
Report No. B03-155-01R2
Date: 07Dec04
Notebook Ref. 424, 457

Development of a Modified Live Bacterial Vaccine Containing a Leukotoxin Deletant Strain of *Mannheimia haemolytica* D153 and an Acapsular Mutant Strain of *Pasteurella multocida* 1062: Immunogenicity Study for *P. multocida*

(APHIS Product Code No. 1861.R0)

Abstract

This immunogenicity study evaluated the efficacy of a modified live bacterial vaccine containing a leukotoxin deletant strain of *Mannheimia haemolytica* D153 (Lkt-) and an acapsular mutant strain of *Pasteurella multocida* 1062 (ΔhyaE) in 9-11 week old calves challenged transtracheally with virulent *P. multocida*. Each deletant strain was at maximum passage from the Master Seed (MSB + 5). The vaccine was produced and tested according to the draft Outline of Production. Calves were randomized into one group of vaccinates (Group B; n = 20) and one group of PBS-vaccinated controls (Group E; n = 20). The vaccine preparation administered to calves in Group B (B03-155-01A1) contained 1.0 x 10^6 cfu/dose *P. multocida* ΔhyaE. The test vaccine was administered subcutaneously as a single 2 mL dose. Fever was the only clinical anomaly observed in calves vaccinated with *P. multocida* ΔhyaE; however, this was not due to the vaccine strain as calves vaccinated with PBS also developed fever. Injection site reactions developed in 35% of the vaccinated calves, with the largest reaction measuring 14x14x3 cm. Most of the other reactions were relatively small, and all the reactions resolved by study day 7. After 21 days, calves were challenged transtracheally with a live culture of *P. multocida* and then monitored for clinical signs of pneumonia including lethargy / depression, persistent coughing, nasal discharge, and increased respiratory rate. The median clinical score for Treatment Group B (13.0; vaccinates) was lower than Treatment Group E (14.0; controls), but not statistically different $P = 0.1401$. After 7 days, calves were euthanized and necropsied. The lungs were examined, swabbed, the percentage of lung consolidation estimated for each lobe, and a lung lesion score calculated. The median lung lesion score for Group B (5.1%; vaccinates) was significantly lower than Treatment Group E (23.3%; controls); $P = 0.0085$.

This study successfully met the test validity and acceptability criteria outlined in the study protocol to demonstrate that a *P. multocida* ΔhyaE dose of 1.0 x 10^6 cfu/dose was 78.1% effective at protecting calves from pneumonia and clinical disease due to virulent *P. multocida*.

addendum 15

Schering Plough Animal Health Corp.

Project No. 98006
Report No. B04-046-01R
Date 11Nov05
Notebook Ref. 457
SB B04-046-01

Study to Evaluate the Safety in Calves Following Administration of One Dose, Administration of an Overdose, and Repeat Administration of One Dose for a Modified Live Vaccine Containing a Leukotoxin Deletant Strain of *Mannheimia haemolytica* D153 and an Acapsular Mutant Strain of *Pasteurella multocida* 1062

(APHIS Product Code No. 1861.R0)

Abstract

Schering Plough Animal Health Corporation (SPAHC) is developing a modified live *Mannheimia haemolytica* / *Pasteurella multocida* vaccine to aid in the prevention of pneumonic pasteurellosis caused by *M. haemolytica* and *P. multocida*. The purpose of this study was to demonstrate the safety of the *M. haemolytica* Lkt- / *P. multocida* ΔhyaE vaccine when administered to 7-8 week old calves. The following parameters were evaluated in this study: (1) safety of the administration of one dose (Groups A1 & A2; n = 5), (2) safety of administration of a 10X overdose (Group B; n = 5), and (3) safety of the repeated administration of one dose (Group A2). On study day 0, calves in Groups A1 and A2 were vaccinated subcutaneously with a 2 mL dose containing 2.2×10^8 cfu *M. haemolytica* Lkt- and 2.0×10^7 cfu *P. multocida* ΔhyaE. Calves in Group B were vaccinated with a 4 mL dose containing 10X the amount of antigen as for Group A (1.3×10^9 cfu/dose *M. haemolytica* Lkt- and 1.6×10^8 cfu/dose *P. multocida* ΔhyaE). A final group of calves were vaccinated with sterile phosphate buffered saline and served as controls (Group C; n = 5). Following the first vaccination, calves were observed for 21 days for signs of systemic and local reactions. Rectal temperatures were recorded at 2 hours (hrs), 4 hrs, and 6 hrs post-vaccination and then daily for 7 days. A greater than 2°C increase over baseline for an individual calf was considered an elevated temperature. Calves in Groups A2 and C were vaccinated again on study day 21 with a 1X dose of vaccine (1.0×10^8 cfu/dose *M. haemolytica* Lkt- and 2.8×10^7 cfu/dose *P. multocida* ΔhyaE) and observed daily for 14 days for signs of systemic and local reactions, with rectal temperatures recorded as above.

Calves were clinically normal throughout the post-vaccination observation periods. There were transient increases in rectal temperatures following vaccination in all four treatment groups, but temperatures returned to within 2°C of baseline by 72 hrs post-vaccination. Swelling at the injection site was expected following vaccination with the modified live *M. haemolytica* Lkt- / *P. multocida* ΔhyaE vaccine, especially in the calves that were given a 10X dose, as the volume of vaccine administered to the calves was doubled to 4 mL. Although the reactions were still detectable after 8-12 days, there was an immediate and dramatic reduction in the size of the reactions within 3-5 days post-vaccination. None of the reactions caused further complications such as abscess formation or tissue damage.

The results of this study indicate that the *M. haemolytica* Lkt- / *P. multocida* ΔhyaE modified live vaccine is safe for use in 7-8 week old calves when administered subcutaneously as a 1X dose, a 10X dose, or a repeat dose. No abnormal systemic reactions developed following vaccination, and acceptable local reactions did not result in any residual tissue damage at the injection site.

addendum 16

Schering-Plough Animal Health Corp.

Project No. 98006
Report No. B07-010-00R
Date: 05 Feb 09
Notebook Ref. Study Binders
B07-010-00, B07-010-01 to -04

Field Safety in Cattle Following Vaccination with a Modified Live *Mannheimia haemolytica* and *Pasteurella multocida* Vaccine

(APHIS Product Code No. 1861.R0)

Summary

Schering-Plough Animal Health Corporation (SPAHC) has developed a modified-live vaccine containing a leukotoxin deletant strain of *Mannheimia haemolytica* D153 (Lkt-) and an acapsular mutant strain of *Pasteurella multocida* 1062 (ΔhyaE) to aid in the prevention of pneumonia due to *M. haemolytica* and *P. multocida*. The objective of this study was to evaluate the safety of the modified live vaccine under field conditions. Calves (n = 803) were enrolled in the study at sites in California, Wisconsin, and Missouri, with 270 of the calves (n = 216 PLS-vaccinates, 54 placebo-vaccinates) between 4 to 8 weeks of age. Calves given antimicrobial medication were not enrolled in the study, or were excluded if mistakenly enrolled. The calves at each site were randomly assigned to Group 1 (placebo-vaccinated controls; ~ 20% of the calves per site) or Group 2 (PLS-vaccinates; n ~ 80% of the calves per site). All calves were vaccinated with 2 mL of the appropriate vaccine by subcutaneous injection in the neck on study day 0. Group 1 calves (n = 161) were vaccinated with a saline-placebo and Group 2 calves (n = 642) were vaccinated with pre-licensing serials 250101 or 250102. All calves were observed daily for 21 days for local or systemic reactions attributable to the vaccine. Injection sites were palpated on all calves on study day 2. Two calves in Group 2 were removed from the study because of prior treatment with antimicrobial medication and 1 calf in Group 2 and 3 calves in Group 1 were removed from the study following antimicrobial treatment. Injection site reactions in Group 2 calves were rare (11 of 639 calves, 1.7%), minor, and with one exception at the Missouri site, resolved within 2 days. Three placebo-vaccinated calves, all at the Missouri site, also had injection site reactions that resolved within 6 days. Extremely wet and muddy conditions may have contributed to the injection site reactions at the Missouri site. However, none of the injection sites were believed to be clinically significant by the Study Investigators. Adverse events were reported for 3 calves in Group 1 and for 2 calves in Group 2. None of the adverse events were attributed to the test vaccines by the Study Investigators. All of the Study Investigators reported that they believed the test vaccines to be safe. The study was conducted according to the Principles of Good Clinical Practice (GCP) as outlined in Veterinary Services Memorandum No. 800.301. The modified live vaccine containing the leukotoxin deletant strain of *Mannheimia haemolytica* D153 (Lkt-) and the acapsular mutant strain of *Pasteurella multocida* 1062 (ΔhyaE) was found to be safe when used under field conditions in cattle 2 months of age or older.

addendum 17

Schering-Plough Animal Health Corp.

Project No. 01005
Report No. 01005-002R
Date: 03Dec03

Category II Summary Information Format for the *Pasteurella multocida* 1062 ΔhyaE Master Seed Lot No. 90113-000

Abstract

Attached is the Summary Information Format (SIF) for the *Pasteurella multocida* ΔhyaE Master Seed Lot No. 90113-000. The SIF outlines important scientific questions and information that should be addressed during the preparation of a U.S. Veterinary Biological Product License Application to the United States Department of Agriculture (USDA) for new biotechnology-derived biologics. The SIF is designed to allow both the participating firm as well as the Center for Veterinary Biologics (CVB) to assess the risk associated with the manufacture and release of biological organisms, specifically the risk associated with genetically modified organisms released into the environment. The purpose of the SIF is to provide specific data about the design, construction, and testing of the biological agent construct and to provide a basis for the Risk Analysis.

Schering-Plough Animal Health Corp.

Project No. 01005
Report No. 01005-002R
Date: 03Dec03

Category II Summary Information Format for the *Pasteurella multocida* 1062 ΔhyaE Master Seed Lot No. 90113-000

Abstract

Attached is the Summary Information Format (SIF) for the *Pasteurella multocida* ΔhyaE Master Seed Lot No. 90113-000. The SIF outlines important scientific questions and information that should be addressed during the preparation of a U.S. Veterinary Biological Product License Application to the United States Department of Agriculture (USDA) for new biotechnology-derived biologics. The SIF is designed to allow both the participating firm as well as the Center for Veterinary Biologics (CVB) to assess the risk associated with the manufacture and release of biological organisms, specifically the risk associated with genetically modified organisms released into the environment. The purpose of the SIF is to provide specific data about the design, construction, and testing of the biological agent construct and to provide a basis for the Risk Analysis.

Category II Summary Information Format

Pasteurella multocida 1062 ΔhyaE
Master Seed Lot No. 90113-000

I. Introduction

A. Objective

1. Identify where the Regulated Biological Agent was constructed and where the product will be made, tested and manufactured. Address the available level of containment.

The Regulated Biological Agent (Pasteurella multocida ΔhyaE Master Seed) was constructed from the agent listed below (II.A) at the National Animal Disease Center (NADC), Ames, IA. The product has been developed at the Schering-Plough Animal Health (SPAH) Research and Development Facility, Elkhorn, NE. Biosafety level 2 containment facilities are available for animal testing at SPAH - Elkhorn, NE, SPAH - Williamsburg, KS, and SPAH - Terre Haute, IN. Suitable contract research organizations with BL2 containment facilities may also be used for animal studies. For US distribution, the final vaccine will be manufactured at SPAH, Elkhorn, NE, U.S. Veterinary License No. 165A.

2. Provide a brief (one sentence) description of the Regulated Biological Agent.

The Regulated Biological Agent (RBA) is an acapsular mutant strain of Pasteurella multocida, biotype A, serotype 3 containing a deletion in the hyaE gene, the first gene in the capsule biosynthetic operon that encodes for the P. multocida capsule.

B. Proposal

1. What is the intended use of the product?

a. Species:

Cattle

b. Proposed claim:

The P. multocida ΔhyaE vaccine is indicated for use in healthy cattle as an aid in the prevention and/or reduction of pneumonic lesions associated with bovine pneumonic pasteurellosis, commonly known as shipping fever.

c. Geographic area:

United States and Canada

d. Route of administration:

Subcutaneous

e. Brief description of the expected safety profile:

The P. multocida hyaE deletant strain is safe for use in calves as young as 3 months of age when administered subcutaneously at a dose of 5.0 x 10^8 cfu (see section III.C). P. multocida serotype A is a common secondary invader in respiratory disease in ruminants. The lungs of animals subjected to environmental stress as well as concurrent or prior infection by viruses or M. haemolytica can be colonized by P. multocida. Once established in the lung, P. multocida can cause chronic or severe and often fatal pneumonia. P. multocida serotype A has been associated with atrophic rhinitis, fowl cholera and respiratory disease in ruminants. However, most P. multocida isolates appear to be specialized for virulence in the host of isolation and do not appear to be able to cause disease in a wide range of species (1).

