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Original Article

Comparison of influenza A virus infection in high- and low-birth-weight pigs using morphometric analysis

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Background Epigenetic studies have shown that low-birth-weight (LBW) and growth restriction has been associated with reduced immune function in humans and reduced passive immunity in pigs. To examine the immune responses of high-birth-weight (HBW) and LBW groups of pigs, influenza A virus infection was used as an exemplifier of neonatal respiratory disease.

Objectives The objectives of this study were (i) to compare clinical, immunological, and pathological outcome of influenza infection in HBW to LBW pigs and (ii) to establish standardized sampling sites, score each site independently with set criteria, and compare scores between sites.

Methods Sixty-eight 4-week-old pigs originating from either HBW or LBW litters were intratracheally inoculated with $10^{6.3}$ $TCID_{50}$/ml of A/swine/Texas/4199-2/1998 H3N2 and euthanized 48 hours later. Samples were collected 2·5 cm from the tip of both cranial and middle lung lobes. The formalin-fixed paraffin-embedded tissue sections were scored in a blinded manner by a single pathologist using established scoring criteria for routine and immunohistochemical stains. Clinical parameters, lung and nasal swab virus titers, and cytokine levels for interferon-alpha and interleukin-1-beta, IL-6, and IL-8 were measured.

Results and Conclusions Lung lesion severity and influenza staining intensity were significantly lower in LBW compared with HBW pigs ($P < 0·05$). Additionally, examining just the LBW group, the significant difference between lobes ($P = 0·009$) showed that the mean score for the right cranial lung lobe was higher compared with the other three lobes.

Keywords Birth weight, epigenetic programming, influenza A virus, morphometry, swine.

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Introduction

In livestock species, lifetime productivity and health may be limited by key events occurring during early embryonic and fetal development. As in humans, intrauterine growth restriction alters post-natal stress response, adipose tissue deposition, muscle development, glucose tolerance, blood pressure, catecholamine response, and the development of all vital organs including liver, spleen, heart, lung, muscle, and thymus.[1–3] Human epidemiological research also suggests that *in utero* growth restriction, associated with maternal undernutrition, may adversely affect humoral and cell-medicated immune responses in adolescence and adulthood. A limited number of studies of full-term, small-for-gestational-age babies in developing countries have demonstrated altered humoral and cell-medicated immune responses resulting in lower likelihood of response to *Salmonella typhi* vaccination,[4] decreased thymopoeitin production (a marker for thymic growth and development),[5] and higher risk of infection-related death.[6]

In swine, the effects of pre-natal maternal stress on post-natal immunity has been most extensively studied using either daily restraint, rough handling, or the administration of adrenocorticotropic hormone (ACTH) during mid-gestation or late gestation. Maternal restraint during late gestation had an immunosuppressive effect on lymphocyte proliferation in response to various mitogens at 1 and 35 days of age, in addition to increasing piglet morbidity and mortality during the lactation period.[7] The same animal model has demonstrated reduced tumor necrosis factor (TNF)-alpha, interleukin (IL)-6, and serum amyloid A production following LPS infusion in 5-week-old progeny[8] after maternal restraint stress. Moreover, eight-week-old pigs derived from ACTH-treated dams demonstrated higher basal cortisol levels and slower healing times.[9] Additionally, rough handling of dams, not the ACTH

treatment, resulted in reduced sickness behavior in these 8-week-old pigs following LPS administration.[10]

Low birth weight and high birth order are well known risk factors for reduced passive immunity and survival in pigs.[11,12] While parity of birth may affect adaptive immunity, piglets born to gilts have reduced humoral response following vaccination;[13] direct evidence for the impacts of birth weight on adaptive immunity and clinical disease severity is lacking and of great relevance to the modern swine industry. To compare severity between piglets born from high-birth-weight (HBW) and low-birth-weight (LBW) litters, swine influenza virus (SIV) infection was used as an exemplifier of neonatal respiratory disease in this study.

The objectives of this study were (1) to compare clinical, immunological, and pathological outcomes of influenza infection in HBW to LBW pigs and (2) to establish standardized sampling sites, score each site independently with set criteria, and compare scores between sites.

