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Age-dependent seroprevalence of antibodies against a *Helicobacter pylori*–like organism and *Helicobacter pylori* in commercially reared swine

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Objective—To determine the prevalence of antibodies against a swine-origin *Helicobacter pylori*–like organism (HPLO) and *H pylori* in conventionally reared swine.

Animals—640 conventionally reared swine of various ages from 16 high-health farms in Canada, 20 sows from Ohio, and 35 gnotobiotic swine.

Procedures—Blood was collected from the cranial vena cava. Sera were collected and tested via ELISA for antibodies against antigen prepared from a swine-origin HPLO and human *H pylori* strain 26695.

Results—Antibodies reactive with a swine HPLO, *H pylori*, or both were detected in 483 of 640 swine from all 16 farms in western Canada. Seroprevalence varied with age and was low (5.6%) in suckling (≤ 4-week-old) swine and increasingly high in swine ranging from > 4 weeks old to adulthood.

Conclusions and Clinical Relevance—Findings suggested that colonization by a swine-origin HPLO, *H pylori*, or both and resultant seroconversion, like that of *H pylori* infection in humans, were common in commercial swine operations. Furthermore, data indicated that gastric infection was acquired at an early age. The relationships to gastric colonization by HPLOs and clinical manifestations of disease such as gastritis and gastroesophageal ulceration remain to be determined. (*Am J Vet Res* 2006;67:1890–1894)

Gastroesophageal ulceration is an important disease entity in swine.[1,2] Erosions, ulcers and accompanying gastritis develop in the glandular and nonglandular gastric mucosa in affected pigs with a prevalence ranging from 5% to 100%.[1] Death losses attributable to GEU may exceed 3%.[1] Although presently poorly documented, subclinical hemorrhage and associated anemia, anorexia, and weight loss result in additional economic losses to the producer.[1]

Unlike humans or other carnivores, the corpus (body) of the porcine stomach contains an extension of the esophageal mucosa into what is termed the pars esophagea or pars. This region of the stomach of pigs is lined by nonglandular stratified squamous epithelium.[3] Not only is it devoid of mucus-secreting cells, it also lacks both an extracellular mucus layer and a local bicarbonate production buffering mechanism thought necessary to neutralize gastric acidity.[3–5] Ulcerative lesions in the nonglandular pars esophageal region are similar to those of so-called acid reflux gastritis in humans.[6] Gastric acid-mediated ulceration of the pars is believed to be potentiated by the physical and nutrient content of the diet, and diet is thought to be the major contributory factor to development of GEU.[1,4,5]

Helicobacter pylori is a gram-negative, motile, microaerophilic, gastric bacterium that colonizes a highly specialized microecological niche in humans comprising the gastric epithelial surfaces and the overlying gastric mucus layer.[7] This bacterium is estimated to infect more than half the human population worldwide, with higher infection rates in developing countries, where 80% of middle-aged adults are infected, compared with rates of only 20% to 50% in developed countries.[7] Overwhelming evidence now causally links *H pylori* with peptic ulcers in humans.[8] As well, *H pylori* is recognized as a contributor to other illnesses ranging from childhood malnutrition and type B gastritis to gastric cancer. Infection with this agent also increases susceptibility to infection with other food and waterborne pathogens.[7] Since the first reports of *H pylori* and its association with disease in humans, related *Helicobacter* organisms have been detected in other species ranging from cats and dogs[9,10] to cheetahs.[11]

A *Helicobacter* sp morphologically distinct from human *H pylori*, *Helicobacter heilmannii* (formerly *Gastrospirillum suis*), is a common gastric commensal organism in swine.[12–14] Infection with *H heilmannii* has been associated with GEU in swine,[15,16] although attempts to reproduce GEU with *H heilmannii* have not been successful.[17] Presently, the role, if any, of *H heilmannii* in GEU is not known. Recently, isolation of an HPLO from swine[18] and experimental reproduction of

Abbreviations

GEU	Gastroesophageal ulceration
HPLO	*Helicobacter pylori*–like organism
PBST	Phosphate-buffered saline solution with Tween 20
OD	Optical density