The P. multocida vaccine strain has a deletion in the hyaE gene that attenuates the wild-type virulence, has no plasmids or mobile chromosomal elements, and would therefore be expected to be much less of an environmental threat than naturally occurring strains of P. multocida.

II. Description of the Regulated Biological Agent Construction

A. The Backbone Biological Agent

1. What organism was used for the Backbone Biological Agent? Are there any known virulence features associated with the Backbone Biological Agent? What happens in the target species?

The parental organism for this vaccine is Pasteurella multocida biotype A, serotype 3. It has been identified as strain NADC-1062. It is a field isolate that was originally cultured from pneumonic bovine lung tissue at the National Animal Disease Center in Ames, IA. The 1062 strain is highly virulent and is capable of replication in the bovine lung. In North America, P. haemolytica biotype A, serotype 1 and P. multocida capsular type A and somatic type 3 are the most frequently recovered serotypes from the lungs of cattle suffering from bovine pneumonic pastuerellosis (2, 3). The capsule of P. multocida helps the bacterium to evade the host's immune defenses by impairing phagocytosis and reducing the action of complement-mediated killing. Response to capsular antigens can be protective; however, because of their chemical structure, they are T-cell independent antigens and do not readily elicit a strong protective immune response unless the antigen is modified. In addition, the presence of a capsule may mask other putative protective antigens such as outer membrane proteins and LPS. Thus, non-capsulated strains should be good candidates for live vaccine strains in that they are devoid of a primary virulence factor, yet still express an array of other putative protective antigens to which an immune response can develop. Spontaneous acapsular variants and genetically defined acapsular mutants of P. multocida have been shown to reach lower titers in the blood of infected animals and have reduced virulence in a number of species (4, 5).

Although P. multocida can be commonly found in the upper respiratory tract (URT) of healthy cattle, exposure of cattle to environmental, managerial, or viral/bacterial stressors leads to an extensive colonization of P. multocida in the lungs. Once established in the lung, P. multocida can cause chronic or severe and often fatal pneumonia (6).

a. What is the previous safe use of the Backbone Biological Agent?

Pasteurella multocida 1062 *wild type strain was isolated from a calf that died of pneumonia. No genetic modifications were made to this strain prior to use as described by this SIF.*

(i) If available provide history of previous safe use using literature or internal documents. Include the recommended CDC/NIH biosafety level for use of the Backbone Biological Agent.

The fourth edition of the CDC/NIH manual, "Biosafety in the Microbiological and Biomedical Laboratories", does not rate the biosafety level for wild-type P. multocida A3. Wild-type P. multocida is not infectious to laboratory personnel. However, working with the methods described in this manual on assigning biosafety levels, wild-type P. multocida can appropriately be handled as a biosafety level 1 agent.

2. Physical characteristics of the Backbone Agent

a. Provide a flow diagram or explanation of the process of how the Backbone Biological Agent was constructed

The Backbone Biological Agent is a field isolate of P. multocida. No modifications were made prior to the construction of the Regulated Biological Agent (Appendix 1, Figure 1).

(i) Describe the proposed site for Reporter Gene insertion.

Not applicable; no Reporter Gene was inserted. However, during the deletion process, 3 - 8 bp SmaI linkers (Serratia marcescens) were inserted into the deletion site of the hyaE gene.

(ii) Do the flanking regions of the proposed insertion site Backbone Biological Agent have any known regulatory elements that could moderate the expression of the inserted Reporter Gene?

Not applicable; no Reporter Gene was inserted.

(iii) Identify unique restriction endonucleases (not more than five) that will give identifiable digestion patterns useful for characterizing the final Backbone DNA.

Not applicable.

(iv) Identify reporter genes if applicable to be inserted into Backbone DNA. Show pertinent sequences or restriction endonuclease sites in the flow diagram above.

Not applicable; no Reporter Gene was inserted. However, during the deletion process, 3 - 8 bp SmaI linkers were inserted into the deletion site. The 8 additional amino acids do not encode a functional protein.

(v) Has there been previous safe use of the reporter gene. Add a citation, if appropriate.

Not applicable; no Reporter Gene was inserted.

B. Construction and Characterization of the Regulated Biological Agent

1. Provide a flow diagram on the construction of the Regulated Biological Agent.

Include the following:

a. Final Backbone Biological Agent:

P. multocida 1062

The hya operon is described in Appendix 1, Figure 1. The P. multocida serogroup A capsule consists of hyaluronic acid, a polymer of alternating sugars, D-glucuronic acid and N-acetyl-D-glucosamine. The P. multocida A:1 capsule biosynthetic locus consists of three distinct regions. Region 1 encodes for four products that serve as transporters of polysaccharide to the bacterial surface. Region 2 encodes for five genes, hyaE, hyaD, hyaC, hyaB and hyaA, whose products synthesize the two polysaccharide monomers of the capsule. Region 3 is comprised of two open reading frames (ORFs) whose products are involved in phospholipid substitution.

b. Heterologous reporter genes (if applicable):

Not applicable

c. All shuttle vectors:

The structural portion of the hyaE gene (including the start and stop codons; 1,869 bp) was obtained by PCR and ligated into the cloning vector pCR2.1. The specific 366 bp deletion within the hyaE gene was achieved by PCR, followed by EcoR V restriction digestion to remove specific sequences from the ends. The resulting fragment was then ligated on itself to generate an in-frame deletion excising nucleotides 718 through 1084. An 8 bp Sma I linker (5'-CCCCGGGG-3') was to be inserted into the EcoR V deletion site to insure that the mutation would result in an acapsular phenotype. However, during the process, 3 Sma I linkers were inserted (Appendix 1, Figure 2). The mutated hyaE fragment was subcloned into plasmid pBCSK followed by insertion of the Tn903 kanamycin resistance element (pBCSKΔhyaEkan^r). Construction of the replacement plasmid

was completed by ligating the ΔhyaEkan^r fragment with the 1.2 kb temperature-sensitive origin of replication of pBB192 (Appendix 1, Figure 2). Because the ColE1 origin of replication is inactive in P. multocida, only the ligation products generating plasmids p192oriΔhyaEkan^r were capable of replicating within the host. The replacement plasmid was introduced into P. multocida 1062 by electroporation. Cells possessing integrated plasmid survived antibiotic selection at the non-permissive temperature for plasmid replication, and could be identified because integration of replacement plasmid resulted in an acapsular phenotype.

d. Host cell lines used:

1062 cells were electroporated with temperature-conditional plasmid vectors containing deleted clones of the hyaE gene.

e. Selection techniques and methods used to evaluate the final Regulated Biological Agent.

The replacement plasmid ĆhyaE was in vitro methylated with Hhal methyltransferase and electroporated into the 1062 parent strain. Transformants were grown with antibiotic selection at a permissive temperature for replication (30°C) then passed to a non-permissive temperature (40°C) on selective solid medium. Colonies were then selected and grown overnight at the permissive temperature but without selection and then streaked for isolation on non-selective media (Appendix 1, Figure 3). At this point, the plasmid is completely lost. Colonies were screened for loss of resistance to kanamycin. Kanamycin sensitive colonies were screened by PCR for the loss of the deleted segment of the hyaE gene, and strains containing the deleted hyaE alleles were recovered. The only heterologous DNA sequences remaining in the deletant strain are the three copies of the synthetic SmaI linker located at the deletion site in the hyaE gene.

2. Describe the laboratory methods or criteria used to evaluate the Regulated Biological Agent.

Colonies were screened by PCR for the loss of the 366 bp segment of the hyaE gene, and strains containing the deleted hyaE alleles were recovered. Wild-type capsular colonies possess hyaluronic acid, the major capsule component, which render colonies mucoid in appearance and when viewed in obliquely transmitted light these colonies exhibit pearl-like iridescence. In contrast, the acapsular deletant is non-mucoid and non-iridescent.

3. Physical characterization of the Regulated Biological Agent

a. Characterize the physical map using unique Reporter Gene and Backbone Biological Agent restriction endonuclease sites and describe resulting restriction fragments and digestion patterns.

Physical mapping of the Backbone Biological Agent vs. the RBA has not been done at this time. However, it is expected that the P. multocida hyaE acapsular mutant strain will be able to be distinguished from the parent strain based on the unique SmaI restriction endonuclease sites that were inserted into the deletion site of the hyaE gene.

b. Devise a PCR or restriction endonuclease test based on the Backbone Biological Agent sequence and the Reporter Gene Sequence that will identify and characterize the Reporter Gene/Backbone Biological Agent construct.

A forward PCR primer, ATGAAAAAGGTTAATATCATTGG (includes start codon of the hyaE gene), and an reverse primer, TTAACCTTGCTTGAATCGTTTACC (includes stop codon of the hyaE gene), will amplify a 1869 base pair product from the wild type capsular gene and a 1503 base pair product from the deleted gene.

c. What will be the criteria for stability and purity of the Regulated Biological Agent Master Seed n and n+5?

Gram stain was used to confirm that the P. multocida ΔhyaE Master Seed was a non-spore forming Gram negative rod. When plated on blood agar, the P. multocida hyaE Master Seed appears non-mucoid and non-iridescent. The Master Seed has been tested by the Quality Control Department at Schering-Plough Animal Health for purity as required by 9 CFR 113.64(a). The P. multocida 1062 parental and ΔhyaE strain were characterized by the BBL Crystal Identification System (Becton Dickinson, Sparks, MD). This system is a miniaturized identification method employing modified conventional and chromogenic substrates for the purpose of identifying bacteria. The BBL Crystal ID panels contain 30 dried biochemical and enzymatic substrates. A bacterial suspension in the inoculum fluid is used for rehydration of the substrates. In general, many of the tests used in the BBL Crystal ID systems are modifications of classical methods. These include tests for fermentation, oxidation, degradation, and hydrolysis of various substrates. In addition, there are chromogen-linked substrates to detect enzymes that microbes use to metabolize various substrates. Briefly, the test consists of preparing a test inoculum that is used to fill 30 wells in a test panel. Following an incubation period, the wells are examined for color changes that result from metabolic activities of the organism. The resulting pattern of the 30 reactions is converted into a ten-digit profile number that is used as the basis for identification. Both strains were identical for all reactions and were identified as P. multocida at the 0.9996 confidence level (Appendix 2). There were no atypical results for either strain. 000008

Molecular characterizations will be performed at Master Seed (n) and highest passage level cells (n+5). These characterizations will include the PCR assay described above followed by DNA sequence analysis of the amplified fragment. Sequence analysis was performed on the Master Seed and confirmed the 366 bp deletion in the hyaE gene and the insertion of 3 - SmaI linkers in the deletion site (Appendix 3).

Backpassage studies will be conducted to evaluate the genetic stability of the P. multocida ΔhyaE Master Seed to ensure that the deletant strain will not revert to virulence when administered to the host animal.

d. Provide the genetic sequence in electronic format for any new or altered genetic sequences, including insertion and flanking sequence.

The electronic file is attached (Appendix 3).

4. What is the recommended NIH/CDC biosafety level for the Regulated Biological Agent?

The recommended biosafety level will be the same as the parent strain (BL1).

5. Provide a short summary or description of genetic motifs that may have resulted as a consequence of the genetic recombination (II.C.3.d). Are there any known motifs that may promote homologous recombination, DNA insertion, or gene expression of existing or new open reading frames?

Since the hyaE gene in the recipient was altered by the removal of part of the genetic material, reversion to the parental hyaE gene is extremely unlikely. There is no known mechanism in the Pasteurella family of bacteria to naturally replace the deleted portion of the gene. The hyaE gene is encoded on the chromosome and not on a plasmid, and there are no phage or conjugative plasmids known for this family of bacteria. No protein encoding DNA has been added or is present anywhere in the recipient organism. The insertion of the 3-SmaI linkers at the deletion site provides an additional hurdle that makes reconstitution of the parent gene extremely unlikely.

III. Biological Properties or Virulence for the Regulated Biological Agent used for Master Seed

A. What are the known phenotypic characteristics or effects of the Regulated Biological Agent identified as Master Seed?

The RBA lacks functional capsular protein, as demonstrated by its non-mucoid and non-iridescent appearance.

B. What are the virulence characteristics of the Regulated Biological Agent used for the Master Seed?

The RBA is attenuated by virtue of a deletion of 366 bp within the capsular hyaE gene which blocks synthesis of the capsular building block N-acetyl-D-glucosamine. Therefore, without a capsule to help P. multocida hide from the immune system, this deletant strain is much more vulnerable to an immune response than its parent equivalent.