Materials and methods

Virus

The challenge virus, influenza A/swine/Texas/4199-2/98 H3N2 (TX98), was grown from stock obtained from Dr. Yan Zhou, Vaccine and Infectious Disease Organization-Intervac (VIDO-Intervac), at the University of Saskatchewan. The virus inoculum was prepared by propagation on Madin–Darby Canine Kidney (MDCK) cells[14] and had a titer of $10^{6.3}$ $TCID_{50}$/ml as calculated by the Spearman–Kärber method.[15] Individual aliquots of inoculum were stored at −80°C until used.

Experimental design

Sixty-eight 4-week-old pigs were purchased from a farm free of SIV, *Mycoplasma hyopneumoniae* (Mhyo), and porcine reproductive and respiratory syndrome virus (PRRSv) in Saskatchewan, Canada, and were transported to a biocontainment level 2 (BSL2) animal care facility at the University of Saskatchewan. This research was approved by the University of Saskatchewan's Animal Research Ethics Board and adhered to the Canadian Council on Animal Care guidelines for humane animal use.

Eligible litters contributing piglets to the study were identified at birth based on weight of all live and stillborn piglets. Piglets were selected from parity one or two dams only and originated from HBW or LBW litters based on being ±0·7 standard deviations (Z-score) from the historical average litter birth weight after controlling for total born litter size and the dam's parity. Two average birth weight male piglets from each selected litter were chosen for the trial. Piglets were in good body condition at weaning (21 days of age). One week prior to weaning, serum samples from selected piglets were sent to Prairie Diagnostic Services, Inc. (PDS) and confirmed negative for antibodies to influenza A virus by H1N1 and H3N2 ELISA (Idexx Laboratories, Westbrook, ME, USA). Additionally, the sera from the pigs were also confirmed to be negative for antibodies to TX98 by hemagglutination inhibition assay at the University of Minnesota Veterinary Diagnostic Laboratory. Upon arrival at the animal care facility, nasal swabs were collected from all pigs and tested by a matrix real-time RT-PCR procedure at PDS using the Canadian Food Inspection Agency (CFIA)-approved protocol.[16,17]

The pigs were given free access to water and were fed an age-appropriate commercial diet (Whole Earth Pig Starter, Federated Co-operatives Ltd., Saskatoon, SK, Canada) *ad libitum* that met or exceeded recommended nutrient requirements. The pigs were acclimated for 5 days, and room temperature was maintained at approximately 27°C. On experimental day 0, all pigs were intratracheally inoculated with TX98 while anesthetized with a single intramuscular dose (up to 20 mg/kg) of ketamine (Ketalean®, Bimeda-MTC, Cambridge, ON, Canada) and 2 mg/kg xylazine (Rompun®, Bayer HealthCare, Toronto, ON, Canada) after sedation with a single intramuscular dose (0·3 ml) of azaperone (Stresnil®, Merial, Baie D'Urfé, QC, Canada). The pigs were inoculated and kept in sternal recumbency for 10 minutes. Four pigs were removed from the study leaving 32 pigs per treatment group. Two pigs were removed from the study due to death from anesthetic complications, one pig died during the acclimation period before inoculation and one pig was removed due to an unrelated lung abscess.

Clinical observations, sampling, and pathological examination

The primary care worker (RG) was blinded to treatment group. In some, but not all cases, there was a visual body weight difference between the HBW and LBW pigs. The pigs were checked for any evidence of injury or illness, and rectal temperatures were obtained twice daily from inoculation until termination of the study. Clinical signs specific to influenza were also monitored twice a day following inoculation and recorded as present or absent (Table 1).

