

Efficacy and safety of a novel DNA immunostimulant in cattle

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Abstract

Four studies were performed to evaluate efficacy (Studies 1 and 2) and safety (Studies 3 and 4) of a novel DNA immunostimulant (Zelnate[®], Bayer Healthcare) in cattle. Zelnate[®] (ZEL) was administered concurrently with (Study 1) or 24 hours after (Study 2) a *Mannheimia haemolytica* (Mh) challenge. Holstein steers (3 to 4 months of age; n = 32/treatment, Study 1; n = 40/treatment, Study 2) received either ZEL or a negative control (CON) on day 0 (Study 1) or day 1 (Study 2). Calves were challenged intratracheally with 60 mL of Mh (10⁷ CFUs/mL, Study 1; 10⁸ CFUs/mL, Study 2) on day 0 and clinically observed to day 5. Lung scores were obtained at the time of premature death or after necropsy on day 5.

ZEL mitigated lung lesions in this Mh model, compared to the CON (Studies 1 and 2), and significantly reduced mortality compared to the CON ($P < 0.05$; Study 2). ZEL was shown to be safe at the injection site (Study 3) and among large populations in different field scenarios (Study 4).

ZEL demonstrated efficacy by mitigating lung lesions (Studies 1 and 2) and decreasing mortality (Study 2) in this Mh disease model. Studies 3 and 4 confirmed that ZEL is safe for use in cattle.

Key words: Zelnate[®], cattle, *Mannheimia haemolytica*, immunostimulant, bovine respiratory disease

Résumé

Quatre études ont été menées afin d'évaluer l'efficacité (Études 1 et 2) et l'innocuité (Études 3 et 4) d'un nouvel immunostimulant à l'ADN (Zelnate[®], Bayer Healthcare) chez les bovins. Le Zelnate[®] (ZEL) a été administré conjointement (Étude 1) ou 24 heures après (Étude 2) une infection de provocation avec *Mannheimia haemolytica* (Mh). Des bouvillons Holstein (3 à 4 mois d'âge; n=32/traitement, Étude 1; n=40/traitement, Étude 2) ont reçu soit le ZEL ou soit un

contrôle négatif au jour 0 (Étude 1) ou au jour 1 (Étude 2). Les veaux ont été infectés par voie intratrachéale avec 60 mL de Mh (10⁷ UFC/mL, Étude 1; 10⁸ UFC/mL, Étude 2) au jour 0 et observés cliniquement au jour 5. Des scores lésionnels pour le poumon ont été obtenus au moment de la mort prématuré ou après la nécropsie au jour 5.

Par rapport au groupe témoin (Études 1 et 2), le ZEL a réduit l'incidence des lésions pulmonaires dans ce modèle avec infection provoquée par Mh et a significativement réduit la mortalité ($p < 0.05$; Étude 2). L'innocuité du ZEL a été démontrée au site d'injection (Étude 3) et au niveau d'une grande population dans différents scénarios sur le terrain (Étude 4).

Le ZEL s'est montré efficace dans la réduction des lésions pulmonaires (Études 1 et 2) et a réduit la mortalité (Étude 2) dans le modèle de la maladie avec Mh. Les études 3 et 4 ont démontré l'innocuité du ZEL chez les bovins.

Introduction

Bovine respiratory disease (BRD) continues to negatively impact economic returns in the beef and dairy industries. The financial impact is driven not only by morbidity and associated incremental treatment costs, but also by feed performance loss, mortality, and reduced carcass quality. Numerous studies have displayed a substantial reduction in average daily gain (ADG) among cattle with lung lesions at slaughter when compared to cattle without evidence of lung pathology.^{5,6,27,28,32,39} Significant mitigation of lung pathology may improve overall performance of feedlot cattle.

The pathogenesis of BRD has been well described.^{7,8,17,41-44} Briefly, viral infections coupled with stressful events are recognized as the primary insult leading to the eventual compromise of the animal's pulmonary immune status.^{1,18,19} Commensal bacteria of the upper respiratory tract subsequently invade and infect the lower respiratory system. This secondary bacterial infection is generally accepted as the root

cause of clinical signs, mortality, and performance losses associated with BRD.^{13,14,31}

Despite its size, the bovine lung is not designed for athletic performance, as compared to the horse. This anatomic reality is exemplified by a small volume of lung tissue relative to body mass.³⁶ When dealing with BRD, efficient clearance of the infection and preservation of viable lung tissue is paramount in order to maximize potential economic value. Further complicating the management of BRD, cattle as a species are highly adept at concealing clinical signs of disease. Once BRD is diagnosed, lung pathology may often be at an advanced stage. Prior studies have observed lung lesions among large proportions of cattle with a history of antimicrobial treatment.^{32,39} These findings suggest that pulmonary damage may have been substantial prior to diagnosis and therapy.^{5,6,27,32,39} Additional treatment modalities may improve the response of cattle treated for BRD.

Mannheimia haemolytica (Mh) has the highest prevalence among bacterial pathogens isolated from feedlot cattle with BRD.^{12,29,31} Although commensal in the upper respiratory tract of healthy cattle, Mh has the propensity to induce severe BRD lesions if allowed to colonize the lower airways. Infection may result in significant lung pathology and possibly death if not effectively managed by the animal's innate immune system, adaptive immune system, or by exogenous methods including antimicrobial therapy.

Existing tools aimed at adaptive immunity (vaccines), antimicrobial therapy, and ancillary BRD treatments have not always met expectations of the cattle industry for BRD management.^{10,21,23} Although these products provide value to the producer, stressful events that occur during the transitional period between the farm of origin and the feedlot stage of production including weaning, shipping, commingling, immunization, castration, and dehorning, may depress the ability of cattle to respond effectively to preventive and therapeutic practices.

