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Serum folate, cobalamin, homocysteine and methylmalonic acid concentrations in pigs with acute, chronic or subclinical *Lawsonia intracellularis* infection

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ABSTRACT

Lawsonia intracellularis is the causative agent of porcine proliferative enteropathy. The clinical presentation can be acute (i.e. proliferative hemorrhagic enteropathy, PHE), chronic (i.e. porcine intestinal adenomatosis, PIA) or subclinical. In humans with chronic enteropathies, low serum folate (vitamin B_9) and cobalamin (vitamin B_{12}) concentrations have been associated with increased serum concentrations of homocysteine and methylmalonic acid (MMA), which reflect the availability of both vitamins at the cellular level. The aim of this study was to evaluate serum folate, cobalamin, homocysteine and MMA concentrations in serum samples from pigs with PHE, PIA or subclinical *L. intracellularis* infection, and in negative controls. Serum folate, cobalamin, homocysteine and MMA concentrations differed significantly among pigs in the PHE, PIA, subclinical and negative control groups. Serum folate concentrations in the PHE and PIA groups were lower than in the subclinical and negative control groups, while serum cobalamin concentrations were lower in the PIA group than in other groups. Serum concentrations of homocysteine were higher in the PHE, PIA and subclinical groups than in the negative control group. Serum concentrations of MMA were higher in the subclinical and PIA groups than in the control group. These data suggest that pigs infected with *L. intracellularis* have altered serum cobalamin, folate, homocysteine and MMA concentrations.

Introduction

Lawsonia intracellularis, an obligate intracellular bacterium, is the causative agent of porcine proliferative enteropathy, which is an important disease in the commercial pig industry (Lawson and Gebhart, 2000). Infection with *L. intracellularis* can lead to three different forms of disease: chronic, acute or subclinical. Pigs with chronic *L. intracellularis* infection (i.e. porcine intestinal adenomatosis, PIA) exhibit diarrhea and poor growth rate, whereas pigs with acute *L. intracellularis* infection (i.e. proliferative hemorrhagic enteropathy, PHE) have bloody diarrhea and may die suddenly (McOrist and Gebhart, 1999). Pigs with subclinical porcine proliferative enteropathy have no detectable clinical signs, but have reduced weight gain during the growth and fattening period (Guedes, 2004; Kroll et al., 2005; Paradis et al., 2005). In all forms of porcine proliferative enteropathy, there is proliferation of intestinal epithelial cells containing intracellular *L. intracellularis* (Lawson and Gebhart, 2000).

Folate (vitamin B_9) and cobalamin (vitamin B_{12}) play important roles in amino acid metabolism and nucleic acid synthesis (Wagner, 1995; Zingg and Jones, 1997; Fenech, 2012; Lopes et al., 2014). Most folate is absorbed in the proximal small intestine, whereas most cobalamin is absorbed in the distal small intestine (Halsted, 1980; Trugo et al., 1985; Said, 2011). Altered serum folate and cobalamin concentrations in humans and dogs have been associated with immunodeficiencies, neuropathies and gastroenteritis (Rutgers et al., 1995; Battersby et al., 2005; Cook et al., 2009; Lanska, 2010; Yakut et al., 2010). In humans, decreased serum folate and cobalamin concentrations have been associated with increased serum concentrations of homocysteine and methylmalonic acid (MMA), which reflect the availability of both vitamins at the cellular level (Ruaux et al., 2009; Berghoff et al., 2011; Fenech, 2012). Hyperhomocysteinemia and hypermethylmalonic acidemia can occur due to deficient function of methionine synthase and/or methylmalonyl-CoA mutase, which are intracellular folate- and/or cobalamin-dependent enzymes (Fenech, 2012; Grützner et al., 2013).

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Folate and cobalamin are essential for genomic stability and, in humans, it has been suggested that controlled supplementation of cobalamin and folate could prevent diseases associated with deficiencies of folate and cobalamin and also prevent developmental abnormalities and degenerative diseases (Blount et al., 1997). In pigs infected with *L. intracellularis*, it has been hypothesized that the bacterium affects the mitotically active cells of the intestinal crypts in order to influence host cell mitosis for its own propagation (Lawson et al., 1993; Smith and Lawson, 2001). The presence of intracellular *L. intracellularis* correlates with microscopically altered epithelium, suggesting that the bacterium affects epithelial cell growth (Rowland and Lawson, 1974; Lawson and Gebhart, 2000).

The aim of this study was to evaluate serum folate, cobalamin, homocysteine and MMA concentrations in pigs with PHE, PIA or subclinical *L. intracellularis* infection. We hypothesized that serum folate, cobalamin, homocysteine and MMA concentrations differ between pigs with different clinical forms (PHE, PIA or subclinical) of *L. intracellularis* infection.

Materials and methods

Samples

Serum samples from pigs with PIA ($n = 10$; 12–16 weeks of age), PHE ($n = 10$; 16–24 weeks of age) and subclinical proliferative enteropathy (subclinical; $n = 10$; 16 weeks of age) were selected from archived sera from controlled *L. intracellularis* challenge trials (PIA and subclinical) or diagnostic submissions to the University of Minnesota. *L. intracellularis* infection was confirmed using an immunoperoxidase monolayer assay (IPMA), as described by Guedes et al. (2002).

