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ORIGINAL ARTICLE

Vitamin E and Selenium Levels are Within Normal Range in Pigs Diagnosed with Mulberry Heart Disease and Evidence for Viral Involvement in the Syndrome is Lacking

H. Shen[1], P. R. Thomas[1], S. M. Ensley[1], W.-I. Kim[1,2], A. T. Loynachan[1], P. G. Halbur[1] and T. Opriessnig[1]

[1] Department of Veterinary Diagnostic and Production Animal Medicine, College of Veterinary Medicine, Iowa State University, Ames, IA, USA

[2] College of Veterinary Medicine, Chonbuk National University, Jeonju, South Korea

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Correspondence:
T. Opriessnig, Department of Veterinary Diagnostic and Production Animal Medicine, College of Veterinary Medicine, Iowa State University, Ames, IA 50011, USA.
Tel.: +1 515 294 1137; Fax: +1 515 294 3564; E-mail: tanjaopr@iastate.edu

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Summary

Mulberry heart disease (MHD) in pigs is characterized by lesions of acute haemorrhagic myocarditis and myocardial necrosis. The objectives of this study were to determine the levels of vitamin E and selenium and 13 other trace minerals in heart and liver tissues and to determine the prevalence of certain viral infections in heart tissues from MHD-affected and MHD-unaffected pigs and the vitamin E and selenium concentration in feed samples from selected farms with MHD. Based on the pathological examination, 114 pigs were separated into MHD lesion-negative (L-NEG) ($n = 57$) and MHD lesion-positive (L-POS) ($n = 57$) groups. Seventy-three samples (40 L-NEG and 33 L-POS) were subjected to chemical analysis, and 66 (32 L-NEG and 34 L-POS) were subjected to PCR detection for viral pathogens. Lower ($P < 0.05$) levels of myocardial copper, lower ($P < 0.05$) levels of hepatic magnesium and higher ($P < 0.05$) levels of myocardial and hepatic sodium were detected in the L-POS cases. Although lower ($P < 0.05$) levels of hepatic selenium were detected in L-POS group, all were within the normal range. Analysis of feed samples ($n = 22$) revealed that selenium levels in all the samples were above the legal limit (0.3 ppm) for pigs. Vitamin E levels in all feed samples were above 20 IU/kg. Among the 66 pigs subjected to PCR detection, there were 19, 4, 13, 8, 2 and 1 animals positive for porcine circovirus type 2, porcine reproductive and respiratory syndrome virus, pan-herpes virus, porcine enterovirus, pan-pestivirus and porcine parvovirus, respectively. Clear evidence of viral association with L-POS was lacking.

Introduction

Mulberry heart disease (MHD) was first described in 1967 (Seffner et al., 1967) and continues to be a common and often frustrating problem in nursery age pigs today (Pallarés et al., 2002). The hallmark microscopic lesions of MHD are transmural haemorrhages in the heart muscle and severe necrosis of heart muscle fibres (Sharp et al., 1972b). This disease and its associated lesions are known by other names such as microangiopathy (MAP) (Rice and Kennedy, 1989; Korpela, 1990a,b, 1991) and white muscle disease (Mahan et al., 1973). In 2007, swine practitioners and producers observed a marked increase in MHD in nursery pigs across the United States. (personal communication Dr. R. B. Baker). Typically, pigs were affected 5–7 days after arrival into the nursery, and the mortality was usually 2–5% regardless of the intervention strategies used such as vitamin E and selenium supplementation of

sows and piglets and preventive antibiotic treatment before and after shipment.

MHD is thought to be associated with vitamin E and/or selenium deficiency. A higher frequency of MHD was observed in pigs fed low-level selenium diets containing torula yeast (Sharp et al., 1972a) and MHD has been experimentally reproduced with diets deficient in vitamin E and selenium (Van Vleet, 1982). In the field, gross lesions such as a heart resembling the appearance of a mulberry, fluid accumulation in the pericardial sac and sudden death are reported conditions of vitamin E and selenium deficiency in pigs (Mahan et al., 1973). A retrospective analysis was conducted on vitamin E and selenium levels in liver in 37 and 26 cases, respectively. Only 25% of the pigs diagnosed as having MHD had low liver levels (<2 ppm) of vitamin E (Pallarés et al., 2002), and all pigs had adequate levels of selenium. Supplemental vitamin E and selenium resulted in a reduction in the incidence of lesions including myocardial degeneration, skeletal muscle degeneration and exudative diathesis by approximately 50% (Sharp et al., 1972b).

