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Distribution of *Salmonella* serovars in breeding, nursery, and grow-to-finish pigs, and risk factors for shedding in ten farrow-to-finish swine farms in Alberta and Saskatchewan

Wendy Wilkins, Andrijana Rajić, Cheryl Waldner, Margaret McFall, Eva Chow, Anne Muckle, Leigh Rosengren

Abstract

The study objectives were to investigate *Salmonella* prevalence, serovar distribution, and risk factors for shedding in 10 purposively selected farrow-to-finish farms in Saskatchewan and Alberta. Pooled fecal samples from the breeding and grow-finish phases and individual fecal samples from breeding, nursery, and grow-finish pigs were cultured for *Salmonella;* serotyping of isolates was performed. Pig and pen characteristics were recorded for each pig and pen sampled.

Overall, 407/1143 (36%) of samples were *Salmonella* positive; within-farm prevalence ranged from 1% to 79%. Sows, nursery, and grow-finish pigs accounted for 43%, 29%, and 28% of positive samples, respectively. More *Salmonella* were detected in pooled pen than individual pig samples ($P < 0.001$). Among 418 *Salmonella* isolates, there were 19 distinct serovars; the most common were *S.* Derby (28.5%), *S.* Typhimurium, var. Copenhagen (19.1%), *S.* Putten (11.8%), *S.* Infantis (6.8%), and *S.* Mbandaka (6.1%). Sows were more likely to shed *Salmonella* than nursery or grow-finisher (OR 2.9, $P < 0.001$) pigs. Pelleted feed (OR 8.2, $P < 0.001$) and nose-to-nose pig contact through pens (OR 2.2, $P = 0.005$) were associated with increased *Salmonella* prevalence. Significant differences in serovar distribution were detected among production phases. The use of pooled pen samples is recommended as a more efficient means for accurate evaluation of *Salmonella* status in different phases of pig production. The breeding herd might be an important source of *Salmonella* persistence within farrow-to-finish farms and should be targeted in control efforts. The latter might also apply to the use of pelleted feed, which remains the most consistently reported significant risk factor for *Salmonella* shedding in pigs.

Résumé

Les objectifs visés étaient d'étudier la prévalence de Salmonella, *la distribution des sérovars, et les facteurs de risque pour l'excrétion dans 10 fermes de naisseurs-finisseurs choisies avec intention en Saskatchewan et en Alberta. Des échantillons de fèces regroupés provenant des groupes de reproducteurs et des animaux en croissance-finition ainsi que des échantillons individuels provenant des porcs reproducteurs, en pouponnière, et en croissance-finition ont été cultivés pour la recherche de* Salmonella; *le sérotypage des isolats a été effectué. Les caractéristiques des porcs et des enclos étaient notées pour chaque porc et enclos échantillonnés.*

De manière globale, 407/1143 (36 %) des échantillons étaient positifs pour Salmonella; *la prévalence intra-ferme variait entre 1 % et 79 %. De tous les échantillons positifs, 43 %, 29 % et 28 % provenaient respectivement des truies, des porcs en pouponnière et des porcs en croissance-finition. Plus de* Salmonella *étaient détectés dans les échantillons regroupés que dans les échantillons de porcs individuels (*$P < 0,001$*). Parmi 418 isolats de* Salmonella, *il y avait 19 sérovars distincts; les plus fréquents étaient* S. Derby *(28,5 %),* S. Typhimurium var. Copenhagen *(19,1 %),* S. Putten *(11,8),* S. Infantis *(6,8 %) et* S. Mbandaka *(6,1 %). Les truies étaient plus susceptibles d'excréter* Salmonella *que les porcs en pouponnière ou en croissance-finition (OR 2,9;* $P < 0,001$*). La nourriture en granule (OR 8,2;* $P < 0,001$*) et le contact nez-à-nez des animaux entre les parcs (OR 2,2;* $P = 0,005$*) étaient associés avec une augmentation de la prévalence de* Salmonella. *Des différences significatives dans la distribution des sérovars ont été détectées parmi les phases de production. L'utilisation des échantillons regroupés est recommandée comme étant un moyen plus efficace pour une évaluation précise du statut de contamination par* Salmonella *dans les différentes phases de production porcine. Le troupeau reproducteur pourrait être une source importante pour la persistance de* Salmonella *à l'intérieur des fermes de naisseurs-finisseurs et devrait être ciblé dans les efforts de réduction de l'infection. Ceci est également applicable à l'utilisation de nourriture en granule qui demeure le facteur de risque le plus fréquemment rapporté pour l'excrétion de* Salmonella *chez les porcs.*

(Traduit par Docteur Serge Messier)

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Introduction

Salmonella is an important challenge to the swine industry worldwide because of its implications for public health. Salmonellosis in humans results in high societal costs that include medical related expenses, losses associated with reduced or lost work productivity, and other costs (1,2). Although in North America pork is not considered a major source for human salmonellosis, *Salmonella* in pigs has become an important research priority over the past decade, primarily as a result of extensive implementation of *Salmonella* surveillance or monitoring programs in Denmark and other European countries. In Canada, Quebec has extensively investigated *Salmonella* in pigs (3,4), and recently initiated a provincial control program for *Salmonella* in pigs. Relatively large baseline studies were conducted in Ontario and Alberta (5,6), where approximately 40% to 60% of finishing swine farms were *Salmonella* positive, with the overall number of positive samples ranging from 11% (individual pigs) to 14% to 18% (pooled samples). A national, abattoir-based baseline study reported an overall pig carcass contamination prevalence of 4.2% (7), demonstrating that *Salmonella* carcass contamination rates within Canada are low when compared to the number of positive animals. Still, further improvement is needed and additional research into the epidemiology of *Salmonella* at both the farm and abattoir levels within Canada is required.

Swine production systems differ substantially among countries (8), and within Canada, among provinces and regions (5). In Canada, limited research has been conducted on the epidemiology of *Salmonella* in pigs, and the research that has been done to date has focused primarily on the finishing pig. Farzan et al (6) found that 46% (37/80) of farms in Ontario were *Salmonella*-positive. In western Canada, Rajić et al (5) reported that among 90 Alberta swine finishing farms producing ≥ 2000 pigs, 26% to 58% of farms studied were *Salmonella*-positive at any given time, and had low to moderate (1 to 4 positive samples, average 15 samples collected per farm) within-farm prevalence. Sorensen et al (9) examined the prevalence of *Salmonella* spp. in Alberta pigs at slaughter, reporting 35% positive cecal samples and 37 different serovars. Most recently, an examination of slaughter pigs from Saskatchewan abattoirs found 13% positive cecal samples (10). Only one study has investigated the distribution of *Salmonella* species in various pig production phases of 2 integrated production systems, where prevalence ranged from 17% to 66% (3). However, no study has investigated *Salmonella* serovar distribution throughout all phases (farrow-to-finish) of pig production in western Canada. The development and implementation of *Salmonella* control programs requires knowledge of the baseline prevalence and serovar distribution in targeted pig populations within a specific region, and thorough knowledge includes investigation of the breeding herd as well as finishing pigs.

Therefore, the objectives of this study were to evaluate *Salmonella* prevalence and serovar distribution in sows, nursery and grow-to-finish pigs, and risk factors for *Salmonella* shedding, using cross-sectional sampling on 10 purposively selected farrow-to-finish swine farms in Saskatchewan and Alberta.

Materials and methods

Farm selection

Ten farrow-to-finish swine herds (herd size $n > 100$ sows) from Alberta (7 farms) and Saskatchewan (3 farms) were purposely selected by swine veterinarians, based on their presumed *Salmonella* positive status ($n = 7$) or *Salmonella* negative status ($n = 3$), and the producer's willingness to participate in the study. Purposeful herd selection was chosen to meet the objectives of a concurrent study evaluating diagnostic tests for *Salmonella* in pigs (unpublished data). Herds were presumed positive if either the herd veterinarian or producer observed clinical salmonellosis within the previous 12 mo, if *Salmonella* species were identified during routine testing, or if replacement breeding stock were purchased from known *Salmonella*-positive farms. Herds were presumed negative if none of these criteria were met. The number of herds and the number of samples used in the study were a function of logistic and financial constraints.

Sample collection

Each herd was visited once from May through August 2004. Samples were delivered to the laboratory either within 2 h of leaving the farm, or held on ice overnight and delivered the following day.

Collection of individual fecal samples. On each farm, feces (minimum 10 g) were collected, from each of 10 randomly selected sows, directly from the rectum or from freshly voided feces on the floor. In the grow-to-finish area, 1 individual sample (minimum 10 g) was similarly collected from 1 pig in each of 30 different randomly selected pens. No individual fecal samples were collected from nursery pigs as most pigs were too small to collect 10 g feces directly from the rectum.

Collection of pooled fecal samples. Twenty pooled samples were taken from the breeding phase in each herd, by collecting a minimum of 5 g of feces from 5 different sows into a single container. Samples from individual sows, as described above, were not incorporated into the pooled sample. In both nursery and grow-to-finish phases, 1 pooled pen floor fecal sample was collected from each of 30 randomly selected pens or all pens on farm if there were < 30 pens. For each pooled pen sample a minimum of 5 g of fecal material was collected from 5 different locations on the pen floor.

Bacteriological culture

Bacteriologic culture for *Salmonella* was performed by the Agri-Food Laboratories Branch (AFLB), Food Safety Division of Alberta Agriculture and Rural Development. All samples were refrigerated and cultured within 24 to 48 h of receiving samples and thoroughly mixed prior to culture.

Ten grams of feces were inoculated into 90 mL of buffered peptone water (BPW) and incubated at 35°C for 20 to 24 h. After incubation, 0.1 mL of BPW was inoculated into 10 mL of Rappaport Vassiliadis broth (RV), placed into a 42°C water bath for 30 min, and then incubated at 42°C for 22 to 24 h. Simultaneously, 1 mL of BPW was inoculated into tetrathionate broth (TT) to which 0.2 mL of iodine solution had been added just prior to use, and placed in a 35°C waterbath for 30 min, then a 35°C incubator for 22 to 24 h.

After incubation, 10 μL of RV and TT were streaked onto XLT4 and Rambach (RAM) selective agar plates and incubated at 35°C for 18 to 24 h, then read. Plates without significant growth of suspect colonies were re-incubated and read after an additional 24 h. At the same time, 0.3 mL of TT (0.1 mL to each of 3 sites) was inoculated onto a modified semi-solid Rappaport-Vassiliadis (MSRV) plate and incubated at 42°C for 20 to 24 h. The halos of growth that occurred on the MSRV plates were streaked to XLT4 and RAM plates and incubated at 35°C for 24 h. Negative plates were reincubated and read again at 48 and 72 h. Suspect colonies were screened using triple sugar iron agar slants, urea agar slants and lysine iron agar slants, and plated to a blood agar plate and MacConkey plate to check for purity then tested with *Salmonella* Poly O and Poly O1 antisera agglutination (Denka Seiken Company, Tokyo, Japan). Unusual or atypical reacting suspect colonies were further tested using Vitek GNI or API-20E (bioMerieux Vitek, Hazelwood, Missouri, USA). All isolates were frozen at –70°C then forwarded to the Laboratory for Foodborne Zoonoses, Public Health Agency of Canada, Guelph, Ontario, for confirmation by serotyping.

Serotyping and phagetyping

One isolate per each *Salmonella*-positive sample, or 2 isolates if they were morphologically distinct, was sent for serotyping and phagetyping at the OIE Reference Laboratory for Salmonellosis, Laboratory for Foodborne Zoonoses, Public Health Agency of Canada, Guelph, Ontario. The serotyping and phagetyping techniques followed standard procedures and have been previously reported (5).

Data collection

During sampling, the primary investigator observed and recorded pen and pig information; sex and age of each individual pig sampled; number of pigs in pen, area and pig density; floor and wall type, and cleanliness of each pen; feed type and feeding method; nose-to-nose contact between pigs through pen separations, and feces characteristics. A list of variables and their distribution is shown in Tables I and II.

Statistical analysis

The pig and pen were *Salmonella*-positive if the fecal sample collected from that pig or pen tested positive. Descriptive statistics were summarized and statistical models were developed using a commercial software program (Stata/SE v9.2; StataCorp, College Station, Texas, USA). Generalized linear mixed models, with a random intercept to account for clustering of individual and pen samples within herd, were used to: 1) examine the difference in *Salmonella* shedding among production phases and the associations between pen-level variables and *Salmonella* shedding; 2) estimate the proportion of variance of *Salmonella* shedding attributable to each production level; 3) compare *Salmonella* recovery from pooled fecal versus individual samples collected from grow-finish pigs; and 4) describe the differences in serovar-specific prevalence among the various production phases. All models used a logit link function, binomial distribution, and an exchangeable correlation structure.

Table I. Distribution of the categorical variables in each production phase that were considered as possible risk factors for the occurrence of *Salmonella* in 10 farrow-to-finish pig farms from Alberta and Saskatchewan

		Distribution		
Variable	Levels of response	Sows (%)	Nursery (%)	Grow-finish
Sex	gilt(s)	na	34	38
	barrow(s)	na	34	37
	mixed pen	na	32	24
	sow(s)	100	na	na
Fecal score	runny	0	0	3
	normal	93	99	96
	hard/dry	7	1	1
Fed pelleted feed	yes	30	51	29
	no	70	49	71
Fed wet feed	yes	44	78	83
	no	56	22	17
Fed on floor	yes	20	0	8
	no	80	100	92
Pen cleanliness	clean	56	76	25
	slightly wet/dirty	24	23	49
	moderately wet/dirty	16	0	9
	very wet/dirty	4	0	16
Pen floor type	full slatted	25	94	16
	part slatted	66	6	80
	not slatted	9	0	4
Concrete floor	yes	79	4	91
	no	21	96	9
Concrete walls	yes	9	4	54
	no	91	96	46
Nose-to-nose contact between pens	yes	79	53	80
	no	21	47	20
Production phase	sows	100	0	0
	nursery/weaners	0	100	0
	grow-finishers	0	0	100

Risk factor analysis was limited to pooled fecal samples to minimize potential bias introduced by different sampling strategies among production phases and because individual samples were only available from 2 of 3 phases. In the first step, the unconditional association between each potential risk factor and whether or not the pooled fecal culture was positive for *Salmonella* was evaluated. All variables with an unconditional *P*-value of ≤ 0.20 were evaluated for inclusion in a multivariable model using a manual forward-stepwise process. Risk factors were defined as confounders if removing or adding the factor changed the effect estimate by more than 20% (11). Variables with $P \leq 0.05$ were considered statistically significant. Biologically reasonable first-order interaction terms were examined

Table II. Distribution of the continuous variables in each production phase that were considered as possible risk factors for the occurrence of *Salmonella* in 10 farrow-to-finish pig farms from Alberta and Saskatchewan

Variable	Production phase	Mean	Median	Minimum	Maximum	s	*n*
Age in weeks[a]	Nursery	6.6	7	3	11	2	236
	Grow-finish	16.6	16	8	27	4.7	255
Number of pigs in pen[b]	Sows	5.3	6	1	10	2.7	39
	Nursery	18.8	18	6	70	9.8	255
	Grow-finish	16.4	14	3	120	12.9	295
Pig density (m^2 per pig)[b]	Sows	2.3	2.25	0.75	4.3	1.1	39
	Nursery	0.25	0.25	0.1	0.53	0.08	255
	Grow-finish	0.85	0.74	0.15	3.12	0.47	295

s — standard deviation.

[a] Observations on age were not recorded for breeding females.

[b] Data from pigs in pens only; does not include observations from sows in gestation/farrowing crates (*n* = 161).

Table III. Proportion of all fecal samples positive for *Salmonella* based on bacterial culture for each phase of pig production in 10 farrow-to-finish herds in Alberta and Saskatchewan

		Number positive					
Farm	Total number of samples	Overall	Sows (pooled)	Sows (ind)	Nursery (pooled)	Grow-finish (pooled)	Grow-finish (ind)
1	120	77	17/20	10/10	17/30	21/30	12/30
2	96	54	14/20	5/10	1/16	17/25	17/25
3	120	95	17/20	5/10	22/30	28/30	23/30
4	120	2	1/21	0/9	0/30	1/30	0/30
5	120	34	13/20	5/10	2/30	11/30	3/30
6	120	46	12/20	3/10	5/30	15/30	11/30
7	119	18	5/19	2/10	9/30	2/30	0/30
8	104	58	17/20	7/10	13/14	15/30	6/30
9	108	11	5/20	0/10	2/19	3/30	1/29
10	116	12	1/20	1/10	10/26	0/30	0/30
All farms	1143	407/1143	102/200	38/99	81/255	113/295	73/294

where > 1 significant risk factor was identified in the final main effects model. Statically significant interaction terms were included in the final model.

To estimate the proportion of variance in *Salmonella* shedding attributable to production phase, a 3-level model was developed, including a random effect for production phase nested within farm. Using pooled samples only, a model with intercept as the only fixed term (null model) was fitted to compute the proportion of the overall variance in *Salmonella* shedding accounted for at the level of production phase and then farm. The proportion of variance that was accounted for by differences between herds was estimated as:

$$\rho_h = \frac{\sigma^2_h}{\sigma^2_h + \sigma^2_p + \sigma^2_\varepsilon} \quad \text{[Equation 1]}$$

where: σ^2_h was the herd-level variance, and σ^2_p was the production phase variance estimated from the null model; and σ^2_ε was the sampling variance estimated according to the latent variable method (12). Likewise, the proportion of variance that was accounted for by differences between production phases was estimated as:

$$\rho_p = \frac{\sigma^2_p}{\sigma^2_h + \sigma^2_p + \sigma^2_\varepsilon} \quad \text{[Equation 2]}$$

To evaluate *Salmonella* recovery from different sampling procedures, the odds of obtaining a *Salmonella* positive sample from a pooled fecal sample were compared to the odds of obtaining a positive culture from an individual sample. The unconditional association between sampling strategy and whether or not the fecal sample was *Salmonella*-positive was evaluated in a model with a random intercept for herd. This analysis was restricted to samples from grow-finish pigs as this was the only production area where both pooled and individual samples were collected from the same pen.