C. Is the Regulated Biological Agent used for the Master Seed virulent for target animals, non-target animals?

1. *Target animal:*

A calf safety study was conducted in accordance with 9CFR 113.41 (Appendix 4; B03-013-01R). Two calves were inoculated subcutaneously at ~3 months of age with 5.0 x 10^9 cfu of a P. multocida ΔhyaE culture that was one passage from the Master Seed. Each vaccinated calf was housed together with a sentinel control calf to maximize nose-to-nose contact. The calves were monitored daily for adverse reactions and then euthanized and necropsied 21 days post-vaccination. Nasal swabs ere collected on study days -3, 1, 3, 7, 14, and 21. Whole blood for culture was collected on study days 0-4. After 21 days, calves were necropsied, the lungs scored for consolidation, and tissue samples collected for isolation of the vaccine strain. At a titer of 5.0 x 10^9 cfu/dose, the P. multocida ΔhyaE deletant strain caused some undesirable adverse reactions such as fever, lethargy, rapid breathing, and large injection site reactions (30x20x3 cm and 8x9x2 on study day 3). The calves quickly recovered from the clinical manifestations of the vaccine without any treatment, but the injection site reactions were still evident 21 days post-vaccination (9x4x2 cm and 4.5x3.5x1 on study day 21). Since the two sentinel control calves did not show any signs of P. multocida ΔhyaE infection (no clinical signs and no isolation from nasal swabs), it was decided to vaccinate these calves on study day 14 with a 10-fold lower dose (5.0 x 10^8 cfu) and evaluate the safety. The lower dose caused a slight increase in rectal temperature in only 1 calf and injection site reactions were much smaller than those induced by the higher dose (9x5x1.5 cm and 5x3x1 cm on study day 15 and 5x2.5x1 cm and 3.5x2x1 on study day 21). After 7 (the 5.0 x 10^8 dose) and 21 days (the 5.0 x 10^9 dose), a pure culture of P. multocida ΔhyaE was isolated from the injection site and pre-scapular lymph nodes. The deletant strain could not be isolated from the blood and did not appear to migrate to and colonize in the nasal passages or lung.

The virulence of the P. multocida ΔhyaE Master Seed will be further investigated later during scheduled backpassage and dissemination in host animal studies.

2. *Non-Target animals:*

P. multocida has a wide range of hosts that it can infect. However, P. multocida A3 is primarily seen in cattle and sheep. The deletant Master Seed is not expected to be any different.

The LD_{50} for the P. multocida ΔhyaE Master Seed was examined in mice. For comparison, the LD_{50} for the parent strain was also examined. The LD_{50} for the P. multocida 1062 parent strain given intraperitoneally was <60 cfu as calculated by the Reed and Muench formula, and the LD_{50} of the deletant strain was 2.4 x 10^4 cfu. It was concluded that the deletion in the hyaE gene has attenuated the P. multocida 1062 strain and made it 400 times less virulent in mice (Appendix 5; B03-001-01R).

Virulence of the P. multocida ΔhyaE Master Seed for other non-host animals has not been investigated yet and will be submitted in the final Risk Assessment to the USDA.

D. Do the Reporter Gene sequences enhance the virulence of the ability to survive in the target animals, non-target animals?

No reporter gene sequences are present in the recipient strain. The SmaI linkers present in the deletion site of the hyaE gene do not encode a functional protein.

1. What is the tissue tropism of the Regulated Biological Agent in target and non-target animals?

The nasopharynx of healthy cattle is commensally colonized with P. multocida. During disease conditions, P. multocida can cause chronic or severe and often fatal pneumonia. P. multocida 1062 wild type strain was isolated from the lung of a calf that died of pneumonia. P. multocida is opportunistic and occasionally has been associated with mastitis in cattle and sheep. In many domestic and wild animals, P. multocida can be found as a commensal in the respiratory and digestive tract. There is no reason to suspect that the tissue tropism of the P. multocida ΔhyaE deletant strain would be altered because of the deletion in the hyaE gene.

Data from a previous calf safety study indicate that the P. multocida ΔhyaE strain can be isolated from the injection site and pre-scapular lymph nodes following subcutaneous vaccination (Appendix 4, B03-013-01R). At a dose of 5.0×10^8 or 5.0×10^9 cfu, P. multocida ΔhyaE could not be isolated from the liver, spleen, lung, mediastinal lymph nodes, or submandibular lymph nodes at 7 days or 21 days, respectively, post-vaccination. Additional studies to evaluate tissue tropism in cattle will be conducted and the data submitted at a later time.

Tissue tropism of the P. multocida ΔhyaE Master Seed for non-host animals has not been investigated yet and will be submitted in the final risk assessment to the USDA.

2. Provide direct scientific evidence, including any relevant scientific publications, if available.

References 2, 3, and 6.

E. Discuss the potential for horizontal gene transfer or recombination of the Regulated Biological Agent.

Horizontal gene transfer has never been reported for the P. multocida 1062 strain. Neither the parental nor the final constructed strain contains autonomously replicating plasmids.

1. Is there any reason to believe that the potential for horizontal gene transfer or recombination is different in target and non-target animals?

There is no reason to believe the potential for horizontal gene transfer or recombination is different in non-target animals.

a. Include contributions from the Backbone Biological Agent and Reporter Gene Sequence in the discussion of the horizontal gene transfer and recombination potential.

Not applicable; there is no potential for horizontal gene transfer or recombination.

b. Reference any relevant scientific publications.

Not applicable.

F. Describe the shed/spread capabilities of the Regulated Biological Agent

Because the reversion to virulence study for the P. multocida ΔhyaE deletant strain has not been completed at this time, there is limited information of what are the actual shed/spread capabilities of the Master Seed. However, deletion of the capsule would most likely reduce shed/spread capabilities due to a decrease in survival in the host and environment. A preliminary safety study indicates that the P. multocida ΔhyaE deletant strain is not shed from animals vaccinated subcutaneously and spread to other calves (Appendix 4, B03-013-01). The deletant strain was administered subcutaneously at a dose of 5.0 x 10^9 cfu to 2, 3-month old calves. Each calf was commingled with an unvaccinated calf for two weeks following vaccination. All calves were observed daily. Nasal swabs and blood cultures were taken throughout the 14-day observation period. None of the nasal swabs or blood cultures were positive for P. multocida ΔhyaE. A dose of 5.0 x 10^9 cfu caused some undesirable adverse reactions such as fever, lethargy, rapid breathing, and large injection site reactions, while the sentinel control calves remained healthy.

1. Include shed/spread potential in non-target animal species.

The host/range specificity of the P. multocida ΔhyaE Master Seed has not been determined at this time. Because the P. multocida ΔhyaE Master Seed is a gene deletion only, it is not expected that the host/range specificity will be any different from the parent strain. More information will be available after the completion of the non-target animal safety studies.

2. Include in the discussion any contributions to shed/spread capabilities that might be associated with the Backbone Biological Agent and Reporter Gene sequence.

Although P. multocida can be commonly found in the upper respiratory tract of healthy cattle, the lungs of animals subjected to environmental stress as well as concurrent or prior infection by viruses of M. haemolytica can be colonized by this bacteria. An increase in colonization of the nasal passages may also be a source for shedding virulent P. multocida organisms to other susceptible animals via nose-to-nose contact.

3. Reference relevant scientific publications

Reference 6.

G. Discuss the expected environmental impact or survivability of the Regulated Biological Agent, and provide available scientific evidence.

P. multocida is widely distributed throughout the world. Survivability of any P. multocida in the environment is dependent upon infection of susceptible animals. The P. multocida ΔhyaE Master Seed and/or subsequent vaccine organisms would not be expected to survive in the environment. The P. multocida ΔhyaE strain is attenuated, has no plasmids or mobile chromosomal element and would therefore be expected to be much less of an environmental threat then naturally occurring strains of P. multocida.

The P. multocida ΔhyaE Master Seed X+1 has been tested and is sensitive to the following antibiotics: ampicillin, ceftiofur (Naxcel), enrofloxacin (Baytril), florfenicol (Nuflor), oxytetracycline (LA200), penicillin, spectinomycin, sulfisoxylin, tetracycline, tilmicosin (Micotil), and trimethoprim sulfadiazine (Tribrissen). Several of these antiboiotics are commonly used in treatment of farm animals, further decreasing the odds of survival in the environment.

1. Do the Reporter Gene sequences or gene sequences associated with the Backbone Biological Agent enhance the ability to survive in the environment or increase resistance to therapeutic agents?

   The SmaI linkers at the deletion site do not encode any functional protein and should have no effect on survivability. Deletion of the capsule may reduce survivability in the environment although this needs to be experimentally determined.

REFERENCES

1. Carter, G. R. 1991. Genus I. *Pasteurella*. pp. 552-558. In: Bergey's Manual of Systematic Bacteriology, Volume 1. N. R. Krieg & J. G. Hot, (Eds), Williams & Wilkins, Baltimore.

2. Carter, G. R. 1967. Pasteurellosis: *Pasteurella multocida* and *Pasteurella haemolytica*. Adv. Vet. Sci. 11:321-379.

3. Collier, J. R. 1968. Significance of Bacteria in Bovine Respiratory Disease. JAVMA. 153:1645-1651.

4. Snipes, K.P., Ghazikhanian, G.Y., Hirsh, D.C. 1986. Fate of *Pasteurella multocida* in the blood vascular system of turkeys following intravenous inoculation: comparison of an encapsulated, virulent strain with its avirulent, acapsular variant. Avian Dis. 31:254-259.

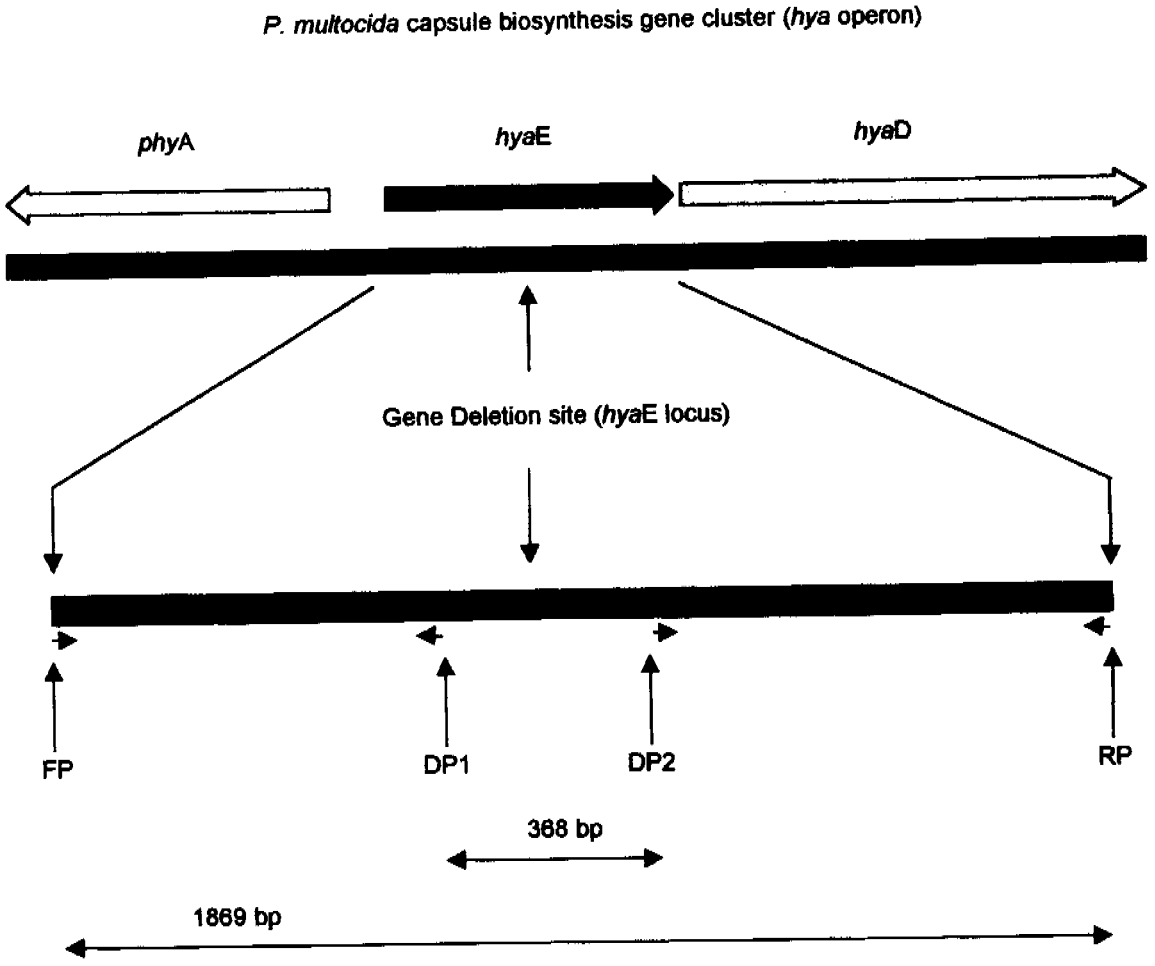
5. Snipes, K.P., Hirsh, D.C. 1986. Association of complement sensitivity with virulence of *Pasteurella multocida* isolated from turkeys. Avian Dis. 30:500-504.