Mineral changes have been investigated in several studies. In pigs that died of MHD or microangiopathy, significantly ($P < 0.001$) higher myocardial and hepatic iron concentrations were found (Korpela, 1990b). Similarly, myocardial and hepatic calcium concentration in MHD-affected pigs was significantly ($P < 0.001$) higher than in pigs with other diseases and in healthy slaughter pigs (Korpela, 1991). On the other hand, myocardial and hepatic magnesium concentrations in pigs with microangiopathy were significantly ($P < 0.001$) lower than in pigs with other diseases and in healthy slaughter pigs (Korpela, 1991). These results suggested that iron, calcium and magnesium concentrations may play a role in the development of MHD (Korpela, 1990b, 1991).

It is possible that there are known and unknown pathogens that may be involved in inducing MHD-like lesions in pigs. Different bacteria have been reported to be isolated in cases with necrotizing myocarditis resembling MHD, including *Escherichia coli*, which is the most common organism (Trapp et al., 1970), and *Streptococcus suis* type 2 from hearts of pigs aged 3–5 weeks (Sanford, 1987). Viral pathogens are also capable of causing MHD-like lesions in pigs, such as porcine circovirus (PCV) type 2 (PCV2) (West et al., 1999) and porcine parvovirus (PPV) (Bolt et al., 1997). Cases of heart failure without the hallmark lymphoid lesions associated with PCV2 (lymphoid depletion and histiocytic replacement of follicles) are rarely tested for the presence of PCV2. However, high amounts of PCV2 in areas of myocardial necrosis in cases consistent with MHD but with normal vitamin E and selenium concentrations have recently been observed (Opriessnig et al., 2006). The prevalence of PPV-associated lesions in growing pigs is not known because laboratory testing for PPV is usually limited to mummified foetuses. Finally, the determination of involvement of porcine enterovirus (PEV) in cases of heart failure is typically not carried out despite having a PCR available and frequently using it on suspected enterovirus-associated CNS cases. Recently, a new disease termed 'porcine myocarditis syndrome' was reported in Australia (McOrist et al., 2004). The cause is believed to be Bungowannah virus (BGWV) that is assumed to be a new species of pestivirus (Kirkland et al., 2007). Nevertheless, the role of these organisms is difficult to assess because many are considered secondary or opportunistic pathogens. It is also possible that many of the cases diagnosed as MHD are not associated with vitamin E or selenium deficiency but are associated with acute viral infection.

The objectives of this study were to determine the relationship between MHD and levels of vitamin E and selenium and 13 other trace minerals in heart and liver tissues from MHD-affected (L-POS) and MHD-unaffected (L-NEG) pigs and to determine the vitamin E and selenium concentration in feed samples from selected farms affected by MHD. Additionally, several PCR assays were conducted on heart tissues to explore a possible relationship between viral pathogens and MHD.

Materials and Methods

Samples

Samples were collected from a total of 114 pigs representing 45 farms and five US States from August in 2007 to June in 2009. Farms ranged in size from 800 to 4500 head and tissues were collected from pigs that ranged in age from 7 to 120 days. Depending on the type of samples collected (liver, heart or both), tissues went for either chemical analysis ($n = 48$; liver only), virus analysis ($n = 41$; heart only) or both ($n = 25$ liver and heart). All farms had a history of increased numbers of MHD cases in nursery pigs with 1–10% of the pigs affected by sudden death and gross lesions of MHD (enlarged heart with transmural haemorrhages, ascites and enlarged liver). Based on the histological examination (myofibre necrosis and haemorrhage in the atrium and ventricular walls and in the interventricular septum), samples were separated into MHD lesion-positive (L-POS; $n = 57$) and MHD lesion-negative (L-NEG; $n = 57$) classifications. Seventy-three of the 114 pigs were used for chemical analysis (40/73 L-NEG; 33/73 L-POS), and 66/114 were tested for viral pathogens (32 L-NEG; 34 L-POS). Differences in numbers tested were because of unavailability of fresh liver (chemical analysis) or fresh heart tissues (viral analysis) in some cases. In addition, 22 feed samples representing 11 farms

were also collected and tested for the presence of vitamin E and selenium.