One factor that could be better leveraged to improve the health of cattle at risk for BRD is the innate immune system. The innate immune system includes cells, such as neutrophils, macrophages, and natural killer cells, that provide immediate defense against infection. When these innate immune cells are activated to fight infection, they produce cytokines and co-stimulatory molecules that are necessary for proper activation of T cells and B cells, which mediate the adaptive immune response.^{22,23} The adaptive immune response then provides the immune memory that allows the host to mount a more rapid and effective defense if it should encounter the same infectious agent in the future. The innate immune response has dual roles: to provide immediate protection against infection and to activate the adaptive immune response to better protect the host against future infection. The innate immune response begins when innate immune cells identify certain molecules that are recognized signatures of certain groups of pathogens. These molecular signatures, or pathogen-associated molecular patterns (PAMPs), bind

to pathogen recognition receptors (PRR) on innate immune cells. The binding of PAMP to PRR "turns on" innate immune cells so they effectively kill pathogens and activate the adaptive immune response.²² One well-known PAMP is lipopolysaccharide (LPS), or endotoxin, which binds to the PRR known as toll-like receptor 4 (TLR4).¹⁵

Although the innate immune response provides a critical component of host defense, specific efforts to improve the innate immune response have not been routinely applied in veterinary medicine. Immunostimulants, also known as immunomodulators, or biological response modifiers, are a group of compounds that have the potential to activate the innate immune response. There are several different classes of immunostimulants, including bacterial products, viruses or viral products, and plant products.^{4,26,30} The mechanism of action of immunostimulants has historically not been well characterized. In recent years it has been recognized that immunostimulants are often PAMPs, and thus activate the innate immune response in a manner similar to that of bacteria, viruses, or other pathogens, without the possibility of infection. An immunostimulant administered to an animal either at risk for infection, post-infection, or clinically ill could activate the innate immune response to help the animal fight against infection. Immunostimulants have been shown to decrease BRD following experimental infection² or natural challenge.⁴⁵

A novel DNA immunostimulant (Zelnate®; ZEL)^a is an innovative cationic lipid delivery system combined with non-coding bacterial DNA that is intended to modulate the innate immune response in cattle. Cationic lipids and bacterial DNA each activate innate immune cells; bacterial DNA is a recognized PAMP^{3,25} while cationic lipids interact with PRR and activate innate immune cells in a manner similar to PAMPs.^{24,38} ZEL is currently indicated for use as an aid in the treatment of BRD due to Mh in cattle 4 months of age or older, when administered at the time of, or within 24 hours after, a perceived stressful event.

The overall objectives of this series of studies were to: 1) determine if ZEL was efficacious in a Mh disease model, 2) determine the safety of ZEL at the site of injection in the target population, and 3) determine if ZEL is safe when administered under field conditions to cattle comprising different populations and production classes. Efficacy was confirmed in the following studies:

1. Study 1 objective: estimate the mitigating effect on lung lesions in cattle when administered concurrently with an experimental challenge with Mh compared to negative controls.
2. Study 2 objective: estimate the mitigating effect on lung lesions in cattle when administered 24 hours after an experimental challenge with Mh compared to negative controls.

Likewise, safety in calves administered ZEL was investigated by the following studies:

3. Study 3 objective: determine if the local inflammatory response at the injection site in cattle ad-

ministered ZEL was consistent with the expected physiological/immunological response to foreign material in the respective tissue, to establish a slaughter withholding period for cattle.

4. Study 4 objective: confirm in-use safety by observing calves administered ZEL for an extended period post-injection in field environments.

Materials and Methods

Studies 1 and 2 (Efficacy)

Study Population

The candidate population consisted of 3 to 4 month-old Holstein steers in each study. Healthy calves were initially screened to identify a population with acceptably low serum Mh antibody titers (whole-cell antibody titers ≤ 1024). Serum Mh antibody status was further categorized as either negative (no serum Mh antibodies) or positive (a serum Mh antibody status of > 0 and ≤ 1024). Among the cohort that met the acceptable serum Mh antibody level, a negative test (immunohistochemistry on skin tissue) for persistent infection (PI) with bovine viral diarrhea virus (BVDV) was further required for inclusion in the study. Screening for other BRD viral or bacterial pathogens was not performed. Cattle not meeting the Mh antibody or PI-BVDV status, and/or displaying clinical signs of infectious/non-infectious disease syndromes, were excluded from enrollment in the study. An overview of the treatment groups in both studies is displayed in Table 1a.

Among the original candidate populations, 64 and 80 calves in Studies 1 and 2, respectively, met these criteria and were tentatively qualified for the study. Upon arrival to the study site and prior to enrollment, all animals were required to be healthy based upon the predefined BRD health criteria outlined in Table 2. All animals were to be free of non-infectious syndromes at the time of arrival at the study site. The study site's Investigational Animal Care and Use Committee (IACUC) approved of the study.

Animal Management and Housing

Calves were housed in 1 of 2 fully enclosed pens that shared a common air space for both studies. Treatment groups were commingled within pens. Pens were constructed of steel pipe fences and gates along with textured concrete floors to minimize the risk of slippage. All pens measured 50 feet (15.24 meters) wide by 60 feet (18.29 meters) long. Each pen contained 5 automatic water tanks and 50 feet (15.24 meters) of bunk space. The room was ventilated with continuous flow, negative-pressure fans.

Upon arrival and for the duration of the study, animals were provided clean water *ad libitum* and fed in accordance with standard industry methods for Holstein calves. Aside from receiving either ZEL or the negative control (CON), no additional products were administered to the study animals.