6. Yates, W. D. G. 1982. A review of infectious bovine rhinotracheitis, shipping fever pneumonia and viral-bacterial synergism in respiratory disease of cattle.

APPENDIX 1

FIGURES

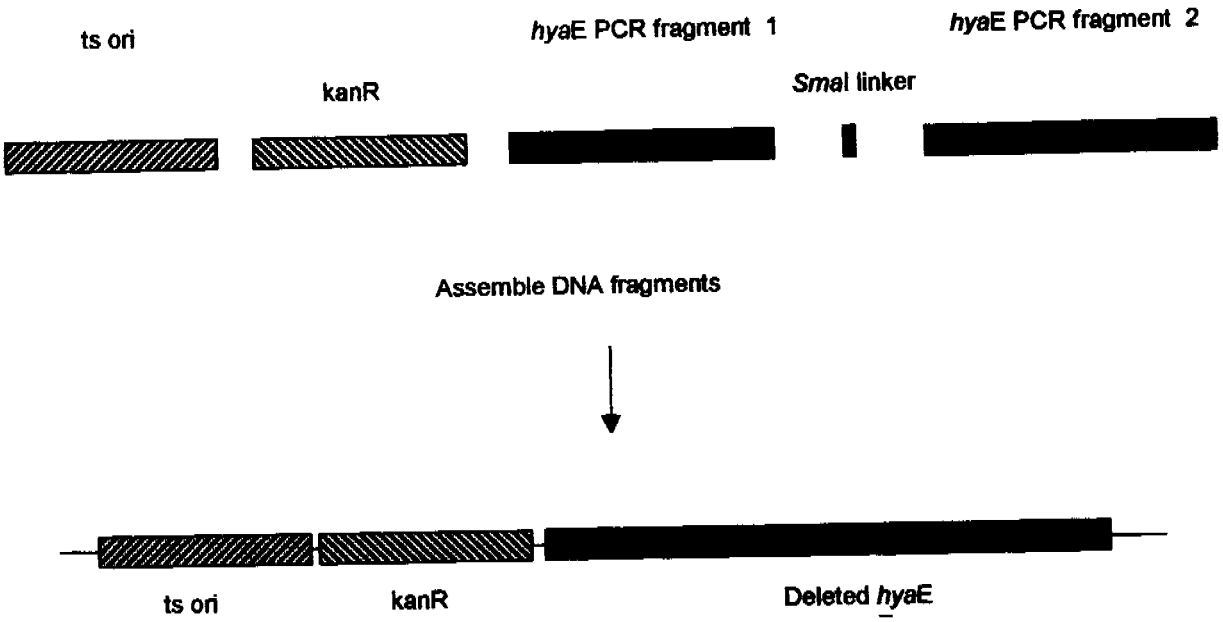
Figure 1. *P. multocida* 1062 Backbone Biological Agent



FP = Forward primer - ATGAAAAAGGTTAATATCATTTGG*
DP1 = Deletion primer 1 - AAAGATATCTTGGTTTACTTCAATAATTTC**
DP2 = Deletion primer 2 - AAAGATATCACTGCATCTGTTCAATCAACGAGC**
RP = Reverse primer - TTAACCTTGCTTGAATCGTTTACC***

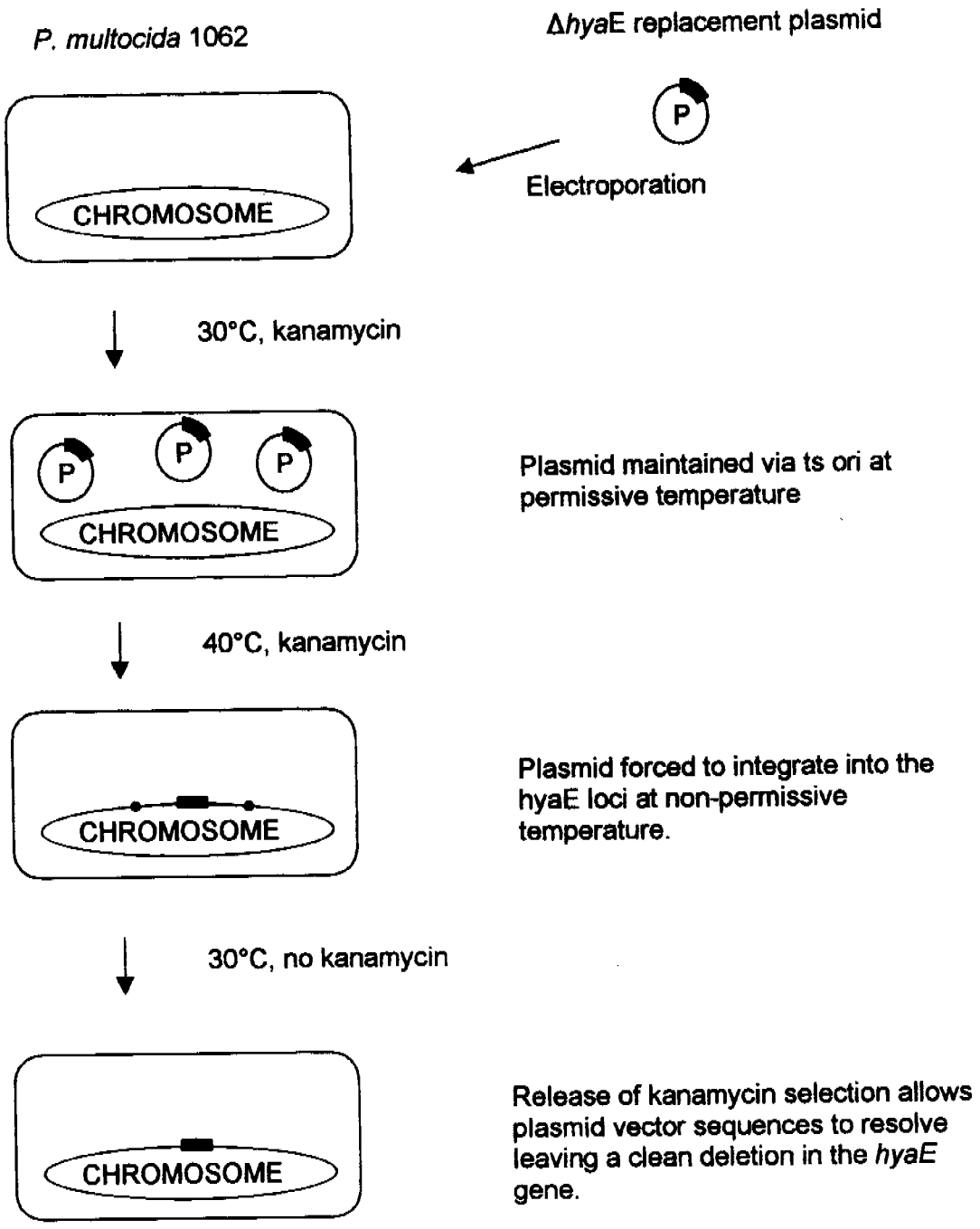
* Contains *hyaE* initiator Met codon ATG
** Contains *Eco*RV site removed during construction
*** Contains *hyaE* terminator codon TAA

Figure 2. Construction of Δ*hya*E replacement plasmid



ts ori	temperature sensitive *Pasteurella* replication origin from pBB192
kanR	kanamycin resistance gene from Tn903
PCR fragment 1	*hya*E PCR fragment primers FP and DP1
PCR fragment 2	*hya*E PCR fragment primers RP and DP2
*Sma*I linker	Synthetic linker CCCCGGGG (3X)

Figure 3. Construction of Regulated Biological Agent



APPENDIX 2

BIOCHEMICAL PROFILE

From Page No.

13Dec02

A. Introduction: Identification and comparison of the P. multocida 1062 Parent and P. multocida Ahp E Master Seed on the BBL Crystal I.D. Kit, Enteric-Nonfermenter panels.

B. Materials & Method:

1. P. multocida 1062 hap E MSB
   lot# 90113-000
2. P. multocida 1062 Parent
   lot# 2162-17-110698

Also Refer to I.D worksheet in 424A binder pg. 258

Results are in 424A binder pgs. 258-259.

Both organisms were identified the same as P. multocida

13Dec02

EXACT COPY
Signed
Date 13Dec02

To Page No.

Witnessed & Understood by me,	Date	Invented by	Date
Rhonda L. LaFlea	19Dec02	Recorded by	13Dec02

Schering-Plough Animal Health
BBL Crystal Identification Systems
RDP No. 7608.0-01
Effective Date: 26 JUL 02

REF. Notebook: 424
Page: 035
Initials:
Date: 13Dec02

BBL Crystal ID Worksheet

Study Number: N/A N/A = Not Applicable

Step III C. TSA II 5% SB Lot # 2240853/1307 Exp. Date: 17Dec02

Incubator Probe # 11

Date/Time In: 11Dec02/1100 Date/Time Out: 12Dec02/1100

Step IV C. BBL Crystal ID Kit Type: Enteric/NF

Lot # 2015171 Exp. Date: 01Feb03

McFarland Standard # : 0.5 Manufacturer: Remel

Lot # 114375 Exp. Date: 06/21/03

Step IV D. Incubator Probe # 11

Date/Time In: 12Dec02/1400 Date/Time Out: 13Dec02/0730

Time Read: 0730

EXACT COPY
Signed
Date 02Dec03

Performed By: Jennifer Johnson 12Dec02
Date

Reviewed By: 19 Dec 02
Date

REF. Notebook: 4242 EE 130ec02
Page: 035
Initials:
Date: 13Dec02

Schering-Plough Animal Health
BBL Crystal Identification Systems
RDP No. 7608.0-01
Effective Date: 26 JUL 02

BBL CRYSTAL Enteric/Nonfermenter ID Form pg. 2 of 2

Study Number: N/A Study Day: N/A N/A = Not Applicable

Sample I.D. P.multocida 1062 Parent Source: Working Seed Date Collected: N/A

N/A Oxidase N/A Indole Positive Result = + Negative Result = o

	A	B	C	D	E	F	G	H	I	J
4	(0) ARA	(+) MNS	(+) SUC	(0) MEL	(0) RHA	(+) SOR	(+) MNT	(0) ADO	(0) GAL	(0) INO
2	(+) PHO	(0) BGL	(0) NPG	(0) PRO	(+) BPH	(0) BXY	(0) AAR	(0) PHC	(0) GLR	(0) NAG
1	(0) GGL	(0) ESC	(0) PHE	(0) URE	(0) GLY	(0) CIT	(0) MLO	(0) TTC	(0) ARG	(0) LYS
ID #	2	4	4	0	2	4	4	0	0	0

Organism I.D.: P.multocida

Sample I.D. P.multocida AlgaE Source: Master Seed Date Collected: N/A

N/A Oxidase N/A Indole Positive Result = + Negative Result = o

	A	B	C	D	E	F	G	H	I	J
4	(0) ARA	(+) MNS	(+) SUC	(0) MEL	(0) RHA	(+) SOR	(+) MNT	(0) ADO	(0) GAL	(0) INO
2	(+) PHO	(0) BGL	(0) NPG	(0) PRO	(+) BPH	(0) BXY	(0) AAR	(0) PHC	(0) GLR	(0) NAG
1	(0) GGL	(0) ESC	(0) PHE	(0) URE	(0) GLY	(0) CIT	(0) MLO	(0) TTC	(0) ARG	(0) LYS
ID #	2	4	4	0	2	4	4	0	0	0

Organism I.D.: P.multocida

Signed
Date 02Dec03

APPENDIX 3

SEQUENCE ANALYSIS

Partial Sequence of Master Seed Lot No. 90113-000
Containing the 366 bp Deletion in the *hyaE* Gene

691 Deletion / *Eco*R V / 3-*Sma*I Linker Junction

5' - ATGGAAATTATTGAAGTAAACCAAGATCCCCGGGGCCCC

*Eco*R V

GGGGCCCCGGGGATCACTGCATCTGTTCAATCAACGAGCAAA

ATACTTTCTGAGAAAGAAAAAGAGATTTCTTGCATAAAAAGT

GAAAATACAAAGATTAAAGAAGAAAAAATTAAAATTGATGAG

GCATACCACTTAACCAAGAAAACCTTGTCGGATAAAGAAAAAGC

CCTCAAAACGCATCAAGATGAAATTGAAGCGCTCAAGATAATT

TTTAATGAAAATATTTCCGTACAAGAAGATATGCNGGNAAAAA

TTTCNGGAAACCAATNTAAGATATCAAGNNCTTGAACAAGAGC

TAAAAGCCATATCGGATAAGAAAGCATTATTA - 3'

Nucleotides in red indicate the 3 - *Sma*I linkers.