Samples utilized for chemical analysis
Overall, liver and heart tissues from 73 pigs representing 22 farms and three states (Iowa, Illinois and Minnesota) were analysed for the presence of vitamin E and selenium and 13 other trace minerals. Mineral panels were completed on all heart and liver tissues using a Varian 820 ICP-Mass Spectrometer (Varian, Inc. Walnut Creek, CA, USA). The panel included tissue levels for cadmium, calcium, chromium, cobalt, copper, iron, magnesium, manganese, molybdenum, phosphorus, potassium, selenium, sodium and zinc. In addition, vitamin E levels were also obtained from heart and liver tissues. Selenium and vitamin E levels were tested in the 22 feed samples collected from 11 different farms.

Samples used for pathogen analysis
A total of 66 heart tissue samples collected from pigs representing 33 farms from five states (Iowa, Illinois, Kansas, Minnesota and Ohio) were analysed for the presence of 11 different viruses/viral families. Specifically, samples were tested using real-time or conventional PCR assays for pan-morbillivirus, pan-coronavirus, pan-pestivirus, PEV, pan-herpesvirus, PCV type 1 (PCV1), PCV2, PPV, European (EU) and North American (NA) porcine reproductive and respiratory syndrome virus (PRRSV), BGWV and West Nile virus (WNV).

Chemical analysis

Vitamin E level analysis
Vitamin E analysis was carried out using high-performance liquid chromatography (HPLC).

Trace mineral level analysis
Trace mineral panels were completed on heart and liver tissues using a Varian 820 ICP-Mass Spectrometer. The panel included tissue levels for calcium, chromium, cobalt, copper, iron, magnesium, manganese, molybdenum, phosphorus, potassium, selenium, sodium and zinc.

Pathogen analysis

Viral RNA/DNA extraction
Heart tissues were mechanically homogenized in 10–35 ml (depending on sample size) sterile Earle's medium (Sigma-Aldrich®, St Louis, MO, USA) by using a stomacher (Seward Stomacher®80; Seward, Sussex, UK) for 120 s. The homogenates were centrifuged at 3220 ***g*** for 30 min at 4°C, and the supernatant was transferred into 5 ml tubes. The tubes were stored at −20°C until the day of the extraction when 50 μl of the homogenate was subjected to total nucleic acid extraction using the 5× MagMAX-96 viral RNA isolation kit (Ambion, Austin, TX, USA) and an automated extraction machine (KingFisher 96; Thermo Scientific, Waltham, MA, USA) following the manufacturers' instructions.

Conventional gel-based PCRs
All the primer sequences are summarized in Table 1. The reverse transcriptase (RT)-PCRs were performed using the QIAGEN® One-step RT-PCR kit (Qiagen, Valencia, CA, USA), and all the other PCRs were performed using the HotStarTaq® Plus DNA Polymerase (Qiagen). The PCR amplicons were analysed using the QIAxcel DNA kit (Qiagen) according to the manufacturers' instructions.

Pan-morbillivirus and pan-coronavirus RT-PCR
Five microlitres of the RNA was added to 45 μl of the reaction mixture containing 28 μl of nuclease-free water, 10 μl of 5× reaction buffer, 2 μl of 10 mM dNTP Mix, 1 μl of each of 20 μM forward and reverse primers, 1 μl of RNase inhibitor and 2 μl of RT-PCR enzyme mix. The reaction conditions were 50°C for 30 min, 95°C for 15 min, followed by 40 cycles of 1 min at 94°C, 1 min at 53°C for pan-morbillivirus and at 50°C for pan-coronavirus, and 1 min at 72°C, and a final extension at 72°C for 10 min.