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GEU with the HPLO were reported.[19] Although the isolate was both pathogenic and ulcerogenic in gnotobiotic swine, the prevalence of gastric infection with the organism and resultant gastritis in commercial swine populations is unknown. As an initial step toward understanding the epidemiologic features of porcine HPLO infection, the study reported here was performed to determine the prevalence of antibodies against a swine-origin HPLO in conventionally reared swine. The same sera were tested in an ELISA by use of *H pylori* as the antigen to determine whether differences in antibody responses represent antigenic differences between 2 closely related organisms[18] that are recognized by the porcine immunologic system.

Materials and Methods

Commercial swine operations and sample collection— Archived sera of apparently clinically normal pigs were obtained from 16 medium-sized high-health farms in Alberta, Saskatchewan, Manitoba, and Quebec, Canada. These herds, usually comprising Landrace–Large White cross pigs, were raised in total confinement and were free of common swine diseases, such as infection with *Mycoplasma hyopneumoniae*, swine dysentery, and atrophic rhinitis, but not necessarily porcine reproductive and respiratory syndrome virus. Forty sera, with 10 from each of 4 age groups (suckling, ≤ 4 weeks of age; nursery, 4 to 12 weeks; grower, > 12 to 20 weeks; and finisher, > 20 to 28 weeks), from each farm were tested. Twenty sow sera from a medium-sized high-health farrow-to-finish commercial swine operation in Ohio were also available for study. Blood was collected by venipuncture of the cranial vena cava. Serum was collected and frozen at –20°C until analyzed as 1 set of assays with an ELISA. Attempted isolation of HPLOs from the swine was not possible, given that these were stored sera.

Gnotobiotic swine antisera—Gnotobiotic swine,[20] derived by caesarean section into sterile pen-tub isolation units, were used as sources of convalescent swine sera of defined specificities for *H pylori*, a swine HPLO, and *H heilmannii* for validation of the ELISA assay. The details of inoculations and pathologic findings have been reported in previous publications.[17,19,20] Uninfected gnotobiotic swine were used for negative control sera.

ELISA—Microtiter plate ELISAs were performed with sonicates of swine HPLO and *H pylori* (0.025 µg of bacterial sonicates/well in basic bicarbonate coating-buffer; pH, 9.6) as antigen, essentially as described[20] with slight modifications. Briefly, alternating rows of a 96-well flat-bottom microtiter plate were coated with 0.025 µg of bacterial sonicates/well of either swine origin HPLO (isolate 1268) or *H pylori* (isolate 26695) in carbonate coating-buffer (pH, 9.6) as antigen. Alternating rows were coated with carbonate coating-buffer only, to serve as a background control for nonspecific binding of either porcine sera or the conjugate. Microtiter plates were then incubated at 25°C overnight. The plates were washed 4 times by immersion in PBST before blocking for 30 minutes at 37°C with PBST containing 0.2% gelatin from bovine skin. The blocking solution was not washed off and was removed only by dumping the plate contents and tapping on paper towels. Porcine sera were diluted 1:100 in PBST containing 0.2% gelatin and plated in duplicate wells (antigen-coated and nonantigen-coated) in 100-µL volumes. Included on every plate was positive control serum (rabbit anti–*H pylori*[a]), negative control serum (normal rabbit serum) at 1:100 in PBST containing 0.2% gelatin, and blank control wells of PBST containing 0.2% gelatin only. The plates were incubated at 37°C for 1 hour and were washed 4 times by immersion in PBST. One hundred microliters of a conjugate used to detect porcine IgG and rabbit IgG, horseradish peroxidase rec protein A[b] diluted 1:5,000 in PBST containing 0.2% gelatin, was added to each well. After incubation for 1 hour at 37°C, each plate was washed 4 times as before, and color was developed with 100 µL of 3, 3′,5, 5′-tetramethylbenzidine substrate[c]/well for 10 minutes at 25°C. Color development was stopped with 50 µL of 1M H_2SO_4. Optical densities at 450 nm were measured with a plate reader interfaced with commercial software.[d]

