Virginiamycin: Lack of interference with *Lawsonia intracellularis* immunization

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Summary

Objective: To determine if concurrent administration of virginiamycin interferes with immunization efficacy of Enterisol Ileitis (Boehringer Ingelheim Vetmedica, Inc, St Joseph, Missouri).

Materials and methods: Eighty-eight weanling pigs were divided into four groups: a strict negative, non-challenged, non-vaccinated, non-medicated group (NCC), a challenged, non-vaccinated, non-medicated group (CC), a challenged, vaccinated, non-medicated group (VC), and a challenged, vaccinated, medicated group (VM). The VM group received in-feed virginiamycin at 11 g per tonne from Day 0 (4 weeks of age) through Day 27. Vaccinated groups were immunized with one dose of Enterisol Ileitis on Day 1. Virginiamycin administration continued for 28 days to determine whether it interfered with development of immunity generated by the vaccine. Challenged groups were administered an oral dose of mucosal homogenate containing virulent *Lawsonia intracellularis* (LI) on Day 31. Pigs were euthanized and necropsied on days 51 and 52 to evaluate intestinal lesions.

Results: Significantly fewer macroscopic jejunal ($P < .01$) and ileal ($P < .05$) lesions were observed in VC and VM groups than in the CC group. Microscopic ileal immunohistochemistry scores in VC and VM did not differ, and both were significantly lower ($P < .05$) than in the CC group. During the vaccination phase when virginiamycin was administered, feed efficiency was best in VM ($P < .001$) and intermediate in VC ($P < .001$), compared to CC.

Implications: Under the conditions of this study, administration of in-feed virginiamycin at 11 g per tonne does not interfere with the efficacy of concurrent immunization with Enterisol Ileitis.

Keywords: swine, virginiamycin, *Lawsonia*, immunization, interference

Received: November 1, 2012
Accepted: January 2, 2013

Resumen - Virginiamicina: Falta de interferencia con la inmunización de *Lawsonia intracellularis*

Objetivo: Determinar si la administración actual de virginiamicina interfiere con la eficacia de la inmunización de Enterisol Ileitis (Boehringer Ingelheim Vetmedica, Inc, St Joseph, Missouri).

Materiales y métodos: Se dividieron ochenta y ocho cerdos recién destetados en cuatro grupos: un grupo negativo estricto, no probado, no vacunado, no medicado (NCC por sus siglas en inglés); un grupo probado, no vacunado, no medicado (CC por sus siglas en inglés); un grupo probado, vacunado, no medicado (VC por sus siglas en inglés); y un grupo probado, vacunado, medicado (VM por sus siglas en inglés). El grupo VM recibió virginiamicina a 11 g por tonelada del Día 0 (4 semanas de edad) al Día 27. Los grupos vacunados fueron inmunizados con una dosis de Enterisol Ileitis en el Día 1. La administración de virginiamicina continuo hasta el día 28 para determinar si la virginiamicina interfiere con el desarrollo de la inmunidad generado por la vacuna. A los grupos retados se les administró una dosis oral de una homogeneizado que contenía *Lawsonia intracellularis* (LI) virulenta el Día 31. Los cerdos fueron sacrificaos y se les practicó la necropsia el día 51 y 52 para evaluar las lesiones intestinales.

Resultados: Se observaron menos lesiones significativas en el yeyuno ($P < .01$) e íleon ($P < .05$) en los grupos VC y VM que en los grupos CC. Las lesiones microscópicas de íleon por inmunohistoquímica en VC y VM no fueron diferentes, y ambas fueron significativamente más bajas ($P < .05$) que en el grupo CC. Durante la fase de vacunación cuando la virginiamicina fu administrada, la eficiencia fue mejor en VM ($P < .001$) en intermedia en VC ($P < .001$), comparadas con CC.

Implicaciones: Bajo las condiciones de este estudio, la administración de virginiamicina en alimento a 11 g por tonelada no interviene en la eficiencia de la vacunación concurrente con Enterisol Ileitis.

Résumé - Virginiamycine: Absence d'interférence lors d'immunisation avec *Lawsonia intracellularis*

Objectif: Déterminer si l'administration concomitante de virginiamycine interfère avec l'efficacité d'immunisation d'Enterisol Ileitis (Boehringher Ingelheim Vetmedica, Inc, St-Joseph, Missouri).

Matériels et méthodes: Quatre-vingt-huit porcelets au sevrage ont été séparés en quatre groupes: un groupe non-challengé, non-vacciné, non-médicamenté (NCC); un groupe challengé, non-vacciné, non-médicamenté (CC); un groupe challengé, vacciné, non-médicamenté (VC); et un groupe challengé, vacciné, médicamenté (VM). Le groupe VM a reçu de la virginiamycine dans sa nourriture à une dose de 11 g par tonne du Jour 0 (4 semaines d'âge) au Jour 27. Les groupes vaccinés ont été immunisés avec une dose d'Enterisol Ileitis au Jour 1. L'administration

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This article is available online at http://www.aasv.org/shap.html.