Pan-pestivirus and PEV nested RT-PCR
For the first-round reaction, the mix and amplification protocol of pan-morbillivirus and pan-coronavirus PCRs were used, with the only change of annealing temperature at 55°C. The nested-PCR was performed with 2 μl of first-round amplification product. The PCR mixture consisted of 40.5 μl of nuclease-free water, 5 μl of 10× PCR buffer, 1 μl of 10 mM dNTP mix, 0.5 μl of each of 20 μM forward and reverse primers and 0.5 μl of HotStarTaq® Plus DNA Polymerase (Qiagen). After initial denaturation at 95°C for 15 min, 40 cycles of PCR were performed (1 min at 94°C, 1 min at 60°C for pan-pestivirus or at 55°C for porcine enterovirus, 72°C for 1 min) and terminated with an elongation step at 72°C for 10 min.

Pan-herpesvirus nested-PCR
Mixed forward primers (HerpCon1F, HerpCon2F, HerpCon3F, and HerpCon4F) and reverse primers (HerpCon7R and HerpCon8R) were used. Five microlitres of sample was added to 45 μl of reaction mix containing 37 μl of nuclease-free water, 5 μl of 10× PCR buffer, 1 μl of 10 mM dNTP Mix, 1 μl of 100 μM forward primer, 0.5 μl of 100 μM reverse primer and 0.5 μl of HotStarTaq® Plus DNA Polymerase (Qiagen). The amplifica-

Table 1. Oligonucleotide primers and probes used in conventional and real-time PCR assays in this study

Virus	Primer/probe	Sequence (5′–3′)	References
Pan-Morbillivirus	P1-F	ATGTTTATGATCACAGCGGT	Jensen et al. (2002)
	P2-R	ATTGGGTTGCACCACTTGTC	
Pan-Coronavirus	Cor-F	ACTCAAATGAATTTGAAATATGC	Quiroga et al. (2008)
	Cor-R	TCACACTTTGGATAATCCCA	
Pan-Pestivirus	324+ F	ATGCCCWTAGTAGGACTAGCA	Vilcek et al. (2003)
	326- R	TCAACTCCATGTGCCATGTAC	
	A11+ F	AGTACAGGGTAGTCGTCAGTGGTTCG	
	A14- R	CAACTCCATGTGCCATGTACAGCAG	
Pan-Enterovirus	PEV-1AF	AGTTTTGGATTATCTTGTGCCC	Zell et al. (2000)
	PEV-1BR	CCAGCCGCGACCCTGTCAGGCAGCA	
	PEV-1CNF	TGAAAGACCTGCTCTGGCGCGAG	
	PEV-1DNR	GCTGGTGGGCCCCAGAGAAATCTC	
Pan-Herpesvirus	HerpCon1F	GAYTTYGCNAGYYTNTAYCC	Ehlers et al. (1999)
	HerpCon2F	TCCTGGACAAGCAGCARNYSGCNMT	
	HerpCon3F	GAYTTYGCIAGYYTITAYCC	
	HerpCon4F	TCCTGGACAAGCAGCARIYSGCIMTIA	
	HerpCon5F	TGTAACTCGGTGTAYGGNTTYCANGG	
	HerpCon6F	TGTAACTCGGTGTAYGGITTYACIGGI	
	HerpCon7R	GTCTTGCTCACCAGNTCNCANCCYTT	
	HerpCon8R	GTCTTGCTCACCAGITCIACICCYTT	
	HerpCon9R	CACAGAGTCCGTRTCNCCRTADAT	
	HerpCon10R	CACAGAGTCCGTRTCICCRTAIAT	
Porcine circovirus type 1	P1570F	TGGCCCGCAGTATTTTGATT	Primers in Opriessnig et al. (2003)
	P1642R	CAGCTGGGACAGCAGTTGAG	
	PCV1 Probe	CAL Fluor® (Biosearch Technologies, Novato, CA, USA) Orange 560-CAGCAATCAGGCCCCCCAGGAAT-Black Hole Quencher™ (Biosearch Technologies, Novato, CA, USA)	
Porcine circovirus type 2	P1570F	TGGCCCGCAGTATTTTGATT	Opriessnig et al. (2003)
	P1642R	CAGCTGGGACAGCAGTTGAG	
	P1591 Probe	FAM™ (Biosearch Technologies, Novato, CA, USA)- CCAGCAATCAGACCCCGTTGGAATG-Black Hole Quencher™	
Porcine parvovirus	461F	CAGAATCAGCAACCTCACCA	Shen et al. (2010)
	566R	GCTGCTGGTGTGTATGGAAG	
	PPV Probe	FAM™-GCAAGCTTAATGGTCGCACTAGACA-Black Hole Quencher™	
Bungowannah virus	BGWF	CAGTTGGTGTGATCCATGATCCT	Finlaison et al. (2009)
	BGWR	GGCCTCACCCTGCAACTTT	
	BGWV Probe	FAM™-AAGTCTTCAGCAGTTAACT- Black Hole Quencher™	
West Nile virus	WN3′NC-F	CAGACCACGCTACGGCG	Lanciotti et al. (2000)
	WN3′NC-R	CTAGGGCCGCGTGGG	
	WN3′NC-Probe	FAM™-TCTGCGGAGAGTGCAGTCTGCGAT-Black Hole Quencher™	