Corrected OD values for positive and negative control sera and for each test porcine serum sample were obtained by subtracting the OD values of the blank (PBST only) from the net OD values (mean OD of antigen coated wells minus mean OD of antigen-free wells). The relative concentration (ie, OD units) of specific antibody in each test sample equaled the percentage of positive control rabbit anti–*H pylori* serum. Briefly, monospecific convalescent sera from *H pylori* and HPLO-infected and uninfected control gnotobiotic piglets were used to establish the parameters of reactivity in an ELISA format. Individual secondary reagents were block-titrated against each other, and optimal concentrations of each (which yielded OD values < 0.300 for negative control sera) were used. Under these conditions, the *H pylori* and HPLO convalescent sera yielded OD values > 1.0. On the basis of these optimal conditions, a commercially available monospecific *H pylori* gold standard serum control (made in rabbits)[a] was used to establish the sensitivity of the ELISA. A cutoff value, 10 units (ie, 10% of positive control value), was set by testing sera from uninfected control gnotobiotic swine that consistently resulted in OD units ≤ 10.

Statistical analysis—The distribution of ELISA OD units among age groups and herds was initially examined descriptively by use of box plots of the data.[e] The association between ELISA OD units and age groups was then examined by use of mixed-effect models.[f] Random intercepts were used to adjust for clustering of observations by herd, and random slopes were introduced to account for differences in the association between age and the ELISA results across herds. The ELISA data were log transformed before analysis because of violations of the assumptions of normality and homogeneous variance of the residuals observed in models constructed from the raw data. The predicted means and 95% confidence intervals were back-transformed for ease of interpretation. Differences with $P < 0.05$ were considered significant.

Results

Validation of ELISA with gnotobiotic convalescent sera—As reported,[17–20] convalescent sera from gnotobiotic pigs orally inoculated with either *H pylori*, a swine HPLO, or *H heilmannii*, 2 to 6 weeks previously, were used to develop optimal test conditions for the ELISAs. In microtiter ELISAs, the *H pylori* and swine-origin HPLO antigen preparations were first tested against individual and pooled convalescent monospecific serum samples from *H pylori*–, HPLO-, and *H heilmannii*–infected gnotobiotic pigs. The patterns of seroconversion in gnotobiotic swine experimentally infected with the 3 *Helicobacter* spp were determined by use of the *H pylori* antigen (**Table 1**). As indicated, pigs infected with either *H pylori* or the swine-origin HPLO seroconverted to *H pylori* antigen with an IgG isotype response, 2 to 3 weeks after infection, and titers persisted thereafter until termination of the study. In contrast, none of the 22 pigs challenged with *H heil-*

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Table 1—Serum ELISA antibody responses (corrected ELISA OD values [range {mean}]) to *Helicobacter pylori* antigen in gnotobiotic swine inoculated with *Helicobacter heilmannii* homogenates,[a] *H pylori*, and a swine-origin HPLO.

Group	No. of piglets	Inoculant[b]	Pre-inoculation	Days after inoculation			
				14	21	35	42
A	3	*H heilmannii*	0	< 0.01–0.013 (0.01)	—	—	—
B	3	*H heilmannii*	0	—	0.0–0.0 (0.0)	—	—
C	3	*H heilmannii*	0	—	—	0.02–0.05 (0.04)	—
D[c]	13	*H heilmannii*	0	—	—	—	< 0.01–0.01 (0.0)
E	4	*H pylori*	0	—	—	2.11–2.92 (1.94)	—
F	6	HPLO swine	0	—	—	0.11–2.51 (0.62)	—
G	3	None	0	—	—	0.0–0.0 (0.0)	—

[a] *Helicobacter heilmannii* was isolated by inoculation into mice and serial passage of isolated parietal cells in gnotobiotic nude mice as described elsewhere.[17] Groups A, B, and C were inoculated with a gastric homogenate from *H heilmannii*-infected gnotobiotic swine. Group D was inoculated with mouse-passage *H heilmannii* as described elsewhere.[17] [b] All pigs were orally inoculated at 3 days of age. [c] The range of OD values (low to high) within each group is given; the mean value is given in parentheses below it. Gross and histologic findings in these pigs were reported previously[17]; stored terminal serum samples obtained from these pigs were tested for antibodies by ELISA in this study.