Both pooled and individual samples were used collectively to estimate differences in serovar-specific prevalence among the different phases of production. A positive outcome was the presence

Table IV. Unconditional associations between predictor variables and the occurrence of *Salmonella* positive pooled fecal samples from pens on 10 farrow-to-finish pig farms from Alberta and Saskatchewan

Variable	Levels of response	β (coefficient)[a]	95% CI (β)	*P*-value
Sex	*overall*			*0.018*
	gilt(s)	−0.85	−1.34 to −0.36	0.001
	barrow(s)	−0.55	−1.03 to −0.67	0.026
	mixed pen	−1.65	−2.39 to −0.92	0.001
	sow(s)	Reference		
Fecal score	*overall*			*0.071*
	normal	−1.48	−3.28 to 0.31	0.11
	hard/dry	−0.40	−2.53 to 1.74	0.72
	runny	Reference		
Fed pelleted feed	yes	0.95	0.19 to 1.71	0.014
	no	Reference		
Fed wet feed	yes	0.57	0.09 to 1.06	0.020
	no	Reference		
Fed on floor	yes	0.57	0.08 to 1.24	0.087
	no	Reference		
Pen cleanliness	*overall*			*0.008*
	slightly wet/dirty	0.34	−0.06 to 0.74	0.10
	moderately wet/dirty	0.19	−0.52 to 0.90	0.60
	very wet/dirty	−1.17	−2.11 to 0.22	0.016
	clean	Reference		
Pen floor type	*overall*			*0.46*
	part slatted	0.65	0.28 to 1.03	0.001
	not slatted	0.15	−1.21 to 1.51	0.83
	full slatted	Reference		
Concrete floor	yes	0.77	0.40 to 1.14	0.000
	no	Reference		
Nose-to-nose contact between pens	yes	0.67	0.16 to 1.18	0.009
	no	Reference		
Production phase	*overall*			*0.089*
	nursery/weaners	−0.73	−1.17 to −0.29	0.001
	grow-finishers	−1.10	−1.56 to −0.63	0.000
	sows	Reference		
Number of pigs in pen	−0.05	−0.07 to −0.03	0.000	Number of pigs in pen
Pig density	0.67	0.28 to 1.06	0.001	Pig density

95% CI — 95% confidence interval.

[a] Log odds ratio from random-effects logistic regression model.

of a specific serovar; any other serovar, or any *Salmonella*-negative sample, was considered a negative outcome. For each of the 5 most prevalent serovars, the association between production phase and whether or not the fecal sample was positive for each of these 5 serovars was investigated. All models were adjusted for sampling strategy (pooled versus individual samples) by including this variable as a fixed effect in each model.

Results

Farm description

Farm size ranged from 130 to 2070 breeding females (mean 531, median 333) and the number of pigs produced for slaughter by each farm ranged from 1100 to 27 000 animals annually (mean 8332,

Table V. Final multivariable regression model for associations between predictor variables and pen *Salmonella* status on 10 farrow-to-finish pig farms from Alberta and Saskatchewan

Variable	β (coefficient)[a]	95% CI (β)	*P*-value
Fed pelleted feed			
Yes	2.1	1.18 to 3.03	0.000
No	Reference		
Nose-to-nose contact			
Yes	0.81	0.24 to 1.37	0.005
No	Reference		
Production phase			
Nursery	−1.4	−1.91 to −0.88	0.000
Grow-finish	−0.84	−1.30 to −0.88	0.000
Sows	Reference		
Grow-finish	0.56	0.09 to 1.02	0.019
Sows	1.40	1.91 to 0.88	0.000
Nursery	Reference		

95% CI — 95% confidence interval.

[a] Log odds ratio from random-effects logistic regression model.

Table VI. *Salmonella* serovars isolated from 10 farrow-to-finish pig farms in Alberta and Saskatchewan, grouped according to production phase

Serovar	Sows, pooled	Sows, individual	Nursery, pooled	Grow-finish, pooled	Grow-finish, individual	Total
S. Derby	20	12	6	48	33	119
S. Typhimurium var. Copenhagen	23	6	24	19	9	81
S. Putten	12	4	7	14	12	49
S. Infantis	8	3	4	7	6	28
S. Mbandaka	0	0	14	8	4	26
S. Give	8	8	1	1	1	19
S. Anatum	5	2	3	5	2	17
S. Ohio	0	0	3	1	0	4
S. Rubislaw	2	1	0	1	0	4
S. Livingstone var. 14+	1	0	3	0	0	4
S. Typhimurium	0	0	0	0	3	3
S. Worthington	3	0	0	0	0	3
S. Give var. 15+	0	0	1	1	0	2
S. Enteriditis	1	0	0	1	0	2
S. Ohio var. 14+	1	0	1	0	0	2
S. Brandenburg	2	0	0	0	0	2
S. Lexington var. 15+	0	0	0	1	0	1
S. Heidelberg	0	0	1	0	0	1
S. Kentucky	1	0	0	0	0	1
Untypeable	15	2	15	10	8	50

median 4300). Three herds primarily produced breeding stock but finished the barrows and cull gilts. Seven herds produced hogs for slaughter only.

Salmonella prevalence (both pooled and individual samples)

Salmonella was isolated from all 10 study farms. Based on total numbers of positive samples, prevalence within presumed-negative herds ranged from 20% to 56%, while prevalence within presumed-positive herds ranged from 2% to 79%. There were 407/1143 (36%) positive fecal samples across all production phases (Table III). Four farms accounted for 70% (284/407) of all positive samples (Table III). The highest proportion was found in the breeding sows, with 38% (38/99), and 51% (102/200) of individual and pooled samples, respectively, positive for *Salmonella*. In the grow-finish population, 25% (73/294) of the individual samples and 38% (113/295)

Table VII. *Salmonella* phage types isolated from 10 farrow-to-finish pig farms in Alberta and Saskatchewan

Serovar	Phage type	Number of isolates	% of isolates
S. Typhimurium var.	UT5	30	34.5%
Copenhagen	21	16	18.4%
	104	13	14.9%
	22	5	5.7%
	208 var	5	5.7%
	135	4	4.6%
	146a var	3	3.4%
	208	1	1.1%
	142 var	1	1.1%
	Untypeable	2	2.3%
	UT3	1	1.1%
S. Typhimurium	27	2	2.3%
	U276	1	1.1%
S. Enteritidis	11b	1	1.1%
	20a	1	1.1%
S. Heidelberg	10	1	1.1%
	Total	*87*	*100%*

of the pooled pen samples tested positive. In the nursery, 32% (81/255) of all pooled pen samples were positive. The occurrence of *Salmonella* positive samples varied significantly among all production phases for the pooled samples ($P < 0.001$) and between the breeding sows and grow-finish population for the individual samples ($P = 0.002$).

Risk factors for shedding *Salmonella* (pooled sample results)

Risk factor variables that were unconditionally associated ($P \leq 0.20$) with *Salmonella* shedding in the pooled samples are summarized in Table IV. Several management factors were specific and uniform to the breeding herd on the farms studied; for example, all breeding females were, naturally, "sex = female," and most breeding females were housed in gestation stalls or farrowing crates. The variable "sex" was therefore perfectly correlated with "production phase — sows" and the variables "number of pigs in pen," and "pig density" were also found to be highly correlated with this production phase. Consequently, these 3 variables were not included in the initial model. A second model was developed to assess the significance of these variables in nursery and grow-finish pigs only.

Only the variables "fed pelleted feed," "production phase," and "nose-to-nose contact" were found statistically significant ($P \leq 0.05$) in either model; thus, the estimates are reported for a single model including these 3 variables and applied to data from all production phases (Table V). In this model, the odds of a positive pooled *Salmonella* culture remained different across the different production phases (Table V). Sows were 2.3 (CI_{OR} 1.5, 3.7) times more likely to shed *Salmonella* than grow-finish pigs, and 4.0 (CI_{OR} 2.4, 6.8) times more likely to shed than nursery pigs; grow-finishers were 1.7 (CI_{OR} 1.1, 2.8) times more likely to shed *Salmonella* than nursery pigs. Pooled samples from pens that received pelleted feed were 8.2 (CI_{OR} 3.2, 20.6) times more likely to be *Salmonella*-positive than samples from pens with non-pelleted feed (Table V). Pens allowing for nose-to-nose contact among pigs were 2.2 (CI_{OR} 1.3, 4.0) times more likely to be *Salmonella*-positive than pens without such contact (Table V).

Variance component estimation (pooled fecal samples)

The estimates of variance in the occurrence of *Salmonella* positive pooled fecal samples at the herd and production phase levels were 2.24 [standard error $(S_{\bar{x}}) = 1.31$] and 1.34 ($S_{\bar{x}}$, 0.57), respectively. Using the latent variable method (12), the proportion of variance residing at the herd level [Equation 1] was 33%, while 20% of total variance was due to production phase [Equation 2].

Salmonella recovery from pooled versus individual samples

Overall, *Salmonella* was isolated from 38% (113/295) of pooled grow-finish samples and 25% (73/294) of individual samples. The odds of *Salmonella* recovery from grow-finishers were 2.9 times (CI_{OR} 1.8, 4.5; $P < 0.001$) higher from pooled than individual samples. In sows, 51% 102/200 of pooled samples and 38% (38/99) of individual samples were *Salmonella*-positive; however, no statistical test for differences between sampling strategies was done for this production phase as paired pooled and individual samples were not collected from the same pen or animals.

Salmonella serovar and phage type distribution (both pooled and individual samples)

The serovar prevalence for each production phase is shown in Table VI. Nineteen distinct serovars were identified. Multiple serovars (2 to 8 per farm) were detected on all but 1 farm. Fewer serovars were detected in individual samples (7 and 8 typed serovars, for sows and grow-finish, respectively) than in pooled samples (13, 12, and 12 typed serovars, for sows, grow-finish, and nursery, respectively). The 5 most common serovars were *S.* Derby (28.5%), *S.* Typhimurium var. Copenhagen (19.4%), *S.* Putten (11.7%), *S.* Infantis (6.7%), and *S.* Mbandaka (6.2%) (Table VI). Phage typing results for all *S.* Typhimurium, *S.* Typhimurium var. Copenhagen, *S.* Enteritidis, and *S.* Heidelberg isolates are presented in Table VII. On the 7 farms where these serovars were found, the number of phagetypes isolated per farm ranged from 1 to 6, with multiple phagetypes found on 4 farms. *Salmonella* Typhimurium PT104 was detected on 2 farms, and on both these farms this was the only phagetype present.

The serovar distributions in various production phases were compared for the 5 most prevalent serovars, with the exception of *S.* Mbandaka. Since this serovar was not isolated from the breeding herd, this comparison was limited to nursery pigs and grow-finishers production phases. In an analysis adjusted for sample type (pooled versus individual), significant differences in serovar distribution were found between production phases; these pair-wise contrasts are presented in Table VIII.

Table VIII. Differences in *Salmonella* serovar distribution between production phases on 10 farrow-to-finish pig farms in Alberta and Saskatchewan

Serovar	Contrast	OR	$CI_{ORlower}$	$CI_{ORupper}$
S. Derby	grow-finish versus nursery	10.2	4.2	24.9
	grow-finish versus sows	1.5	0.9	2.5
	sows versus nursery	6.7	2.6	16.9
S. Infantis	sows versus nursery	3.1	0.9	10.8
S. Putten	sows versus nursery	3.2	1.2	9.0
S. Typhimurium var. Copenhagen	nursery versus grow-finish	3.0	1.4	6.4
	sows versus grow-finish	3.3	1.7	6.4
S. Mbandaka	nursery versus grow-finish	4.4	1.7	11.3

Discussion

Existing research on the epidemiology of *Salmonella* in pigs has focused primarily on finishing pigs due to their proximity to the consumer. Still, pigs of other ages can play an important role in the maintenance and dissemination of *Salmonella* on-farm, as well as contribute to food safety issues themselves. In this study we investigated the epidemiology of *Salmonella* throughout all levels of swine production and reported on production phase level factors which could potentially influence the *Salmonella* status of pigs, an important contribution to future surveillance and control efforts for *Salmonella* in western Canada.

Three herds included in this study were initially presumed to be free of *Salmonella*; however, all 10 herds were ultimately found to be *Salmonella*-positive. Although only *S.* Typhimurium and *S.* Choleraesuis commonly cause clinical salmonellosis in pigs, infection by other serovars causes prolonged carrier states and intermittent shedding (13). Even when *S.* Typhimurium and *S.* Choleraesuis are present within a herd, infection may remain primarily subclinical without outbreaks of clinical salmonellosis. In these cases, and in the absence of regular testing, the presence of *Salmonella* goes unsuspected and undetected. Our observations then emphasize clinical history is not an accurate indicator of herd *Salmonella* status.

The current study parallels previous studies that sows were more at risk for shedding *Salmonella* than both nursery and grow-finish pigs (8,14–17). Cull sows are usually shipped to slaughter immediately after weaning, when increased shedding has been observed (16). Additionally, transport and lairage practices may contribute to increased shedding of *Salmonella* by sows immediately prior to slaughter (18,19). For these reasons, potential control efforts should be placed on this population both on-farm and at slaughter to reduce the on-farm *Salmonella* reservoir as well as minimize potential food safety risks.

The use of pelleted feed and nose-to-nose pig contact through pens were 2 other significant risk factors detected in this study. Other researchers, both in Canada and elsewhere, have also reported strong associations between the use of pelleted feed and farm *Salmonella* status (15,20–22). Other research groups reported that the use of acidifying rations reduced the prevalence of *Salmonella* in market-age pigs (23), and that pelleted feed decreased stomach acidity in the pig compared with coarser feed (24) or increased mucin secretion, contributing to the survival of ingested *Salmonella* and colonization of the pig (25). Efforts to reduce *Salmonella* at the farm level could incorporate acidification of water or rations or changing feed to coarser-grind rations. Nose-to-nose contact between pigs through pens is a less likely target for intervention, since this is a feature inherent to barn design and unlikely to be easily changed. However, consideration of the possibility of transmission of *Salmonella* and other important pathogens between pens and production units should be taken into consideration when designing and building new barns.

Approximately 1/3 of the estimated variance of *Salmonella* shedding resided at the farm level, suggesting that farm-level factors may exert the greatest influence on the outcome (26). Others have reported farm type as a significant risk factor for *Salmonella* shedding (27), which further supports the premise that farm-level management factors significantly impact the *Salmonella* status of pigs. Within farms, 20% of the variance of *Salmonella* shedding was attributable to production phase, suggesting that production phase specific factors might also be important and concurs with our finding that production phase is a significant risk factor when included as a fixed effect in the regression model. However, previous studies investigating risk factors for *Salmonella* have focused primarily on finishing pigs and little information regarding risk factors for pigs of other ages is available. Further research into production phase level factors, which could potentially influence the *Salmonella* status of pigs, is required.

One-time sampling of individual pig feces (compared with repeated or pooled samples) has been identified, among other reasons, for poor sensitivity of *Salmonella* culture (28). Similarly, in our study, more positives were found in pooled pen samples than from individual pigs. Furthermore, more positive farms were identified when sampling pigs from all production phases. Consequently, the use of pooled pen samples, from all phases of pig production, is recommended as a more reliable means of accurately of establishing the prevalence of *Salmonella* in swine herds.

The observed distribution in *Salmonella* serovars was similar to other findings within Canada (3,5,9,29) and the United States (30,31), except for one notable exception. *Salmonella* Putten, a serovar that has not been reported by any of these studies, was the 3rd most common serovar in the study, and was found only in 3 farms in Saskatchewan; these farms also accounted for over 80% of all untypeable isolates. Taken together, this is suggestive of either possible geographical differences in serovar distribution in pigs in western Canada or other common factors that contributed to the transmission of specific serovars between these herds. Molecular methods, such as those used to document transmission of *S.* Typhimurium DT 104 between

geographically related herds in Denmark (32), would be necessary to further investigate this observation and further our understanding of the spread of *Salmonella* within and between herds.

Significant differences were observed in serovar prevalence between production phases. Surveillance efforts which focus solely on finisher pigs, either on-farm or at slaughter, would not have detected the full range of serovars present on these farms. As other researchers have noted, an understanding of serovar type and distribution is important because certain serological tests, such as the Danish-mix enzyme-linked immunossorbent assay (ELISA), detect antibodies against serogroups B, C1, and D1 only (5). Serological response to serovars such as *S.* Mbandaka, *S.* Anatum, or *S.* Putten, would not have been detected by this ELISA. The changes in serovar distribution as pigs progress through the production cycle presents a challenge to *Salmonella* surveillance and control efforts that use serological tools only; cost-effective complementary bacteriologic testing of samples from all levels of pig production is necessary for accurate evaluation of *Salmonella* status in swine herds.