Two days post-inoculation (DPI), all pigs were euthanized with a lethal dose of pentobarbital (Euthanyl Forte®; Bimeda-MTC, Cambridge, ON, Canada) for necropsy. A nasal swab and serum were collected from all pigs immediately after euthanasia, and both samples were stored at −80°C. All necropsies were performed following blood collection. The lungs were removed with the trachea attached and evaluated for the percentage of the lung affected with purple, lobular consolidation typical of influenza infection in swine. The percentage of consolidation for each lobe was calculated using weighted proportions of the total lung volume.[18] The proportions were 10% each for the cranial, middle, and accessory lobes and 25% each for the caudal

Table 1. Clinical signs of the respiratory disease caused by influenza A virus infection were assessed for each pig

Score	Clinical signs
Attitude and responsiveness	
0	Alert and active
1	Alert, but slower than penmates
2	Reluctant to move, but gets up by stimulation
3	Down, doesn't respond with stimulation or demonstrates seizures (leg paddling, recumbency, opisthotonos)
Respiratory rate and effort	
0	Normal
1	Increased respiratory rate
2	Increased respiratory rate, slight abdominal breathing (dyspnea)
3	Increased respiratory rate and marked abdominal breathing (dyspnea)
Appetite and body condition	
0	Normal appetite and body condition
1	Reduced feed intake, normal body condition
2	Anorexic, slight loss of body condition and weight (>10% loss of body weight)
3	Anorexic, moderate loss of body condition and weight (>15% loss of body weight)
Coughing and sneezing	
0	No coughing or sneezing
1	Observed coughing/sneezing once while in the room
2	Observed coughing/sneezing 2–3 different times while in the room
3	Observed continual coughing/sneezing while in the room

lobes for a total of 100%. The bronchoalveolar lavage fluid (BALF) sample was collected as previously described[19] using phosphate-buffered saline (PBS). After gently massaging the lungs, the PBS was aspirated, and 5-ml aliquots of the aspirated fluid were stored at −80°C.

Lung samples were collected 2·5 cm from the tip of the left cranial (A), left middle (B), right cranial (C), and right middle (D) lung lobes (Figure 1) and labeled with a beaded safety pin (Figure 2). An additional 1-cm sample of each lobe was collected, individually labeled, and stored at −80°C until used for further testing. The tissue from the tip of the lobes was fixed in 10% formalin for 24 hours and trimmed, so that the histological sample examined came from approximately 2 cm from the tip of the lobe and paraffin-embedded for histopathology by standard techniques. The first 4-μm serial section was used for the hematoxylin and eosin staining, and the second section was used for immunohistochemistry with anti-influenza A ribonucleoprotein antibody (National Institute of Allergy and Infectious Disease, Bethesda, MD, USA) at 1:5,000 dilution as previously described with 3,3-diaminobenzadine (IMMpact™ DAB; Vector Laboratories, Burlingame, CA, USA) chromagen.[20]

The hematoxylin-and-eosin-stained lung sections were examined microscopically for bronchiolar epithelial changes

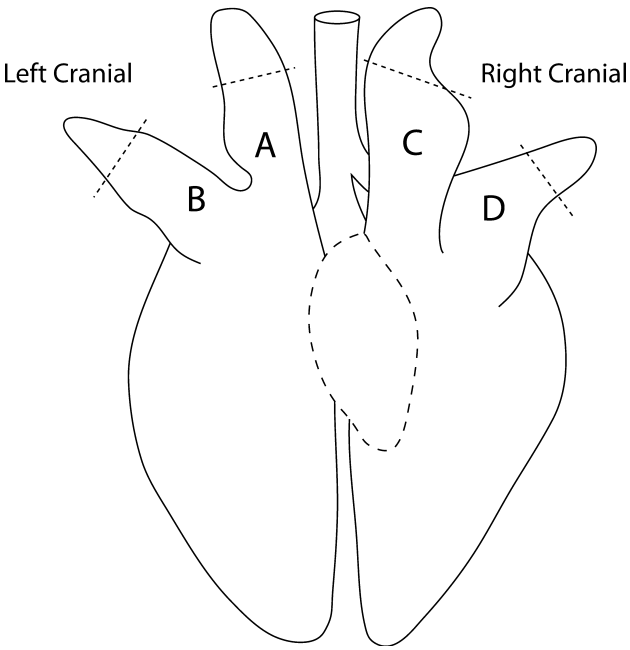

Figure 1. Diagram of the dorsoventral aspect of the lungs showing lung lobe designations (A, B, C, and D) and the approximate location of sampling.