— = Not done.

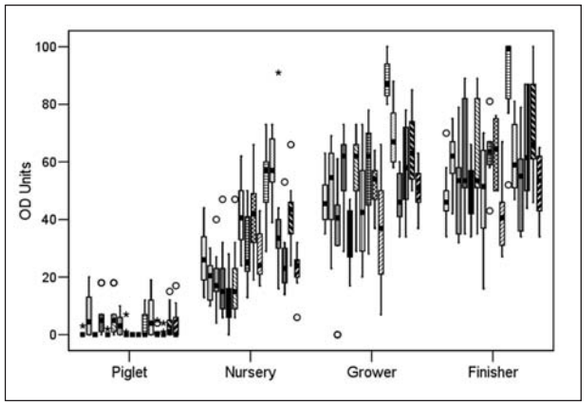

Figure 1—Box-and-whisker plot of the association between mean ELISA OD units obtained by use of a swine-origin HPLO antigen and age group in 16 commercial herds (overall n = 640). For each herd, the box represents interquartile range, the dark center line represents the median value, and the whiskers represent the range, excluding outliers. Circles indicate outliers from 1.5 to 3 box lengths from the upper or lower limit of the box. Asterisks represent extreme outliers > 3 box lengths from the upper or lower limit of the box

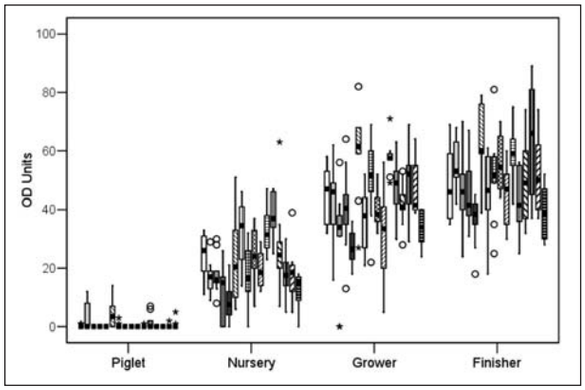

Figure 2—Box-and-whisker plot of the association between mean ELISA OD units obtained by use of *Helicobacter pylori* antigen and age group in the same swine as in Figure 1. *See* Figure 1 for key.

mannii, 2 through 6 weeks previously, developed ELISA-detectable antibodies against *H pylori*.

Conventionally reared pigs—Overall, 483 of the 640 (75.4%) pigs from the commercial swine operations in Canada were seropositive (≥ 10 units), compared with values from uninfected controls, via 1 or both of the ELISAs (**Figures 1 and 2**). The majority (148/160 [92.5%]) of suckling pigs were seronegative via both ELISAs. In contrast, 151 of 160 (94.4%) nursery pigs and all grower and finisher pigs were seropositive to both *Helicobacter* antigens. After accounting for clustering of observations by herd and variation in responses among herds, there was a significant ($P < 0.001$) age-dependent difference in swine-origin HPLO and *H pylori* mean ELISA OD units among all age groups except growing and finishing pigs. All 20 of the adult sows from Ohio were strongly seropositive to both *H pylori* and HPLO antigens (ELISA OD values, > 10 OD units).

Results of the 2 ELISAs were compared (**Figure 3**). The 2 assays were highly correlated (Spearman ρ, 0.94; $P < 0.001$), and agreement between the 2 assays was good (Lin concordance coefficient, 0.87; 95% confidence interval, 0.86 to 0.88). After herd effects were accounted for, the OD units were slightly higher when the swine origin HPLO antigen was used in the ELISA (β, 1.12 OD units; 95% confidence interval, 1.09 to 1.15; $P < 0.001$), suggesting greater specificity of antibody responses against the infecting organism.