In this study, only 3 significant risk factors were identified, possibly because the study did not have sufficient power to detect other significant risk factors due to the small number of studied farms. The main study limitation was the use of purposeful selection of farms, which was necessary to meet the objectives of a concurrent study. For these reasons, no conclusions based on this study should be made regarding *Salmonella* prevalence in western Canadian swine farms in general. This study does, however, indicate that the breeding herd plays an important role in the persistence of *Salmonella* infection within pig herds, as suggested by other researchers (8,16). Molecular fingerprinting methods are needed to confirm clonal spread of *Salmonella* from sows to other production phases within these herds. In summary, the study herein has contributed to future surveillance and control efforts by providing important insight into the on-farm epidemiology of *Salmonella* in western Canada.

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Hemotropic mycoplasma prevalence in shelter and client-owned cats in Saskatchewan and a comparison of polymerase chain reaction (PCR) — Results from two independent laboratories

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Abstract

The primary objective of this study was to determine the prevalence of subclinical hemotropic mycoplasma (HM) infections in 2 distinct feline populations: cats from a local shelter and client-owned cats presented for elective procedures (vaccination, ovariohysterectomy, orchiectomy) at the Western College of Veterinary Medicine — Veterinary Teaching Hospital (WCVM-VTH). The second objective of this study was to evaluate the inter-test agreement of 2 independent conventional polymerase chain reaction (PCR) assays used for the diagnosis of feline HM-infections.

Fifty-eight clinically healthy shelter cats and 57 clinically healthy client-owned cats were screened for subclinical HM-infection using a conventional PCR assay to detect the 16S rRNA of *Mycoplasma haemofelis* and "*Candidatus* M. haemominutum." All cats in both groups had normal physical examinations. Sex, age (estimated for shelter cats), breed, reproductive status and the presence or absence of ectoparasites were determined. Packed cell volume (PCV), total protein, retroviral status, and blood smear evidence of HM-infection were evaluated. Subclinical HM-infection was identified by PCR assay in 12% (7/58) of the shelter cats and 4% (2/57) of the client-owned cats. *M. haemofelis* was found in 3/7 HM-infected shelter cats and 2/2 of the HM-infected client-owned cats; "*Candidatus* M. haemominutum" was found in 4/7 of the HM-infected shelter cats. There was no significant difference in prevalence of HM-infection between the populations (OR 3.8, 95% CI 0.75 to 19, $P = 0.16$), and no risk factors for infection were identified in either population.

Blood samples from 44 cats with known PCR results (26 cats sampled in the prevalence study and 18 clinical cases) were submitted to a second independent laboratory for HM PCR assay to assess inter-laboratory agreement. There was substantial, but not complete agreement between the 2 independent laboratories for PCR detection of *M. haemofelis* ($\kappa = 0.66$) and "*Candidatus* M. haemominutum" ($\kappa = 0.70$).

Résumé

L'objectif premier de la présente étude était de déterminer la prévalence d'infection sous-clinique associée au mycoplasme hémotropique (HM) dans 2 populations félines distinctes : des chats provenant d'un refuge local et des chats appartenant à des clients et présentés pour des procédures électives (vaccination, ovario-hystérectomie, orchiectomie) au Western College of Veterinary Medicine-Veterinary Teaching Hospital (WCVM-VTH). Le deuxième objectif de l'étude était d'évaluer l'accord inter-test de deux épreuves indépendantes conventionnelles de réaction d'amplification en chaîne par la polymérase (PCR) utilisées pour le diagnostic d'infections félines à HM.

Cinquante-huit chats de refuge cliniquement en santé et 57 chats cliniquement en santé appartenant à des clients ont été éprouvés pour une infection à HM sous-clinique à l'aide d'une épreuve PCR conventionnelle pour détecter l'ARNr 16S de Mycoplasma hemofelis *et «*Candidatus *M. haemominutum». L'examen physique de tous les chats dans les deux groupes ne révéla rien d'anormal. Le sexe, l'âge (estimé pour les chats de refuge), la race, l'état reproducteur et la présence ou l'absence d'ectoparasites ont été déterminés. L'hématocrite (PCV), les protéines totales, l'état rétroviral et une évidence d'infection par HM au moyen d'un frottis sanguin ont été évalués. L'infection sous-clinique à HM a été identifiée par épreuve PCR chez 12 % (7/58) des chats de refuge et 4 % (2/57) des chats de propriétaire.* M. haemofelis *a été retrouvé chez 3/7 des chats de refuge infectés par HM et 2/2 des chats de clients infectés par HM; «*Candidatus *M. haemominutum» a été trouvé chez 4/7 des chats de refuge infectés par HM. Il n'y avait aucune différence significative dans la prévalence d'infection par HM entre les populations (OR 3,8, 95 % CI 0,75 à 19,* P *= 0,16), et aucun facteur de risque pour l'infection n'a été identifié dans les deux populations.*

Des échantillons sanguins provenant de 44 chats avec des résultats connus de PCR (26 chats échantillonnés dans l'étude de prévalence et 18 cas cliniques) ont été soumis à un deuxième laboratoire indépendant pour une épreuve PCR pour détecter HM afin d'évaluer l'accord inter-laboratoire. Il y avait un accord marqué mais incomplet entre les deux laboratoires indépendants pour la détection par PCR de M. hemofelis *(*$\kappa = 0,66$*) et «*Candidatus *M. haemominutum» (*$\kappa = 0,70$*).*

(Traduit par Docteur Serge Messier)

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Introduction

Hemotropic mycoplasmas (HM), a subset of the *Mycoplasma* genus, are gram-negative, uncultivable, epierythrocytic parasites (1). Infection of cats with HM can result in subclinical disease or can cause severe hemolytic anemia (1). The development and severity of clinical disease in cats depend on host factors, the infecting species of HM, and potentially the infecting strain (1–4). There are currently 4 species known to infect cats: *Mycoplasma haemofelis*, "*Candidatus* Mycoplasma haemominutum," "*Candidatus* Mycoplasma turicensis," and "*Candidatus* Mycoplasma haematoparvum" (2). Testing is not available on a commercial basis for the latter 2 newly recognized species. The proposed routes of natural transmission include arthropod vectors (fleas and ticks) as well as saliva and feces (5–7). Currently, the diagnosis of HM-infection is based on either observation of the coccoid bacteria on erythrocytes during blood smear evaluation or polymerase chain reaction (PCR) assay. Polymerase chain reaction technology has been used to demonstrate a global distribution of HM-infection in domestic and wild felids (2–4,8–11). The prevalence of HM-infection in cats in Canada has not been reported. In addition to aiding in the diagnosis of feline HM-infection, PCR assays may also be useful for monitoring response to therapy.

Polymerase chain reaction assays are reported to offer increased diagnostic accuracy over traditional microscopic visualization and provide a valuable diagnostic option, particularly given the uncultivable nature of HM (12). Many consider PCR assay to be the "gold standard" for HM diagnosis; however, this diagnostic test has not been standardized among laboratories. Diagnostic sensitivity and specificity data have not been reported, and inter-laboratory agreement has not been evaluated. Polymerase chain reaction assays performed at different laboratories are often, incorrectly, assumed to be equivalent. Different primer sets may be used to identify the same organism by different diagnostic laboratories and this, combined with varying protocol, can lead to discordant results.

The primary objective of this study was to determine if there was a difference in the prevalence of subclinical HM-infection in cats from 2 distinct populations: shelter cats and client-owned cats presented for elective procedures (vaccination, ovariohysterectomy, or ochiectomy). We hypothesized that cats from the shelter population may be at higher risk of infection based on the presence of risk factors which have been previously identified for feline HM-infection (time spent outdoors, external parasites, positive retroviral status) (2,3,9). A secondary objective of our study was to evaluate the inter-laboratory agreement of 2 independent validated conventional PCR assays used at separate diagnostic laboratories for the diagnosis of HM-infection.

Materials and methods

A convenience sample of 58 clinically healthy cats from the local animal shelter (Society for the Prevention of Cruelty to Animals — SPCA) and 57 clinically healthy, client-owned cats were selected for the study. The client-owned cats had been presented to the Veterinary Teaching Hospital at the Western College of Veterinary Medicine (WCVM-VTH) over a 6-month period during 2006 for routine physical examination and vaccination. The protocol for this study was approved by the Animal Care Committee, University of Saskatchewan, and written consent was obtained before sampling all client-owned cats. The following information was recorded for all cats: age (estimated based on dentition for the shelter cats), sex, neuter status, breed, and the presence or absence of ectoparasites determined following flea combing and otoscopic examination. Cats were grouped by age into 2 categories: < 2 years and ≥ 2 years. Cats were also categorized as purebred or as a domestic breed. For client-owned cats, information on their origin, access to outdoors, and number of cats within the household was also collected. Client-owned cats that originated from the SPCA or from households that had more than 3 cats were excluded from the study. All cats were assessed as clinically healthy, based on a routine physical examination.

Blood was collected from the jugular or saphenous vein for each cat. Fresh blood smears were made immediately to decrease the chance of detachment of mycoplasma organisms from the red blood cells (RBC) following exposure to ethylenediamine tetra-acetic acid (EDTA). Blood samples were collected into EDTA tubes and serum tubes. Blood in the serum tubes was spun within 2 h of collection, and the serum was harvested and stored frozen at −20°C in plastic tubes. Blood collected into EDTA tubes was used to determine packed-cell volume (PCV) and plasma total protein for each cat using microhematocrit tubes and refractometry, respectively. Serum (or plasma when serum was not available) from each cat was tested for feline leukemia virus (FeLV) antigen and feline immunodeficiency virus (FIV) antibody, using a commercially available enzyme-linked immunosorbent assay (ELISA) kit (Snap FIV Antibody/FeLV Antigen Combo; IDEXX Laboratories, Westbrook, Maine, USA) according to the manufacturer's instructions.

Blood smear evaluation for all 115 study cats was performed by a senior clinical pathologist (board certified by the American College of Veterinary Pathologists) and a senior clinical pathology resident who were both blinded to the population source and PCR assay results for each cat. Samples were classified as positive on smear evaluation if there were ≥ 3 conclusive HM organisms present on examination of 10 to 100 × oil immersion fields (hpf), negative if no HM organisms were visible, and equivocal if non-conclusive inclusions were seen or if there were < 3 HM organisms present on all 10 hpf. The reported sensitivity and specificity of blood smear evaluation for detection of *M. haemofelis* are 0% and 98%, respectively, and for "*Candidatus* M. haemominutum" 10% and 87%, respectively (13).

Aliquots of EDTA blood from each cat (n = 115) were stored at −20°C. Samples were submitted in batches to Prairie Diagnostic Services (PDS) (Saskatoon, Saskatchewan) for evaluation by a previously validated conventional HM PCR assay for both species (14).

Evaluation of the inter-laboratory agreement of 2 independent conventional HM PCRs involved submission of 44 aliquots of feline EDTA blood to a second independent laboratory, Animal Health Laboratory (AHL) (Guelph, Ontario) for analysis using a previously validated, comparable conventional HM PCR assay (15,16). The 44 samples submitted to AHL included: 26 samples collected from cats enrolled in the subclinical prevalence study where adequate sample volume remained to permit a second PCR assay, and samples collected from 18 additional cats. The 26 cats from the prevalence study included 6 cats that were positive on HM PCR assay at PDS (3 for *M. haemofelis* and 3 for "*Candidatus* M. haemominutum"), and

Table I. Comparison of the various features of shelter and client-owned cats in Saskatoon, Saskatchewan that were evaluated for hemotropic mycoplasma organisms by polymerase chain rection assay at Prairie Diagnostic Services during 2006

	Shelter cats (N = 58)	Client-owned cats (N = 57)
Mean age (minimum – maximum) (years)	2.0 y (0.3–10 y)	2.1 y (0.3–11 y)
Male cats (*n*)	37	35
Female cats (*n*)	21	22
Spayed or neutered cats (*n*)	16	26
Domestic cat breeds (*n*)	51	52
Purebred cats (*n*)	7	5
Ectoparasites		
Fleas (*Ctenocephalides felis*, *C. canis*) (*n*)	0	0
Earmites (*Otodectes cynotis*) (*n*)	13	1
FeLV positive (*n*)	1	0
FIV positive (*n*)	1	1
Median PCV (%) (minimum – maximum)	39 (21–48)	44 (29–60)
Median TP (g/L) (minimum – maximum)	75 (60–98)	74 (58–100)
HM positive on smear evaluation (*n*)	0	1
M. haemofelis – positive by PCR (*n*)	3	2
"*Candidatus* M. haemominutum" – positive by PCR (N)	4	0

FeLV — feline leukemia virus; FIV — feline immunodeficiency virus; PCV — packed-cell volume; TP — total protein; HM — hemotropic mycoplasma; PCR — polymerase chain reaction.

20 negative cases. The 18 additional cat samples included 5 cats that were positive for *M. haemofelis* and 6 cats that were positive for "*Candidatus* M. haemominutum" on PCR at PDS and 7 cats that were negative for both species on PCR at PDS.

The association between subclinical HM-infection (as determined by the PCR assay from PDS) and each of the following parameters was evaluated using a series of 2-tailed Fisher's exact tests (Statistix 8; Analytical Software, Tallahassee, Florida, USA): population represented by the cat (shelter or client-owned), age (< 2 y or ≥ 2 y), breed (domestic or purebred), sex, neuter status, presence of external parasites, and retroviral infection status. The associations between HM-infection and PCV as well as total protein were evaluated using Wilcoxon rank sum tests (Statistix 8; Analytical Software). All associations where $P < 0.05$ were considered statistically significant. The inter-laboratory agreement for the 2 independent qualitative HM PCR assays as well as the agreement between blood smear evaluation and PCR assay were assessed using the kappa statistic (κ) (STATA 9.0; StataCorp, College Station, Texas, USA). A kappa value > 0.6 was interpreted as substantial agreement and kappa > 0.8 was interpreted as almost perfect agreement (17). The difference in the odds of a sample being classified as positive on 2 different tests was assessed using exact McNemar's chi-squared tests (STATA version 9.0; StataCorp).

Results

A summary of the signalment, hematologic, and diagnostic test results for the 115 cats (58 shelter and 57 client-owned) is presented in Table I. Based on PCR assay, the overall prevalence of subclinical HM-infection when both populations were considered together was 8% [95% confidence interval (CI) = 3.6% to 14.3%; 9/115]. The prevalence of subclinical HM-infection in the shelter cat population was 12% (7/58), compared with 4% (2/57) in the client-owned cat population. Three of the HM-infected shelter cats were infected with *M. haemofelis* and the other 4 HM-infected shelter cats were infected with "*Candidatus* M. haemominutum." Both of the client-owned HM-infected cats were infected with *M. haemofelis*. The overall prevalence of *M. haemofelis* in both populations (based on PCR assay) was 4.3% (95% CI = 1.4 to 9.9%; 5/115).

There was no difference between the prevalence of HM-infection in the shelter cat population versus the client-owned cat population [$n = 115$, odds ratio (OR) = 3.8; 95% CI = 0.75 to 19, $P = 0.16$). There was also no significant difference in the prevalence of HM-infection between cats ≥ 2 y and cats < 2 y (OR = 3.3; 95% CI = 0.78 to 13.9; $P = 0.15$). None of the other factors evaluated, including sex ($P = 0.48$), neuter status ($P = 0.99$), breed ($P = 0.23$), presence of external parasites ($P = 0.99$), or retroviral status ($P = 0.99$) were associated with HM-infection. The median PCV was significantly lower in the HM-infected cats (median: 35%, range: 29% to 49%) than in the HM negative cats (median: 46%; range: 29% to 60%) ($P = 0.01$). However, none of the HM-infected cats was anemic (based on the PCV reference interval at PDS: 24% to 45%).

Prevalence of HM by blood smear evaluation was 0.9% (95% CI = 0.02% to 4.7%). Blood smear evaluation was significantly less likely to be positive for HM-infection ($P = 0.008$) than PCR assay [$\kappa = 0.19$, standard error ($S_{\bar{x}}$) = 0.05].

Inter-laboratory results for the 2 PCR assays ($n = 44$) showed there was substantial, but not complete, agreement between the 2 independent HM PCR assays for *M. haemofelis* ($\kappa = 0.66$, $S_{\bar{x}} = 0.15$) and "*Candidatus* M. haemominutum" ($\kappa = 0.70$, $S_{\bar{x}} = 0.15$). The AHL PCR identified 6 positive samples for *M. haemofelis* and although the PDS PCR agreed for 5 of these 6 samples, an additional 3 samples

were positive for *M. haemofelis* with the PDS assay. Similarly, the AHL PCR identified 7 positive samples for "*Candidatus* M. haemominutum" and the PDS PCR results agreed with 6 of these samples, but the PDS PCR also identified 3 additional positive samples. Though the PDS PCR assay was significantly more likely to be positive than the AHL PCR ($P = 0.03$), there was no significant difference in the odds of being positive between the two PCR tests for either *M. haemofelis* or *Candidatus* M. haemominutum ($P = 0.63$). The PCR assay used by the AHL detected co-infection in 2 cats, while the PDS PCR assay did not identify any co-infected cats.