Beckler DC, Segal MU, Weiss DL, et al. Virginiamycin: Lack of interference with *Lawsonia intracellularis* immunization. *J Swine Health Prod.* 2013;21(5):253–260.

de virginiamycine continua pendant 28 jours afin de déterminer si la virginiamycine interférait avec le développement de l'immunité engendrée par le vaccin. Les animaux des groupes challengés reçurent une dose orale d'un homogénat de muqueuse contenant une souche virulente de *Lawsonia intracellularis* (LI) au Jour 31. Les porcs furent euthanasiés et soumis à une nécropsie aux jours 51 et 52 afin d'évaluer les lésions intestinales.

Résultats: On observa significativement moins de lésions macroscopiques au niveau jéjunal ($P < .01$) et iléal ($P < .05$) dans les groupes VC et VM que dans le groupe CC. Les pointages en immunohistochimie au niveau de l'iléon des groupes VC et VM n'étaient pas différents, et les deux étaient significativement inférieurs ($P < .05$) à celui du groupe CC. Pendant la phase de vaccination et que la virginiamycine était administrée, l'efficacité alimentaire était meilleure pour le groupe VM ($P < .001$) et intermédiaire pour le groupe VC ($P < .001$), comparativement au groupe CC.

Implications: Dans les conditions expérimentales de la présente étude, l'administration de virginiamycine dans l'alimentation à une concentration de 11 g par tonne n'interfère pas avec l'efficacité d'une immunisation concomitante avec Enterisol Ileitis.

Porcine proliferative enteropathy (PPE) is a highly prevalent and costly swine disease[1] caused by infection with the obligate intracellular bacterial pathogen *Lawsonia intracellularis* (LI).[2] All forms of PPE can be controlled by a single vaccination with an avirulent live oral LI vaccine (Enterisol Ileitis; Boehringer Ingelheim Vetmedica, Inc, St Joseph, Missouri) that is administered to weaned pigs in the drinking water over a 6-hour period. Previous work has demonstrated the efficacy of this vaccine.[3-6]

Sick et al[3] showed that average daily gain (ADG) was 4.7% higher ($P < .001$) and mortality was numerically lower in vaccinated barns than in unvaccinated barns, using the barn as the statistical unit. Kolb and Sick[6] used a dataset containing more than 46,900 vaccinated pigs and demonstrated higher ADG ($P < .05$) as well as numerically improved cull and mortality rates. Hardge et al[4] demonstrated higher ADG ($P < .05$) and greater feed intake ($P < .05$) in vaccinated pigs, as well as numerically lower mortality. Pejsak et al[5] tested vaccination administration via oral drench and via the drinking water. This work demonstrated that in vaccinated groups the number of pigs that required further therapeutic intervention during the finishing phase was lower ($P < .05$) than in non-vaccinated groups. In vaccinated groups, the number of pigs culled and the number of *Lawsonia*-positive pigs were numerically lower and ADG was numerically higher than in non-vaccinated groups.

Limited replication of the Enterisol Ileitis vaccine bacteria in pig enterocytes is required for production of interferon gamma and stimulation of a protective immune response.[7] In-feed antimicrobials are often used in weaned or growing pigs for treatment, control, or prevention of disease and to improve growth rates and feed-conversion efficiency. It is unknown to what extent the use of various antimicrobials fed concurrently with an avirulent live oral bacterial vaccine may inactivate the avirulent LI cells contained within the vaccine, and thereby prevent production of interferon gamma and interfere with the stimulation of immunity generated by the vaccine. Thus, it has been recommended that medication be removed from feed at least 3 days prior to and 3 days after Enterisol Ileitis vaccination.[6] Previous work has demonstrated that concurrent in-feed administration of colistin sulfate does not interfere with the immunization efficacy of Enterisol Ileitis.[8]

Virginiamycin is a streptogramin antibiotic with excellent gram-positive activity. The antibiotic is composed of two factors, M and S, that function synergistically in control of susceptible bacterial organisms.[9,10] Virginiamycin is used in multiple food-animal species both as a therapeutic agent and to improve feed efficiency and rate of weight gain. As a therapeutic agent, virginiamycin is approved for swine in the United States to aid in control of swine dysentery in animals or on premises with a history of swine dysentery, but where clinical signs of the disease have not yet occurred. In the United States, virginiamycin is approved in broiler chickens for prevention of necrotic enteritis caused by *Clostridium perfringens* susceptible to virginiamycin, and in cattle for reduction of liver abscesses.