Discussion

The serologic data indicated that exposure to and subsequent IgG-dominant antibody responses against

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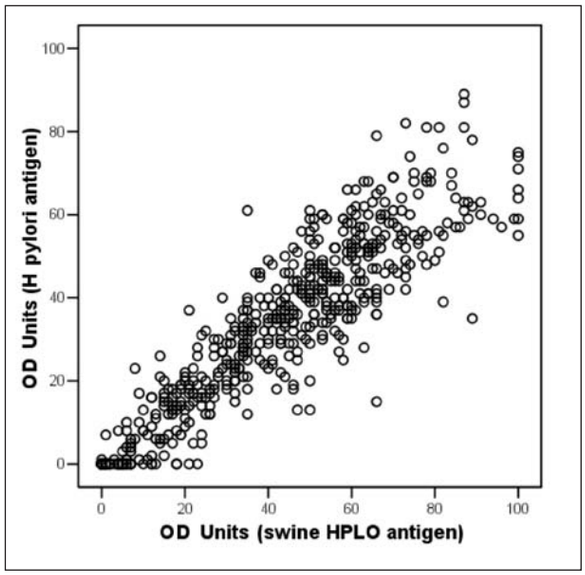

Figure 3—Comparison between antibody concentrations measured in ELISA using *H pylori* as the antigen or swine origin HPLO as the antigen (n = 640; n = 16). The ELISA OD units are a percentage of the OD of positive control rabbit anti–*H pylori* serum.

the HPLO and *H pylori* were common in the commercial swine herds studied. In addition, infection appeared to be acquired early in life because IgG antibodies were detectable in > 90% of nursery-age pigs. On the basis of the expected time to development of serum antibodies in experimentally infected pigs,[18,19] extrapolation backward indicated that gastric colonization by *Helicobacter* spp was initiated at 4 to 6 weeks of age in most swine. This pattern of exposure to *Helicobacter* spp in young swine and the increasing seroconversion rates with age were similar to the infection and seroconversion pattern detected in humans exposed to *H pylori* in endemically infected areas of the developing world.[21,22] In humans and swine, this likely reflects the relative ease of fecal-oral transmission that must occur in host-microbe situations and likely involves healthy adults.[23]

Surprisingly, convincing evidence for passively acquired maternal antibodies was not regularly detected in the young suckling pigs tested in this study, even though consistent seroconversion with high concentrations of HPLO-reactive antibody was detected in older swine on the Canadian farms and in sows from Ohio. This could simply be a function of the conditions (eg, dilutions) of the ELISA. As well, it is possible that seropositive neonatal pigs were colonized by the HPLO shortly after birth and that maternal antibodies against the HPLO were adsorbed to these microbes in the gastric lumen and thereby depleted from circulation. Alternatively, if only modest amounts of colostral antibodies were transferred to piglets, the passive decay rate would result in most young swine being seronegative by 4 weeks of age. In this study, colostrum samples were not available for testing, nor were serum samples serially collected from swine from 1 day of age onwards. Such samples need to be examined before definitive statements can be made regarding the amount of HPLO-reactive maternal antibodies that piglets may acquire during suckling and at what rate. Regardless, most piglets appeared to be colonized by the HPLO at 4 weeks of age or younger.

Gastric colonization by *H heilmannii* is thought by some to result in GEU in pigs.[15,16,24] The suggestion that *H heilmannii* infection in swine is a causal factor in the pathogenesis of GEU is based on association; that is, swine with mucosal ulcers and GEU at slaughter have a higher prevalence of colonization than do swine without GEU. However, not all *H heilmannii*–colonized swine have GEU, and not all GEU-affected swine are colonized by *H heilmannii*.[15,16,24] A cause-and-effect relationship between gastric colonization by *Helicobacter* spp, including *H heilmannii*, can only be established in an environment in which other gastric infectious agents are excluded. Gnotobiotic technologies are ideally suited for this purpose. Experimental challenge studies[17,19] with *H heilmannii* have been conducted in gnotobiotic swine. In those experiments, gnotobiotic swine were inoculated with *H heilmannii*–containing gastric homogenates. Gastric colonization was established, but neither mucosal ulcers nor GEU was detected, even when the pigs were provided with fermentable carbohydrates in the diet.[17,19] Thus, the experimental evidence suggests that *H heilmannii* is a gastric commensal organism of minimal or no pathogenic potential for swine.