Discussion

The reported prevalence of HM-infection (4% to 30%) varies widely by geographic location (3,18,19). The prevalence of 8% identified in this study is higher than that reported in Ontario, Canada (4%). The relatively low prevalence in the current study, as well as the study in Ontario, might be expected because both studies looked at subclinical disease in a healthy cat population. The high prevalence of HM-infection at 30% was identified in a Spanish study of clinically ill cats (18). In the Kewish (14) study, used to validate a HM PDS PCR assay, HM-infection was documented in the same geographic region as the current study. In the Kewish (14) study, 38% (23/60) of cats were positive for HM on PCR testing. Of the cats that were tested in the Kewish (14) study, 30% (18/60) were suspected to have HM-infection based on blood smear examination and a regenerative anemia, and 72% (13/18) of those cats were subsequently positive for HM on PCR assay. A subpopulation of cats in the Kewish (14) study group with normal complete blood (cell) counts had a 10% prevalence of HM-infection (2/20), which is similar to the prevalence identified in this current larger study.

There was no detectable difference in the prevalence of infection between the 2 populations of cats (shelter versus client-owned) evaluated in this study. These 2 groups were compared based upon the hypothesis that risk factors for HM-infection were likely to be different between client-owned and shelter cats. The natural mode of transmission for HM is not known but is suspected to involve arthropod vectors as well as direct cat to cat transmission. Shelter cats are expected to have a higher prevalence of ectoparasitic infestations and have a higher rate of exposure to other cats. Client-owned cats acquired from the shelter or belonging to households with > 3 cats were excluded to maximize the difference in risk factors between the 2 populations. Though the percentage of HM-infected shelter cats (12%) was 3 times higher than the percentage of HM-infected client-owned cats (4%), the study did not have sufficient power, likely related to the relatively small sample size, to demonstrate a statistically significant difference.

Outdoor access has been previously associated with an increased risk of HM-infection in cats (3). All of the shelter cats presumably had outdoor access as a risk factor in their history. However, the assumption that all the shelter cats had outdoor access may have been incorrect, as their history was not known and some may have been surrendered indoor cats from private homes. Of the client-owned cats, 33% were known to have outdoor access, including the 2 client-owned cats that tested positive for HM.

Choice of a different breakpoint for age may have identified a significant association between age and HM-infection. In previous prevalence studies, older cats with an average age of 10 y are reported to be more at risk for presentation with clinical HM-infection (2). In the current study, the shelter cats were primarily young adults, which may have diminished the ability to detect age as a significant risk factor. Additionally, sample size may not have been adequate to demonstrate an age association.

None of the cats in the study had detectable flea or tick infestations, reflecting the low prevalence of these ectoparasites in this region. Ear mites, *Otodectes cyanotis*, were found in a number of shelter cats. As expected, *Otodectes cyanotis* infection was not associated with HM-infection as these mites do not engage in hematophagus activity, which is presumed to be needed to transmit HM.

Retroviral infection is an established risk factor for HM-infection and clinical disease (1). There were 2 FeLV positive cats identified in the study (one client-owned cat and one shelter cat) and both were negative on PCR for HM-infection. There was 1 client-owned FIV-positive cat and this cat was also negative on PCR for HM-infection. The lack of association between retroviral infection and HM-infection in this study likely reflects the small sample size and possibly the low prevalence of these infections in this area as well as the low prevalence in the study group related to the selection of clinically healthy cats.

Blood smear evaluation identified only 11% (1/9) of the HM-infections identified by PCR which is very similar to previous reports (13). Blood smear evaluation is reported to have relatively low sensitivity as demonstrated by a recent study that found the sensitivity of this technique to be 0.0% for *M. haemofelis* and 10.3% for "*Candidatus* M. haemominutum" compared to quantitative PCR (13). Reliance on blood smear evaluation for a diagnosis of HM will underestimate prevalence and lead to missed clinical cases.

A difference in PCV between HM PCR-positive and negative cats has been reported in some studies (2,13). The biological significance of the trend towards a lower PCV in the HM-positive cats is questionable in the current study as none of the cats had a PCV below the reference interval.

Inter-laboratory agreement for the 2 HM PCR assays was substantial but results were not identical. Polymerase chain reaction assays for the same organism often use different primers and are therefore not equivalent, as they target different non-conserved portions of the 16S rRNA. Prairie Diagnostic Services used the same primers as AHL plus one additional primer each for *M. haemofelis* and "*Candidatus* M. haemominutum." The PDS laboratory was more likely to find positive *M. haemofelis* samples compared to AHL. The higher number of positive results at PDS may result from the additional primers, subtle differences in the assay, or may represent false positives. At the same time, AHL found dual infections where PDS found none. Once again, the use of additional primers and other subtle differences in the assay may have affected the ability of PDS to detect a dual infection or the dual infections may represent false positives at AHL. This study did not have sufficient data to calculate diagnostic sensitivity and specificity for the 2 PCR tests using either traditional methods or latent class models which do not require a gold standard to estimate test accuracy. Even if we could assume blood smear evaluation is a reasonable proxy for a gold standard, given that

there was only one positive sample, meaningful estimations could not be generated. Different detection rates between PCR assays is relevant to patient care, as a practitioner may withhold treatment if HM-infection is not confirmed by testing. Also, in screening blood-donor cats for HM-infection, a positive cat may inadvertently be screened as negative. Different detection rates are also relevant from a research standpoint, as prevalence studies are more difficult to interpret if between test agreement is low and diagnostic accuracy is unknown.

The biggest limitation of this prevalence study was that it lacked power. Preliminary calculations to determine the number of cats to be included in the study were based on a pilot study (using the same PDS PCR assay as the current study) that showed a much higher prevalence of HM-infection in the cat shelter population than was identified in this study. One possible explanation for the difference in prevalence of HM-infections found between this study and the original pilot study would be a problem with the PCR assay. Specifically, it is possible that a problem with the assay may have led to either false positive results in the pilot study or false negative results in the present study. Although the differences were not statistically significant, the odds ratios and their associated confidence intervals for both population source and age do not rule out differences among these groups.

In conclustion, there was no detectable difference observed in the prevalence of subclinical HM-infection between the clinically healthy shelter and client-owned cat populations in this study. The power to detect differences between the 2 populations was low because the prevalence was lower than expected. There was substantial but not perfect agreement between the results of the HM PCR assays for the 2 laboratories accessed in this study. The need for continued attention to standardization of PCR testing is emphasized.

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Evidence of cell-mediated immune response and specific local mucosal immunoglobulin (Ig) A production against *Lawsonia intracellularis* in experimentally infected swine

Roberto M.C. Guedes, Connie J. Gebhart

Abstract

The purpose of this study was to detect cell-mediated and local humoral immune responses to *Lawsonia intracellularis* in pigs inoculated with a pure culture of the pathogenic isolate or with an intestinal mucosa homogenate. Twenty-four 5-week-old pigs were inoculated with a pure culture of *L. intracellularis* (n = 10), an intestinal mucosa homogenate from proliferative enteropathy diseased pigs (n = 10), or a control solution (n = 4). All animals were bled 0, 7, 14, and 20 d post-inoculation (pi). Serum was tested for immunoglobulin (Ig) G against *L. intracellularis* and for the production of interferon (IFN)-γ by peripheral blood mononuclear cells (PBMC) after inoculation with *L. intracellularis* total proteins. Delayed-type hypersensitivity (DTH) reactions were evaluated 24 and 48 h after intra-dermal injection of different concentrations of *L. intracellularis* antigen 20 d pi. All animals were euthanized on day 22, intestinal lavages of ileum and IgA titrations were done. Weak IFN-γ production was detected in 1 pig from the pure culture group and 2 pigs from the mucosal homogenate group 14 d pi, and in 2 animals from both groups 20 d pi. All pigs, in both inoculated groups, were seropositive for IgG on day 20. Inoculated pigs from both groups showed very weak dose-dependent DTH reactions, which were more evident at 24 h than 48 h pi. Eight pigs from the pure culture group and 7 from the mucosa homogenate group had detectable IgA titers in the intestinal lavage 22 d pi. In conclusion, specific local intestinal humoral and weak cell-mediated immune responses can be detected in pigs experimentally infected with *L. intracellularis*.

Résumé

L'objectif de la présente étude était de détecter les réponses immunitaires à médiation cellulaire et humorale locale à Lawsonia intracellularis *chez des porcs inoculés avec une culture pure de l'isolat pathogène ou avec un homogénat de la muqueuse intestinale. Vingt-quatre porcs âgés de 5 semaines ont été inoculés avec une culture pure de* L. intracellularis (n = *10), un homogénat de la muqueuse intestinale de porcs atteints d'entéropathie prolifératives (*n = *10), ou une solution témoin (*n = *4). Un prélèvement sanguin a été obtenu de tous les animaux aux jours 0, 7, 14 et 20 post-inoculation (pi). On vérifia la présence dans le sérum d'immunoglobulines (Ig) G envers* L. intracellularis *et la production d'interféron (IFN)-γ par les mononucléaires du sang périphérique (PBMC) après l'inoculation de protéines totales de* L. intracellularis. *Les réactions d'hypersensibilité retardée ont été évaluées 24 et 48 h après injections intra-dermiques de différentes concentrations d'antigène de* L. intracellularis *20 j pi. Tous les animaux ont été euthanasiés au jour 22, des lavages de l'iléon et la détermination des titres d'IgA ont été effectués. Une faible production d'IFN-γ a été détectée chez 1 porc inoculé avec une culture pure et 2 porcs inoculés avec l'homogénat de muqueuse14 j pi, et de 2 animaux des deux groupes 20 j pi. Tous les porcs, dans les deux groupes inoculés, étaient séropositifs pour la présence d'IgG au jour 20. Les porcs inoculés des deux groupes ont montré de très faibles réactions de DTH dose-dépendante qui étaient plus évidentes à 24h qu'à 48h. Huit porcs inoculés avec une culture pure et 7 inoculés avec un homogénat de muqueuse avaient des titres d'IgA détectables dans le lavage intestinal 22 j pi. En conclusion, des réponses locales spécifiques d'immunité humorale et de faibles réponses à médiation cellulaire peuvent être détectées chez des porcs infectés expérimentalement avec* L. intracellularis.

(Traduit par Docteur Serge Messier)

Introduction

Proliferative enteropathy (PE) is an infectious enteric disease caused by *Lawsonia intracellularis*, an obligate intracellular gram-negative bacterium. Based on current knowledge, intracellular organisms usually stimulate a cell-mediated immune response (1). Although *L. intracellularis* is an intracellular organism, much is known about systemic humoral immune response (serum IgG) in experimentally and naturally infected pigs, which is likely not protective. However, little is known about the cell-mediated immune (CMI), delayed type hypersensitivity (DTH), or local mucosal IgA immune responses of pigs infected with the organism.

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The enzyme-linked immunosorbent spot (ELISPOT) T-cell assay detects the secretion of interferon-gamma (IFN-γ) produced by memory or activated T-lymphocytes and is used to evaluate the T-helper 1 (Th 1) response on the single-cell level (2). The ELISPOT T-cell assay was recently used to assess the onset and duration of cell-mediated immune response to *L. intracellularis* in pigs challenged with a pathogenic isolate or exposed to a commercial vaccine (Enterisol Ileitis; Boehringer Ingelheim, St. Joseph, Missouri, USA) (3). Results of this study showed the induction of cell-mediated immune response started at 21 dpi; however, as this was the first and only study using this assay, more data are necessary to corroborate these results.

The DTH reaction can be induced by a variety of intracellular bacterial pathogens (such as *Brucella abortus, Listeria monocytogenes, Mycobacterium bovis*), but it has not yet been studied during *L. intracellularis* infection. This reaction happens as a consequence of the antigen activation of T-helper lymphocytes (1). A granulomatous inflammatory reaction, in the intestines of Iberian pigs, was associated with *L. intracellularis* infection (4). Thus, use of intradermal injections of different types and concentrations of *L. intracellularis* antigens in experimentally infected pigs may demonstrate the existence of a DTH response involved in PE. The presence of a cell-mediated immune response against *L. intracellularis* infection suggests that further studies relating the DTH response to protection from infection are warranted.

Secretory IgA binds to bacteria and viral surface antigens in the lumen of the intestine and prevents pathogens from attaching to mucosal cells (1). In addition, it has been suggested that IgA is involved in the neutralization of intracellular organisms in the lamina propria and when passing through infected enterocytes (5). Enterocytes, mainly from the aboral part of the small intestines, are the permissive cells for the entry and maintenance of *L. intracellularis* resulting in persistent infection (6). Understanding the role of mucosal IgA, specific against *L. intracellularis*, may help explain the mechanism of protection from infections in future studies of PE.

The immune response induced by *L. intracellularis* infection was studied in pigs challenged with pure culture of a pathogenic isolate or with an intestinal mucosa homogenate extracted from PE-diseased pigs. In addition to the ELISPOT T-cell assay, 3 other methods were used to evaluate immune response: a skin test measuring the DTH reaction, titration of secretory IgA specific to *L. intracellularis* in intestinal lavages measuring local humoral immune response, and titration of serum IgG specific to *L. intracellularis* measuring systemic humoral immune response.

Materials and methods

Study design

All procedures were conducted in accordance with the guidelines of the Animal Care and Use Manual from the University of Minnesota and approved by the Institutional Animal Care and Use Committee. Twenty-four 5-week-old pigs weighing between 9 and 13.6 kg (20 and 30 lb) were assigned to one of 3 groups. The study design implemented was described in detail by Guedes and Gebhart (7). Briefly, 1 d prior to inoculation, the pigs were divided into 3 groups randomized by weight: 4 pigs in the control group; 10 pigs in the pure culture group; and 10 pigs in the intestine homogenate group. Each group was housed in different rooms in the isolation barns at the University of Minnesota. All animals were intragastrically dosed with 40 mL of inoculums, according to their respective group, using a stomach tube. On day 0, pigs in the control group received sucrose-potassium glutamate solution (SPG; 0.218M sucrose, 0.0038M KH_2PO_4, 0.0072M K_2HPO_4 and 0.0049M of glutamate, pH 7.0) with 5% fetal bovine serum (FBS). Each pig in the pure culture group received 8.9×10^8 *L. intracellularis* organisms in a SPG solution with 5% FBS. The 3rd group received a solution of 2.9×10^{10} *L. intracellularis* organisms per pig, scraped from the affected mucosa of a PE-affected gilt, diluted 1:1 w/v SPG with 5% FBS.

Fecal and blood sampling

Two days before challenge, all pigs were bled and serum was tested for exposure to *L. intracellularis* by an immunoperoxidase monolayer assay (IPMA) (8,9). In addition, fecal samples were collected and tested for *L. intracellularis* DNA using polymerase chain reaction (PCR) (10) to assure animals were negative for PE.

Serum and heparinized whole blood samples were collected from all pigs on days 7, 14, and 20 post-inoculation (pi) and tested by IPMA for IgG specific to *L. intracellularis* (8,9) and by ELISPOT assay for interferon-gamma (IFN-γ) (3).

Delayed-type hypersensitivity reaction

On day 20, all animals were sedated and 200 μL of 10 different solutions were injected intradermally in 10 different areas between the nipples. Solutions were as follows: (A) sterile phosphate buffer saline (PBS, pH 7.2); (B) 165 μg/mL of sonicated noninfected McCoy cell suspension; (C) 250 μg/mL of Phytohemagglutinin (PHA; Sigma, St. Louis, Missouri, USA) (positive control); (D) 10^9 *L. intracellularis* formalin-fixed organisms per mL; (E) 10^8 *L. intracellularis* formalin-fixed organisms per mL; (F) 10^7 *L. intracellularis* formalin-fixed organisms per mL; (G) 250 μg/mL of sonicated *L. intracellularis*; (H) 25 μg/mL of sonicated *L. intracellularis*; (I) 75 μg/mL of outer membrane protein of *L. intracellularis*; and (J) 7.5 μg/mL of outer membrane protein of *L. intracellularis*. The purification of *L. intracellularis* from an infected McCoy cell line and the preparation antigens for the DTH and ELISPOT tests are described in detail by Guedes and Gebhart (3). Outer membrane preparations were obtained as previously described (11,12). Twenty-four and 48 h later, the delayed-type hypersensitivity immune response was measured using a manual caliper evaluating double skin fold thickness and the erythema diameter, when present.

Intestinal mucosal IgA

All pigs were euthanized 22 d pi. Intestinal lavage of the aboral 25 cm of the small intestine (ileum) of each pig was done using 20 mL of cold PBS, which was then centrifuged at $150 \times g$ for 5 min to eliminate solid material and the supernatant was frozen for posterior secretory IgA titration (described below).

The technique used to quantitate intestinal mucosal IgA was the immunoperoxidase monolayer assay (IPMA) (8,9), with some modifications. Briefly, 96-well plates (Nunclon, 167008; Nunc, Rochester, New York, USA) containing an acetone fixed monolayer of McCoy cells highly infected with *L. intracellularis* were rehydrated

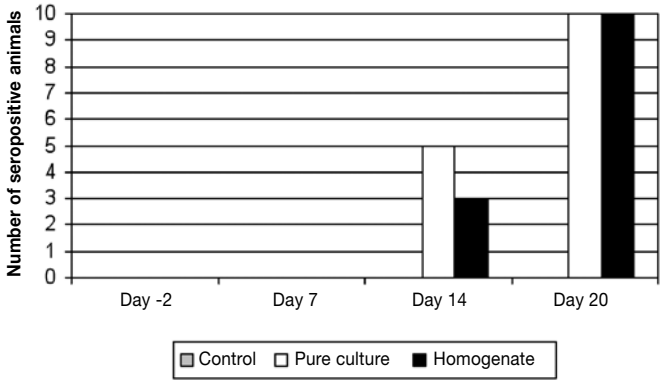

Figure 1. Immunoperoxidase monolayer assay results for serum IgG against *L. intracellularis* in pigs from the control (n = 4), pure culture (n = 10), and intestinal homogenate (n = 10) groups on days –2, 7, 14, and 20 post-inoculation (pi).