Partial Sequence the *hyaE* gene of *P. multocida* 1062

691
5' - ATGGAAATTATTGAAGTAAACCAAGAT *TTATCTCACCAA

GAAGAATGTCCACTGTCTAACTTTATTGTTAGCCAAATTATAAAA

AATTCTCCTACTGTTACGCAGGTATATGAAGAATTACAGTCGCAT

GCTGATCTGCCTTATATTTCAGAACAAAAATTAGTAAATGATGCC

GATTTTGCTCTCCTTGCATGGAAAGATATGATTCAAAAACAAGTC

GATGCAAATCAATATCAACATGAAAAAGAATTAGAACTTAGCACA

ATAAAAGAACGTCAATTAGAGGTCACAGAGAAATATCAATTGACG

GAACAAAAACTGTCAGAAACACAAAAAGAAATCGAACAAATTAAA

GATGAAAATAGAAAAGTAAAATCTGAAAAAGCAAAACTC *ACT

GCATCTGTTCAATCAACGAGCAAAATACTTTCTGAGAAAGAAAAA

GAGATTTCTTGCATAAAAAGTGAAAATACAAAGATTAAAGAAGAA

AAAATTAAAATTGATGAAGCATACCACTTAACCAAGAAAACCTTG

TCGGATAAAGAAAAAGCCCTCAAAACGCATCAAGATGAAATTGAA

GCGCTCAAGATAATTTTTAATGAAAATATTTCCGTACAAGAAGAT

ATGCAAGAAAAATTTCAGGAAACCAATAAAAGAAAACAAGAACTT

1396
GAACAAGAGCTAAAAGCCATATCGGATAAGAAAGCATTATTA - 3'

*Nucleotides in red indicate the 366 bp deletion.

13. **Shewen, P. E., and B. N. Wilkie.** 1985. Evidence for the *Pasteurella haemolytica* cytotoxin as a product of actively growing bacteria. Am. J. Vet. Res. **46**:1212-1214.

14. **Shewen, P. E., and B. N. Wilkie.** 1988. Vaccination of calves with leukotoxin culture supernatant from *Pasteurella haemolytica*. Canad. J. Vet. Res. **52**:30-36.

15. **Whiteley, L. O., S. K. Maheswaran, D. J. Weiss, and T. R. Ames.** 1990. Immunohistochemical localization of *Pasteurella haemolytica* A1-derived endotoxin, leukotoxin, and capsular polysaccharide in experimental bovine pasteurella pneumonia. Vet. Pathol. **27**:150-161.