along with the severity and extent of inflammation within and surrounding large, medium, and small bronchioles. The following scoring stratagem was used: 0 = no airways affected, 0·5 = only a few isolated airways affected, 1 = localized cluster of affected airways (in 1 or 2 lobules), 1·5 = several airways affected throughout section plus minimal interstitial infiltrates, 2 = several airways affected throughout section plus mild to moderate interstitial infiltrates, 2·5 = several airways affected, often severely plus moderate interstitial and alveolar infiltrates, and 3 = many airways affected, often severely plus moderate interstitial and alveolar infiltrates.[19]

The immunostained lung sections were examined microscopically for the amount of immunoreactivity present in both airways and alveoli. Immunoreactivity was strongest and most frequently observed in the nucleus of bronchiolar epithelial cells, but was also observed in the cytoplasm of bronchiolar epithelial cells and in the nucleus and cytoplasm of type II pneumocytes. The following scoring stratagem was used: 0 = no immunoreactivity, 1 = rare to occasional immunoreactivity, 2 = scattered immunoreactivity (<25% of area), 3 = moderate immunoreactivity (25–50% of area), and 4 = abundant immunoreactivity (>50% of area). The separate scores for immunoreactivity in the airways and alveoli were added together for the final score.[21]

Virus titration

The nasal swabs collected at necropsy were placed in a vial containing 1 ml of Eagle's minimal essential medium (MEM;

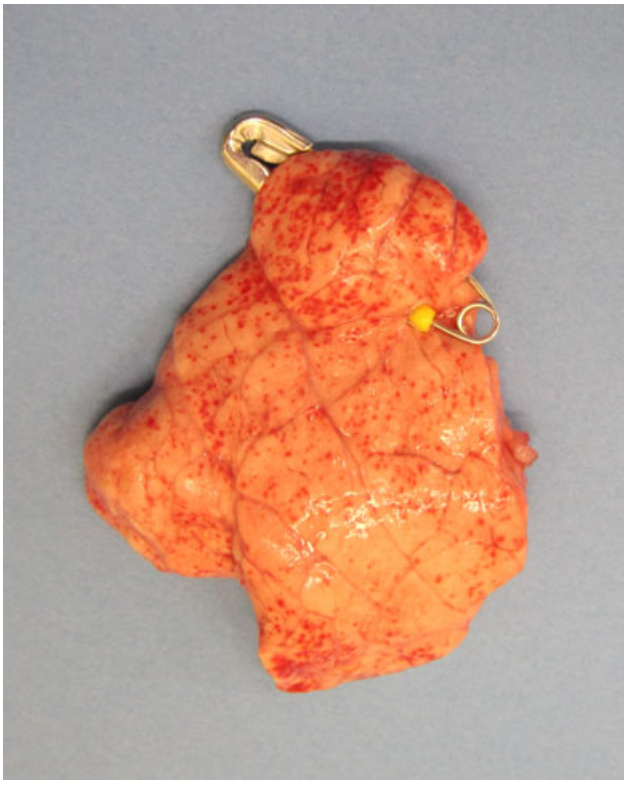

Figure 2. Photograph of a lung sample for histopathology demonstrating the individual lobe labeling using a safety pin and a colored bead. Each lung lobe was identified with a different color.

Invitrogen, Camarillo, CA, USA) with 0·5% gentamicin and stored at −80°C. The 10% w/v lung tissue homogenates were made in a screw-top vial containing a steel ball (VWR #97007-688) and MEM with 0·5% gentamicin using a Retsch MM400 homogenizer (Retsch GmbH, Haan, DE) for 2 cycles of 2 minutes at 30 Hz followed by centrifugation for 15 minutes at 2272 *g*. The homogenates for lobes A, B, C, and D were pooled, aliquoted, and stored at −80°C.