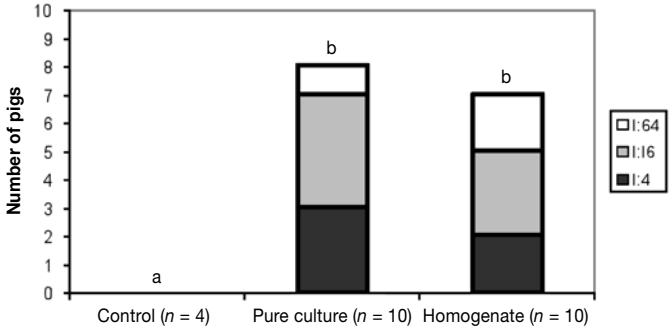

Figure 2. Number of pigs, from the control, pure culture, and intestine homogenate groups, showing various levels of specific IgA titers against *L. intracellularis* by ileum lavage, 22 d post-inoculation (pi).
[ab] Significant difference between groups ($P < 0.05$)

in a solution of PBS with 5% skim milk for 10 min at 37°C to block nonspecific reactions. The intestinal lavage samples were diluted in the same block solution in serial 4-fold dilutions (1:4, 1:16, 1:64, and 1:256). Then, 50 μL of each diluted sample was added to the test well. The plate was incubated for 30 min at 37°C and then washed 5 times with PBS containing 0.05% Tween 20 (PBST, Tween 20; Sigma). Goat anti-porcine IgA-peroxidase conjugate (A100-102P-11; Bethyl Laboratories, Montgomery, Texas, USA), diluted 1:1,000 in PBST, was added at 30 μL/well, and incubation proceeded for 45 min at 37°C. The plate was washed 5 times with PBST and 100 μL of pre-diluted chromogen (3-amino-9-ethyl-carbazole; AEC; A-6926; Sigma) solution was added to each well and incubated at room temperature for 20 min. The plate was washed with PBS 3 times, allowed to dry, and examined using an inverted light microscope. The presence of red stained *L. intracellularis* indicated a positive result.

Results

Intragastric inoculation of 5-week-old pigs with either a pure culture of *L. intracellularis* (PHE-MN/01) or an intestinal homogenate resulted in clinical signs of PE, pathological lesions, and infection determined using immunohistochemistry, as described in detail in a previous study (7). In brief, diarrhea was most commonly observed in inoculated pigs in the 3rd wk pi and reduced average daily gain in pure culture and homogenate groups (440 and 490 g, respectively) compared to the control group (650 g, $P < 0.05$). Macroscopic lesions typical of PE, ranging from 4 to 125 cm and from 5 to 140 cm, were observed in the small intestine (jejunum and ileum) in all pigs inoculated with pure culture and intestinal homogenate, respectively. Characteristic PE histologic lesions and positive immunohistochemistry results were observed in all ileum sections of animals from both inoculated groups. No lesions or signs of infection were detected in any of the control animals. All animals were negative by PCR analysis of fecal samples and by IPMA serology 2 d prior to inoculation.

Serum conversion was first detected in some animals in both challenged groups at 14 d pi (Figure 1). All pigs in both challenged groups were seropositive on day 20 pi with titers ranging from 1:30 to 1:480 in the pure culture group and from 1:30 to 1:1920 in the intestinal homogenate group. Eight out of 10 pigs from the pure culture group and 7 out of 10 pigs from the intestine homogenate group had IgA titers specific for *L. intracellularis* in the intestinal lavage ranging from 1:4 to 1:64 (Figure 2). Using Mann-Whitney to compare 2 groups at a time, there was a statistically significant difference between the control and the 2 challenge groups ($P < 0.05$).

One pig in the pure culture group (6 spots) and 2 pigs in the intestine homogenate group (11 and 8 spots) had detectable production of IFN-γ in vitro, as measured by ELISPOT using 10 μg/mL of bacteria antigen on day 14 pi. Two animals in the pure culture group (6 and 5 spots), including 1 that had been detected on day 14, and the same 2 animals in the homogenate group (15 and 6 spots) had detectable production of IFN-γ on day 20 pi. No skin lesions were observed in control animals. The results of the DTH response are summarized in Figure 3. The skin reaction was more evident at 24 h than 48 h pi and only the higher concentration of whole bacteria (10^9 *L. intracellularis* organisms) induced detectably different ($P < 0.01$) results compared to PBS and sonicated McCoy cells injection sites in both *L. intracellularis* inoculated animal groups using ANOVA. No skin reaction was detected at either 24 or 48 h pi with 10^8 and 10^7 *L. intracellularis* organisms, 250 and 25 μg/mL of sonicated *L. intracellularis*, or 75 and 7.5 μg/mL of outer membrane protein of *L. intracellularis*. No difference in erythema diameter was observed among the pure culture, intestinal homogenate, or control groups.

Discussion

Serum IgG titers detected in all challenged animals during the study were similar to those reported in other studies using intestine homogenate (8,9,13) or pure culture inocula (3,14), starting 2 wk pi and peaking at the 3rd wk. Specific *L. intracellularis* local intestinal humoral immune response, represented by IgA titers in intestine lavages, was demonstrated in the majority of the animals in both challenged groups using a modified IPMA technique, but it was absent in the control group. Large accumulations of IgA of unknown specificity in the apical cytoplasm of proliferating enterocytes in intestinal sections from pigs affected with the acute and chronic forms of PE was previously described (15,16). Immunoglobulin A accumulation was also described in the cytoplasm of plasma cells

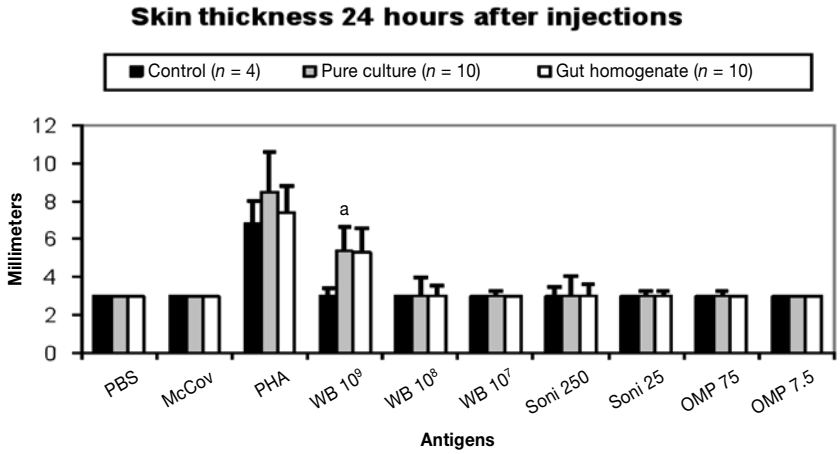

Figure 3. Delayed-type hypersensitivity (DTH) response against 10 different antigens in pigs from the control, pure culture, and intestinal homogenate groups on day 20 post-inoculation (pi). Skin thickness was measured 24 h pi.
[a] Significant difference ($P < 0.01$) between inoculated (pure culture and intestinal homogenate) and control groups, and between PBS and sonicated McCoy cells injection sites and 10^9 whole *L. intracellularis* sites in inoculated animal groups.
Antigens used in injection sites. PBS — Phosphate buffered saline (pH 7.2); McCoy — non-infected sonicated McCoy cells; PHA — phytohemagglutinin; WB 10^9–10^9 whole *L. intracellularis* organisms; WB 10^8–10^8 whole *L. intracellularis* organisms; WB 10^7–10^7 whole *L. intracellularis* organisms; Soni 250–250 μg of total *L. intracellularis* proteins after bacterial sonication; Soni 25–25 μg of total *L. intracellularis* proteins after bacterial sonication; OMP 75–75 μg of *L. intracellularis* outer-membrane proteins; OMP 7.5–7.5 μg of *L. intracellularis* outer-membrane proteins.

underlying proliferative lesions (17) and in peripheral areas of lymphoid Peyer's patches (15). In addition to the well-known effect of specific IgA against infectious agents in the mucus of the intestinal lumen, IgA secreted by lymphocytes in the lamina propria can be effective against microorganisms present in the lamina propria and also in the cytoplasm of enterocytes, during IgA translocation through the epithelial cell layer toward the intestinal lumen (5). *Lawsonia intracellularis* is an obligate intracellular organism that enters intestinal epithelial cells and is found in the cytoplasm of enterocytes and often in the lamina propria (8). As a result, IgA probably plays an important role in protecting the intestine against *L. intracellularis* invasion and intracellular proliferation. This is the first report of detection of an intestinal IgA specific for *L. intracellularis* in infected animals. Future studies must be conducted to determine whether there is a correlation between intestinal lavage IgA titers and protection.

A weak production of IFN-γ by T helper cells using 10 μg/mL of *L. intracellularis* test antigen purified from pure cultures of the bacteria was initially detected 2 wk pi in just a few animals from both challenge groups in this study. These results are in agreement with a preliminary study (3) that showed that the onset and peak of cell-mediated immune response in the majority of the animals infected with *L. intracellularis* are delayed in relation to the systemic humoral immune response (serum IgG). In a mouse *L. intracellularis* challenge model, infection of IFN-γ receptor knockout animals was substantially higher than in the wild type mice (18). In this same study, infection level peaked on day 21 pi in both wild type and IFN-γ receptor knockout mice, but it was more prolonged in the latter, up to 35 d pi. The authors concluded that IFN-γ may play a significant role in limiting infection and cell proliferation of *L. intracellularis*. Further studies must be conducted to evaluate the peak response, duration of response, and correlation between cell-mediated immune response and protection in pigs.

The fact that a significant DTH reaction was detected with *L. intracellularis* whole bacteria, at a concentration of 10^9 organisms, suggests that this kind of immune response can be induced in PE-diseased pigs as early as 22 d pi. The non-responsiveness to lower concentrations of whole cell bacteria and bacterial antigens reflects the fact that the response is easily titrated out and so may not be easily detected. Granulomatous inflammatory reactions associated with *L. intracellularis* infection in pigs have been described (4), but there was no proof of a cause and effect association between them. Based on the results of the present study, further research should be conducted in this area using a higher concentration of bacteria for a longer period of time.

The ability to detect systemic cell-mediated immune response measured by the IFN-γ T-cell assay and local intestinal humoral immune response (secreted IgA) in intestinal lavages measured by the modified IPMA technique will certainly contribute to a better understanding of the immune response involved in PE. Future studies will address whether these specific immune responses reflect immune protection to *L. intracellularis* infection.

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Article

Development of an immunocapture–polymerase chain reaction assay using IgY to detect *Mycobacterium avium* subsp. *paratuberculosis*

Linda W. Chui, Robin King, Jeong Sim

Abstract

A diagnostic assay using immunomagnetic separation was developed to capture *Mycobacterium avium* subsp. *paratuberculosis* (MAP) from bovine feces by means of IgY derived from chicken eggs. The antibody was coupled directly onto the surface of MagaCell cellulose/iron oxide beads or indirectly by being mixed with MagaBeads and a rabbit IgG linker against chicken antigen. Optimization parameters for the immunocapture included incubation time, temperature, volume, and type of immunocapture beads. Analytical sensitivity and specificity were determined by extracting DNA from the captured bacteria and amplifying it by polymerase chain reaction (PCR). The 2 bead preparations had the same analytical sensitivity, and the detection level of MAP cells in spiked bovine feces was 2×10^4 cells/g. No PCR inhibition was observed with DNA from the organisms captured with use of the MagaCell-IgY beads.

Résumé

Une épreuve diagnostique utilisant la séparation immuno-magnétique a été développée afin de capturer Mycobacterium avium *ssp.* paratuberculosis *(MAP) à partir de fèces bovines au moyen d'IgY provenant d'œufs de poule. Les anticorps ont été couplés directement sur la surface de billes MagaCell composées de cellullose/oxyde de fer ou indirectement en étant mélangées avec des MagaBeads et des IgG de lapin dirigés contre les antigènes de poulet. L'optimisation des paramètres pour l'immunocapture incluait le temps d'incubation, la température, le volume et le type de billes. La sensibilité analytique et la spécificité ont été déterminées par extraction de l'ADN des bactéries capturées et en l'amplifiant par réaction d'amplification en chaîne par la polymérase (PCR). Les 2 préparations de billes avaient la même sensibilité analytique, et le degré de détection des cellules de MAP dans des échantillons inoculés intentionnellement était de 2×10^4 cellules/g de fèces bovines. Aucune inhibition du PCR n'a été observée avec l'ADN des microorganismes capturés en utilisant les billes MagaCell-IgY.*

(Traduit par Docteur Serge Messier)

Introduction

Paratuberculosis, or Johne's disease, was first reported by Johne and Frothingham in 1895 (1). It is a contagious, progressive, chronic digestive disorder of both wild and domestic ruminants caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP) (2–9). Fecal culture is still considered the "gold standard" for detecting MAP, but this method depends on the bacterial load in the specimen and the stage of the disease, and results may not be available for 16 to 20 wk. Enzyme-linked immunosorbent assay (ELISA) has commonly been used in the diagnostic laboratory, but its sensitivity has been reported as 87% in clinical cases, 75% in subclinical cases with heavy fecal shedding, and 15% in subclinical cases with light fecal shedding (10). Consequently, ELISA is suitable only for detecting herd-level status. In a mixed herd of subclinically infected and noninfected cattle, the herd-level sensitivity of ELISA and fecal culture is about 45% (11) and 45% to 55% (12), respectively. The sensitivity of both methods in cattle has also been reported to be around 35% (13).

Molecular testing gives a rapid diagnosis of Johne's disease; several polymerase chain reaction (PCR) assays have been developed (14–19). However, inhibitory factors in bovine fecal specimens coextract with the target DNA and interfere with the assay. To circumvent this problem, we have developed an immunocapture [or immunoseparation (IMS)] assay using IgY to capture the organisms in the fecal sample prior to DNA extraction and PCR. The objective of this study was to optimize the conditions required for liquid- and solid-phase IgY-immunocapture of MAP in the presence and absence of fecal material.

Materials and methods

Preparations of IgY

The IgY was purified from chicken egg yolks as previously described (20). Purified IgY with an ELISA titer greater than 1/5000 was pooled and used for the following experiments. MagaBeads (Cortex BioChem, San Leandro, California, USA) of rabbit IgG against chicken antigen were mixed with IgY in a ratio of 1 mg of beads to 1.5 μg of IgY and incubated at room temperature for 30 min, as recommended by the manufacturer; this preparation

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was designated Beads A. In addition, purified IgY was coupled to magnetizable cellulose/iron oxide beads (Cortex BioChem) in a ratio of 18 mg of IgY to 1 g of beads, for a concentration of 3×10^8 beads/mL. This preparation was designated MagaCell-IgY.

Sensitivity determination for PCR assay without immunocapture

The concentrations of 3 field and 2 reference strains of MAP [11992, EA4146, 2945, American Type Culture Collection (ATCC) 12258, and ATCC 19698] were adjusted to 2×10^7 cells/mL. Dilutions of this standardized cell suspension were made from 10^{-3} to 10^{-7} in 12 mM Tris-HCl (pH 7.4), and 200 μL of each dilution was extracted with the MagaZorb DNA isolation kit (Cortex BioChem) according to the manufacturer's instructions. The DNA was eluted in 200 μL of 12 mM Tris-HCl, and 10 μL was used as a template for PCR. Titration of each MAP strain was performed in triplicate.

Sensitivity determination for PCR assay with Beads A immunocapture

A field strain and a reference strain of MAP (EA4146 and ATCC 19698) were selected on the basis of differences in their restriction fragment length polymorphism (20), thereby allowing comparison of binding affinity. Aliquots (1 mL) of each strain, consisting of 2×10^4 cells in 12 mM Tris-HCl and 6% sodium dodecyl sulfate (SDS), were shaken on an Eberbach model 6000 shaker (Eberbach Labtools, Ann Arbor, Michigan, USA) at low speed for 30 min. A duplicate aliquot of cells was processed without SDS for comparison. The cells were pelleted by centrifugation at 13 000 × *g*, washed with 1 mL of 12 mM Tris-HCl (pH 7.4), and resuspended in 1 mL of the same buffer. A 200-μL aliquot of the resuspended cells was centrifuged at 13 000 × *g* for 3 min, and the cell pellet was resuspended in 990 μL of phosphate-buffered saline (PBS), pH 7.4. After the addition of 10 μL of Beads A, the tubes were incubated at 37°C for 1.5 h. After incubation, the cell/bead suspension was placed on an MPC-S unit (Invitrogen, Burlington, Ontario) for 5 min, after which the supernatant was discarded. The beads were resuspended in 1 mL of 12 mM Tris-HCl and returned to the MPC-S unit for 5 min, after which the supernatant was again discarded. This wash step was repeated 4 more times. The captured bacteria were released from the beads when the pellet was resuspended in 200 μL of 0.1% SDS at room temperature (RT) for 15 min. The reaction tubes were placed in the MPC-S unit for 15 min to remove the beads. Next, DNA was extracted from the supernatant by means of the MagaZorb DNA isolation kit, according to the manufacturer's instructions. Similarly, DNA was extracted without immunocapture from 200 μL of the MAP cells treated or not treated with 0.6% SDS. All DNA was diluted from 10^{-1} to 10^{-7} in 12 mM Tris-HCl (pH 7.2), and 10 μL of the 10^{-3} to 10^{-7} dilutions was used as the template for the PCR assay.