Withholding of feed antimicrobials for a peri-vaccination period of at least 7 days is recommended to avoid potential interference with Enterisol Ileitis efficacy. However, it can be difficult for pig production systems to consistently and precisely control the flow of medicated and non-medicated feeds during such narrowly defined periods of time. It would simplify the management of both vaccination and in-feed medication if lack of interference between specific antimicrobial-vaccine combinations were known. The objective of this study was to determine if concurrent in-feed medication with 11 g per tonne virginiamycin (Stafac; Phibro Animal Health Corporation, Teaneck, New Jersey) interferes with protective immunity induced by an avirulent live oral LI vaccine as measured by differences in *Lawsonia*-specific lesion scores after oral experimental challenge with virulent LI.

Materials and methods

The Gut Bugs, Inc, Institutional Animal Care and Use Committee (IACUC) approved all animal use, handling, and sampling techniques described herein.

Animals and housing

Eighty-eight 4-week-old castrated male segregated early weaned Landrace × Yorkshire pigs weighing approximately 15.8 pounds (7.2 kg) were purchased from a commercial sow herd in eastern South Dakota and delivered to Gut Bugs, Inc (GBI; Fergus Falls, Minnesota), an independent contract research organization. All pigs were weaned at 18 to 21 days of age at the source farm. Upon arrival at GBI, pigs were housed in 1.5 m × 1.8 m pens with slatted plastic nursery floors (Double L Group, Ltd, Dyersville, Iowa) and metal bar fences, designed to provide at least 0.40 m^2 of space per pig. Each pen was equipped with one stainless steel five-hole self-feeder and one bowl self-drinker.

Rations and feed assays

Water and corn-soybean rations were provided ad libitum. The corn-soybean ration was formulated (Form-A-Feed, Inc, Stewart, Minnesota) to meet or exceed National Research Council[11] recommendations. The ration was formulated appropriately according to the age and nutrient requirements of the pigs. Stage 1 ration and stage 2 through stage 4 rations were fed as described in Tables 1 and 2, respectively. Stage 1 and stage 2 rations were pelleted rations, while stage 3 and stage 4 rations were provided in meal form.

Medicated and non-medicated feeds were identical except for inclusion of virginiamycin

Table 1: Dietary composition of stage 1 diet (as fed) and selected nutrients composition*

Ingredients	Composition
Whey (%)	31.13
Oat groats (%)	17.50
Breakfast cereal fines (%)	13.65
Soy (%)	12.66
Protein supplement (%)	10.00
Base/pig starter 50 (%)	2.50
Fish meal (%)	2.50
Milk chocolate product (%)	2.50
Animal fat/choice white (%)	1.75
Vitamins, minerals, other trace ingredients (%)†	5.81
Total	**100**
Calculated composition	
Crude protein (%)	21.99
Calcium (%)	0.73
Phosphorus (%)	0.70
Lysine (%)	1.70
Methionine + cysteine (%)	1.01
Tryptophan (%)	0.30
Threonine (%)	1.09
Metabolizable energy (kcal/kg)	3720

* An 88-pig trial was conducted to measure the effect of in-feed virginiamycin administration at 11 g/tonne on the efficacy of immunization with an avirulent live oral *Lawsonia intracellularis* vaccine. Diets were formulated to provide for the nutrient requirements of growing pigs throughout the experiment. Pigs were 4 weeks old on Day 0. Stage 1 diet was fed from Day -5 through Day -2.

† Stage 1 diet was formulated so that each kg of ration contained vitamin A, 13792 IU; vitamin D, 2288 IU; vitamin E, 111.03 IU; copper, 129.05 mg; iodine, 0.72 mg; manganese, 72.88 mg; zinc, 3712.87 mg; and iron, 202.41 mg.

at 11 g per tonne in the medicated rations. Feed samples were collected from each ration prior to trial initiation and analyzed (Eurofins AvTech Laboratories, Inc, Kalamazoo, Michigan) to verify that the virginiamycin concentration in the feed was at least 11 g per tonne. Feed samples were collected at the feed mill after the rations were mixed, during the bagging process. Pooled feed samples were generated by collecting a sample from each bag of feed. Samples were collected from this pooled sample and submitted for virginiamycin assay.

Experimental design

Pigs were randomly allocated to four treatment groups using a random number generator as follows. Ten pigs were allocated to two pens of five pigs to serve as a strict negative non-challenged control group (NCC). Seventy-two pigs were allocated to three treatment groups: a challenged control group (CC), a vaccinated challenged group (VC), and a vaccinated medicated challenged group (VM). Groups CC, VC, and VM each consisted of 24 pigs divided into four pens of six pigs (Table 3). Kroll et al[12] previously demonstrated that the Enterisol Ileitis vaccine does not induce macroscopic or microscopic lesions in the ileum or colon. Since these were the parameters used to measure interference with vaccine efficacy in this study, in keeping with IACUC recommendations, no vaccinate-only control group was included in this study.