tion conditions were 95°C for 15 min, followed by 35 cycles of 94°C for 1 min, 46°C for 1 min and 72°C for 1 min and finally extension at 72°C for 10 min. The nested-PCR was performed with 2 μl of the first-round product, forward primers (HerpCon5F and HerpCon6F) and reverse primers (HerpCon9R and HerpCon10R). The

PCR mixture consisted of 40.5 μl of nuclease-free water, 5 μl of 10× PCR buffer, 1 μl of 10 mM dNTP mix, 0.5 μl of each of 100 μM forward and reverse primers and 0.5 μl of HotStarTaq® Plus DNA Polymerase (Qiagen). The cycling conditions were 95°C for 15 min, 35 cycles of 94°C for 1 min, 46°C for 1 min and 72°C for 1 min and terminated with elongation at 72°C for 10 min.

Real-time PCR or real-time RT-PCR

Specimens were considered positive when the C_t value was below 40. All primer–probe combinations are summarized in Table 1. All the probes were labeled with different reporter dyes at their 5′ end (FAM™ or CAL Fluor® Orange 560) and their 3′ end (Black Hole Quencher™).

PCV1, PCV2 and PPV real-time PCR assays

The reaction mix consisted of 12.5 μl of ABI 2× PCR master mix (Applied Biosystems, Foster City, CA, USA), 0.5 μl of 10 μM probe, 1 μl each of 10 μM forward and reverse primers, 7.5 μl of nuclease-free water and 2.5 μl of extracted DNA, for a total volume of 25 μl. The amplification conditions were 2 min at 50°C, 10 min at 95°C and 40 cycles of 15 s at 95°C and 1 min at 60°C.

PRRSV one-step real-time RT-PCR

The reaction was performed using the TaqMan® NA and EU PRRSV reagents (Applied Biosystems). Eight microlitres of extracted RNA was added to 17 μl of reaction mixture containing 12.5 μl of 2× multiplex RT-PCR buffer, 2.5 μl of 10× PRRSV primer–probe mix, 1.25 μl of 20× multiplex enzyme mix and 0.75 μl of nuclease-free water. The amplification conditions were 10 min at 45°C, 10 min at 95°C and 40 cycles of 15 s at 95°C and 70 s at 60°C.

BGWV real-time RT-PCR

The reaction was performed using the AgPath-ID™ One-Step RT-PCR Reagents (Applied Biosystems). Extracted RNA (2.5 μl) was added to 22.5 μl of reaction mixture containing 12.5 μl of 2× RT-PCR Buffer, 1 μl of 25× RT-PCR enzyme mix, 0.5 μl of BGWV probe, 1 μl of BGWF primer, 1 μl of BGWR primer and 6.5 μl of nuclease-free water. The amplification conditions were 10 min at 45°C, 10 min at 95°C and 40 cycles of 15 s at 95°C and 45 s at 60°C.