Optimization of Beads A for the immunocapture assay

Aliquots of 10, 20, 30, or 40 μL of Beads A suspension were added to 1.5-mL microcentrifuge tubes containing 4000 MAP cells (EA4146 or ATCC 19698) in a total volume of 1 mL of PBS. The mixture was held at 37°C for 1.5 h, then washed 5 times, and DNA was extracted as described above. For the PCR assay 10 μL of undiluted and of 1:10 and 1:100 dilutions of template DNA was used.

Table I. End-point titration results for *Mycobacterium avium* subsp. *paratuberculosis* (MAP) cells in polymerase chain reaction with or without treatment with sodium dodecyl sulfate (SDS) and subsequent immunocapture

Dilution of MAP cells	Without immunocapture		With immunocapture	
	With SDS	Without SDS	With SDS	Without SDS
10^{-3}	+	+	+	+
10^{-4}	+	+	+	+
10^{-5}	+	+	+	–
10^{-6}	+	+	–	–
10^{-7}	–	–	–	–

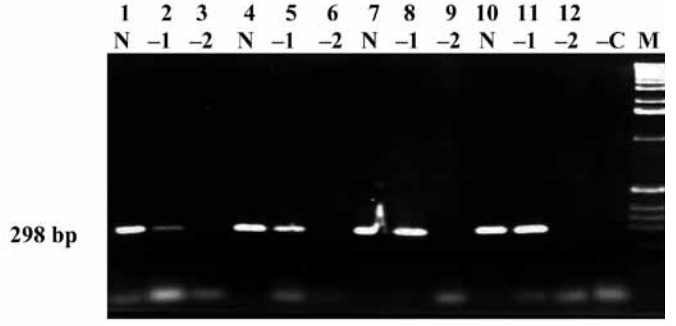

Figure 1. Determination of Beads A volume required for immunocapture of American Type Culture Collection (ATCC) strain 19698 of *Mycobacterium avium* subsp. *paratuberculosis* (MAP). For each Beads A volume used, DNA was run undiluted (N) and at dilutions of 1:10 (−1) and 1:100 (−2). The bead volumes were as follows: lanes 1 to 3, 10 μL; lanes 4 to 6, 20 μL; lanes 7 to 9, 30 μL; lanes 10 to 12, 40 μL. Lane –C — negative water control; lane M — 1-kb molecular weight marker; bp — base pairs.

To determine the optimal incubation time and temperature required for the immunocapture assay, 4000 MAP cells of each strain in 1 mL of PBS were captured with 40 μL of Beads A, and the suspensions were mixed with a MyLab Rotamix SLRM1 (MJS BioLynx, Brockville, Ontario) at 37°C and RT for 15, 30, or 60 min. The Beads A mixture was washed and the DNA extracted as previously described.

Limit of detection using the immunocapture assay and bovine feces spiked with MAP

Bovine feces were provided by Agri-Food Laboratories Branch, Alberta Agriculture and Rural Development, Edmonton, Alberta. All samples had tested negative for MAP by culture and had been stored at –80°C. The MAP strains used for spiking were the same field and reference strains as used for the PCR sensitivity determination without immunocapture. Aliquots of frozen feces (1 g) were thawed overnight at 4°C and mixed with 1 mL of the MAP strains containing 2×10^3, 2×10^4, or 2×10^5 cells. An unspiked fecal sample was included as a negative control. Each sample was added to a conical centrifuge tube containing 24 mL of 0.6% (w/v) SDS in water. Each suspension was mixed by vortex for 1 min and then placed for 30 min on a horizontal shaker (Eberbach model 6000) set at low speed. After the tubes had stood for 30 min at RT to allow the

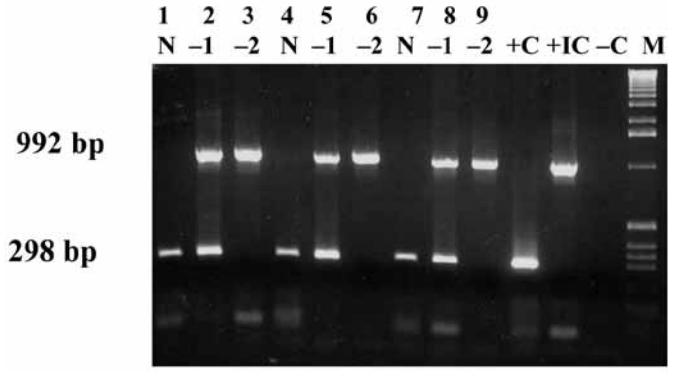

Figure 2A. Polymerase chain reaction (PCR) results with different concentrations of DNA extracted from ATCC 19698 MAP cells immunocaptured by 40 μL of Beads A incubated at room temperature (RT) for different times; DNA was run undiluted (N) and at dilutions of 1:10 (−1) and 1:100 (−2). The incubation times were as follows: lanes 1 to 3, 15 min; lanes 4 to 6, 30 min; lanes 7 to 9, 60 min. Lane +C — positive-control DNA (ATCC 19698); lane +IC — positive internal-control DNA; lane –C — negative water control; lane M — 1-kb molecular weight marker.

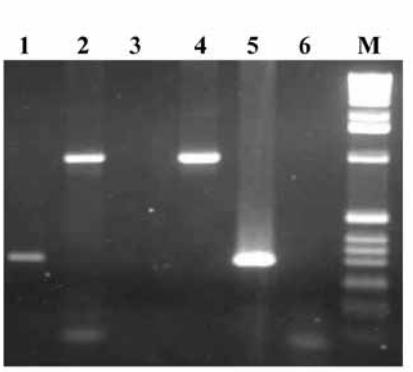

Figure 2B. Results of PCR with the use of 40 μL of Beads A to immunocapture MAP ATCC 19698 spiked in bovine feces (2×10^4 cells/g) incubated at RT for 15 min. Lanes 1 and 2 — DNA diluted 1:10 and 1:100; lanes 3 and 4 — negative-control bovine feces with DNA diluted 1:10 and 1:100; lane 5 — positive-control DNA (ATCC 19698); lane 6 — negative water control; lane M — 1-kb molecular weight marker.

particulate matter to settle, 20 mL of the supernatant was removed and centrifuged in a Beckman centrifuge G120 (Beckman Coulter, Brea, California, USA) at 2380 × *g* for 30 min. The supernatant was discarded and 24 mL of sterile, purified water added to the pellet. After being mixed by vortex for 1 min, the suspension was centrifuged at 2380 × *g* for 30 min. The cell pellet was washed once more in the same way and resuspended in 1 mL of PBS (pH 7.4). All suspensions were then stored at 4°C.

For the immunocapture, 200 μL of each prepared spiked suspension was centrifuged, the pellet was resuspended in 160 μL of PBS, and then 40 μL of Beads A was added. After incubation with gentle mixing at RT for 15 min, the cell/bead suspension was placed on the MPC-S unit for 5 min, after which the supernatant was discarded. The beads were resuspended in 1 mL of PBS and returned to the MPC-S unit for 5 min, after which the supernatant was again discarded. This washing process was repeated 4 more times. The captured bacteria were released from the beads when the pellet was resuspended in 200 μL of 0.1% SDS in PBS at RT for 15 min. The reaction tubes were placed in the MPC-S unit for 3 min to remove the beads. DNA was extracted from the entire 200 μL of supernatant with use of the MagaZorb DNA isolation kit according to the manufacturer's instructions.The same immunocapture and DNA extraction protocols described for Beads A were used to test the MagaCell-IgY beads. To optimize the assay, 4 replicate sets of spiked fecal samples containing 2×10^3, 2×10^4, and 2×10^5 MAP cells per gram of feces were run with 5, 10, 15, or 20 μL of MagaCell-IgY beads instead of the 40-μL volume used for Beads A. All other steps were replicated as described above.

Detection of amplicons

The PCR was performed with primers that produce an amplicon 298 base pairs (bp) long that targets the insertion element IS900 of MAP (21). Amplification conditions were those described previously (20). Briefly, the PCR master mix consisted of 1X PCR buffer, 2.0 mM magnesium chloride, 150 μM of dNTPs, 1.0 μM of each primer, 1 unit of Taq polymerase and 2 μL of template in a total volume of 50 μL. All reagents were purchased from Invitrogen. The PCR cycling parameters were as follows: 95°C for 5 min; [95°C for 15 s, 55°C for 15 s, and 72°C for 15s] for 25 cycles; [95°C for 15 s, 65°C for 30 s, and 72°C for 15 s] for 25 cycles; and a final extension at 72°C for 7 min. The PCR product was subjected to 1% agarose gel electrophoresis, and the bands were visualized after staining with 0.5% ethidium bromide. An internal control that gave an amplified product of 992 bp was included in each PCR tube to monitor for inhibitors (20). A 1-kb marker was used for size verification.

Results

The PCR titration of DNA from the 3 field and 2 reference strains of MAP gave identical end-point results in triplicate titrations with each of the strains (data not shown). The PCR analytical sensitivity was detected at a cell dilution of 10^{-6}, or 20 cells/mL, which is equivalent to ≤ 1 cell per PCR assay. No difference was observed in the end-point PCR detection level between the presence or absence of 0.6% SDS without immunocapture of MAP ATCC 19698 (Table I). The sensitivity of immunocapture using 10 μL of Beads A was determined by capturing 4000 cells of 2 MAP strains, EA4146 and ATCC 19698. With SDS treatment followed by immunocapture, the PCR end-point detection was at the dilution of 10^{-5}. There was a log difference between the treated and untreated cells, as shown for MAP ATCC 19698 in Table I; identical results were observed with strain EA4146 (data not shown). The PCR end-point titrations with various dilutions of DNA extracted from 4000 cells of MAP ATCC 19698 captured with different amounts of Beads A are illustrated in Figure 1. Again the results were identical to those obtained using MAP EA4146 (data not shown). The intensity of the PCR band increased as the volume of Beads A increased from 10 to 30 μL (lanes 2, 5, and 8), but there was no difference in intensity between volumes of 30 and 40 μL (lanes 8 and 11).

PCR titration results showed that incubation times of 15, 30, or 60 min make no difference to immunocapture efficiency at RT with the use of 4000 cells of MAP ATCC 19698 (Figure 2A) or MAP EA4146 (data not shown). In addition, results were duplicated when

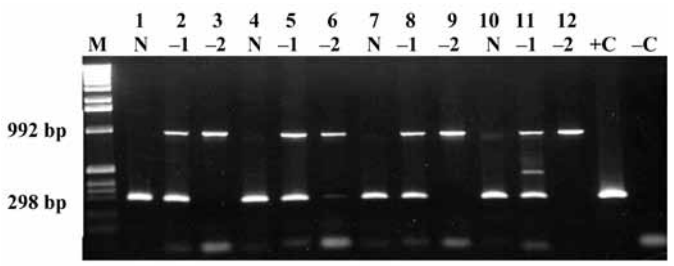

Figure 3. Determination of MagaCell-IgY beads volume required for immunocapture of MAP ATCC 19698 spiked in bovine feces (2×10^4 cells/g) and incubated at RT for 15 min. For each MagaCell-IgY beads volume used, DNA was run undiluted (N) and at dilutions of 1:10 (−1) and 1:100 (−2). The bead volumes were as follows: lanes 1 to 3, 5 μL; lanes 4 to 6, 10 μL; lanes 7 to 9, 15 μL; lanes 10 to 12, 20 μL. Lane M — 1-kb molecular weight marker; lane +C — positive-control DNA (ATCC 19698); lane –C — negative water control.

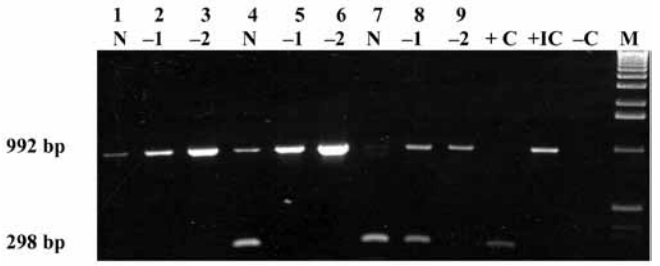

Figure 4. Results of PCR with the use of 10 μL of MagaCell-IgY beads to immunocapture MAP strain ATCC 19698 spiked in bovine feces at concentrations of 2×10^3 cells/g (lanes 1 to 3), 2×10^4 cells/g (lanes 4 to 6), and 2×10^5 cells/g (lanes 7 to 9). End-point titration was performed with extracted DNA run undiluted (N) and at dilutions of 1:10 (−1) and 1:100 (−2). Lane +C — positive-control DNA (ATCC 19698); lane +IC — positive internal-control DNA; lane –C — negative water control; lane M — 1-kb molecular weight marker.

the incubation temperature was 37°C (data not shown). Figure 2B demonstrates that 40 μL of Beads A at RT for 15 min was sufficient to capture MAP ATCC 19698 at a concentration of 2×10^4 cells per gram of bovine feces; results were identical for spiking with MAP field strains 11992, EA4146, and 2945 and ATCC 12258 (data not shown). Inhibition was observed when undiluted DNA from spiked and unspiked fecal samples captured with Beads A was used as the template for PCR. When 1:10 dilutions of DNA from the spiked and negative samples were used as the template, the internal control (IC) was not amplified, although the MAP gene was amplified in the spiked sample. At a 1:100 dilution of DNA, the IC was amplified in both cases but the MAP gene was not amplified.

Figure 3 illustrates that there was an insignificant difference in the PCR detection level with the use of 10 μL of MagaCell-IgY beads as opposed to 5, 15, or 20 μL. Again, amplification of the IC was absent or weak with undiluted DNA but was amplified when DNA was diluted 1:10 or 1:100. The limit of detection for the capture assay was 2×10^4 cells/g of bovine feces with the use of 10 μL of MagaCell-IgY beads (Figure 4) or 40 μL of Beads A. This limit of detection was the same when MAP field strains 11992, EA4146, 2945 and ATCC 12258 were used (data not shown).

Discussion

Different reports have been published on the use of IgG purified from rabbits for immunocapture assays of MAP (22–25), but this is the first study to show the utility of IgY prepared from chicken eggs for this purpose. The detection of pathogens by IMS has been used in conjunction with culture enrichment to increase cell number before immunocapture and, consequently, increase assay sensitivity (26,27). Owing to the slow growth of MAP organisms, this approach is inadequate for timely diagnosis. None the less, IMS can be used to immobilize and concentrate the number of organisms in a sample and enable their removal from the sample matrix so that, theoretically, the sensitivity of an assay can be improved. Although the ability to detect 1 MAP cell per reaction tube by PCR amplification afforded excellent sensitivity in this study, there is a problem with inhibition of the PCR when fecal samples are tested. Simple dilution of the template DNA does reduce the effect of these inhibitors, but it also reduces the amount of MAP DNA available for amplification as well as the sensitivity of the assay. The detection level of IMS-PCR increased to 200 cells per reaction tube in the presence of bovine feces. Decreased sensitivity of IMS-PCR for MAP cells in spiked sheep feces as compared with direct PCR on the same fecal sample was reported by Mason et al (25). This decrease is most likely due to the presence of free DNA from degraded MAP cells that is amplified by direct PCR but is not captured during IMS.

Bovine feces are a very heterogeneous and complex medium. Another reason for a decrease in the sensitivity of IMS-PCR with this type of suspension is the presence of nonspecific material that causes steric interference with the binding of the MAP cells to the antibody or actually traps the beads so they are lost during the wash steps. These effects were minimized in this study by increasing the volume of Beads A from 10 to 40 μL when applying IMS to MAP cells in the presence of bovine feces. If one assumes that the majority of MAP cells binding to the IgY must be intact so the integrity of the binding site is maintained for the antibody–antigen reaction to occur during IMS, the sensitivity of the IMS-PCR assay likely represents detection of intact organisms only. Intact microorganisms cannot be differentiated from free DNA fragments in a fecal sample by means of direct PCR assays, which is important if the shedder status of an animal is being determined for purposes of risk management. Our data corroborate the finding of Mason et al (25) of an IMS-PCR sensitivity of 10^4 MAP cells per gram of spiked feces with PCR primers directed to the IS900 sequence.

Bead-coating methods were also evaluated in this investigation. Whether the coating was done directly (as with the MagaCell-IgY beads) or indirectly via an antibody linker (as with Beads A), the immunocapture capability of the IMS was the same, as illustrated by the PCR end-point titrations. In the study by Mason et al (25), the antibody to MAP was directly coated onto the immunomagnetic beads by means of tosyl chemistry or indirectly coated via an antibody linker. That study also showed no difference in the capture capability of the 2 types of beads. However, in our investigation we observed PCR inhibition if MAP cells spiked in bovine feces were captured by Beads A. The 0.6% SDS shaking step of the National Veterinary Services Laboratory standardized protocol for MAP culture was incorporated into our study. This detergent may disrupt

the aggregation of MAP cells from the fecal material and help to free them for subsequent binding to the beads. The SDS treatment may have little or no effect on the release of intracellular MAP cells. Freezing and thawing of fecal samples will lyse macrophages and free the MAP cells for subsequent binding to the beads. Other detergents, such as Tween, have been used and most likely serve the same purpose (25).

The IgY used in this study to coat the immunocapture beads was a purified polyclonal antibody raised against a pool of 4 heat-inactivated MAP strains (20). Polyclonal antibodies are not as highly specific as monoclonal antibodies because they are directed to a number of bacterial surface antigens rather than a single surface antigen. Thus, the antibody may recognize other MAP strains that were not included in the original pool. However, cross-reactivity with *M. avium* has been observed and was eliminated after adsorption with *M. avium* cells (20). Grant et al (24) recommended a higher dilution of the IgG used to coat the beads to circumvent the problem of cross-reactivity with other mycobacterial strains.