An additional six pigs used in this study served as sentinel pigs. These six pigs were divided among the treatment groups such that two sentinel pigs were included in the NCC group, one pig in each pen; two sentinel pigs were included in the VC group, one pig in each of two of the pens; and two sentinel pigs were included in the VM group, one pig in each of two of the pens. Sentinel pigs were treated exactly the same as their pen-mates. Sentinel pigs were selected at random (by a random number generator) from the pens that included sentinel pigs. On Day 28, in the same manner described for all other pigs at study termination, fecal and blood samples were collected from the sentinel pigs, which were then euthanized, and tissue samples were collected for immunohistochemistry (IHC) analysis.

On Day -3, fifteen pigs from the CC treatment group, 10 pigs from the VM treatment group, six pigs from the VC treatment group, and five pigs from the NCC treatment group were randomly selected by a random number generator for collection of fecal and blood samples. All samples were confirmed negative by fecal polymerase chain reaction (PCR)[13] for LI and by immunoperoxidase monolayer assay (IPMA)[14] for serum antibody against LI. All pigs were vaccinated for circovirus with one dose of Circoflex (Boehringer Ingelheim Vetmedica, Inc, St Joseph, Missouri), for *Salmonella choleraesuis* with one dose of SC-54 (Boehringer Ingelheim Vetmedica, Inc), and for *Erysipelothrix rhusiopathiae* with one dose of Ery-ALC (Boehringer Ingelheim Vetmedica, Inc) on Day -1 to minimize the opportunity for unintended diseases to confound the results and data analysis.

This study consisted of two phases: a vaccination phase from Day 0 through Day 27 and a challenge phase from Day 28 through Day 51. During the vaccination phase, the VM group received feed targeted to contain at least 11 g per tonne virginiamycin. The VC and VM groups were orally drenched on Day 1 with one dose of Enterisol Ileitis vaccine. Although the label-approved method of administration of this vaccine in the United States is via the drinking water, this method of administration was chosen to ensure that each pig received an equal dose of vaccine, as well as to validate using the individual pig as the statistical unit for analysis.

On Day 28, fecal and blood samples were collected from the same 15 pigs from the

Table 2: Dietary composition of rations (as fed) and selected nutrients composition*

Ingredients	Stage 2 ration†	Stage 3 ration‡	Stage 4 ration§
Corn (%)	26.10	42.66	62.35
Soy (%)	19.25	26.35	29.23
Whey (%)	22.25	10.00	0.00
Breakfast cereal fines (%)	7.50	5.00	0.00
Oat groats (%)	7.50	5.00	2.50
Fish meal (%)	5.00	3.00	0.00
Animal fat/choice white (%)	3.15	2.93	2.35
Base/pig starter 50 (%)	2.50	2.50	0.00
Milk chocolate product (%)	2.50	0.00	0.00
Vitamins, minerals, other trace ingredients (%)	4.25¶	2.56**	3.57††
Total	**100**	**100**	**100**
Calculated composition			
Crude protein (%)	20.17	20.49	20.27
Calcium (%)	0.69	0.70	0.66
Phosphorus (%)	0.65	0.65	0.60
Lysine (%)	1.50	1.40	1.30
Methionine + cysteine (%)	0.92	0.85	0.78
Tryptophan (%)	0.26	0.25	0.24
Threonine (%)	0.95	0.91	0.85

* An 88-pig trial was conducted as described in Table 1.

† Stage 2 diet was fed from Day -1 through Day 6.

‡ Stage 3 diet was fed from Day 7 through Day 27.

§ Stage 4 diet was fed from Day 28 through Day 51.

¶ Stage 2 diet was formulated so that each kg of ration contained vitamin A, 13,789 IU; vitamin D, 2286 IU; vitamin E, 115.17 IU; vitamin K, 5.94 mg; vitamin B_2, 16.30 mg; vitamin B_{12}, 0.06 mg; pantothenic acid, 57.33 mg; copper, 131.22 mg; iodine, 0.72 mg; manganese, 75.99 mg; zinc, 3718.17 mg; and iron, 217.39 mg.

** Stage 3 diet was formulated so that each kg of ration contained vitamin A, 13,788 IU; vitamin D, 2286 IU; vitamin E, 117.03 IU; vitamin K, 5.94 mg; vitamin B_2, 13.86 mg; vitamin B_{12}, 0.05 mg; pantothenic acid, 56.13 mg; copper, 130.77 mg; iodine, 0.72 mg; manganese, 79.74 mg; zinc, 3718.87 mg; and iron, 242.85 mg.

†† Stage 4 diet was formulated so that each kg of ration contained vitamin A, 11,028 IU; vitamin D, 1828 IU; vitamin E, 81.93 IU; vitamin K, 4.75 mg; vitamin B_2, 11.42 mg; vitamin B_{12}, 0.04 mg; pantothenic acid, 44.54 mg; copper, 52.85 mg; iodine, 0.62 mg; manganese, 50.49 mg; zinc, 528.01 mg; and iron, 157.20 mg.