Table 2. Number of pigs that were found to have deficient, adequate or toxic levels of selenium, and mean levels of selenium for MHD lesion-positive (L-POS) and MHD lesion-negative (L-NEG) pigs

			Classification			
Tissue	Status	*n*	Deficient	Adequate	Toxic	Mean ± SE
Heart	Reference values[a]		<0.1	0.2–0.3	0.4–4.1	
	L-POS	33	0	10	23	0.48 ± 0.03
	L-NEG	40	0	12	28	0.48 ± 0.02
Liver	Reference values[a]		<0.1	0.1–2.9	3.0–120	
	L-POS	33	0	33	0	0.73 ± 0.05
	L-NEG	40	0	38	2	1.15 ± 0.11

MHD, mulberry heart disease.
[a]Ching et al., 2002; Chung et al., 1992;.

Table 3. Number of pigs that were found to have deficient, adequate or high levels of Vitamin E, and mean levels of Vitamin E for MHD lesion positive (L-POS) and MHD lesion negative (L-NEG)

			Classification				
Tissue	Status	*n*	Deficient[a]	Deficient[b]	Adequate[a,b]	High[a,b]	Mean ± SE
Heart	Reference values		<2		2–3.5	>3.5	
	L-POS	33	8		9	16	1.66 ± 0.27
	L-NEG	40	23		11	6	1.18 ± 0.19
Liver	Reference values		<2	<3.8	3.8–10	>10	
	L-POS	33	9	11	13	10	4.08 ± 0.68
	L-NEG	40	21	29	8	3	2.11 ± 0.32

MHD, mulberry heart disease.
[a]Ching et al., 2002; Chung et al., 1992;.
[b]Puls, 1994.

WNV real-time RT-PCR

The reaction was performed using the AgPath-ID™ One-Step RT-PCR Reagents (Applied Biosystems). Extracted RNA (2.5 μl) was added to 22.5 μl of reaction mixture containing 12.5 μl of 2× RT-PCR buffer, 1 μl of 25× RT-PCR enzyme mix, 0.5 μl of probe, 1 μl of each of forward and reverse primers (Table 1) and 6.5 μl of nuclease-free water. The amplification conditions were 10 min at 45°C, 10 min at 95°C and 40 cycles of 15 s at 95°C and 45 s at 60°C (Lanciotti et al., 2000).

Results

Chemical analysis

Tissue samples

All results are reported in ppm. Vitamin E and selenium levels are summarized in Tables 2 and 3 for L-POS and L-NEG pigs. Among L-POS ($n = 33$) and L-NEG ($n = 40$) pigs, no significant differences were detected in levels of magnesium and selenium in heart, copper in liver and potassium, calcium, chromium, manganese, iron, cobalt, zinc, molybdenum and cadmium in both heart and liver. However, significant differences ($P < 0.05$) were observed in levels for sodium (1615.8 ± 25.2 in L-POS versus 1441.8 ± 39.4 in L-NEG pigs), copper (3.9 ± 0.2 in L-POS versus 4.6 ± 0.2 in L-NEG pigs) and vitamin E (4.4 ± 0.6 in L-POS versus 2.4 ± 0.6 in L-NEG pigs) in heart tissues. Significant differences were also observed in sodium (1805.0 ± 55.3 in L-POS versus 1586.6 ± 44.8 in L-NEG pigs), magnesium (159.2 ± 3.0 in L-POS versus 177.8 ± 4.1 in L-NEG pigs), selenium (0.7 ± 0.1 in L-POS versus 1.1 ± 0.1 in L-NEG pigs) and vitamin E (7.9 ± 1.3 in L-POS versus 3.7 ± 1.3 in L-NEG pigs) in liver tissues.