Although a simple technique, IMS is labor-intensive, especially when a large number of specimens are to be processed in the absence of automation for the washing steps. Beads may be lost during the sequential washing steps when the supernatant is being aspirated. Another potential drawback is that nonspecific material can bind to the beads and also be attracted to the magnet. Consequently, it is difficult to wash the beads and remove all of the nonspecific material. We did not observe any sliding of the beads with aspiration of the supernatant, as observed by Grant et al (28). The washing steps were time-consuming and labor-intensive and required special attention and care to avoid aspirating the beads along with the supernatant.

This study demonstrated the use of IMS capture assay for detecting MAP in bovine feces. The IgY purified from chickens was used to coat the beads directly or indirectly via an antibody linker. For IMS plus conventional PCR with gel-based detection, the reporting time for 10 samples was less than 8 h. The detection level for cells in spiked bovine feces was 200 cells per PCR assay after capture with MagaCell-IgY beads. The inhibitory problem for the PCR was overcome, as illustrated by positive amplification of the internal control. However, future work is required to improve the sensitivity of the assay before implementation as a routine diagnostic tool for the detection of MAP in the diseased animals.

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Article

Field efficacy of an inactivated bivalent influenza vaccine in a multi-site swine production system during an outbreak of systemic porcine circovirus associated disease

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Abstract

Swine influenza (SI) is a disease of significance for the swine industry, and vaccination is often recommended as a way to reduce its impact on production. The efficacy of SI vaccines is well established under experimental conditions, but information about field efficacy is scarce. The objective of this study was to evaluate the efficacy of a commercial inactivated bivalent (H1N1/H3N2) vaccine under conditions of natural exposure to a field SI variant. To accomplish our goal we used a randomized, blinded, field trial in 2 cohorts of finisher pigs in a multi-site swine production system located in southern Ontario. During the trial, this herd experienced an outbreak of porcine circovirus associated disease (PCVAD). The efficacy of the SI vaccine was assessed through its effect on average daily weight gain, and serological responses to SI over time. The effect of vaccination on pig growth was different in the 2 cohorts. Weight gain was higher in vaccinated pigs than in control pigs in Cohort 1, but was numerically higher for control pigs than for vaccinated pigs in Cohort 2. Vaccination against swine influenza, in a herd experiencing an outbreak of PCVAD, was of questionable value.

Résumé

L'influenza porcin (SI) est une maladie importante pour l'industrie porcine, et la vaccination est souvent recommandée comme moyen pour réduire son impact sur la production. L'efficacité des vaccins contre SI est bien établie lors de conditions expérimentales, mais les informations quant à l'efficacité en condition de terrain sont rares. L'objectif de la présente étude était d'évaluer l'efficacité d'un vaccin bivalent inactivé commercial (H1N1/H3N2) dans des conditions naturelles d'exposition à un variant de champs de SI. Afin de réaliser notre objectif nous avons réalisé un essai clinique randomisé, à l'aveugle dans 2 cohortes de porcs en finition dans un système de production porcine en multi-sites dans le sud de l'Ontario. Durant l'essai, ce troupeau a vécu une épidémie de maladie associée au circovirus porcin (PCVAD). L'efficacité du vaccin contre SI a été évaluée par son effet sur le gain de poids quotidien moyen, et les réponses sérologiques envers SI dans le temps. L'effet de la vaccination sur la croissance des porcs était différent dans les 2 cohortes. Le gain de poids était plus élevé chez les porcs vaccinés comparativement aux porcs témoins dans la Cohorte 1, mais était numériquement plus élevé pour les porcs témoins comparativement aux porcs vaccinés dans la Cohorte 2. La vaccination contre SI, dans un troupeau au prise avec une épidémie de PCVAD, avait une valeur questionnable.

(Traduit par Docteur Serge Messier)

Introduction

Swine influenza virus (SIV) is a prevalent respiratory pathogen of swine with worldwide distribution. In addition to its significance as an important pathogen of swine, it is of concern for its potential human health risk (1). Infection with SIV in naïve swine herds usually manifests as an outbreak of febrile respiratory disease with high morbidity and low mortality. Loeffen et al (2) attributed 7 of 16 investigated outbreaks of respiratory disease in finisher pigs in The Netherlands solely to SIV infection. Circulation of H1N1 and H3N2 SIV among pigs is documented in herds experiencing atypical, mild, or no clinical signs (2–4). Additionally, SIV infection is a component of the porcine respiratory disease complex (PRDC) (5). Since the late 1990's, both antigenic drift and shift have been recorded in SIVs circulating in the US swine population, causing concern from a production perspective. Swine influenza vaccines appear effective in preventing clinical disease under experimental conditions (6,7) but their efficacy has been questioned in field reports (8). In addition, vaccine efficacy under conditions of natural exposure to SIV has been rarely evaluated.

The objective of the study was to evaluate the direct effect of a commercial inactivated bivalent (H1N1/H3N2) influenza vaccine on growth of finisher pigs. To accomplish this we used a randomized, blinded, field trial in a commercial multi-site swine production system in Ontario, in which the producer reported persistent coughing in successive finisher groups. Further objectives were to evaluate serological response, mortality, and development of clinical signs suggestive of SI over time, with the expectation of comparing these

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parameters between treatment groups to better understand within-herd SI dynamics. However, during the trial, the study herd had an outbreak of porcine circovirus type-2 associated disease (PCVAD), characterized by weight loss, increase in mortality, and respiratory distress of growing pigs.

Materials and methods

Farm and management description

The farm selected for this study was a multi-site swine production system consisting of 1 nursery barn and 3 finisher barns, each located at a different site. The nursery barn was divided into 2 major sections. The section used for the first stage nursery consisted of 5 rooms, each divided into 16 pens, housing approximately 35 pigs per pen. The section for the second stage nursery consisted of 4 rooms, each divided into 8 pens with slatted floors, housing approximately 70 pigs per pen. Nursery rooms were ventilated with a negative pressure system. The finisher barn used for this study was located approximately 2 km from the nursery barn, and consisted of 4 equally sized rooms, each with a capacity of 550 pigs. Doors between rooms were kept closed. Each room contained 5 pens on each side of a central alley. Rooms were individually ventilated with a combination of negative pressure (winter ventilation) and an electronically monitored natural ventilation system with curtains (summer ventilation). All pens in the nursery and finisher barns were washed with hot water and disinfected between groups. The nursery barn and the finishing barn selected for this study were intended to be managed as all-in/all-out (AIAO) by room facilities. Once weekly, the nursery barn received 500 to 600 pigs weaned at 17 to 19 days of age from 3 different supplier farms. Weaned pigs stayed in the first-phase nursery for 4 wk, and were then sorted by size and moved to the second-phase nursery for an additional 3 wk. The second-phase pen housing the smallest pigs was also used to house pigs that were treated during this phase. Once per week, pigs at the end of the nursery phase were shipped by truck to a finisher barn and redistributed into finisher pens by size. The finisher barn used in this study was populated with pigs that had come from 4 consecutive weekly cohorts of pigs. Once populated, pigs from the nursery barn would be shipped to other finisher sites. Frequently, 1 or 2 rooms in the finisher barn were filled with light-weight pigs from previous cohorts waiting to be shipped to market, and pigs entering the finisher barn from the nursery were moved through these rooms. Each finisher room contained 1 pen that housed light-weight pigs and pigs that required treatment, extra care, or both (room-hospital pen). An additional pen in the storage area of the barn was used for pigs that required extra heat, care, and feed (barn-hospital pen).

Pelleted feed was fed in the first-phase nursery and mash feed in the second phase. Nursery feed was medicated with penicillin (trade name not recorded) and 60 mg/kg of salinomycin (Posistac 6% Premix; Phibro Animal Health, Regina, Saskatchewan). However, in nursery-week 3 for Cohort 1, a new protocol was introduced so that feed contained 275 mg/kg of chlortetracycline hydrochloride (Chlor 100 Granular Premix; Bio Agri Mix LP, Mitchell, Ontario) and 31 mg/kg of tiamulin (Denagard Medicated Premix; Novartis Animal Health Canada, Mississauga, Ontario). Finisher pigs were fed liquid feed, pulse-medicated for 14 d at a time with chlortetracycline hydrochloride (Chlor 100; 550 mg/kg feed) as required in response to respiratory problems. Weaned pigs were vaccinated with modified-live PRRS vaccine (Ingelvac PRRS ATP; Boehringer Ingelheim Vetmedica, St. Joseph, Missouri, USA) at the time of entry into the nursery barn.

History of clinical problems prior to the study period

Finisher pigs in this herd experienced outbreaks of respiratory disease during several months prior to this study; the disease started at approximately 110 days of age (4 wk after entering the finisher barns). Clinical signs included a persistent barking cough and lack of response to treatment. Diagnosis of SI was confirmed by isolation of influenza A virus using Madin-Darby canine kidney cells, typed as H1N1, and the demonstration of almost 100% seropositivity to H1N1 SIV by enzyme-linked immunosorbent assay (ELISA) in finisher pigs prior to the study. At the same time, seropositivity to porcine reproductive and respiratory syndrome virus (PRRSV) in finisher pigs was also 100%, with sample-to-positive (SP) ratios ranging from 0.5 to 4 when tested by HerdChek PRRS ELISA, (IDEXX Laboratories, Westbrook, Maine, USA).

The SI status of the first-phase nursery pigs before study initiation was dependent on the source. While first-phase nursery pigs from the first source barn were 80% positive, the pigs coming from the second and the third source barn were 25% and 0% positive, respectively.

Study design, randomization and baseline measurements

Only one finisher barn was selected for this study in an attempt to minimize the potential effect of site-specific variables on the investigated associations and because of concerns regarding biosecurity. The study site was selected because it started receiving nursery pigs at the time the study was initiated. This field trial was designed to determine if there was a difference of at least 3.5 kg in the market live body weight between the influenza-vaccinated (vaccinated) and non-vaccinated (control) animals with 95% confidence and 80% power, and under the assumption that the standard deviation *(s)* in live body weight for this farm was assumed to be 12 kg and the mean final market body weight was 115 kg. The initial estimate of 185 animals per intervention group was increased to 224 animals per group to accommodate for possible losses; to equalize the number of pigs per pen due to stratification; and, to adjust for clustering of pigs within a finisher pen. For logistical reasons, and to minimize the potential effect of herd immunity, the required number of pigs was divided into 2 cohorts. Cohort 1 entered the trial January 20, 2004, and Cohort 2, 4 wk later. Each cohort included 112 vaccinated and 112 control pigs (Table I).

A sampling frame was constructed, whereby pigs were randomized into intervention groups, and a subset from each intervention group was selected to be blood sampled. All randomization procedures were performed in MINITAB 14 (Minitab, State College, Pennsylvania, USA). All pigs housed in each of the 7 pens of the second-stage nursery that met the inclusion criteria were marked with sequential numbers to facilitate random selection (target

Table I. Description of target and study population of pigs in a swine influenza virus vaccine trial followed in the nursery and finisher barn[a]

		Cohort 1		Cohort 2	
		Vaccine		Vaccine	
Week	Total population	469	Control	533	Control
0	Number of pigs included	112	112	112	112
	Number of pens[b]	7	7	7	7
	Number of pigs blood sampled[c]	31	34	33	33
	Mean body weight ± s (kg)	16.0 ± 2.4	16.0 ± 2.8	14.0 ± 1.9	13.9 ± 1.9
	Gilts (%)	20.5	23.2	16.2	19.6
	Mean SIV SP ratio	0.09	0.11	0.06	0.10
	SIV positive (%)	3.2	5.9	0.0	3.0
6	Number of pigs weighed[d]	108	110	106	108
	Number of pens[b]	9	9	10	10
	Number of pigs blood sampled	31	34	32	33
	Mean body weight ± s (kg)	42.6 ± 8.0	43.0 ± 7.9	45.7 ± 6.6	46.2 ± 6.4
	Mean SIV SP ratio	0.85	0.25	0.75	0.28
	SIV positive (%)	90.3	20.6	87.5	27.3
12	Number of pigs weighed[d]	100	109	100	104
	Number of pens	9	9	10	10
	Number of pigs blood sampled	31	34	30	31
	Mean body weight ± s (kg)	81.2 ± 11.9	79.6 ± 11.9	84.2 ± 11.8	86.2 ± 9.9
	Mean SIV SP ratio	0.58	0.55	0.56	0.41
	SIV positive (%)	64.5	58.8	56.7	35.5
15	Number of pigs weighed[d]	90	97	88	92
	Number of pens	9	9	10	10
	Number of pigs blood sampled	31	32	28	31
	Mean body weight ± s (kg)	98.9 ± 12.7	96.5 ± 13.4	99.4 ± 11.2	103.1 ± 8.4
	Mean SIV SP ratio	0.41	0.52	0.41	0.34
	SIV positive (%)	45.2	53.1	46.4	32.3
All	Number of pigs (%) diseased[e]	23 (10.3)	21 (9.4)	17 (7.6)	13 (5.8)
	Number died (%)	12 (5.4)	8 (3.6)	8 (3.6)	8 (3.6)
	Number necropsied[f] (trial)	8	3	3	2
	Number (%) died[g]	43 (9.2)		28 (5.2)	
	Number necropsied[f,g]	18		6	

s — standard deviation.

[a] Animals were followed from approximately 6.5 weeks of age (week 0) to approximately 21.5 weeks of age (week 15).

[b] Number of pens with pigs weighed for the trial. Pigs stayed 3 wk in the nursery and were then moved to the finisher barn. Integrity of pens was not maintained when pigs were moved from 7 nursery pens into 9 and 10 pens in the finisher barn.

[c] Only sera from pigs that were blood sampled > 1 time were submitted for ELISA testing. Consequently number of tested sera was < 34 in some treatment groups.

[d] Reduced number of pigs weighed because of mortality or moving to the barn-hospital pen, and pigs sent to market earlier at week 15.

[e] Pig was treated as diseased if it was treated with antimicrobials at least one time.

[f] Necropsies performed at the Animal Health Laboratory (University of Guelph).

[g] Figures apply to the overall population in the 2 cohorts.

population). Pigs that had been in the hospital pen, pigs with hernias or that appeared sick on the day of selection, and non-castrated male pigs were excluded from the study. Thirty-two pigs per pen for a total of 224 pigs per cohort (study population) were randomly selected from the target population using numbers from the sampling frame and the numbers recorded on pigs. These pigs were randomly allocated to the intervention group (vaccinated and control). In addition, 5 pigs per pen per intervention group were randomly selected for the sequential blood sampling for a total of 35 pigs per intervention group per cohort. This number was designed to detect the seroconversion of at least 10% in the control group with 95% confidence (assuming a perfect test and accounting for possible losses). Hence, pigs included for both weight and serology evaluation were selected by a stratified random sampling

based on pen strata. Animals were tagged with 4-colored, uniquely numbered ear tags whereby the colors represented the vaccination and blood sampling status of the pig.

Pigs in the control group, pigs not selected for the study, and pigs excluded from the study were vaccinated with *Mycoplasma hyopneumoniae* bacterin (RespiSure; Pfizer Animal Health, Exton, Pennsylvania, USA) according to the manufacturer's instructions. Pigs in the vaccinated group were vaccinated according to the manufacturer's instructions with FluSure/RespiSure RTU (Pfizer Animal Health), which is a combination of *Mycoplasma hyopneumoniae* bacterin and SI. The SI component is a bivalent (H1N1/H3N2) inactivated vaccine with an oil-in-water adjuvant containing antigens from strains claimed to match the ones currently circulating in North American swine. Booster doses were administered in the control and vaccinated groups 3 wk later, just before the pigs were moved to the finisher barn.

Only 2 observers were aware of the experimental designation of the pigs, and neither of them participated in the initial marking or weighing of pigs. Observers who weighed pigs and farm personnel were blinded throughout the study. Baseline measurements for each pig included body weight, gender, and for the pigs in the sequential blood sampling group, SIV serological status. This protocol was approved by the Animal Care Committee of the University of Guelph and was in accordance with guidelines for the care and use of experimental animals (9,10).

Measurements

Each pig ($n = 2 \times 224$) was weighed 4 times: baseline (6.5 wk of age) and 44, 88, and 108 d later, corresponding to approximately 0, 6, 12, and 15 study wk, respectively. Pigs were weighed on an electronic scale (Pelouze Model 4010; Pelstar LLC, Bridgeview, Illinois, USA) for the baseline weight and on a spring scale for all other weights. For the spring scale, frequent weighing of a constant weight was performed throughout the duration of the weighing, and observers recording weight were blinded with respect to vaccination status. A standardized approach was used to read the scale. Sixteen pigs in Cohort 1 (7.9%) and 22 pigs in Cohort 2 (10.9%) were shipped to market 3 to 9 d before the final weighing. These 38 pigs were weighed just before shipping, and they accounted for 9.2% and 6.7% of vaccinated and control pigs, respectively, in Cohort 1; and 11.1% and 10.7% of vaccinated and control pigs, respectively, in Cohort 2. Some of these pigs were faster growing pigs shipped earlier to avoid penalties; the other pigs in this group could be considered as a random sample since they were sold to a niche market.