CC treatment group, 10 pigs from the VM treatment group, six pigs from the VC treatment group, and five pigs from the NCC treatment group as were sampled on Day -3. Samples were tested for LI by fecal PCR and serum IPMA. All samples were negative for LI, verifying the adequacy of the biosecurity measures performed in this study and also confirming that no vaccinated pigs were shedding fecal LI organisms at this time. On Day 31, the CC, VC, and VM groups received one dose of LI challenge inoculum administered via oral gavage.

During the vaccination phase, the VC group was housed in Barn 1, the NCC and CC groups in Barn 2, and the VM group in Barn 3. Groups were housed in this fashion so that the VC and the VM groups were each housed in separate barns from the other groups to minimize the chance of treatment errors and contamination; to prevent contamination, fecally or otherwise, of the NCC and CC groups with LI from the Enterisol Ileitis vaccine; and to prevent contamination of the non-medicated groups (NCC, CC, VC) with virginiamycin. For the challenge phase, pigs were moved such that the NCC group was housed in Barn 1 while the CC, VC, and VM groups were housed in Barn 2. Treatment groups were moved to ensure that the NCC group was provided its own air space to prevent contamination of the NCC group from the challenged groups (CC, VC, VM).

From Day -5 through Day 30, all animal handling procedures began in Barn 2 where the non-vaccinated treatment groups (NCC, CC) were housed, to minimize contamination with vaccinal LI organisms from the

Table 3: Treatment group permutations for a study examining lack of interference with Enterisol Ileitis vaccination efficacy when concurrently administered in-feed virginiamycin at a dose of 11 g/tonne*

	No. of pens	No. of pigs/pen	Total pigs	Vaccinated	Medicated	Challenged
Non-challenged controls (NCC)	2	5	10	No	No	No
Challenged controls (CC)†	4	6	24	No	No	Yes
Vaccinated controls (VC)†‡	4	6	24	Yes	No	Yes
Vaccinated medicated (VM)†‡§	4	6	24	Yes	Yes	Yes

* All pigs were 4 weeks old at the beginning of the study (Day 0). Treatment groups included a total of 82 pigs. In addition, six sentinel pigs were divided among the treatment groups.

† Pigs were challenged with 1×10^8 virulent *Lawsonia intracellularis* (LI) cells/pig delivered in 60 mL of carrier buffer administered individually by gastric gavage using a size 18 French esophageal tube feeder on study day 31. The LI mucosal homogenate challenge inoculum was harvested from infected donor pigs.

‡ Vaccinated pigs were orally drenched with one dose of Enterisol Ileitis vaccine (Boehringer Ingelheim Vetmedica, Inc, St Joseph, Missouri) on Day 1.

§ Medicated and unmedicated feeds were identical except for the addition of virginiamycin (Stafac; Phibro Animal Health Corporation, Teaneck, New Jersey) at a dose of 11 g/tonne in the feed of the VM group from Day 0 through Day 27.

vaccinated treatment groups (VC, VM). Throughout the challenge phase, from Day 28 through Day 51, animal handling procedures began in Barn 1, which contained the strict negative non-challenged control group (NCC), to prevent contamination with challenge LI organisms from the challenged treatment groups (CC, VC, VM). Additionally, coveralls, boots, and gloves were changed when moving between barns to minimize contamination of the NCC treatment group. During the challenge phase, pens in Barn 2 were stratified in blocks using a random number generator such that each block contained one pen of each treatment group (CC, VC, VM). Pen body weights were measured before and after each trial phase: Day -1 prior to the inclusion of virginiamycin in the feed at the beginning of the vaccination phase, Day 28 at the beginning of the challenge phase, and Day 51 prior to necropsy at the end of the challenge phase. Leftover feed was vacuumed out of the feeders and quantified at each diet change, as well as on Day 28 at the beginning of the challenge phase and on Day 51 prior to trial termination. This enabled determination of feed consumed per pen during the vaccination phase (Day 0 to 27) and during the challenge phase (Day 28 to 51).

Challenge inoculum

The LI mucosal homogenate challenge inoculum was harvested from infected donor pigs from the same source farm and of the same genetic line as the experimental pigs. The mucosal homogenate was prepared using the method described previously by Guedes and Gebhart.[15] The mucosal homogenate was harvested by scraping LI lesions from the mucosal surface of the small intestine. Mucosal contents were homogenized in a Warring blender with a cryopreservative buffer. An aliquot of the homogenate was submitted to a diagnostic laboratory to test for extraneous agents. The LI in the homogenate was enumerated by quantitative PCR and stored at -80°C. The inoculum was thawed immediately prior to use, administered to individual pigs by gastric gavage using a size 18 French esophageal tube feeder, and delivered in 60 mL of carrier buffer with a total challenge dose of 1×10^8 virulent LI cells per pig.