Feed samples

When tested for vitamin E, 2/22 diets contained between 20 and 30 IU/Kg, 11/22 between 45 and 100 IU/Kg and 9/22 between 100 and 350 IU/Kg. Concentrations for selenium in feed were as follows: 3/22 between 0.4 and 0.6 ppm, 17/22 between 0.7 and 2.0 ppm and 2/22 between 2.1 and 3.5 ppm, giving a total of 22/22 diets above the 0.3 ppm legal (National Research Council, 1998) supplementation limit for selenium.

Pathogen analysis

Pig level

A total of 19/66 pigs were positive for PCV2 (10 L-POS; nine L-NEG), four were positive for the North American PRRSV strain (one L-POS; three L-NEG), 13 were positive for pan-herpesvirus (seven L-POS; six L-NEG), eight were positive for porcine enterovirus (six L-POS; two L-NEG), two were positive for pan-pestivirus and one pig was positive for PPV (one L-NEG). There were no positive pigs for any of the other viruses tested.

Farm level

A total of 33 farms had pigs that were positive for one or more of the viruses tested. The distribution for viruses among farms was as follows: PPV 1/33, PCV2 12/33, NA PRRSV 3/33, pan-herpesvirus 10/33, pan-pestivirus 2/33 and PEV 5/33. While many of the viruses were detected from several pigs among the groups, they did not appear to be associated with MHD lesion status in the pigs. However, L-POS pigs had a trend for a higher incidence of PCV2 ($P = 0.45$), PEV ($P = 0.25$) and pan-herpesvirus ($P = 0.25$).

Discussion

The lesions associated with MHD have historically been called dietetic microangiopathy, hepatosis dietetica or exudative diathesis. It is generally accepted that MHD is caused by a deficiency of selenium and/or vitamin E in feed (Sharp et al., 1972a,b; Mahan et al., 1973; Van Vleet, 1982). However, this is not always the case as shown in a study comparing the concentration of vitamin E and selenium in feed for MHD (microangiopathy)-affected pigs and control pigs where both diets contained adequate amounts of vitamin E and selenium (Rice and Kennedy, 1989). Similarly, in Denmark, MHD persisted among young pigs even though abundant supplies of selenium and vitamin E were added to feedstuffs for sows and pigs (Nielsen et al., 1989). In the present study, analysis of feed samples revealed that the selenium concentration for all samples was above the 0.3 ppm legal supplementation limit, and all vitamin E levels were above 20 IU/Kg. Based on these results, it is not likely that a deficiency of selenium and vitamin E is the cause of MHD in the present study. MHD may be a vitamin E- and selenium-responsive condition in pigs but evidence continues to build indicating MHD is not because of a deficiency of either vitamin E or selenium in the diet.

The level of vitamin E (alpha-tocopherol) in tissue is an important parameter to measure to further investigate the role of vitamin E in the occurrence of MHD. In spite of apparently adequate amounts of dietary alpha-tocopherol, previous results indicate that pigs with MHD (microangiopathy) had lower tissue alpha-tocopherol concentrations than the control pigs (Rice and Kennedy, 1989). Investigation of MHD cases revealed that mean liver vitamin E concentrations were lower in pigs with MHD than in pigs that died of causes other than MHD (Pallarés et al., 2002). In cases submitted to the Veterinary Diagnostic Laboratory at Iowa State University from

1999 to 2009, only 24.6% (30/122) of the MHD cases were actually deficient in liver concentrations of vitamin E, and similarly only 5.6% (6/106) cases were deficient in liver concentrations of selenium. The concentrations of selenium and vitamin E in the liver and heart tissues of young pigs that died suddenly and had characteristic lesions of MHD were not significantly different from the concentrations found in pigs of the same age that had died from other causes (Nielsen et al., 1989). In the present study, in heart tissues, more pigs in the L-NEG group had deficient levels of vitamin E compared to L-POS pigs (8/33 L-POS; 23/40 L-NEG). This pattern was similar for liver tissues where a total of 40 pigs were deficient in vitamin E (11 L-POS; 29 L-NEG). This study does indicate a close association of heart and liver concentrations of vitamin E. As the concentration of vitamin E in the liver changed, the heart concentration also changed. With this information, it is difficult to associate MHD with a vitamin E deficiency as this study clearly provides evidence that pigs affected by MHD (L-POS) had high levels of vitamin E in tissues similar to levels in pigs with no lesions of MHD (L-NEG).