Randomization

Cattle meeting inclusion criteria were randomized to treatment groups and pens on day -1 in both studies. Briefly, calves were stratified by source (i.e. ranch of origin) and Mh antibody status (i.e. Mh antibody-negative or positive). Random numbers (between 0 and 1) were generated for each calf within each source and antibody status. The allocation of calves to pen and treatment group was then dependent on the number of treatments in the study. For example, in Study 1, the smallest 2 random numbers were assigned to pen 1 and the smallest to largest random number assigned to treatment groups 1 and 2, respectively. Then the calves with the next-smallest random numbers were assigned to pen 2, and so on. This process was repeated until all calves were randomly allocated to 1 of the 2 pens and 1 of the 2 treatment groups. This randomization procedure attempted to evenly distribute both source and antibody titer status as evenly as possible across pens and treatment groups.

Product Preparation and Administration

On study days 0 (Study 1) and 1 (Study 2), the study population received its predetermined treatment of ZEL or the CON. ZEL was prepared for administration according to the package insert by simply rehydrating the lyophilized cake and then re-suspending the mixture in a final proprietary stabilizing diluent. All ZEL injections were administered at the USDA-approved minimum protective dose, intramuscularly in the neck, and in a 2 mL volume. The proprietary stabilizing diluent acted as the negative control (CON) treatment in these studies.

Study Days -1 and 0 Procedures

Clinical observations, depression, respiratory scores, and rectal temperatures of enrolled animals occurred on both days -1 and 0 (prior to the Mh challenge on day 0). Animals were observed, clinically scored, and rectal temperatures collected at approximately the same time each day (± 3 hours). Body weight was collected on all enrolled calves on day -1. A summary of the clinical scoring system is displayed in Table 2. All rectal temperatures were collected by a digital thermometer calibrated prior to the study.

Study Day 0: Mannheimia haemolytica Challenge

The Mh challenge bacteria used in both studies consisted of an isolate obtained from the lung tissue of a feedlot calf that died acutely of BRD in 2007. The calf had not been treated with antimicrobials at the time of death. The Mh isolate was well characterized based on the morphological characteristics and biochemical tests. Antimicrobial susceptibility testing was performed using the bovine/porcine minimum inhibitory concentration (MIC) format (BOPO6F) in accordance with the manufacturer's instructions and Clinical and Laboratory Standards Institute guidelines.³⁷ The antibacterial agents included in the selected plate format

Table 1a. Studies 1 and 2 were designed as clinical trials to evaluate the efficacy of ZEL* to mitigate lung lesions in calves when administered simultaneously with (Study 1) and 24 hours after (Study 2) a *Mannheimia haemolytica* (Mh) intratracheal challenge. The table below displays the respective number of calves in each treatment group across both studies, the day of treatment administration, and their corresponding body weights on day 0. The Mh challenge was performed on day 0 in both studies. The CON† consisted of the proprietary stabilizing diluent used to dilute the product after reconstitution.

Study	Treatment	n	Gender	Day of treatment administration	Study duration	Calf age
Study 1	ZEL	32	Steers	Day 0	5 days	3-4 months
	CON	32				
Study 2	ZEL	40		Day 1		
	CON	40				

*ZEL = Zelnate®, Bayer Animal Health, Shawnee Mission, KS

†CON = control

Table 1b. Study 3 was designed to determine if the local inflammatory response at the injection site was consistent with the expected physiological/immunological response to foreign material.

Treatment number	Treatment	Side of neck	n	Gender	Day of treatment administration	Study duration	Calf age
1	ZEL*	Right	5	Steers	Day 0	21 days	3-4 months
	CON†	Left	5				
2	ZEL	Left	5				
	CON	Right	5				
3	ZEL	Right	5			28 days	
	CON	Left	5				
4	ZEL	Left	5				
	CON	Right	5				

*ZEL = Zelnate®, Bayer Animal Health, Shawnee Mission, KS

†CON = control

Table 1c. Study 4 was designed to determine if safety concerns became evident upon observing calves administered ZEL* for an extended period post-injection.

Study site (state)	Breed	n	Gender	Day of ZEL treatment administration	Study duration	Calf age
IN	Holstein	212	Steers & heifers	Day 0	21 days	3 months
MO	Beef-cross	200	Steers			6 months
NE	Beef-cross	200	Steers			> 6 months

*ZEL = Zelnate®, Bayer Animal Health, Shawnee Mission, KS

were ampicillin, ceftiofur, chlortetracycline, clindamycin, danofloxacin, enrofloxacin, florfenicol, gentamicin, neomycin, oxytetracycline, penicillin, spectinomycin, sulfadimethoxine, tiamulin, tilmicosin, trimethoprim-sulfamethoxazole, and tulathromycin. Although neomycin is not labeled for treating cattle for BRD, it was included for quality control purposes. *Mannheimia haemolytica* isolate was grown on 5% sheep blood agar^b and incubated overnight at 95 to 98.6 °F (35 to 37 °C). Several isolated colonies were suspended in trypticase soy broth or demineralized water, and the suspension was adjusted to approximate a 0.5 McFarland turbidity standard. A 10 µl volume of the adjusted suspension was then transferred into 10 mL of Mueller-Hinton broth to give a final inoculum density of approximately 5 × 10⁵ colony-forming units/mL in the wells of the broth microdilution trays. The inoculums

(50 µl) were dispensed into each well of the 96-well plate.^c The plates were sealed and incubated at 95 to 98.6 °F (35 to 37 °C) for 18 to 24 hr. The MIC value was defined as the lowest antimicrobial concentration that resulted in no growth of the isolate. The MIC results were interpreted according to the breakpoints of the Clinical and Laboratory Standards Institute guidelines.³⁷ The antibiogram for this Mh isolate is displayed in Table 3.

Prior to the day of challenge, 1 vial of Mh culture was removed from the ultra-low freezer and thawed in a cold water bath for 5 minutes. A loop of bacteria was streaked onto 5% sheep blood agar plates and incubated at 98.6 °F (37 °C) overnight in a 5% CO₂ incubator. On the following day, the purity of the culture was evaluated based on the colony morphology, gram staining, and biochemical tests.