Lesion collection and analysis

Due to laboratory handling capabilities, half of the pigs were humanely euthanized via lethal injection and necropsied on Day 51, and the remaining pigs on Day 52. Gross intestinal lesions were measured and scored by an expert evaluator who had extensive experience performing necropsies and evaluating LI lesions and was blinded to treatment. Gross lesions in the jejunum, ileum, cecum, and colon were scored using the following scale described previously[16] by Guedes and Gebhart: 0 = normal; 1 = mild mucosal proliferation, edema, hyperemia; 2 = moderate mucosal proliferation, edema, hyperemia; 3 = severe mucosal proliferation, edema, hyperemia. In addition, the length (cm) of each lesion was measured.

Composite scores for jejunum and ileum were calculated by multiplying the length of the lesion with the severity score of the lesion (reported as cm). Composite scores for cecum and colon were calculated by multiplying the length and width of the lesion by the severity score of the lesion (reported as cm^2). While lesions in the small intestinal sections (jejunum and ileum) generally covered the entire circumference of the intestinal mucosa, lesions in the cecum and colon were more patchy and tended not to cover the entire circumference of the intestinal mucosa. Thus, for lesions in the cecum and colon, the area of the lesion, generated by multiplying the length of the lesion by the width of the lesion, was more suitable for lesion analysis and comparison.

Uniform sections of ileum were collected from the gastrointestinal tract of each pig, 1 inch proximal to the ileo-cecal junction, and placed in a sufficient volume of 10% formalin to provide a 10:1 formalin-to-tissue ratio to ensure adequate tissue fixation. These tissues were shipped to the University of Minnesota Veterinary Diagnostic Laboratory (St Paul, Minnesota) for LI immunohistochemistry (IHC) staining and analysis of microscopic lesions by another consultant blinded to treatment. Tissue sections were assessed for the estimated percent of colonized crypts and scored using the following scale: 0 = negative; 1 = > 0% to 25%; 2 = > 25% to 50%; 3 = > 50% to 75%; 4 = > 75% to 100%.

Statistical analysis

The NCC pigs were included in this study in limited numbers and housed separately to serve as biosecurity sentinels to ensure that no diseases were present upon pig arrival or were introduced during the study that could

confound data analysis, and also to validate the challenge model. Accordingly, the NCC pigs were not included in the statistical analyses. Pigs were vaccinated and challenged individually to justify *Lawsonia*-specific lesion-score comparisons on an individual pig basis (n = 24 pigs per treatment group). Since pigs were housed and fed by pen, growth rate and feed conversion outcomes were evaluated on a pen basis (n = 4 pens per treatment group). Performance data were analyzed using one-way analysis of variance (ANOVA). If the ANOVA *P* value for treatment effects was significant ($P < .05$), Tukey's honestly significant difference (HSD) was used to discern differences among groups. Individual pig composite gross and microscopic lesion-score data, as well as the percent of pigs with positive lesions, were analyzed using the Kruskal-Wallis rank sum test.

Results

All fecal PCR, serum IPMA, and intestinal IHC samples tested from the sentinel pigs were negative for LI. Table 4 shows the results of gross intestinal lesion evaluation as well as microscopic IHC intestinal evaluation. Gross intestinal lesion scores were significantly lower in both the jejunum ($P < .01$) and ileum ($P < .05$) of vaccinated treatment groups (VC, VM) compared to the unvaccinated challenged treatment group (CC). The macroscopic jejunal and ileal lesion scores of the VC and VM treatment groups did not differ. The percentages of pigs with macroscopic lesions in the jejunum ($P < .01$) and ileum ($P < .05$) were also significantly lower in the VC and VM groups than in the CC group. The percentages of pigs with macroscopic lesions in the ileum and jejunum in the VC and VM treatment groups did not differ. Macroscopic lesion scores and percentages of pigs with macroscopic lesions in the cecum and colon in the CC group were not high enough for vaccination to confer a statistically significant advantage.

Immunohistochemistry scores are regarded as the reference test for evaluation of LI infection status.[12,17-19] Mean ileum IHC scores were significantly lower ($P < .05$) in VC and VM treatment groups than in the CC treatment group. Mean ileum IHC scores in the VC and VM treatment groups did not differ. The percentages of pigs with a positive ileum IHC score were significantly lower ($P < .05$) in the VC and VM treatment groups than in the CC treatment group. The percentages of pigs with positive ileum IHC scores in the VC and VM treatment groups did not differ. No macroscopic or microscopic lesions were detected in the gastrointestinal tracts of the NCC treatment group. As a result, this group has been excluded from Table 4 and was not included in the statistical analysis.