APPENDIX 1

FIGURES

Figure 1. *M. haemolytica* A1 Backbone Biological Agent

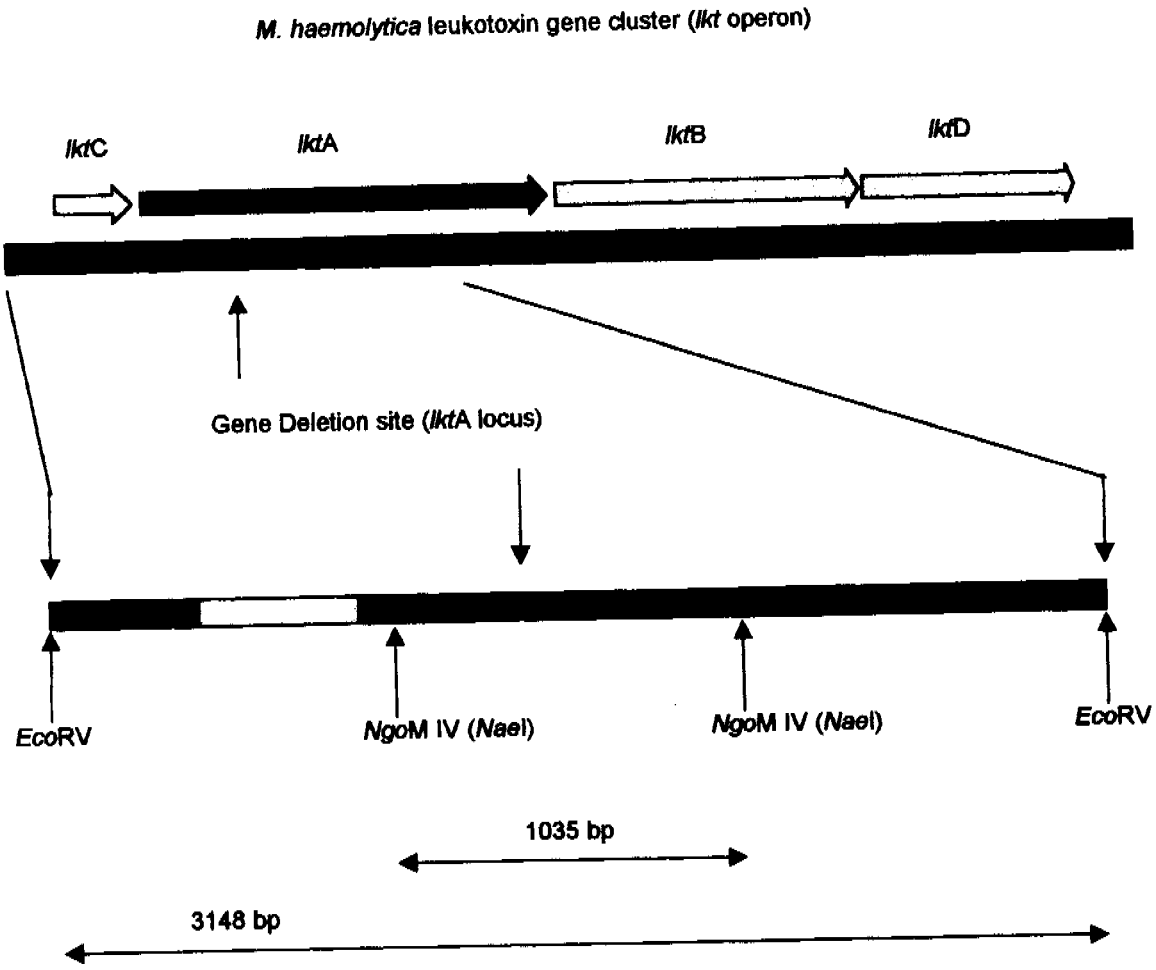


Figure 2. Construction of pBB80CΔlktA shuttle vector

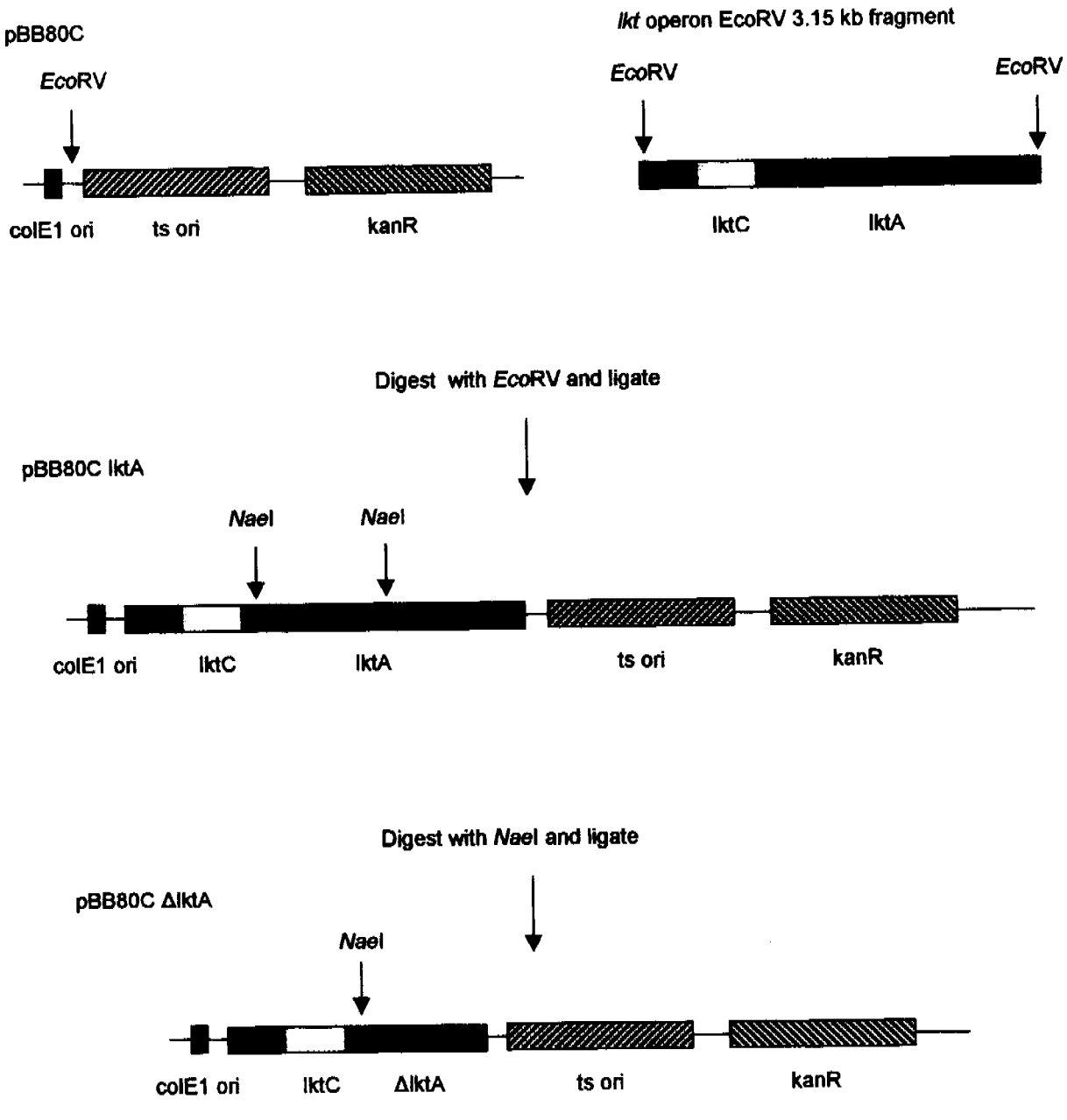


Figure 3. Construction of Regulated Biological Agent

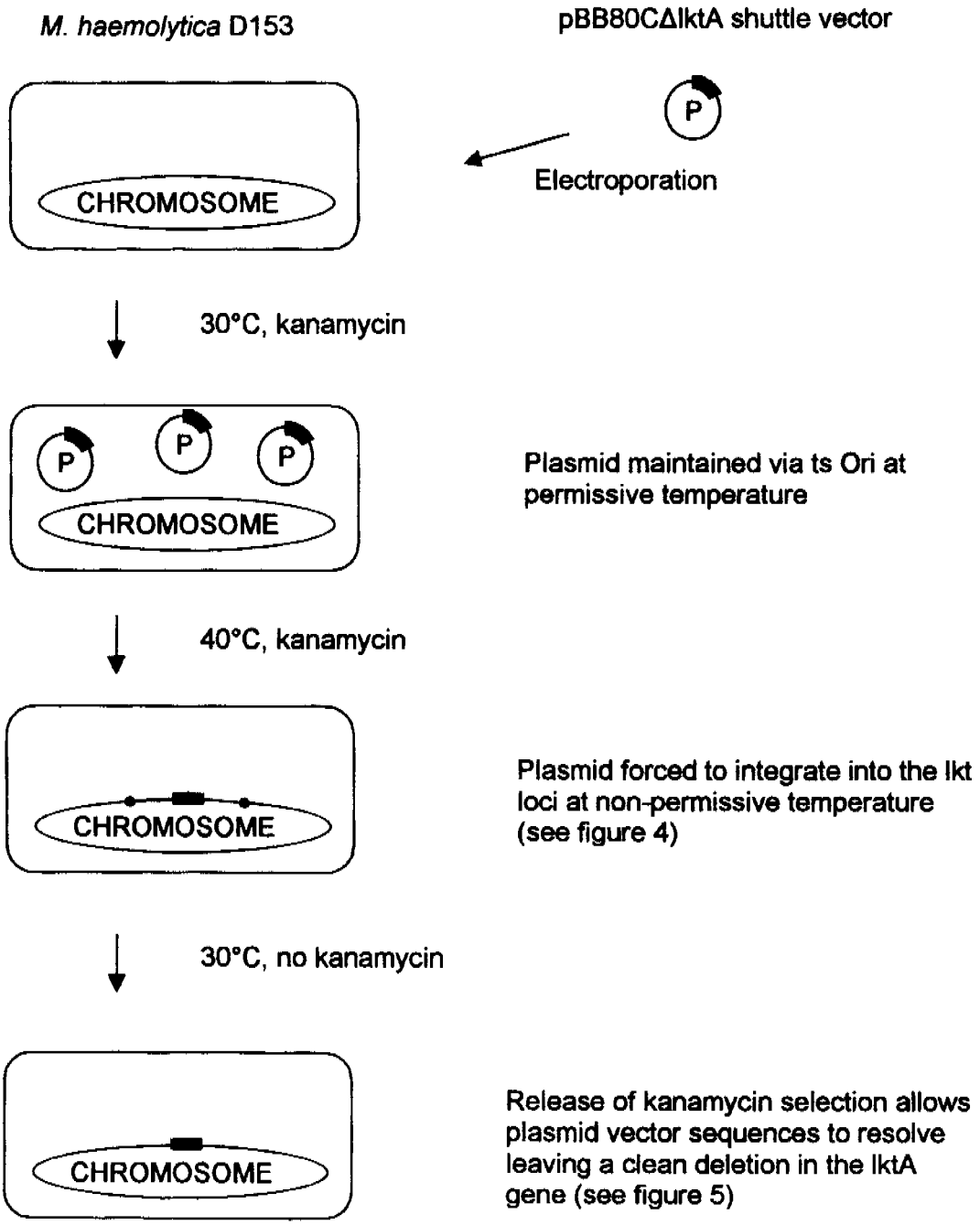


Figure 4. Integration of shuttle vector pBB80CΔ*lktA*

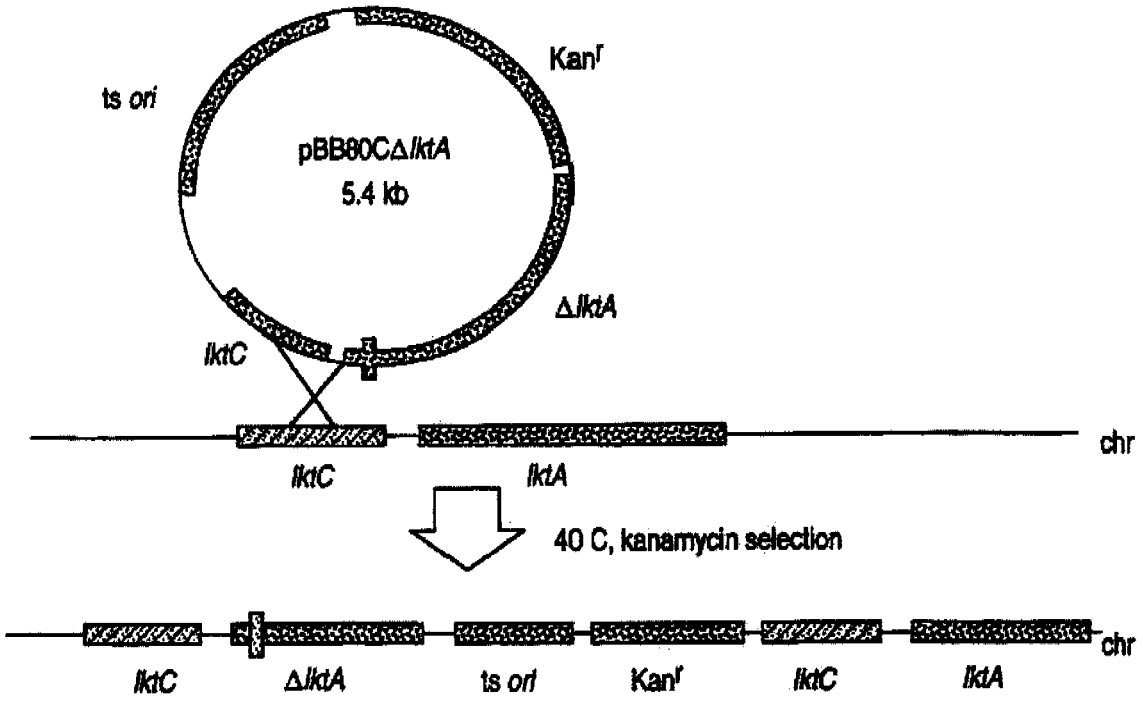
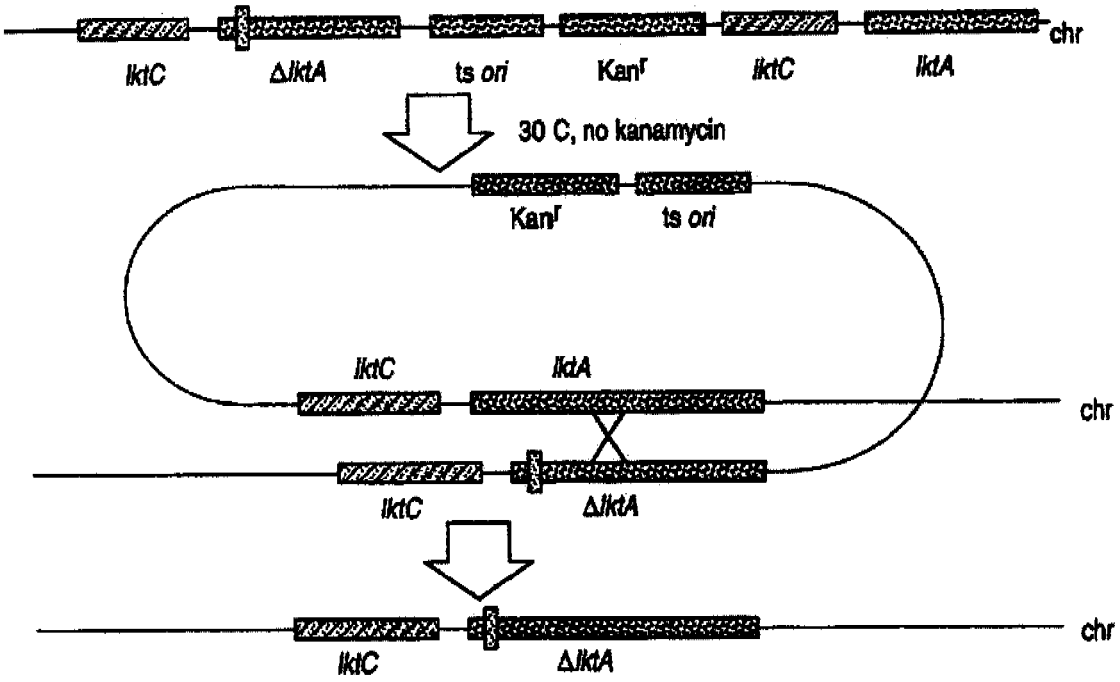


Figure 5. Vector sequences resolved from chromosome



APPENDIX 2

BIOCHEMICAL PROFILE

Biochemical Profile of *M. haemolytica* D153 Parent Strain and *M. haemolytica* Lkt- Deletant Strain Using the BBL Crystal Identification System

Substrate	Parent Strain	Deletant Strain
Arabinose	-	-
Mannose	-	-
Sucrose	+	+
Melibiose	-	-
Rhamnose	-	-
Sorbitol	+	+
Mannitol	+	+
Adonitol	-	-
Galactose	-	-
Inositol	+	+
p-n-p phosphate	+	+
p-n-p-α-β-glucoside	-	-
p-n-p-β-galactoside	+	+
Proline-p-nitroanilide	-	-
p-n-p-bis-phosphate	+	+
p-n-p-xyloside	-	-
p-n-p-α-arabinoside	+	+
p-n-p-phosphorylcholine	-	-
p-n-p-β-glucuronide	-	-
p-n-p-N-acetyl-glucosaminide	-	-
γ-L-glutamyl-p-nitroanilide	-	-
Esculin	-	-
p-nitro-DL-phenylalanine	-	-
Urea	-	-
Glycine	-	-
Citrate	-	-
Malonate	-	-
Tetrazolium	-	-
Arginine	-	-
Lysine	-	-

APPENDIX 3

SEQUENCE ANALYSIS

M. haemolytica lktA gene (partial sequence)

TAAAAATGAATTTAATTTTATTAAAAAATAGAAGGAGACATCCCTTATGGGA
ACTAGACTTACAACCCTATCAAATGGGCTAAAAAACACTTTAACGGCAAC
CAAAAGTGGCTTACATAAAGCCGGTCAATCATTAACCCAA*GCCGGC*TCGG
NgoM IV
TTATTGCTTCACCGATTGCCTTATTAGTATCTGGGATTACCGGTGTAATT
TCTACGATTCTGCAATATTCTAAACAAGCAATGTTTGAGCACGTTGCAAA
TAAAATTCATAACAAAATTGTAGAATGGGAAAAAAATAATCACGGTAAGA
ACTACTTTGAAAATGGTTACGATGCCCGTTATCTTGCGAATTTACAAGAT
AATATGAAATTCTTACTGAACTTAAACAAAGAGTTACAGGCAGAACGTGT
CATCGCTATTACTCAGCAGCAATGGGATAACAACATTGGTGATTTAGCTG
GTATTAGCCGTTTAGGTGAAAAAGTCCTTAGTGGTAAAGCCTATGTGGA
TGCGTTTGAAGAAGGCAAACACATTAAAGCCGATAAATTAGTACAGTTG
GATTCGGCAAACGGTATTATTGATGTGAGTAATTCGGGTAAAGCGAAAA
CTCAGCATATCTTATTCAGAACGCCATTATTGACGCCGGGAACAGAGCA
TCGTGAACGCGTACAAACAGGTAAATATGAATATATTACCAAGCTCAATA
TTAACCGTGTAGATAGCTGGAAAATTACAGATGGTGCAGCAAGTTCTAC
CTTTGATTTAACTAACGTTGTTCAGCGTATTGGTATTGAATTAGACAATG
CTGGAAATGTAACTAAAACCAAAGAAACAAAAATTATTGCCAAACTTGGT
GAAGGTGATGACAACGTATTTGTTGGTTCTGGTACGACGGAAATTGATG
GCGGTGAAGGTTACGACCGAGTTCACTATAGCCGTGGAAACTATGGTGC
TTTAACTATTGATGCAACCAAAGAGACCGAGCAAGGTAGTTATACCGTA
AATCGTTTCGTAGAAACCGGTAAAGCACTACACGAAGTGACTTCAACCC
ATACCGCATTAGTGGGCAACCGTGAAGAAAAAATAGAATATCGTCATAG
CAATACC...

Addendum 18

Schering-Plough Animal Health Corp.

Project No. 00011
Report No. 00011-003R
Date: 03Dec03

Category II Summary Information Format for the *Mannheimia (Pasteurella) haemolytica* D153 Lkt- Master Seed Lot No. 19039-001

Abstract

Attached is the Summary Information Format (SIF) for the *Mannheimia haemolytica* leukotoxin gene-deleted (Lkt-) Master Seed Lot No. 19039-001. The SIF outlines important scientific questions and information that should be addressed during the preparation of a U.S. Veterinary Biological Product License Application to the United States Department of Agriculture (USDA) for new biotechnology-derived biologics. The SIF is designed to allow both the participating firm as well as the Center for Veterinary Biologics (CVB) to assess the risk associated with the manufacture and release of biological organisms, specifically the risk associated with genetically modified organisms released into the environment. The purpose of the SIF is to provide specific data about the design, construction, and testing of the biological agent construct and to provide a basis for the Risk Analysis.

Schering-Plough Animal Health Corp.

Project No. 00011
Report No. 00011-003R
Date: 03Dec03

Category II Summary Information Format for the *Mannheimia (Pasteurella) haemolytica* D153 Lkt- Master Seed Lot No. 19039-001

Abstract

Attached is the Summary Information Format (SIF) for the *Mannheimia haemolytica* leukotoxin gene-deleted (Lkt-) Master Seed Lot No. 19039-001. The SIF outlines important scientific questions and information that should be addressed during the preparation of a U.S. Veterinary Biological Product License Application to the United States Department of Agriculture (USDA) for new biotechnology-derived biologics. The SIF is designed to allow both the participating firm as well as the Center for Veterinary Biologics (CVB) to assess the risk associated with the manufacture and release of biological organisms, specifically the risk associated with genetically modified organisms released into the environment. The purpose of the SIF is to provide specific data about the design, construction, and testing of the biological agent construct and to provide a basis for the Risk Analysis.