Table 2. Clinical scoring system used to evaluate calves in studies 1 through 4. To be eligible for enrollment, calves had to display a depression and respiration score = 0. The case definition of BRD was defined as a depression score = 1 or 2 OR a respiratory score = 2 OR a respiratory or depression score = 3. Calves meeting the case definition for BRD prior to or on study day -1 were not enrolled in the study.

Score	Depression	Respiration
0	Bright, alert, and responsive	No abnormal respiratory symptoms. Respiratory rate and effort are appropriate for the environment.
1	May stand isolated with its head down or ears drooping, but will quickly respond to minimal stimulation.	Serous nasal or ocular discharge and/or cough.
2	May stand isolated with its head down and may show signs of muscle weakness (standing cross-legged or knuckling when walking). Shows a delayed response to minimal stimulation or requires greater stimulation before showing a response.	Mucous or mucopurulent nasal or ocular discharge and/or increase in respiratory rate or effort.
3	May be recumbent and reluctant to rise, or if standing isolated, may be reluctant to move. Ataxia, knuckling or swaying may be evident when moving. Head carried low with eyes dull and ears drooping. Possible excess salivation and/or lacrimation.	Marked increase in respiratory rate or effort, with one or more of the following: open mouth breathing, abdominal breathing and/or extended head.

Bacteria were transferred from the blood agar plate to 10 mL of pre-warmed tryptic soy broth (TSB); a uniform suspension was prepared by pipetting thoroughly. The 10 mL uniform suspension was then transferred to 4000 mL of pre-warmed TSB and the culture incubated at 98.6 °F (37 °C) on a rotary shaker at approximately 100 rpm for 8 hours.

The bacterial culture was centrifuged in 250 mL tubes at 3500 rpm in a refrigerated centrifuge for approximately 10 minutes. The supernatants were discarded and the pellet was washed 3 times with phosphate buffered saline. The final pellet was dissolved in 500 mL of PBS. The optical density (OD) was calculated using the spectrophotometer.⁴ The OD of the culture was 1.32 at 560nm.

A total of 11 liters of challenge material with a target of 10^6 to 10^8 CFUs/mL was prepared in tryptic soy broth. Pre- and post-challenge aliquots were prepared and retained in the laboratory for enumerating the bacterial counts. The remaining challenge material was maintained on ice until the time of challenge.

Table 3. Antibigram and Clinical and Laboratory Standards Institute breakpoint interpretation† for the *Mannheimia haemolytica* isolate used as the challenge organism in Studies 1 and 2.

Antibiotic name	MIC (µg/mL)	Sensitivity
Ceftiofur	<0.25	Susceptible
Tiamulin	8	No interpretation*
Chlortetracycline	<2	Susceptible
Gentamicin	>16	No interpretation
Florfenicol	8	Resistant
Oxytetracycline	>8	Resistant
Penicillin	<0.12	Susceptible
Ampicillin	<0.25	No interpretation
Danofloxacin	<0.12	Susceptible
Sulphadimethoxine	>256	No interpretation
Neomycin	32	No interpretation
Trimethoprim/ Sulfamethoxazole	<2/38	No interpretation
Spectinomycin	>64	Resistant
Tylosin tartrate	>32	No interpretation
Tulathromycin	32	Intermediate
Tilmicosin	32	Resistant
Clindamycin	>16	No interpretation
Enrofloxacin	<0.12	Susceptible

*No interpretation indicates no CLSI breakpoints are available for that respective antibiotic.

†Watts JL, Shryock TR, Apley M. Performance standards for antimicrobial disk and dilution susceptibility tests for bacteria isolated from animals; Second Informational Supplement. *Clinical Laboratory Standards Institute*. 2013.

At the time of challenge, all calves were administered an intratracheal volume of 60 mL. Briefly, each calf was individually challenged with Mh while restrained, yet unsedated, in a hydraulic chute. An endoscope was passed through the nares of the respective calf to the point of the tracheal bifurcation. Sixty milliliters of the Mh inoculum was then administered through the endoscope, carefully distributing the inoculum evenly between both lung fields. This was then followed by injecting 60 mL of air through the endoscope. Across both studies, bacteria within the challenge inoculum were enumerated at 3 different time points: pre-challenge, mid-challenge, and post-challenge.

Study Days 1 to 5 Procedures

Clinical observations (depression, respiratory scores, and rectal temperatures) of animals occurred daily from days 1 through 5. Calves that died prior to day 5 were weighed prior to necropsy. Lung scores were collected on all calves dying prior to day 5.

Study Day 5 Procedures

A body weight was collected on all calves on day 5. Euthanasia was performed by an initial overdose of pentobarbital followed by a captive bolt to the cranium and subse-

quent exsanguination. Necropsies were then performed on all calves, but focused solely on assessment of the respiratory tract. Individual lung lobe scores were determined by blinded study personnel at the time of necropsy, which was the time of premature death or on day 5. A lung lesion estimate for each individual animal was calculated in the manner outlined in Table 4.¹⁶ Briefly, each lobe was examined by a blinded and experienced diagnostician using visual and tactile parameters to estimate the volumetric percentage of consolidation within each lung lobe. The composite estimation of lung pathology was then calculated by multiplying the estimated percentage of pathologic involvement of each lobe by an adjustment factor which reflects that individual lobe's proportion of overall lung tissue, thus resulting in a "weighted average" of lung consolidation. Lung tissue samples were collected from each individual calf in whirl-pack bags, labeled, and kept cooled on ice until being submitted to the Midwest Veterinary Services diagnostic laboratory. The lung tissue samples were processed in the laboratory by following the laboratory standardized procedures. A section of the lung sample where the pneumonic lesions were prominent was seared under the biosafety hood using a heated spatula. A sterile scalpel blade was used to cut the surface of the lung tissue, and a sterile swab was stab-inserted into the cut surface. The swabs were used to inoculate the blood, chocolate, and MacConkey agar plates which were incubated at 98.6 °F (37 °C) in a 5% CO₂ incubator for 24 h. The bacteria were identified based on the colony morphology on the plates, gram staining, and biochemical tests (indole, catalase, and oxidase).