In addition, 10 serum samples from negative control pigs (12 to 16 weeks of age) were obtained during training courses for third year veterinary students at the College of Veterinary Medicine at Texas A&M University. These pigs were considered to be healthy based on a thorough physical examination. Sera from these pigs were tested by IPMA for antibodies against *L. intracellularis*. No other diseases were documented at the pig farm during the time of sample collection. The protocol for sample collection was reviewed and approved by the Texas A&M Institutional Animal Care and Use Committee (AUP 2009-143; 2012-13).

All pigs were fed commercial diets that met or exceeded all nutrient requirements established by the National Research Council (NRC, 1998). However, dietary intake of folate or cobalamin was unknown. Serum samples were collected by venipuncture of a jugular vein and all sera were stored in polypropylene tubes at –20 °C.

Folate and cobalamin immunoassays

Serum folate and cobalamin concentrations were measured using an Immulite 2000 assay (Siemens Healthcare Diagnostics) using methods previously validated by Grützner et al. (2011, 2012).

Homocysteine and methylmalonic acid assays

Serum homocysteine and MMA concentrations were measured using a gas chromatography-mass spectrometry method as described by Stabler et al. (1987) and Ruaux et al. (2001), respectively.

Statistical analysis

Data were evaluated for normality using the Shapiro–Wilk *W* test. Serum folate, cobalamin, homocysteine and MMA concentrations were compared among groups by analysis of variance (ANOVA), with a Tukey's post hoc test or non-parametric Kruskal–Wallis test with a Dunn's post-hoc test, and using the Spearman rank sum correlation coefficient (ρ). GraphPad Prism5 was used to perform statistical analyses.

Results

Serology for Lawsonia intracellularis

Sera from all 10 control pigs were negative for antibodies against *L. intracellularis*.

Table 1
Means (±standard deviations, SD) or medians (ranges) for serum folate (μg/L), serum cobalamin (ng/L), serum homocysteine (μmol/L) and methylmalonic acid (nmol/L) for the four groups of pigs included in this study: proliferative hemorrhagic enteropathy (PHE), porcine intestinal adenomatosis (PIA), subclinical proliferative enteropathy (Subclinical) and unaffected (Control).

Pigs	Folate (mean ± SD)	Cobalamin (median and range)	Homocysteine (mean ± SD)	Methylmalonic acid (mean ± SD)
PIA	25.5 ± 5.7	All <149	20.4 ± 4.4	804 ± 168
PHE	29.4 ± 8.1	195 (<149–527)	17.9 ± 3.2	649 ± 179
Subclinical	51.9 ± 17.1	169 (<149–379)	15.3 ± 2.6	1327 ± 408
Control	46.0 ± 11.4	198 (150–456)	11.0 ± 2.9	351 ± 134

Folate and cobalamin concentrations

Serum concentrations of folate were significantly different among groups; mean folate concentrations were 25.5 μg/L in the PIA group, 29.4 μg/L in the PHE group, 51.9 μg/L in the subclinical group and 46.0 μg/L in the control group ($P < 0.0001$; Table 1, Fig. 1). The post-hoc test indicated that serum folate concentrations in the PHE and PIA groups were significantly lower than the subclinical and control groups ($P < 0.05$).

Serum concentrations of cobalamin were significantly different among groups; median cobalamin concentrations were <149 ng/L in the PIA group, 195 ng/L in the PHE group, 169 ng/L in the subclinical group and 198 mg/L in the control group ($P < 0.01$; Table 1, Fig. 1). Dunn's post-hoc test indicated that serum cobalamin concentrations in the PIA group were significantly lower than the PHE, subclinical and control groups ($P < 0.05$).

Homocysteine and methylmalonic acid concentrations

Data were available for nine pigs only in the PIA group, since insufficient serum was collected for testing in one group. Serum concentrations of homocysteine were significantly different among groups; mean homocysteine concentrations were 20.4 μmol/L in the PIA group, 17.9 μmol/L in the PHE group, 15.3 μmol/L in the subclinical group and 11.0 μmol/L in the control group ($P < 0.0001$; Table 1, Fig. 2). The post-hoc test indicated that serum homocysteine concentrations in the PIA, PHE and subclinical groups were significantly higher than the control group ($P < 0.001$, $P < 0.01$ and $P < 0.05$, respectively).

Serum concentrations of MMA were significantly different among groups; mean MMA concentrations were 804 nmol/L in the PIA group, 649 nmol/L in the PHE group, 1327 nmol/L in the subclinical group and 351 nmol/L in the control group ($P < 0.0001$; Table 1, Fig. 2). The post-hoc test indicated that serum MMA concentrations were significantly higher in the subclinical group compared with the PIA, PHE and control groups ($P < 0.001$). Serum concentrations of MMA were significantly higher in the PIA group than in the control group ($P < 0.01$).

Correlations

A moderate correlation was observed between serum folate and homocysteine concentrations as well as between serum concentrations of cobalamin ($\rho = -0.50$, $P < 0.01$) and homocysteine ($\rho = -0.52$, $P < 0.001$; Table 2, Fig. 3). No correlation was observed between serum folate and MMA concentrations ($\rho = -0.01$, $P = 0.9$) or between serum cobalamin and MMA concentrations ($\rho = -0.27$, $P = 0.1$; Table 2, Fig. 3). There was a weak correlation between serum homocysteine and MMA concentrations ($\rho = 0.33$, $P = 0.04$; Fig. 4).

Discussion

To our knowledge this is the first study that investigated B-vitamins (folate and cobalamin) and B-vitamin metabolites