Table 4: Macroscopic and microscopic lesion scores and percent positive pigs*

Response variable	CC	VC	VM	*P*
Mean ileum composite lesion score (cm)	32.26[a]	9.18[b]	11.26[b]	.01
Ileum % positive pigs	56.52[c]	17.39[d]	27.27[d]	.01
Mean jejunum composite lesion score (cm)	244.57[a]	37.05[b]	52.78[b]	< .01
Jejunum % positive pigs	47.83[c]	13.64[d]	13.04[d]	< .01
Mean cecum composite lesion score (cm^2)	3.04	0.00	0.00	.38
Cecum % positive pigs	4.35	0.00	0.00	.37
Mean colon composite lesion score (cm^2)	2.17	0.00	0.00	.14
Colon % positive pigs	8.7	0.00	0.00	.13
Mean ileum IHC lesion score	1.78[a]	0.82[b]	0.83[b]	.02
Ileum % IHC-positive pigs	73.91[c]	40.91[d]	43.48[d]	< .05

* Treatment groups were vaccinated, medicated, and challenged as described in Table 3. Pigs were humanely euthanized over a 2-day period on Day 51 and Day 52 and necropsied to evaluate macroscopic and microscopic intestinal *Lawsonia intracellularis* (LI) lesions. Gross macroscopic lesions were evaluated on a four-point scale: 0 = normal; 1 = mild mucosal proliferation, edema, hyperemia; 2 = moderate mucosal proliferation, edema, hyperemia; 3 = severe mucosal proliferation, edema, hyperemia. IHC-stained tissue sections were assessed for the estimated percent of colonized crypts and scored as follows: 0 = negative; 1 = > 0% to 25%; 2 = > 25% to 50%; 3 = > 50% to 75%; 4 = > 75% to 100%. Composite lesion-score values represent macroscopic lesion scores. Jejunum and ileum composite scores were calculated as lesion length × severity score (reported as cm). Cecum and colon composite scores were calculated as lesion length × width × severity score (reported as cm^2). IHC lesion-score values represent microscopic lesion score analyses. The line below each lesion score analysis represents the percent of pigs (n = 24 pigs per treatment group) identified as positive using the lesion analysis method in the preceding line. No macroscopic or microscopic lesions were observed in the nonchallenged control group. Statistical analysis was performed using the Kruskal-Wallis rank sum test.

[ab] Wilcoxon nonparametric comparison was used to determine statistical difference between pairs of values. Values within rows with different superscripts are statistically different ($P < .05$).

[cd] Analysis of means was used to determine statistical difference between pairs of values. Values within rows with different superscripts are statistically different ($P < .05$).

CC = challenged control group; VC = vaccinated challenged group; VM = vaccinated medicated group; IHC = immunohistochemistry.

Table 5 shows the performance results analyzed on a pen basis (n = 4 pens per treatment group). During the vaccination phase (Day 0 through Day 27), when virginiamycin was included in the feed, the VM treatment group had the best feed efficiency ($P < .001$), outperforming both the VC and CC treatment groups. During the vaccination phase the VC group outperformed the CC treatment group, with an intermediate feed efficiency ($P < .001$). Over the entirety of the trial (Day 0 through Day 51) the vaccinated treatment groups (VC, VM) outperformed the CC treatment group in feed efficiency ($P = .001$).

The results of all virginiamycin feed concentration assays were above the desired in-feed level of 11 g per tonne. Assay samples averaged 13.8 g per tonne and ranged from 11.4 to 15.7 g per tonne.

Discussion

Although there was no specific vaccinate-only control group, the two sentinel pigs included

Table 5: Performance results on a pen basis (n = 4 pens per treatment group)*

Response variable	NCC†	CC	VC	VM	*P*‡
Day 0 mean BW (lb)	16.16	15.50	15.94	15.69	.76
Day 0-27 ADG (lb)	1.26	1.09	1.28	1.23	.12
Day 0-27 F:G	1.49	1.58[a]	1.46[b]	1.30[c]	< .001
Day 28-51 ADG (lb)	1.91	1.30	1.50	1.43	.17
Day 28-51 F:G	1.75	1.96	1.80	1.75	.08
Day 0-51 ADG (lb)	1.52	1.16	1.38	1.35	.06
Day 0-51 F:G	1.61	1.76[a]	1.62[b]	1.51[b]	.001

* Treatment groups were vaccinated, medicated, and challenged as described in Table 3. This study was divided into two phases: a vaccination phase (Day 0 through Day 27) and a challenge phase (Day 28 through Day 51).

† Not included in the statistical analysis; raw means are included for reference only.

‡ Analysis of variance.

[abc] Values within rows with different superscripts are statistically different ($P < .05$).

NCC = non-challenged control group; CC = challenged control group; VC = vaccinated challenged group; VM = vaccinated medicated challenged group; BW = body weight; ADG = average daily gain; F:G = feed-to-gain ratio (feed efficiency).

in the VC treatment group had not yet been orally challenged with gut homogenate LI inoculum and thus essentially served as a vaccinate-only control group. The absence of positive IHC scores for *Lawsonia* in these pigs validates this research model and the ability to draw meaningful conclusions without a vaccinate-only control group.