All nasal swab supernatant and lung homogenate samples were titered using 10-fold serial dilutions and 4 wells per dilution that were inoculated in 48-well plates containing monolayers of MDCK cells and using MEM containing 4% bovine serum albumin, trypsin, and antibiotics (MEM+). All cultures were incubated at 37°C under a 5% CO_2 atmosphere and examined daily for 5 days under an inverted light microscope to observe cytopathic effects (CPEs). Virus titers calculated by the Spearman–Kärber method.[15] All negative titers were confirmed negative by matrix real-time RT-PCR procedure using RNA extraction kit and a real-time RT-PCR kit (Qiagen Inc., Valencia, CA, USA).[16,17]

Cytokines and additional diagnostic testing

One aliquot of BALF from each piglet was sent to Aushon BioSystems (Billerica, MA, USA) to determine the cytokine concentrations of interleukin-1-beta (IL-1β), interleukin-6 (IL-6), and interleukin-8 (IL-8) using their SearchLight Array Technology. Interferon-alpha (IFNα) was measured using an in-house fluorescent microsphere immunoassay (FMIA) as previously described[22] with several modifications. Firstly, magnetic rather than polystyrene beads were used. Secondly, BALF samples, negative control sample, and standards were diluted 1:3 in BALF collected from a healthy pig from the same source farm as the experimental pigs. The lung collected from the healthy pig had no significant macroscopic or microscopic lesions (same A, B, C, and D samples), and was PCR negative for SIV (as described in the previous section), PRRSv, porcine circovirus type 2 (PCV2), and Mhyo (all tested by PDS).

Additional diagnostic testing was performed to determine the status of all inoculated pigs for North American and European (NAEU) variants of PRRSv, PCV2, and Mhyo. DNA and RNA were extracted from 30 mg of the right middle lung lobe using a commercial kit (AllPrep DNA/RNA Mini Kit, Qiagen, Toronto, ON, Canada). Extracted product was stored at −20°C. RNA was tested for PRRSv using a commercial kit as per manufacturer's instructions (Tetracore RT-qPCR, Gaithersburg, MD, USA). DNA from lung samples was tested for PCV2 by SYBR green qPCR[23] and Mhyo by conventional PCR.[24]

Statistical analysis

Multilevel mixed-effects regression models were used to determine whether influenza outcome variables were associated with HBW or LBW group, Z-score, or parity from which the inoculated pigs were born using STATA 12 (STATA Corp, College Station, TX, USA). Body weights, Z-score, wean age, lactational growth rate, rectal temperature, BALF cytokine levels, the percentage of total lung with lesions, microscopic lesion severity scores, influenza antigen staining intensity, and virus titers from nasal swabs and lungs were evaluated using the XTMIXED function. The presence or absence of respiratory clinical signs (coughing and sneezing, nasal discharge) was evaluated using XTMELOGIT. In all models, piglet was the experimental unit with litter of birth and experimental repetition included as random effects. Dichotomization and natural log transformation (lnskew0 function in STATA) were used as required for non-normally distributed data. Univariate analysis was first performed, and experimental group or parity was included in the full models if $P < 0{\cdot}2$. Final models were developed using a stepwise backward selection, and variables were retained in the final model if $P < 0{\cdot}05$. Final models were verified for normality and homogeneity, and occasional outliers regardless of group were removed from the data set, and the models rerun as required. Data collected from the pigs removed from the study were not used for statistical analysis.

Results

The birth weight of HBW and LBW pigs selected was 1·61 ± 0·07 and 1·29 ± 0·17 kg, respectively, and the average Z-scores of the high- and low-birth-weight litters were 1·0 and −1·9 standard deviations, respectively ($P < 0·05$ for both). The average weaning age was 21·5 days. Across both groups, the mean growth rate during lactation was 0·214 ± 0·043 kg/day, which did not differ by group. Average wean weights, 6·2 ± 1·1 for HBW and 5·9 ± 1·0 for LBW, did not differ by group, but the numerical difference (0·3 kg) was similar to the numerical difference in birth weight.

Clinical observations and pathology

There were no between-group statistical differences in clinical parameters including rectal temperature (peak temperature or temperature over baseline) or respiratory clinical signs. The percent of gross lesions observed in the lung lobes were compared between HBW and LBW groups (Table 2). The HBW group had significantly higher mean total gross lung lesions (14·2% ± 9·2%) than the LBW group (10·1 ± 8·3%) due to more severe lesions in the cranial, middle, and accessory, but not the caudal lobes (Table 2).