Nevertheless, it is not clear whether colonization with *H heilmannii* and subsequent antibody responses to *H heilmannii* (or another closely related microbe) contributed to the serum antibody titers detected by HPLO and *H pylori* ELISAs in the present seroepidemiologic survey. Resolution of this question was hampered by the fact that *H heilmannii*, unlike either *H pylori* or HPLOs, is not culturable in vitro. *Helicobacter heilmannii* will react with monospecific rabbit antiserum against *H pylori* heat-stable antigen in tissue sections,[19] and therefore, it is possible that a portion of the antibodies that were reactive against the HPLO and *H pylori* antigens in this study were an indication of a cross-reactive *Helicobacter* spp–specific antibody response. The stomachs of swine used in this serosurvey were not available for histologic analysis, so the prevalence of gastric colonization in these swine was not known, although it was likely, on the basis of results of other surveys,[15,16,24] that at least 50% of the swine were colonized by *H heilmannii*. Yet to be investigated is the prevalence of coinfection (eg, by *H heilmannii* and HPLOs) in swine and how this may affect serologic data and the ability to detect both bacterial species in the porcine stomach. In this regard, it is noteworthy that sera from gnoto biotic pigs infected with *H heilmannii* for up to 6 weeks were all IgG seronegative via the *H pylori* and HPLO ELISAs, even when these sera were tested for IgM isotype antibodies.[18,19] Pooled sera from *H heilmannii*–infected gnotobiotic pigs did give a faint positive reaction to several protein bands in *H pylori* and HPLO western blot analyses.[18] These data suggest that *H heilmannii* infection may contribute to the ELISA titers to HPLO in older swine. However, definitive determination of the role, if any, that *H heilman-*

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nii infection may have on serologic reactivity to *Helicobacter* spp cannot be accomplished unless or until *H heilmannii* can be recovered in pure culture for use as an ELISA antigen. As reported,[18] there are apparent antigenic differences between the swine-origin HPLO and a prototype isolate of human-origin *H pylori*. These apparent differences were again detected in the similar, but disparate, results when the 2 isolates were used as antigens in ELISA to examine the serologic responses in naturally infected, conventionally reared swine. Duplicate sera tested with equal amounts of both antigens revealed higher OD units when the swine-origin HPLO was used as antigen than when *H pylori* was used in the same assay. How such antigenic differences reflect biologically important constitutive differences between the 2 organisms that might relate to observed differences in pathogenicity[19] remains to be determined.

Regardless of the level of cross-reactivity among the porcine helicobacters, which may ultimately account for a portion of the total antibody response and for seropositivity in some swine, these data and data from a recent study[25] of gastric samples from slaughtered pigs strongly suggest that infection with an HPLO or closely related bacteria is a common occurrence in commercially reared swine. The larger issues, such as the role of each bacterial species in the serologic profile of infection as well as the pathogenesis of clinically important bacterial gastritis and GEU, remain to be determined in further epidemiologic and experimental studies, which will take into account the interaction of infection with HPLO and recognized cofactors such as diet.

a. Novocastra Laboratories Ltd, Newcastle upon Tyne, UK.
b. Zymed Laboratories Inc, San Francisco, Calif.
c. KPL Inc, Gaithersburg, Md.
d. Microplate Manager III software, BioRad Laboratories Canada Ltd, Mississauga, ON, Canada.
e. SPSS for Windows, version 12.0, SPSS Inc, Chicago, Ill.
f. PROC MIXED, SAS, version 8.2, SAS Institute Inc, Cary, NC.

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