The NCC group remained negative for LI infection as verified by fecal PCR and blood serum IPMA on Day 28, as well as by the complete absence of both macroscopic and microscopic intestinal lesions at necropsy evaluation upon study termination, validating the adequacy of internal biosecurity measures. The CC pigs developed severe intestinal lesions compared to the NCC pigs, validating the adequacy of the LI challenge model. Both the VC and VM groups had significantly lower lesion scores than the CC group, confirming immunization efficacy. Importantly, lesion scores were not significantly different between the VC and VM groups, confirming the lack of interference between virginiamycin medication and immunization. On the basis of the primary criterion of comparative LI-specific ileum IHC scores, statistical equivalence between the VC and VM treatment groups confirms a lack of interference with immunization efficacy of Enterisol Ileitis when concurrently administered with virginiamycin at 11 g per tonne.

During the vaccination phase (Day 0 through Day 27), feed efficiency was significantly better ($P < .001$) in the VM treatment group than in either the CC or VC groups. Performance results were numerically better for the NCC treatment group than for the challenged groups (CC, VC, VM) during both the challenge phase (Day 28 through Day 51) and the study overall. The authors attribute this to the morbidity caused by LI challenge.

It is unknown if virginiamycin at higher feed inclusion rates or feeding other antimicrobials would interfere with immunization efficacy of this LI vaccine. Enteric mucosal and luminal-content virginiamycin concentrations corresponding to the virginiamycin feed inclusion rate used in this study are unknown. Previous work has demonstrated in vitro[20,21] and in vivo[22] efficacy of virginiamycin against LI. Virginiamycin minimum inhibitory concentration for two LI isolates was previously reported to be 1 µg per mL for both intracellular and extracellular assay methodologies, and the minimum bactericidal concentration for LI NCTC strain 12656 was previously reported to be < 2 µg per mL.[20,21]

After oral administration to animals, virginiamycin is minimally absorbed and causes no adverse effects.[9,23] Diffusion across the intestinal mucosa is a function of the size of the molecule, with only compounds with lower molecular weights diffusing without first being metabolized.[24,25] Virginiamycin is composed of two factors, M and S. Factor M has a molecular weight of approximately 500 Da; factor S has a molecular weight of approximately 800 Da.[26] Lambert[25] defined intestinal integrity in animals as the ability to restrict passive diffusion of molecules larger than 150 Da. Earlier work by Loehry et al[24] indicated that in rabbits, water-soluble molecules larger than 180 Da do not readily diffuse across the intestinal mucosa. DiCuollo[27] demonstrated poor absorption in pigs of virginiamycin across the intestinal mucosa.

Lawsonia intracellularis is an obligate intracellular organism that resides in porcine enterocytes. In the gastrointestinal tract, LI cells associate with the enterocyte cell membrane and quickly enter the enterocyte via an entry vacuole.[1] Since virginiamycin is minimally absorbed from the gastrointestinal lumen, it is possible that LI cells in the Enterisol Ileitis vaccine did not have enough contact time with virginiamycin prior to endocytosis into enterocytes to affect the efficacy of the vaccine, despite virginiamycin's previously reported efficacy against LI.

In a normal production setting, pigs might receive in-feed virginiamycin for more than one day prior to Enterisol Ileitis vaccination. However, virginiamycin is not absorbed from the gastrointestinal lumen and would not be expected to accumulate in the gastrointestinal tract. Thus, a longer period of in-feed virginiamycin administration prior to vaccine administration should not be necessary to mimic the luminal gastrointestinal concentration of virginiamycin that would be expected in a production setting.

Implications

- Under the conditions of this study, administration of in-feed virginiamycin at the labeled dose of 11 g per tonne does not interfere with the efficacy of concurrent immunization of pigs with an avirulent live oral LI vaccine.
- The performance data validates the benefits of virginiamycin in improving feed efficiency under the conditions of this study, a valuable benefit of increased significance at higher feed costs.
- Under the conditions of this study, off-label use of the Enterisol Ileitis vaccination as an oral drench is also an efficacious means of immunization.

Acknowledgements

The authors would like to thank Dr Keith Kinsley and Dr Connie Gebhart for their contributions to the conduct of this study.

Conflict of interest

Dr Beckler owns the contract research organization (Gut Bugs, Inc) that performed the trial work and acted as a paid consultant both on trial design and on provision of the LI challenge inoculum. Dr Segal is employed by Phibro Animal Health Corporation as a Research Veterinarian. Dr Weiss, Dr Nimmo, and Dr Guggenbiller are employed by Phibro Animal Health Corporation as Technical Services Managers.

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