Blinding/Masking

All study personnel involved in clinical scoring and lung lesion measurement were masked to study treatment. The treatment dispenser and any study personnel witnessing or administering treatments or allocating cattle to specific pens were not masked, and were the only study personnel with access to the randomization schedule or the ZEL used in this study. Unmasked personnel were not involved in clinical observations, including recording of those observations, measurement, or recording of the lung lesion scores.

Study 3 (Injection Site Safety Study)

Study Population

Study 3 was designed per Veterinary Services (VS) Memorandum No. 800.51.³⁴ The inclusion criteria for this study required that eligible calves be healthy on the day of arrival. It was required that all enrolled calves be 3-to-4 months of age. No gender or breed restrictions were placed on this study population. Any calf that exhibited clinical signs of infectious or non-infectious disease on day 0, the day of treatment administration, was excluded from the study population. The final study population was composed of 20 Holstein bulls that were 3-to-4 months of age at the time of enrollment. An overview of treatment groups is provided in Table 1b.

Table 4. Lung lesion scoring system used to evaluate the extent of lung lesions in calves in Studies 1 and 2. The proportion of lung pathology in each individual lung lobe was estimated and then multiplied by an adjustment factor which reflects that individual lobe's volume proportion. The overall lung lesion score for each individual calf constitutes the summation of all lobe adjusted estimates.

% lung lobe consolidation		Adjustment factor
% left apical lobe consolidation	x	0.05
+		
% left cardiac lobe consolidation	x	0.06
+		
% left diaphragmatic lobe consolidation	x	0.32
+		
% right apical lobe consolidation	x	0.06
+		
% right cardiac lobe consolidation	x	0.07
+		
% right diaphragmatic lobe consolidation	x	0.35
+		
% accessory lobe consolidation	x	0.05
+		
% intermediate lobe consolidation	x	0.04
Overall lung lesion score		

Animal Management and Housing

Animal housing and management in Study 3 was identical to that in Studies 1 and 2 with the exception that calves from all treatment groups shared 1 large pen.

Study Days -5 to -1 Procedures

Animals arrived to the study site during this time frame. Animals were observed for any health concerns at approximately the same time each day (± 3 hours). On day -1, the rectal temperature was collected from each calf and the injection site was shaved on either side of each calf's neck. Any pre-existing lesions on the calf's neck were documented by study personnel.

Study Day 0 Procedures

All calves were clinically observed to ensure they were in acceptable health. Prior to treatment administration, a rectal temperature was recorded and each injection site was palpated in every animal. Both treatments were then administered to each calf in the predetermined side of the neck. Two hours post-administration, all calves were clinically observed and rectal temperatures were once again measured.

Study Days 1 to 7 Procedures

During this time frame, all calves were clinically observed for adverse health issues. Rectal temperatures were collected from all calves on each of these 7 days post-administration. The injection site on both sides of each calf's neck was palpated to assess for any swelling.

Study Days 8 to 28 Procedures

General health observations continued for the entirety of the study. If injection site lesions were observed during study days 1 to 7, daily palpation of those respective animals continued until they resolved. No rectal temperatures were collected during this time frame.

The method of euthanasia in Study 3 was performed in the same manner as described in Studies 1 and 2. Necropsies on calves allocated to treatment groups 1 and 2 occurred on day 21, while necropsies among cattle in treatment groups 3 and 4 occurred on day 28. Tissue sections were harvested from both sides of each calf's neck (corresponding to the site of injection of the either ZEL or CON) and evaluated for evidence of gross pathology. Tissue specimens were then placed in formalin for histopathology and examination by a board-certified veterinary pathologist. Tissue specimens from both days 21 and 28 were shipped to the veterinary pathologist on day 28 so that all specimens were reviewed at the same time.

Randomization

On day 0 of Study 3, cattle meeting inclusion criteria were randomized to 1 of 4 treatment groups (Table 1b). All randomizations utilized a unique pre-designed randomization table. One random number was generated for each qualified animal. The smallest 4 numbers were assigned to treatments 1 to 4. Then the next smallest 4 numbers were assigned to treatments 1 to 4 until all 20 calves were randomized to treatment groups.

Preparation and Administration of the DNA Immunostimulant

The preparation of ZEL in Study 3 was identical to that in Studies 1 and 2. In Study 3, administration of both treatments was performed in a similar fashion to Studies 1 and 2 (intramuscular and in 2 mL volumes). However, per the guidelines of VS Memorandum 800.51, each calf in Study 3 served as its own control.³⁴ Briefly, each calf received both ZEL and the CON treatment in either the right or left side of the neck (Table 1b).

Blinding/Masking

The treatment administrator and study participants responsible for generating the treatment allotment were separated from study participants responsible for daily general health observations, clinical observations, injection site observations, rectal temperatures, and necropsy to maintain blinding. The pathologist examining histopathologic samples was also blinded to treatment groups.

Study 4 (Field Safety Study)

Study Population

Study 4 was designed per the recommended guidance outlined in VS Memorandum 800.204.³⁵ This study was conducted at 3 independent sites: a Holstein calf growing facility (Indiana), a cow-calf operation (Missouri), and a commercial

feedlot (Nebraska). At each site, the supervising veterinarian/investigator enrolled only healthy, post-weaned, commercial cattle. The study population (n = 612) was composed of calves ranging from the minimum age of 3 months (totaling approximately one-third of the study population; n = 212) to > 6 months. Both genders were evenly represented in this study population. An overview of treatment groups is displayed in Table 1c. Note that negative controls were not incorporated into this study per VS Memorandum 800.204.³⁵

Animal Management and Housing

At all study sites, calves were commingled and housed according to the normal husbandry practice of the respective site.