Microscopic lesion severity scores were compared per lobe between HBW and LBW groups and were compared between lobes within HBW and LBW groups (Tables 2 and 3). LBW piglets had significantly lower total microscopic lung scores ($P = 0·009$), mainly associated with decreased lesions in left cranial ($P = 0·005$) and right middle lobes ($P = 0·02$). The microscopic lesion scores for the within-group comparisons by lobe were not significant for the HBW pigs. Within the LBW group, the right cranial lung lobe had a significantly higher ($P < 0·001$) lesion severity score than all other lobes (Table 3). Immunoreactivity for the anti-influenza antibody was lower in the LBW pigs in the left ($P = 0·025$) and right ($P = 0·014$) middle lung lobes. However, there was no difference in total immunoreactivity between the two groups ($P = 0·13$).

Cytokines and additional diagnostic testing

For the HBW pigs, the mean cytokine levels (pg/ml) in the BALF were 306 ± 231 for IL-1β, 57 ± 49 for IL-6, 63 ± 51 for IL-8, and 1068 ± 1247 for IFN-α. For the LBW pigs, the mean cytokine levels in the BALF were 300 ± 258 for IL-1β, 55 ± 50 for IL-6, 49 ± 46 for IL-8, and 843 ± 1193 for IFN-α. There were no statistical differences in cytokine production in BALF ($P > 0·05$). All pigs were negative for PRRSv, PCV2, and Mhyo by PCR.

Virus titers

The mean concentration of influenza virus isolated from the lung trended lower in the LBW (3·0 ± 1·5 $\log_{10}$ $TCID_{50}$/ml) compared with HBW pigs (3·5 ± 1·4 $\log_{10}$ $TCID_{50}$/ml) ($P = 0·09$, Table 2). There was no significant difference in the virus levels isolated from nasal swabs. The concentration

Table 2. Pathological lesion assessments in low- compared with high-birth-weight (BW) piglets 48 hours post-inoculation

	Low BW ($n = 32$)		High BW ($n = 32$)		*P* value
	Mean	SD	Mean	SD	H versus L
Gross lung lesion percentages					
Cranial lobes	3·2	3·0	4·3	2·5	**0·03**
Middle lobes	3·8	3·0	6·0	4·5	**0·02**
Accessory lobe	1·3	1·7	1·8	1·3	**0·05**
Caudal lobes	1·8	1·8	2·0	1·8	$P > 0·05$
Total lung	10·1	8·3	14·2	9·2	**0·03**
Microscopic lesion severity (scored 0–3)					
Left cranial	1·6	0·6	1·8	0·6	**0·005**
Left middle	1·5	0·7	1·7	0·7	$P > 0·05$
Right cranial	1·9	0·5	2·0	0·5	*P > 0·05*
Right middle	1·4	0·7	1·8	0·6	**0·02**
Total left lobes	3·0	1·2	3·5	1·1	**0·009**
Total right lobes	3·4	1·0	3·8	1·0	$P > 0·05$
Total lung	1·6	0·5	1·8	0·5	**0·009**
Influenza antigen staining intensity in lung (scored 0–4)					
Left cranial	1·4	0·9	1·4	0·9	$P > 0·05$
Left middle	1·3	1·1	1·8	0·9	**0·025**
Right cranial	1·4	0·9	1·4	0·9	$P > 0·05$
Right middle	1·3	0·9	1·8	0·9	**0·014**
Total lung	1·3	0·8	1·6	0·7	0·13
Viral concentration isolated from lung and nasal cavities					
Lung (log 10)	3·0	1·5	3·5	1·4	0·09
Nasal cavity (log 10)	2·0	1·6	1·9	1·5	$P > 0·05$

Bolded *P* values are significant ($P < 0·05$) based on multilevel regression (STATA; xtmixed).