Several studies investigated the selenium concentration of tissues originating from MHD-affected pigs. In one experimental study, tissue selenium concentration did not appear to be related to the incidence of MHD-related lesions (Sharp et al., 1972b). In Western Australia, hepatic selenium concentrations were not low in pigs with MHD (Moir and Masters, 1979). In field cases, heart and kidney selenium concentrations were similar in pigs of either group, i.e. spontaneous MHD (microangiopathy) and control pigs (Rice and Kennedy, 1989), and liver selenium concentrations were adequate in all pigs including MHD cases and non-MHD cases (Pallarés et al., 2002). In pigs that died of MHD (microangiopathy), hepatic selenium concentration in pigs was lower than levels found in healthy pigs (Korpela, 1990a). In the present study, there were no significant differences in selenium levels in heart tissue; however, there was a significant ($P < 0.05$) difference in selenium in liver tissues. Even though the significant difference exists, the level of selenium for MHD pigs is not considered deficient, in fact it is considered within the adequate range (0.1–2.9 ppm). Of all the livers tested, the selenium levels were considered adequate in 71/73 and within the toxic range (3.0–120 ppm) in 2/73. This is not surprising when looking at the 22 feed samples, where all were above the legal supplementation limit. Selenium does occur naturally in corn and soy diets, which are the most common in swine rations; however, the level of selenium that naturally occurs in these diets is not higher than 0.1 ppm. When taking this into consideration, 19/22 feed rations were still above the legal supplementation limit as defined by the food and drug administration (FDA). It is most likely the excess selenium observed in these rations was because of added selenium, not naturally occurring selenium in the diet.

Some mineral changes, including iron, (Korpela, 1990b), calcium (Korpela, 1991) and magnesium (Korpela, 1991), have been reported to be related to MHD. In the present study, significantly higher levels of sodium in heart and liver, lower levels of copper in heart, magnesium in liver and phosphorus in liver were detected in L-POS pigs. However, more information is needed to associate these findings with the presence of MHD.

Bungowannah virus, recently described in MHD cases in Australia (Kirkland et al., 2007), was not identified in any of the samples tested. In this study, none of the pathogens detected could be clearly linked to MHD; however, there are a number of viruses in humans that have been shown to cause lesions in humans similar to MHD in pigs (Chariot et al., 1997; Ramanathan and Taylor, 1997; Beck et al., 2003). These viruses, which also cause selenium deficiencies in the infected persons, include human immunodeficiency virus (HIV) 1 and HIV2, Coxsackievirus B3 and several haemorrhagic fever viruses, most notably *Ebola Zaire* (Chariot et al., 1997; Ramanathan and Taylor, 1997; Taylor et al., 1997; Zhang et al., 1999; Beck et al., 2003). Furthermore, while the data were not strong enough to point to a definitive pathogen associated with MHD, there did seem to be a relationship between MHD and PEV and pan-herpesvirus. This is especially interesting because of the link between certain viruses in other species and their associated clinical signs that are common to MHD (Chariot et al., 1997; Ramanathan and Taylor, 1997; Taylor et al., 1997; Zhang et al., 1999; Beck et al., 2003).

While the aetiology of MHD and its relationship to vitamin E and selenium is still not completely understood, the data generated from this study provide evidence for the association of MHD with lower, but still within the currently defined normal range, selenium levels in tissues. Interestingly, we found that supplementation of vitamin E and selenium in feed above recommended and in some cases legal limits was not uncommon on farms experiencing MHD, and in some cases, these were approaching levels where it may become toxic to the pig. It is possible that there are several different causes of MHD-like lesions based on our observation of significantly higher levels of sodium in heart and liver, and lower levels of copper in heart, magnesium in liver and phosphorus in liver in MHD pigs. We also found some evidence that may warrant further investigation of the relationship between MHD and PEV and pan-herpesvirus.

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