Category II Summary Information Format

Mannheimia (Pasteurella) haemolytica D153 Lkt-
Master Seed Lot No. 19039-001

I. Introduction

A. Objective

1. Identify where the Regulated Biological Agent was constructed and where the product will be made, tested and manufactured. Address the available level of containment.

The Regulated Biological Agent (Mannheimia haemolytica leukotoxin gene-deleted (Lkt-) Master Seed) was constructed from the agent listed below (II.A) at the National Animal Disease Center (NADC), Ames, IA. The product has been developed at the Schering-Plough Animal Health (SPAH) Research and Development Facility, Elkhorn, NE. Biosafety level 2 containment facilities are available for animal testing at SPAH - Elkhorn, NE, SPAH - Williamsburg, KS, and SPAH - Terre Haute, IN. Suitable contract research organizations with BL2 containment facilities may also be used for animal studies. For US distribution, the final vaccine will be manufactured at Schering-Plough Animal Health (SPAH), Elkhorn, NE, U.S. Veterinary License No. 165A.

2. Provide a brief (one sentence) description of the Regulated Biological Agent.

The Regulated Biological Agent (RBA) is a leukotoxin gene-deleted strain of Mannheimia haemolytica, biotype A, serotype 1 that produces a truncated and ineffective leukotoxin capable of eliciting a protective immune response.

B. Proposal

1. What is the intended use of the product?

a. Species:

Cattle

b. Proposed claim:

The M. haemolytica Lkt- vaccine is indicated for use in healthy cattle as an aid in the prevention and/or reduction of pneumonic lesions associated with bovine pneumonic pasteurellosis, commonly known as shipping fever.

c. Geographic area:

United States and Canada

d. Route of administration:

Subcutaneous

e. Brief description of the expected safety profile:

The M. haemolytica Lkt- deletant strain is safe for use in calves as young as 3 months of age when administered subcutaneously at a dose of 5.0 x 10^8 cfu (see section III.C). Mannheimia haemolytica A1 is widely distributed throughout the world and naturally inhabits cattle, sheep, pigs, rabbits, poultry and many other domestic and wild animals. The M. haemolytica Lkt- vaccine strain has a deletion in the leukotoxin A gene attenuating the wild-type virulence, has no plasmids or mobile chromosomal elements, and would therefore be expected to be much less of an environmental threat than naturally occurring strains of Mannheimia.

II. Description of the Regulated Biological Agent Construction

A. The Backbone Biological Agent

1. What organism was used for the Backbone Biological Agent? Are there any known virulence features associated with the Backbone Biological Agent? What happens in the target species?

The parental organism for this vaccine is Mannheimia haemolytica biotype A, serotype 1. It has been identified as strain NADC-D153. It is a field isolate that was originally cultured from bovine pneumonic lung tissue at the National Animal Disease Center in Ames, IA. Although M. haemolytica possesses an array of potential virulence factors, a large body of evidence points to the leukotoxin (Lkt) as one of the most important factors contributing to lung injury. Leukotoxin is only cytolytic to ruminant leukocytes and platelets (2, 4, 12) and is presumed to destroy leukocytes at the site of infection, thus reducing the animal's capacity to launch an effective immune response (3). Several observations point to an important role of Lkt in the pathogenesis of bovine pneumonic pasteurellosis. First, in experimental pasteurellosis with cattle, the clinical and pathophysiologic events are dose-dependently reproduced by intratracheal or intrapulmonic administration with the live logarithmic phase of M. haemolytica as opposed to stationary phase organisms (1, 10). This enhanced pathogenicity may be related to the amount of Lkt and other virulence determinants produced by these different bacterial populations as it has been demonstrated that logarithmic phase cells produce far greater amounts of Lkt than stationary cells (2, 13). Second, other studies have shown that cattle with high Lkt neutralizing antibody titers have higher resistance to the natural disease and experimental pneumonic pasteurellosis than animals with low antibody titers (5, 7). Third, the presence of Lkt in situ in acute pneumonic lesions from M. haemolytica-induced experimental pneumonic pasteurellosis and from natural disease (15) has been previously demonstrated. Fourth, a Lkt-rich, cell-free vaccine prepared from actively growing M. haemolytica has been demonstrated to induce serum neutralizing antibodies to the Lkt as well as serotype-specific agglutinating antibodies (9). Calves immunized against the Lkt and soluble cell surface antigens

contained in the vaccine are protected against M. haemolytica challenge whereas non-immunized calves succumb to fibrinous pneumonia (14). Finally, studies with the mutant M. haemolytica strain S9B0071, which does not produce detectable Lkt have demonstrated reduced virulence in both goats and cattle in comparison with the parent wild-type strain (11). Both reduced mortality and reduced lung lesion scores were found following infection with doses of the mutant strain that exceeded the parent wild-type challenges by as much as 1,000-fold (11).

Although M. haemolytica is commonly found in the upper respiratory tract (URT) of healthy cattle, exposure of cattle to environmental, managerial, or viral stressors leads to an explosive proliferation and colonization of M. haemolytica serotype A1 in all areas of the upper respiratory tract (6, 8). This heavily colonized URT then becomes the source for lung exposure via aspiration of nasopharyngeal secretions which ultimately leads to the fibrinonecrotic pleuropneumonia commonly associated with M. haemolytica (6).

a. What is the previous safe use of the Backbone Biological Agent?

M. haemolytica D153 wild type strain was isolated from a calf that died of pneumonia. No genetic modifications were made to this strain prior to use as described by this SIF.

(i) If available provide history of previous safe use using literature or internal documents. Include the recommended CDC/NIH biosafety level for use of the Backbone Biological Agent.

The fourth edition of the CDC/NIH manual, "Biosafety in the Microbiological and Biomedical Laboratories", does not rate the biosafety level for wild-type M. haemolytica A1. Wild-type M. haemolytica is not infectious to laboratory personnel. However, working with the methods described in this manual on assigning biosafety levels, wild-type M. haemolytica can appropriately be handled as a biosafety level 1 agent.

2. Physical characteristics of the Backbone Agent

a. Provide a flow diagram or explanation of the process of how the Backbone Biological Agent was constructed

The Backbone Biological Agent is a field isolate of M. haemolytica. No modifications were made prior to the construction of the Regulated Biological Agent (Appendix 1, Figure 1).

(i) Describe the proposed site for Reporter Gene insertion.

Not applicable; no new DNA or genetic markers are present in the recipient strain.

(ii) Do the flanking regions of the proposed insertion site Backbone Biological Agent have any known regulatory elements that could moderate the expression of the inserted Reporter Gene?

Not applicable; no additional DNA has been inserted into the recipient strain.

(iii) Identify unique restriction endonucleases (not more than five) that will give identifiable digestion patterns useful for characterizing the final Backbone DNA.

In the final Backbone DNA, the deletion site is contained on a 3148 bp EcoRV restriction fragment (Appendix 1, Figure 1). Within this fragment the 1035 bp to be deleted are flanked by two NgoM IV sites.

(iv) Identify reporter genes if applicable to be inserted into Backbone DNA. Show pertinent sequences or restriction endonuclease sites in the flow diagram above.

Not applicable; no additional DNA has been inserted into the recipient strain.

(v) Has there been previous safe use of the reporter gene. Add a citation, if appropriate.

Not applicable; no additional DNA has been inserted into the recipient strain.

B. Construction and Characterization of the Regulated Biological Agent

1. Provide a flow diagram on the construction of the Regulated Biological Agent.

Include the following:

a. Final Backbone Biological Agent:

M. haemolytica D153

The lkt operon is described in Appendix 1, Figure 1. The genetic organization of the secreted leukotoxin is comprised of four genes: lktA, lktC, lktB, and lktD. The lktA gene encodes the toxin, lktC encodes a protein that post-translationally activates the toxin, and the lktB and lktD gene products are required for leader-independent transport of Lkt from the cell.

b. Heterologous reporter genes (if applicable):

Not applicable

c. All shuttle vectors:

A 3.15 kb EcoRV fragment containing lktC and approximately 75% of the lktA coding region was cloned into the unique EcoRV site of the cloning vector pBB80C (Appendix 1, Figure 2). Using the two unique NgoM IV restriction enzyme sites in the lktA gene, a 1033 bp fragment was removed near the N-terminal coding region of that gene. Plasmid pBB80C, a temperature sensitive plasmid, was electroporated into the D153 serotype A1 strain, and isolates were selected for lack of hemolytic activity on blood agar plates indicating attenuation of the lktA gene. No foreign DNA remains in this newly constructed M. haemolytica strain.

d. Host cell lines used:

D153 cells were electroporated with temperature-conditional plasmid vectors containing deleted clones of the lktA gene.

e. Selection techniques and methods used to evaluate the final Regulated Biological Agent.

The shuttle plasmid pBB80CΔlktA was methylated in vitro with HhaI methyltransferase and electroporated into the D153 parent strain. Transformants were grown with antibiotic selection at a permissive temperature for replication (30°C) then passed to a non-permissive temperature (40°C) on selective solid medium. Colonies were then selected and grown overnight at the permissive temperature but without selection and then streaked for isolation on non-selective media (Appendix 1, Figure 3). At this point, the plasmid is completely lost. Colonies were screened by PCR for the loss of the deleted segment of the lktA gene, and strains containing the deleted lktA alleles were recovered. The resulting deletants have no plasmids, residual pieces of DNA from the cloning process, or any inserted foreign or heterologous nucleic acids.

2. Describe the laboratory methods or criteria used to evaluate the Regulated Biological Agent.

The following evidence supports the successful deletion of the leukotoxin A gene. First, the deleted strain produces no detectable leukotoxin activity against bovine lymphoma (BL-3) target cells (<1:2 for the mutant compared to 1:1024 for the parent). Second, SDS-PAGE and Western blot analysis revealed a new protein of 65,000 molecular weight, which is consistent with the predicted molecular weight of the deleted product. Third, the anti-LktA monoclonal antibody did not react with any proteins at the 100-105 kd molecular weight of native LktA. Fourth, PCR products from the putative leukotoxin mutant and its parent were found to be 2 kb and 3 kb in size, respectively, indicating a deletion had been introduced into lktA. Fifth, digestion of the PCR products revealed 2 - 1 kb bands from the mutant and 3 - 1 kb bands from the parent strain, indicating the deletion is in frame to lktA. Finally,

when grown on sheep blood agar plates, the deletant strain was unable to produce hemolysis due to lack of a functional leukotoxin.

3. Physical characterization of the Regulated Biological Agent

a. Characterize the physical map using unique Reporter Gene and Backbone Biological Agent restriction endonuclease sites and describe resulting restriction fragments and digestion patterns.

The Backbone Biological Agent includes a 3148 bp EcoRV fragment containing the deletion site. In the Regulated Biological Agent, this EcoRV fragment has been reduced in size to 2113 bp.

b. Devise a PCR or restriction endonuclease test based on the Backbone Biological Agent sequence and the Reporter Gene Sequence that will identify and characterize the Reporter Gene/Backbone Biological Agent construct.

A sense PCR primer, CTGGCGATAGACGTTGGATTA, and an anti-sense primer, CGCTTTACCCGAATTACTCACATC, will amplify a 1855 base pair product from the wild type leukotoxin gene and a 822 base pair product from the deleted gene.

c. What will be the criteria for stability and purity of the Regulated Biological Agent Master Seed n and n+5?

Gram stain was used to confirm that the M. haemolytica Lkt- Master Seed was a non-spore forming Gram negative rod. When plated on blood agar, the M. haemolytica Lkt- Master Seed lacks hemolysis. The Master Seed was tested by the Quality Control Department at Schering-Plough Animal Health for purity as required by 9 CFR 113.64(a). The M. haemolytica D153 parental and Lkt- strains were characterized by the BBL Crystal Identification System (Becton Dickinson, Sparks, MD). This system is a miniaturized identification method employing modified conventional and chromogenic substrates for the purpose of identifying bacteria. The BBL Crystal ID panels contain 30 dried biochemical and enzymatic substrates. A bacterial suspension in the inoculum fluid is used for rehydration of the substrates. In general, many of the tests used in the BBL Crystal ID systems are modifications of classical methods. These include tests for fermentation, oxidation, degradation, and hydrolysis of various substrates. In addition, there are chromogen-linked substrates to detect enzymes that microbes use to metabolize various substrates. Briefly, the test consists of preparing a test inoculum that is used to fill 30 wells in a test panel. Following an incubation period, the wells are examined for color changes that result from metabolic activities of the organism. The resulting pattern of the 30 reactions is converted into a ten-digit profile number that is used as the basis for identification. Both strains were identical for all reactions and were identified as P. haemolytica at the 0.9996 confidence level (Appendix 2). There were no atypical results for either strain.