Study Days -14 to -1 Procedures

Upon animal arrival, calves were clinically observed for signs of infectious or non-infectious disease.

Study Day 0 Procedures

On day 0, calves were clinically observed and necks were palpated prior to administration of ZEL to each calf. ZEL was then administered to all calves.

Study Day 3 Procedures

The injection site was palpated on all calves. No further palpation was performed on calves that exhibited no evidence of injection site swelling on day 3. Calves with palpable injection site lesions on day 3 were further assessed on days 7 and 14.

Study Days 1 to 21 Procedures

All calves were clinically observed for any adverse events throughout this time frame. The study concluded on day 21.

Randomization

In accordance with VS Memorandum 800.204, no randomization was utilized in this study.³⁵

Preparation and administration of the DNA Immunostimulant

The preparation of ZEL in Study 4 was identical to that in Studies 1 through 3. Each calf was administered ZEL intramuscularly, in either the right or left side of the neck (Table 1c).

Blinding/Masking

Maintaining blinded study personnel was not necessary for this study.

Statistical Methods

Data were entered into a commercial software package^e and descriptive statistics were calculated in all studies. Inferential statistics were performed in Studies 1 and 2. Calves living to day 5, as well as calves that died prematurely, were

included in the respective data sets. The experimental unit was the calf in all analyses. An alpha of 0.05 was considered significant for all analyses.

In Studies 1 and 2, non-parametric analyses (mitigated fractions) were performed on lung lesion data. Mitigated fractions and 95% confidence intervals (2.5 and 97.5 percentiles) were generated by a commercial software program.^f

Mixed models were employed to evaluate the remaining continuous outcome, rectal temperatures. A generalized linear mixed model (GLMM) was used to evaluate categorical outcome variables, morbidity and mortality. In these GLMMs, a binomial distribution was assumed and a logit link was used. Mixed models and GLMMs were analyzed by a commercial software program.^g

Results

All calves included in the final sample populations for all studies were healthy on day 0. The breakdown of calf numbers and their respective genders and ages on day 0 for each treatment group, within each study, is provided in Tables 1a-c.

Study 1 (Efficacy)

On day 0, all calves were challenged with 60 mL (10^7 CFUs/mL, Study 1) of the Mh inoculum simultaneously with the respective treatment. The Mh inoculum CFU counts stayed consistent throughout the pre-challenge to post-challenge time frame. Two calves died prior to day 5 (peritonitis (n = 1; ZEL), severe BRD (n = 1; CON)). Because of the low cumulative incidence of mortality (1.25%), and the lack of variability within the morbidity parameter, further inferential analyses between treatment groups were not performed for these 2 specific outcome variables in Study 1. By day 5, 100% of the study population met the case definition of BRD.

No significant findings ($P > 0.05$) were observed with regard to BRD morbidity, rectal temperature, or ADG between treatment groups.

ZEL was observed to mitigate lung pathology by 40.3% compared to the negative control (95% confidence interval (CI); 13.9%, 65.3%). These findings are provided in Table 5a. Lung cultures revealed that 43.8% (n = 14) and 56.3% (n = 18) of calves within the ZEL and CON groups, respectively, were positive for Mh at the time of lung collection and scoring.

Study 2 (Efficacy)

On day 0, all calves were challenged with 60 mL of the Mh inoculum (10^8 CFUs/mL). On day 1, all calves received their predetermined treatment. Nine calves (11.3% of the study population; ZEL: (n = 1), CON: (n = 8)) died prior to day 5. All mortalities were diagnosed as fibrinous bronchopneumonia. Cattle administered ZEL on day 1 displayed a significant reduction in mortality compared to cattle receiving the negative control on day 1 ($P = 0.0394$; Figure 1).

By day 5, 93% (n = 37) of remaining calves in each treatment group had been classified as morbid based upon

the case definition of BRD. No significant differences were observed across treatment groups for BRD morbidity, rectal temperature, or ADG ($P > 0.05$).

ZEL treatment mitigated lung pathology by 36% compared to CON (95% CI; 3%, 64%; Table 5b). Lung cultures revealed that 90% (n = 36) and 97.5% (n = 39) of calves within the ZEL and CON groups, respectively, were positive for Mh at the time of lung collection and scoring.

Study 3 (Injection Site Safety Study)

On day 0, all calves were administered both ZEL and the negative control (on opposite sides of the neck) by intramuscular injection and in 2 mL volumes. The average rectal temperature across the study population, pre-administration, was 101.7 °F (38.7 °C). The average rectal temperature across the study population 2 hours post-administration was 102.1 °F (38.4 °C). The average rectal temperature from days 1 through 7 were 102.2 °F (39 °C), 101.8 °F (38.8 °C), 101.6 °F (38.7 °C), 101.6 °F (38.7 °C), 101.5 °F (38.6 °C), 101.7 °F (38.7 °C), and 101.5 °F (38.6 °C), respectively.

No observable changes in clinical signs were noted immediately after administration of the ZEL on day 0. Indeed, no clinical signs were observed across the study population from days 0 to 7. Additionally, no injection site lesions were palpated from days 0 to 7. From day 8 to the predetermined necropsy day (day 21 or 28), all general health observations were normal.

Of the 40 tissue sections (2 tissue specimens, 1 on each side of the neck, for each of the 20 enrolled calves) collected, no gross lesions were observed at the time of tissue harvest. The veterinary pathologist reported that some (n = 15) tissue specimens from individual calves displayed skeletal muscle with very small areas exhibiting a white refractile appearance.