Table 3. Microscopic lesion scores by lung lobes within the LBW and HBW groups

	LBW ($n = 31$)* ($P < 0·001$)		HBW ($n = 32$) ($P > 0·05$)	
Lesions by lobe	Mean	SD	Mean	SD
Left cranial	1·58[a]	0·62	1·81	0·56
Left middle	1·53[a]	0·67	1·72	0·69
Right cranial	1·98[b]	0·38	1·95	0·46
Right middle	1·46[a]	0·68	1·81	0·64

*One outlier pig was removed from LBW group for the statistical analysis as indicated by model diagnostics.
Superscripts within the LBW column indicate significantly different lesion scores ($P < 0·05$) based on multilevel regression (STATA; xtmixed).

of influenza virus isolated from nasal swabs, however, was significantly lower in progeny from parity 2 compared with parity 1 sows ($P = 0{\cdot}01$).

Discussion

This experiment provides clear, albeit unexpected, insight into the impact of litter birth weight on the severity of swine influenza infection. Based on the research indicating that low birth weight is a risk factor for nosocomial influenza A infections,[25] our hypothesis was that the severity of influenza infection in the LBW pigs would be more severe than in the HBW pigs. The opposite occurred, in which severity was lower in LBW compared with HBW piglets. Significant differences, however, were limited to pathology and only in specific lobes. Cytokine protein production, virus shedding, and clinical respiratory signs did not differ between groups. We suspect that this is partly due to the relatively low experimental power (0·32–0·68 depending on outcome variable) and the considerable between-pig variation. To demonstrate statistical differences across other variables, a similar future experiment would need sample sizes of 40–50 litters (selecting 4 piglets per litter) or 60–100 litters (selecting 2 piglets per litter) based on the group means and variances generated in the present study.

There are many reasons why a sow may deliver a low- or high-birth-weight litter, and some sows flip-flop delivering high- and low-birth-weight litters in successive parities. However, a small proportion of sows consistently deliver high- or low-birth-weight litters. Ongoing research conducted by our group has demonstrated that the litter birth-weight phenotype is not fully expressed until sows have repeatedly delivered multiple consecutive high- or low-birth-weight litters.[26] Although evaluating the progeny from these repeatable sows is our overarching research goal, in this study, we were unfortunately not able to include progeny from sows older than second parity. So although litter birth weight and Z-score differed significantly by group, we were unable to specifically select progeny from dams delivering repeatable phenotypes. This may or may not have impacted our results. The exact mechanisms related to the delivery of repeatable HBW or LBW phenotypes are not fully understood at this time, but it may involve epigenetic programming, and we have studies underway to investigate this phenomenon.

A number of studies have linked influenza susceptibility to low- birth-weight and epigenetic programming. Firstly, it is proposed that the explosive mortality associated with the 1918 H1N1 pandemic may be associated with acquired epigenetically mediated immunological differences among birth cohorts in combination with the emergence of a new strain/subtype.[27] It is difficult to prove whether or not this theory is valid; however, epigenetic programming has been linked to the metabolic syndrome (hypertension, insulin resistance, dyslipidemia, obesity),[28] and obesity indirectly increases the susceptibility to a number of community-borne pathogens, including influenza, through number of potentially altered immunological mechanisms.[29]

Direct epigenetic links to respiratory health have also been reported. Pre-natal and early childhood environmental programming is directly associated with the risk of asthma and allergic airway disease.[30,31] Moreover, host defenses in response to influenza infection involve a number of antiviral mechanisms including pro-inflammatory interleukin-32 (IL-32), cyclooxygenase-2 (COX-2), and prostaglandin E2 (PGE2). It has been demonstrated that epigenetic changes that upregulate the DNA methyltransferase (DNMT) enzymes act to silence genes and decrease expression of IL-32, COX-2, and PGE2, thus reducing host defenses and viral clearance.[32,33] In theory, epigenetic programming events that alter the methylation of genes responsible for pro-inflammatory or other immunological pathways may alter susceptibility or severity of influenza in humans and possibly in animals. Why influenza severity was greater in HBW pigs in the present study is unclear and warrants further investigation. While it is possible that our findings are spurious, it is also possible that mechanisms affecting influenza susceptibility in pigs differ from those of humans.