Molecular characterizations will be performed at Master Seed (n) and highest passage level cells (n+5). These characterizations will include the PCR assay described above followed by DNA sequence analysis of the amplified fragment. Sequence analysis was performed on the Master Seed and confirmed the deletion of the 1033 bp NgoM IV fragment (Appendix 3)

The absence of a functional leukotoxin in the M. haemolytica Lkt- deletant strain may also be tested by a cytotoxicity assay.

Backpassage studies will be conducted to evaluate the genetic stability of the M. haemolytica Lkt- Master Seed to ensure that the deletant strain will not revert to virulence when administered to the host animal.

d. Provide the genetic sequence in electronic format for any new or altered genetic sequences, including insertion and flanking sequence.

The electronic file is attached (Appendix 3). The deletion junction is at the underlined NgoM IV site.

4. What is the recommended NIH/CDC biosafety level for the Regulated Biological Agent?

The recommended biosafety level will be the same as the parent strain (BL1).

5. Provide a short summary or description of genetic motifs that may have resulted as a consequence of the genetic recombination (II.C.3.d). Are there any known motifs that may promote homologous recombination, DNA insertion, or gene expression of existing or new open reading frames?

Since the lktA gene in the recipient was altered by the removal of part of the genetic material, reversion to the parental lktA gene is extremely unlikely. There is no known mechanism in the Pasteurella family of bacteria to naturally replace the deleted portion of the gene. The lktA gene is encoded on the chromosome and not on a plasmid, and there are no phage or conjugative plasmids known for this family of bacteria. The M. haemolytica Lkt- deletant strain contains no plasmids, residual pieces of DNA from the cloning process, or any inserted foreign or heterologous nucleic acids.

III. Biological Properties or Virulence for the Regulated Biological Agent used for Master Seed

A. What are the known phenotypic characteristics or effects of the Regulated Biological Agent identified as Master Seed?

The RBA lacks functional leukotoxin activity as demonstrated by the absence of hemolysis on blood agar plates.

B. What are the virulence characteristics of the Regulated Biological Agent used for the Master Seed?

Virulence of the RBA has been attenuated by virtue of a 1035 bp deletion within the leukotoxin A gene, resulting in a non-functional leukotoxin. Leukotoxin is one of the most important virulence factors that contribute to the lung injury seen in bovine pneumonic pasteurellosis.

C. Is the Regulated Biological Agent used for the Master Seed virulent for target animals, non-target animals?

1. *Target animal:*

A calf safety study was conducted in accordance with 9CFR 113.41. Two groups of calves (11 vaccinates + 2 sentinel controls per group) were inoculated either subcutaneously or intranasally at ~3 months of age with 5.0 x 10^8 cfu of a M. haemolytica Lkt- culture that was one passage from the Master Seed. The calves were monitored daily for adverse reactions and then euthanized and necropsied 28 days post-vaccination. None of the calves displayed any adverse reactions that could be attributed to the vaccine (Appendix 4, B00-150-01R1).

A feasibility study was conducted to evaluate the efficacy of the M. haemolytica Lkt- deletant strain in 2-3 month old calves challenged trans-tracheally with virulent M. haemolytica. The experimental vaccine was a lyophilized, avirulent live culture of M. haemolytica Lkt- that was 3 passages from the Master Seed. The vaccine was tested at 2 levels (1.6 x 10^8 cfu/dose and 1.1 x 10^7 cfu/dose) with each level administered subcutaneously as a single 2 mL dose. All vaccinated calves were normal throughout the post-vaccination period, with a few elevated temperatures during the first 24 hr post-vaccination. Injection site reactions were only observed in 27% of the calves vaccinated with the high dose of M. haemolytica Lkt-. These reactions were relatively small (average 18 cm^3) and lasted no longer than 48 hr. post-vaccination (Appendix 5, B01-187-01R).

The virulence of the M. haemolytica Lkt- Master Seed will be further investigated later during scheduled backpassage and dissemination in host animal studies.

2. *Non-Target animals:*

The host range of M. haemolytica A1 is strictly limited to ruminants. M. haemolytica A1 isolates appear to be host specific and neither bovine nor ovine isolates readily cause disease in other species. The M. haemolytica Lkt- Master Seed is not expected to be any different.

The LD_{50} for the M. haemolytica Lkt- Master Seed was examined in mice. For comparison, the LD_{50} for the parent strain was also examined. The LD_{50} for the M. haemolytica parent strain given intraperitoneally was 7.6 x 10^8 cfu as calculated by the Reed and Muench formula, and the LD_{50} of the deletant strain was 4.1 x 10^9 cfu.

While not particularly virulent in mice, it was concluded that the M. haemolytica Lkt- deletant strain is less virulent than the parent strain (Appendix 6, B01-065-01R).

A mouse safety test in accordance with 9CFR 113.33 was performed on the Master Seed by the Quality Control department at the Elkhorn, NE facility. The test passed, and the results are presented on Form 2008.

Virulence of the M. haemolytica Lkt- Master Seed for other non-host animals has not been investigated yet and will be submitted in the final Risk Assessment to the USDA.

D. Do the Reporter Gene sequences enhance the virulence of the ability to survive in the target animals, non-target animals?

No new DNA or genetic markers are present in the recipient strain.

1. What is the tissue tropism of the Regulated Biological Agent in target and non-target animals?

The nasopharynx of healthy cattle is commensally colonized with M. haemolytica A1. During disease conditions, M. haemolytica is able to form fibrinous pneumonic lesions in the lungs. M. haemolytica D153 wild type strain was isolated from the lungs of a calf that died of pneumonia. There is no reason to suspect that the tissue tropism of the M. haemolytica Lkt- deletant strain would be altered because of the clean deletion in the lktA gene. When administered intranasally, the M. haemolytica Lkt- deletant strain can colonize the nasal passages and spread to other calves by nose to nose contact, but without causing any adverse reactions (Appendix 4, B01-150-01R1).

Tissue tropism in the susceptible host has not been examined for the M. haemolytica D153 Lkt- Master Seed at this time. A study to determine the dissemination in host animal will be performed and submitted to the USDA at a later time.

The host range of M. haemolytica A1 is strictly limited to ruminants. M. haemolytica A1 isolates appear to be host specific and neither bovine nor ovine isolates readily cause disease in other host species. Tissue tropism of the M. haemolytica Lkt- Master Seed for other non-host animals has not been investigated yet and will be submitted in the final Risk Assessment to the USDA.

3. Provide direct scientific evidence, including any relevant scientific publications, if available.

Frank, G. H and R. E. Briggs. 1992. Colonization of the tonsils of calves with *Pasteurella haemolytica*. Am. J. Vet. Res. 53:481-484.

Frank, G. H. 1989. Pasteurellosis of cattle. p. 197-222. *In* C. Adlam and J. M. Rutter. *Pasteurella* and pasteurellosis. Academic Press Ltdl, London.

Wessman, G. E. 1967. Susceptibility of mice, guinea pigs and hamsters to challenge with *Pasteurella hemolytica* and its enhancement by microbial polysaccharides and related compounds. J. Infect. Dis. 117:421-428.

E. Discuss the potential for horizontal gene transfer or recombination of the Regulated Biological Agent.

Horizontal gene transfer has never been reported for the M. haemolytica D153 strain. Neither the parental nor the final constructed strain contain autonomously replicating plasmids.

1. Is there any reason to believe that the potential for horizontal gene transfer or recombination is different in target and non-target animals?

Because the host range of M. haemolytica A1 is strictly limited to ruminants, and neither bovine nor ovine isolates readily cause disease in other host species, there is no reason to believe the potential for horizontal gene transfer or recombination is different in non-target animals.

a. Include contributions from the Backbone Biological Agent and Reporter Gene Sequence in the discussion of the horizontal gene transfer and recombination potential.

Not applicable; there is no potential for horizontal gene transfer or recombination.

b. Reference any relevant scientific publications.

Not applicable.

F. Describe the shed/spread capabilities of the Regulated Biological Agent

Because the reversion to virulence study for the M. haemolytica Lkt- has not been completed at this time, there is limited information of what are the actual shed/spread capabilities of the Master Seed. However, preliminary safety/feasibility studies indicate that the M. haemolytica Lkt- deletant strain is not shed from animals vaccinated subcutaneously and spread to other calves (Appendix 4, 5; B01-150-01R1 and B01-187-01R, respectively). Culture analysis of waste collected from M. haemolytica Lkt- vaccinated calves indicates that the deletant strain is not shed from the urine or feces. Calves vaccinated intranasally with the deletant strain are capable of shedding the organism and spreading it to other calves via nose to nose contact (Appendix 4, B01-150-01R1). There were no signs of infection or disease in calves vaccinated either subcutaneously or intranasally 3 and 7 days post-vaccination.

1. Include shed/spread potential in non-target animal species.

The host range of wild type M. haemolytica A1 is limited to ruminants (cattle and sheep). The host/range specificity of the M. haemolytica D153 Lkt- Master Seed has not been determined at this time. Because the M. haemolytica D153 Lkt- Master Seed is a gene deletion only, it is not expected that the host/range specificity will be any different from the parent strain. More information will be available after the completion of the non-target animal safety studies.

2. Include in the discussion any contributions to shed/spread capabilities that might be associated with the Backbone Biological Agent and Reporter Gene sequence.

Although M. haemolytica is commonly found in the upper respiratory tract (URT) of healthy cattle, exposure of cattle to environmental, managerial, or viral stressors leads to an explosive proliferation and colonization of M. haemolytica serotype 1 in all areas of the upper respiratory tract. This heavily colonized URT then becomes the source for lung exposure via aspiration of nasopharyngeal secretions which ultimately leads to the fibrinonecrotic pleuropneumonia commonly associated with M. haemolytica. The nasopharyngeal secretions can also be a source for shedding virulent M. haemolytica organisms to other susceptible animals via nose to nose contact.

4. Reference relevant scientific publications

Frank, G. H. and P. C. Smith. 1983. Prevalence of *Pasteurella haemolytica* in transported calves. Am. J. Vet. Res. 44:981-985.

Gonzalez, C. T., S. K. Maheswaran, and M. P. Murtaugh. 1995. *Pasteurella haemolytica* serotype 2 contains the gene for a noncapsular serotype 1-specific antigen. Infect. Immun. 63:1340-1348.

G. Discuss the expected environmental impact or survivability of the Regulated Biological Agent, and provide available scientific evidence.

The M. haemolytica D153 Lkt- Master Seed is only capable of replication in mammalian tissues and in the laboratory in/on select media. Survivability of wild-type M. haemolytica A1 strain in the environment is dependent upon infection of susceptible animals. Culture analysis of waste collected from M. haemolytica Lkt- vaccinated calves indicates that the deletant strain is not shed from the urine or feces. Calves vaccinated subcutaneously do not shed the M. haemolytica Lkt- deletant strain, thus providing no opportunity for this deletant strain to impact the environment. Because there is a clean deletion in the gene of M. haemolytica's primary virulent factor, any potential release of this deletant strain into the environment would be no worse than the release of the naturally occurring wild type strain.

The M. haemolytica Lkt- Master Seed X+1 has been tested and is sensitive to the following antibiotics: ampicillin, ceftiofur (Naxcel), enrofloxacin (Baytril), florfenicol (Nuflor), oxytetracycline (LA200), penicillin, spectinomycin, sulfisoxylin, tetracycline, tilmicosin (Micotil), and trimethoprim sulfadiazine (Tribrissen). Several of these antibiotics are commonly used in treatment of farm animals, further decreasing the odds of survival in the environment.

1. Do the Reporter Gene sequences or gene sequences associated with the Backbone Biological Agent enhance the ability to survive in the environment or increase resistance to therapeutic agents?

   Not applicable; no additional DNA has been inserted into the recipient strain.

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2. **Baluyut, C. S., R. R. Simonson, W. J. Bemrick, and S. K. Maheswaran.** 1981. Interaction of *Pasteurella haemolytica* with bovine neutrophils: Identification and partial characterization of a cytotoxin. Am. J. Vet. Res. **42**:1920-1926.

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8. **Gonzalez, C. T., S. K. Maheswaran, and M. P. Murtaugh.** 1995. *Pasteurella haemolytica* serotype 2 contains the gene for a noncapsular serotype 1-specific antigen. Infect. Immun. **63**:1340-1348.

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11. **Petras, S. F., M. Chidambaram, E. F. Illyes, S. Froshauer, G. M. Weinstock, and C. P. Reese.** 1995. Antigenic and virulence properties of *Pasteurella haemolytica* leukotoxin mutants. Infect. Immun. **63**:1033-1039.

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