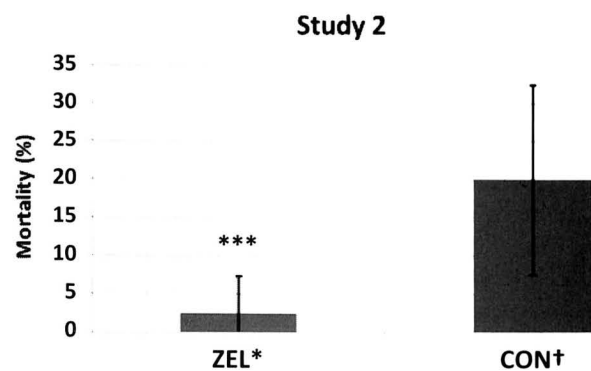


Figure 1. Study 2 mortality outcomes among calves receiving either ZEL* (n = 40) or CON† (n = 40) 24 hours after a *Mannheimia haemolytica* intratracheal challenge. Calves were followed for 5 days post-challenge; therefore, all deaths occurred during that time frame. Error bars denote 95% confidence intervals.

*ZEL = Zelnate®, Bayer Animal Health, Shawnee Mission, KS

†CON = control

***Denotes a statistical difference of $P < 0.05$

Table 5a. Study 1: Mitigated fraction estimates (and confidence intervals) among calves receiving either ZEL* (n = 32) or CON† (n = 32) simultaneously with an intratracheal challenge with *Mannheimia haemolytica* (Mh). Lung lesion estimates were collected at necropsy at the time of premature death or after euthanasia 5 days after Mh challenge.‡

Treatment group	n	Mitigated fraction	95% confidence intervals	
			Lower bound	Upper bound
ZEL	32	40.3%	13.9%	65.3%
CON	32	NA	NA	NA

*ZEL = Zelnate®, Bayer Animal Health, Shawnee Mission, KS

†CON = control

‡Note: Only lung lesion data from Study 1 are reflected in the current Zelnate® label.

Table 5b. Study 2: Mitigated fraction estimates (and confidence intervals) among calves receiving either ZEL* (n = 40) or CON† (n = 40) 24 hours after an intratracheal challenge with *Mannheimia haemolytica* (Mh). Lung lesion estimates were collected at the time of premature death or 5 days after Mh challenge.

Treatment group	n	Mitigated fraction	95% confidence intervals	
			Lower bound	Upper bound
ZEL	40	36%	3%	64%
CON	40	NA	NA	NA

*ZEL = Zelnate®, Bayer Animal Health, Shawnee Mission, KS

†CON = control

Of these 15 samples, 9 were associated with ZEL administration (5 samples collected on day 21 and 4 samples collected on day 28) and 6 were associated with the control product (3 samples collected on day 21 and 3 samples collected on day 28). However, despite these findings, the veterinary pathologist deemed these observations to be not relevant. Among the remaining tissue samples (n = 25), the pathologist did not observe any gross lesions. Further histopathologic examination of the tissue specimens revealed no evidence of significant microscopic findings, such as necrosis, inflammation, and fibrosis, across all study animals on either days 21 or 28.

Study 4 (Field Safety Study)

On day 0, healthy calves were administered a 2 mL dose of ZEL intramuscularly in the neck. No injection site lesions were observed on day 3 among calves across all study sites. The cumulative incidence of mortality across study sites was 0.3% (n = 2), and was observed in the population of calves comprising the feedlot segment of the study. Necropsy findings revealed severe tracheal edema and fibrinous bronchopneumonia in the 2 calves, respectively. The cumulative incidence of morbidity across study sites was 8% (n = 49). However, the majority of morbidity was observed among the younger segment of the study population (i.e., 3-month-old Holstein calves; n = 32) and was attributed to BRD (14.6%;

n = 31). All Investigators provided affidavits stating that the level of morbidity and mortality was not uncommon for each respective class of cattle, and that all morbidity and mortality was not likely related to the administration of ZEL. Additionally, all investigators provided statements indicating that ZEL was safe for use in these respective classes of cattle.

Discussion

The overall objectives of this series of studies were to: 1) determine if ZEL was efficacious in a Mh disease model (Studies 1 and 2); 2) determine the safety of ZEL at the site of injection in the target population (Study 3); and 3) determine if ZEL is safe when administered under field conditions to cattle comprising different populations and production classes. The results of these studies indicate that ZEL is both efficacious and safe when administered to cattle.

As previously discussed, stressful events compromise the pulmonary immune status of animals, thereby allowing commensal bacteria of the upper respiratory tract (predominately Mh) to infect the lower respiratory system and cause BRD.^{1,18,19} In Studies 1 and 2, stress and the subsequent bacterial infection of the lung tissue were simulated by transport of the calves from Indiana to the study site, and subsequently challenging each calf with a large concentration and volume of Mh. This challenge system was observed to be highly pathogenic given the level of clinical illness observed during Studies 1 and 2 and the degree of lung pathology in the CON groups. Despite the severity of the Mh challenge, ZEL significantly mitigated lung pathology when administered concurrently with, or 24 hours after, a Mh challenge (Studies 1 and 2, respectively). Given prior data that demonstrate the negative impact of lung lesions on feedlot performance,^{5,11,27,28,32,39} the findings from both studies are noteworthy as they provide insight into the potential advantage that ZEL may afford to managing BRD among populations of calves experiencing stressful situations.

In Study 2, the Mh concentration in the challenge inoculum was increased by 1 log compared to Study 1 (10^7 CFU/mL and 10^8 CFU/mL in Studies 1 and 2, respectively). As expected, the degree of lung pathology observed in the CON group was elevated, likely resulting in increased mortality during the 5-day study. Despite the elevated disease pressure and the time lag in administration of ZEL relative to the Mh challenge (ZEL administration 24 hours after Mh challenge), the use of ZEL significantly reduced the risk of BRD mortality compared to CON. Findings from Study 2 are relevant, as prior data evaluating the economic impact of lung lesions at slaughter do not reflect the risk of mortality. Although we do not know the threshold of lung pathology a calf can endure prior to death, in Study 2 the observed average lung lesion estimates among calves dying up through day 5 with the CON and ZEL-treated groups was 55.3% and 17.6%, respectively (data not shown). The effect of ZEL to preserve viable lung tissue may increase the likelihood of survival, minimize

performance loss, and maximize the economic value of an animal affected with BRD.