In spite of the fact that the birth weights differed by group by approximately 0·3 kg, weaning weight did not differ statistically. This is not surprising because there are numerous factors affecting average daily gain (ADG) during the lactation period including litter size, parity of dam, sow feed intake, cross-fostering procedures, and room temperature. Piglets selected for this study suckled their birth sow and were of average weight at birth compared with their siblings. Although group differences in weaning weight were identical to the differences in birth weights (0·3 kg), there was considerably more variation in weaning weight than in birth weight, resulting in the lack of statistical significance. To evaluate whether lactational ADG was related to influenza severity, it was tested in all statistical models as a potential predictor variable, but was found to be non-significant in all. Although excessive childhood weight gain following fetal and infant malnourishment is a risk factor for metabolic syndrome in humans,[34] it was not significantly associated with influenza severity in the present study.

A limitation of this study was the lack of a non-inoculated reference control group. Although the primary objective of the study was to compare high- and low-birth-weight pigs, the addition of a reference control group would have primarily served to verify whether the source farm remained free of swine influenza as well as the absence of pneumonic lesions and BALF cytokines in non-infected pigs. The experiment used small batches of pigs over a number of months, and each successive batch tested negative for swine

influenza by serology a week before arrival and by PCR at arrival. We are confident that the source farm remained negative throughout the experiment due to the absence of respiratory disease in the source farm and lack of seroconversion. Moreover, the negative control BALF was collected from a pig that came from the same source farm and was kept in the BSL2 facility for 4 weeks. This animal was SIV, PRRSv, PCV2, and Mhyo negative at necropsy.

Another interesting finding in this study involved the morphometric techniques that we applied to limit variation in sampling and scoring methods. Using systematic sampling of the lung lobes at 2·5 cm from the lobar tip regardless of lesions seen was especially helpful in this study because we were sampling the animals at 48 hours after inoculation (3 days earlier than is customary). For several pigs, there were minimal to no gross lesions seen in the lungs, but these animals had both microscopic lesions and corresponding influenza immunoreactivity. This is a conundrum faced in experimental studies when treatments are compared, and there are minimal to no lesions seen at necropsy. In most studies, the investigator often collects one or two samples containing a lesion, and these sites vary from animal to animal. For the lungs that have no lesions, a pre-established sample, such as a section from the right middle lobe, may be collected. In an attempt to standardize the sampling process for influenza studies, specific sites were selected for this study.

The sampling sites for this study were chosen based on the personal observations by the pathologist (SED) on over 250 necropsies of experimentally inoculated pigs (both intratracheal and intranasal) where the majority of lesions spanned the area approximately 2–3 cm from the tip of each cranial and middle lobe.[19,35,36] As a pilot study, the sampling method and scoring were also compared for 60 of these pigs (comparison not published[35]).

Using the individual lung lobes and summative scores, the findings of significant differences were variable in location, but most consistent in the right middle lung lobe where both the microscopic and immunohistochemistry scores were significantly different between LBW and HBW groups. However, when the microscopic scores were compared between the lobes and within the birth weight groups, the right cranial lobe was significantly different. This finding is most likely related to the fact that pigs have a tracheal bronchus that enters the right cranial lung lobe and is cranial to the carina.[37] This anatomical variation in pigs could allow a higher concentration of the inoculum to enter the right cranial lung lobe than the other lobes. It also points to the fact that it is important to make sure that the animals are consistently placed in the same position for waking from the anesthetic after being inoculated, so that the gravitational pull on inoculum in the airways is similar between pigs.

Another reason for standardizing the tissue collection procedures is to enable the use of more advanced applications in our analysis. In general, the microscopic and immunohistochemistry scores are assigned by pathologists that are blinded to the treatment groups. However, there is always a certain degree of subjectivity to these scores. If we apply morphometric scoring techniques, such as using a computer program to measure the color intensity from microphotographs of an immunohistochemistry slide,[38] then we have a quantitative, instead of a qualitative score for the statistical analysis.

In summary, there were two key findings in this study. Using the SIV infection model, we found that HBW pigs had more severe lesions than LBW pigs when infected with TX98 for 48 hours. The results of this study also demonstrated a standardized method that could be applied in future studies where morphometric scoring techniques are used. However, further assessment is needed to determine the repeatability and accuracy of the proposed sampling and assessment methods for influenza studies.

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