In Study 2, ZEL was administered 24 hours after Mh challenge. At this time, 72.5% (n = 29) of calves within the sample population met the case definition for BRD (data not shown), indicating that ZEL was administered to the population in the face of clinical disease. Therefore, ZEL administration was not only associated with a significant reduction in the extent of lung lesions when given prior to infection (Study 1), but also when it was given in the midst of clinical BRD (Study 2). Additionally, like lung lesions, mortality was also significantly decreased when calves were treated in the face of active disease.

Without a primary viral insult, a large concentration of Mh is necessary to induce BRD and subsequent lung pathology. The sequelae to this challenge system is a relatively acute endotoxemic insult that manifests in severe depression, anorexia, and pyrexia. In both studies, nearly all animals met the BRD case definition at least once post-challenge. Therefore, given the severity of this direct-challenge Mh disease model, which was necessary to ensure adequate lung pathology and consistent manifestation of clinical disease across both study populations (in lieu of the remaining components of the BRD complex), it is not surprising that differences were not observed among the remaining of outcome parameters, such as BRD morbidity, rectal temperature, and ADG. Future field studies will be helpful to address the effect of ZEL on these parameters in field scenarios.

It is notable that the significant reduction of lung lesions (Studies 1 and 2) and mortality (Study 2) among ZEL-treated cattle was observed in a test system in which the Mh isolate is known to be multi-drug resistant (florfenicol, spectinomycin, and tilmicosin; Table 3). Additionally, this Mh isolate also displayed intermediate sensitivity to tulathromycin. These observations may suggest that the efficacy afforded by the innate immune system is independent of the antimicrobial resistance status of the bacterium. Further data is necessary to clarify the effect of antimicrobial resistance on the efficacy of an innate immune response.

The exact mechanism by which ZEL works to mitigate lung pathology and mortality following Mh challenge is currently under evaluation. Similar components of ZEL have been shown to activate immune responses in cattle and other animals.^{3,9,20,24,25,38} In general, ZEL is expected to be protective against intracellular infections (e.g. viruses or intracellular bacteria).²⁵ However, because Mh is an extracellular bacterial pathogen, it may be that ZEL exerts more than 1 mechanism of action that contributes to the protective effect against Mh challenge. Further research is necessary to characterize ZEL's mechanism of action in cattle exposed to experimental or natural infectious challenge.

A potential limitation of this series of studies was the decision to exclude a non-challenged/non-treated sentinel group within the sample populations of Studies 1 and 2. The potential value of this group may be to estimate the potency

of the Mh challenge and to estimate the prevalence of pre-existing lung pathology within the sample population. These assessments were indeed performed while developing the Mh model (data not shown). Given the clinical outcomes observed in Studies 1 and 2 (as described above), the authors were comfortable with regard to the potency of the challenge. Additionally, it can be assumed that the randomization process evenly distributed any unmeasured parameters that may cause bias, like pre-existing lung pathology, evenly across treatment groups. Finally, given the terminal outcome of all enrolled calves, it was the authors' intention to minimize the number of animals that were used in the development of this product.

Aside from ruling out PI with BVDV, no additional screening for BRD pathogens took place during Studies 1 and 2. The development of ZEL was targeted at BRD, but more specifically, towards the most prevalent BRD bacterial pathogen, *Mannheimia haemolytica*. Despite the need for evaluating efficacy within an artificial challenge, the product will be used in field disease scenarios, therefore it was necessary to attempt to maintain a level of "real-life" practices within the development program to optimize external validity. It is understood that numerous viral and bacterial agents are present in most cohorts; nonetheless, aside from some BVDV testing, practicing veterinarians and producers don't routinely screen incoming cohorts for BRD pathogens. It is assumed that the randomization process evenly distributed any pre-existing BRD pathogens evenly across the sample population. Collectively, by allowing this potential degree of variability to enter into the model while still observing a difference in the primary outcome variable (lung lesions) provides encouragement that this product may have field applicability.

Despite the efficacy of ZEL in Studies 1 and 2, it was important to also address the first principle of veterinary medicine; namely, that treatment should not impose harm on an animal. Therefore, animal safety was comprehensively assessed. Studies 3 and 4 indicate that ZEL is safe for cattle as there were neither observations of injection site lesions nor gross display of adverse events attributed to the administration in field scenarios. Additionally, Study 3 indicates that beef carcass quality appears to be maintained despite the intramuscular route of administration.

Conclusion

In summary, ZEL was observed to mitigate the lung lesions resulting from experimental Mh challenge when administered concurrently with (Study 1) or 24 hours after challenge with Mh in the face of clinical disease. Additionally, a significant reduction in mortality was observed in Study 2 among cattle administered ZEL relative to the CON group. These findings are relevant given the high incidence of BRD in the beef and dairy sectors, its negative impact on the economic returns in those respective businesses, and

ongoing efforts to identify additional preventive and treatment modalities for BRD.

Endnotes

- ^aZelnate[®], Bayer Animal Health, Shawnee Mission, KS
^bRemel Inc., Lenexa, KS
^cSensititre[®], Trek Diagnostic Systems, Cleveland, OH
^dSpectronic 21, Bausch and Lomb M# 33-22-02
^eMicrosoft[®] Excel 2010, Microsoft Corp., Redmond, WA
^fR Software 2.15.2, The R Foundation for Statistical Computing, 2012
^gSAS version 9.3, SAS Institute Inc., Cary, NC

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