Blood was collected from the orbital venous sinus after weight was recorded from pigs selected for blood sampling (70 per cohort). Sera were submitted to the Animal Health Laboratory (AHL; University of Guelph, Guelph, Ontario) for testing with a commercial SIV H1N1 ELISA assay (IDEXX Laboratories). A sample-to-positive ratio of ≥ 0.4 was considered positive. This assay was used because only H1N1 was detected by virus isolation prior to the study.

Herd managers in the nursery and finisher barns were provided with a log book to record information about mortality, medication, and moving of pigs into room-hospital pens. For any treated animal, the date of treatment, reason for treatment, dosage of drug used, and identification of the treated animal were recorded. Pigs that were moved into the barn-hospital pen were not weighed nor blood sampled, but treatments were recorded in the log book.

Table II. Random coefficient Poisson regression estimates of log incidence rate ratios for cough development at the pen level[a] in the finisher barn in 2 cohorts of pigs in a swine influenza vaccine trial

Parameter	Estimate[b]	$S_{\bar{x}}$	*P*
Fixed effects			
Intercept	−10.35	0.38	< 0.01
Week	0.19	0.06	< 0.01
Cohort 2	−0.55	0.28	0.06
Covariance parameters			
Intercept (pen)	1.31	0.66	0.02
Covariance	−0.20	0.11	0.06
Week (pen)	0.03	0.02	0.04
Residual	1.81	0.21	< 0.01

Akaike's information criterion = 690.2; $n = 179$

$S_{\bar{x}}$ — standard error.

[a] Two cohorts entered the finisher barn 4 weeks apart. Only measurements from week 7 of the trial (13.5 weeks of age) until week 15 of the trial (21.5 weeks of age) were included. The predictions from the same model were graphically interpreted in Figure 1.

[b] Estimates are on the natural log scale.

Investigators visited the farm each week to observe clinical signs, to score coughing, and to collect dead pigs if they were in suitable condition for postmortem examination. Mortalities were submitted to AHL for necropsy. Two observers recorded the number of pigs per pen and counted the number of coughs per pen per 5-min period after pigs were encouraged to move. One episode of coughing was counted as a single cough. Pigs showing clinical signs of a barking or paroxysmal cough were marked, and nasal swabs were taken. If no barking or paroxysmal coughing was observed, nasal swabs were collected from pigs that showed any kind of coughing or other respiratory distress, regardless of whether or not they were part of the trial. Swabs were transported in virus transport media (VTM; AHL, University of Guelph) at 4°C. At least 3 swabs per week were collected until SIV was detected. Swabs were frozen at −80°C and submitted the following week to AHL for SIV isolation in Madin-Darby canine kidney cells and embryonated eggs. Isolated SIVs were typed by reverse-transcriptase polymerase chain reaction.

Data processing and statistical analyses

Data were entered into a database (Access; Microsoft Corporation, Redmond, Washington, USA) and imported into SAS version 9.1 (SAS Institute, Cary, North Carolina, USA) for further manipulation and analysis. The effect of influenza vaccine on average daily gain (ADG) was analyzed using a linear mixed effect model (Proc Mixed; 11), with randomly varying intercepts and slopes among individual pigs nested within the finisher pens. The model assumptions for the "jth" pig nested within the "ith" finisher pen, at the "kth" measurement occasion are listed in Equation 1. The outcome consisted of 3 sequential weight measurements in grams, and the effect of covariates of interest on weight gain over time was evaluated using their interaction with the time variable measured as days since the initial

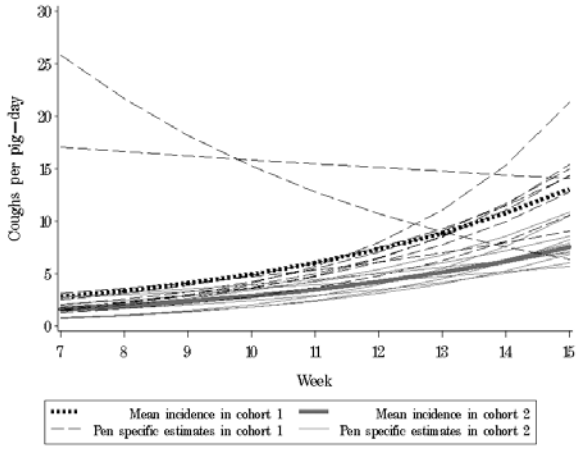

Figure 1. Conditional (pen-specific) estimates of development of incidence of cough at the pen level in 2 cohorts (Cohorts 1 and 2) of pigs used in a swine influenza field trial from week 7 of the trial (13.5 weeks of age) until week 15 of the trial (21.5 weeks of age). Estimates are obtained after anti-log transforming the expectations obtained from the random coefficient Poisson regression model and calculating the expected number of coughs per pig-day. Each line represents the development of incidence of cough for each pen. Mean incidence in both cohorts is the expected incidence obtained from the fixed part of the model and represents the expected number of coughs per pig day in an "average" pen of the cohort.

weight measurement (11). Hence, the coefficients containing the interaction with time was interpreted as average daily gain in grams.

$$y_{ijk} = \beta_0 + \beta_1 x_{1ij} + \beta_2 x_{2ijk} + (\beta_1 x_{1ij} * \beta_2 x_{2ijk}) + b_{0i} + b_{0ij} + b_{2ijk} x_{2ijk} + \varepsilon_{ijk}$$

Linear mixed effect model used to assess the pig's weight over time

where:

- y_{ijk} = the weight of the jth pig in the ith pen at the kth measurement.
- x_{1ij} = time-invariant covariates (vaccine status, gender, cohort, weight at day 0, interactions)
- x_{2ijk} = time-variant covariates (time in days since vaccination)
- beta = coefficients associated with fixed effects (vaccine, gender, cohort, initial weight, interaction terms)
- b = random effects (intercept and random coefficient of days since the first vaccination)
- i = pen id
- j = pig id
- k = repeated measurement (days: 44, 88, 108)

Outcome is measured as weight in grams. Slope of interaction terms between the time variables measured in days and other fixed effects is interpreted as average daily gain in grams.

The final fixed effect model was fitted using the maximum likelihood method (ML). Contrasts were performed to test for equality of slopes (ADG). Outliers and influential observations were assessed for fixed and random effects at the pig and pen level (12). After identifying observations with undue influence, models were reanalyzed without them.

Changes in the SP ratio over time were modeled using a linear mixed effect model, with unstructured covariance to account for repeated measurements within a pig (SP ratio model). Least square means of SP ratios were evaluated at each time point that sera were taken. Logistic regression, with pen as a random effect using penalized quasilikelihood (Glimmix macro) was used to assess the mean probability of seroconversion to SIV between weeks 0 and 6, and between weeks 6 and 12, for each cohort (Empty model). The intra-cluster correlation coefficient was calculated using a latent class model approach (13) and interpreted as the proportion of variation in seroconversion that resided at the pen level. Seroconversion was defined as a change from negative status ($SP < 0.4$) to positive status ($SP \geq 0.4$).

Effect of vaccine and cohort on mortality was evaluated using a Cox's proportional hazard model. In addition, effect of cohort on mortality in the target population was evaluated by logistic regression in a model that included all pigs (Table I).

The incidence rate of coughing in the finisher barn was evaluated in a Poisson regression, using penalized quasilikelihood approach (Glimmix macro), and with the random intercept and time at the pen level.

Results

Observers noted a paroxysmal, barking cough in pigs even when they were resting. The mean incidence rate of coughing in finisher pens tended to be higher [incidence rate ratio, 1.73; 95% confidence interval (CI), 1.0–3.0] in Cohort 1 than in Cohort 2 throughout the study (Table II). In addition, the incidence rate of coughing increased consistently by 21% (95% CI, 7.5%–36%) on a weekly basis. However, there was significant random variation both in the initial rate of coughing and in its development over time (Table II, Figure 1). The pattern of coughing in 2 pens of Cohort 1 differed both from the pattern in most of the other pens in either cohort, and from the pattern in the average pen in each cohort predicted by the model (Figure 1). The pigs in one of these pens were 1 wk younger than the others in that room. Nasal swabs collected in week 9 from 2 coughing pigs in this pen were positive for type A SIV by virus isolation, typed as H1N1.

Other pathogens identified during the course of the trial included porcine parvovirus (PPV; virus isolation in cell culture) from pigs exhibiting severe dyspnea; PRRSV (RT-PCR); and porcine circovirus type-2 (PCV-2; immunohistochemistry) in lungs, spleen, liver, and kidney of vaccinated and control pigs. *Salmonella enterica*, *Actinobacillus suis*, *Streptococcus suis*, *Haemophillus parasuis*, and *Pasteurella multocida* were cultured in a subset of pigs submitted for complete necropsy. In pigs submitted for gross examination, the 4 most common tentative diagnoses were pneumonia, serositis, wasting, and lymphadenopathy. Room-hospital pens were filled with pigs with severe (expiratory) dyspnea and tachypnea, which was especially noticeable after nursery pigs were moved to the finisher barn. Enlarged inguinal lymph nodes were observed in almost all pigs examined in the room-hospital pen. Severe wasting was observed in pigs with and without respiratory signs. Clinical signs and lesions discovered on gross and histological examination and immunohistochemistry were consistent with PCVAD (14). The start of the PCVAD outbreak in the study finisher barn apparently coincided with the start of the trial, although clinical signs consistent with the systemic PCVAD were observed in nursery pigs prior to randomization.

Table III. The association between swine influenza vaccine and pig weight (g) between 6.5 and 21.5 weeks of age in 2 cohorts (Cohorts 1 and 2) that entered the nursery 4 weeks apart, adjusted for initial weight and gender[a]

Parameter	Estimate[b]	$S_{\bar{x}}$	*P*
Intercept	3619.2	856.2	0.00
Cohort 2	3868.6	1213.3	0.00
Vaccine	−2161.2	991.0	0.03
Cohort 2 × Vaccine	2317.8	1421.2	0.10
Day	856.4	14.1	< .0001
Vaccine × Day	44.0	20.5	0.03
Cohort 2 × Day	60.5	20.2	0.00
Cohort 2 × Vaccine × Day	−68.6	29.2	0.02
Initial weight (centered at 15 kg)	1945.0	115.1	< .0001
Gilts	−85.2	611.5	0.89
Intercept[c] (Finisher pen)	183 514 7	1067149	0.04
Intercept[c] (Pig)	240 158 24	421 983 4	< .0001
Day[c] (Pig)	17 564	1567.23	< .0001
Covariance (Intercept and day)	−463 544	70 386	< .0001
Residual	765 692 2	552 750	< .0001
Model fit	AIC = 24 294.5; *n* = 1212		
Difference in slopes in Cohort[d] 1	44.0	20.5	0.03
Difference in slopes in Cohort[d] 2	−24.6	20.8	0.24
Equation[e] for vaccinated pigs (Cohort 1)	1458.0 + 900.4 × day = g/d		
Equation[e] for control pigs (Cohort 1)	3619.2 + 856.4 × day = g/d		
Equation[e] for vaccinated pigs (Cohort 2)	7644.4 + 892.3 × day = g/d		
Equation[e] for control pigs (Cohort 2)	7487.7 + 916.9 × day = g/d		

$S_{\bar{x}}$ — standard error.

[a] Initial weight and gender were forced in the model. Interaction with time was not considered.

[b] Model is based on observations of pigs not placed in the room-hospital pen when they entered the finisher barn. Weight is recorded in grams so that slopes could be interpreted as average daily gain in grams.

[c] Intercept was random at the pig and the finisher-pen level. Week was random at the pig-level.

[d] Difference between expected slopes in vaccinated and control pigs tested by contrast.

[e] Equations are based on fixed effects in the model.

AIC = Akaike's information criterion.

Descriptive statistical results are presented in Table I. Coefficients of variation (CV) of initial body weight at room level were 16.2 in Cohort 1 and 13.6 in Cohort 2. At the pen level, body weight CV ranged from 12 to 15.3 in Cohort 1 and from 9.3 and 13.1 in Cohort 2.

Rate of growth of vaccinated and control pigs differed between Cohorts 1 and 2, as suggested by the significant three-way interaction between vaccine, cohort, and time variable (day). Evaluation of residuals and influential statistics at the pig- and pen-levels revealed that pigs coming from the room-hospital pens had an undue impact on the model estimates. The final model, therefore, was fitted without animals that originally occupied room-hospital pens at Week 6 of the trial (7 pigs), but it still indicated a different vaccine effect in the 2 cohorts (Table III; $P = 0.02$). Least squares differences in mean average daily gain of vaccinated and control pigs are presented in Table III. Briefly, the model suggested that vaccinated pigs in Cohort 1 grew 44.0 g/day faster than control pigs ($P = 0.03$). In contrast, Cohort 2 control pigs grew at 24.6 g/day faster rate than the vaccinated pigs, but this difference was not statistically significant ($P = 0.24$).

The final model for the development of a serological response contained the linear and quadratic effect of time represented by week ($P < 0.01$), interaction of vaccination with the linear and quadratic effect of week ($P < 0.01$), and three-way interaction of cohort, vaccination, and linear effect of week ($P = 0.01$). The expected SP ratios in different groups predicted by this model are shown in Figure 2. The least squares means of SP ratios of vaccinated pigs did not differ ($P > 0.5$) at weeks 6, 12, and 15 of the trial, although at week 0, vaccinated pigs in Cohort 1 tended to have higher SP ratios ($P = 0.06$) than vaccinated pigs in Cohort 2. However, the least squares means of SP ratios of control pigs tended to be higher in Cohort 1 than in Cohort 2 at Week 6 ($P = 0.09$), and were higher at weeks 12 ($P = 0.03$) and 15 ($P = 0.02$).

The proportions of vaccinated and control pigs that were positive for SI at each measurement are presented in Table I. For each cohort,

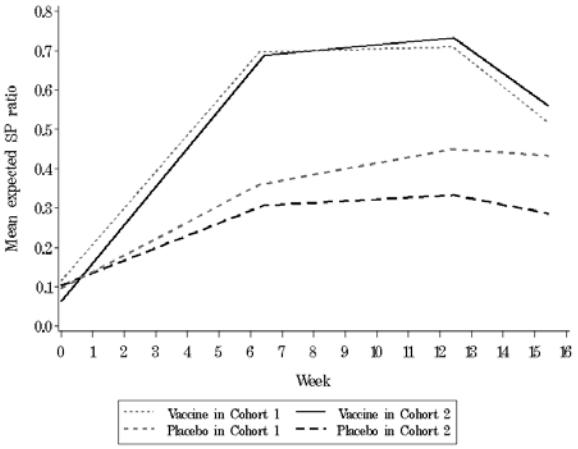

Figure 2. Expected SIV SP ratio in vaccinated and control pigs used in a swine influenza vaccine trial between week 0 and 15 of the trial (6.5 and 21.5 weeks of age) in 2 cohorts (Cohorts 1 and 2) that entered the nursery 4 weeks apart. Expectations are obtained from the linear mixed effect model, with unstructured covariance to account for repeated measurements within pigs.

the mean probability of seroconverting to SI between weeks 0 and 6 and between weeks 6 and 12, and the approximate proportion of variation at the finisher-pen level, are listed in Table IV. The highest mean probability of seroconversion occurred in Cohort 1 in the period between weeks 6 and 12.

In the study population, the hazard of dying did not differ between vaccinated and control pigs ($P > 0.05$). In the overall cohorts, the total mortality was higher in Cohort 1 than in Cohort 2 (OR = 1.81; 95% CI, 1.12–2.98). Development of clinical signs indicative of PCVAD over time was reported elsewhere (15).

Discussion

The major clinical problem reported by the producer that motivated this study was persistent coughing in successive finisher groups; however, during the study period, the most important disease concern was an outbreak of systemic PCVAD. In addition, overall disease dynamics in the 2 cohorts were different, as indicated by a higher mortality risk in Cohort 1 pigs.

Under experimental conditions, the efficacy of influenza vaccine is usually evaluated by measurements related to the expression of clinical signs and to viral replication (6,7). Both types of measurements provide information about varying degrees of vaccine protection against clinical disease and SIV infection (6,7). Under field conditions, however, these measurements are impractical. Hence, we decided to evaluate vaccine efficacy by measuring growth, which is, from the producers' perspective, the most practical parameter.

Efficacy of vaccination in 2 cohorts was different, as indicated by significant interaction between vaccine, cohort, and time in a model for ADG. In Cohort 1 vaccinated pigs had higher ADG compared with control pigs, whereas in Cohort 2 vaccinated pigs had numerically lower ADG than control pigs. The following factors may have contributed to the observed measures of vaccination efficacy in 2 cohorts: 1) spread of influenza virus in the study populations and consequent level of exposure, 2) initial level of maternally derived antibodies against influenza virus, 3) similarity between the vaccine and the field strain, 4) potential detrimental effect of vaccination, and 5) effect of other co-infections on vaccine efficacy.

The spread of influenza virus was assessed using serological response and further supported by evaluating counts of coughs over time. The SP ratios of vaccinated pigs developed similarly in both cohorts. This was expected, as vaccination commonly induces high HI titers which are protective (6,7,16). However, the SP ratios of the control pigs developed differently in the 2 cohorts, suggesting that Cohort 1 had a greater exposure to SIV. Our estimates of seroconversion risks for both six-week periods in Cohort 2 (0 to 6 wk and 6 to 12 wk) and for the initial six-week-period in Cohort 1 were in agreement with the results of the study by Loeffen et al (17), who estimated an incidence risk of 16% to 17% for a four-week period in nursery pigs. Seroconversion risk in the second six-week period of Cohort 1 was higher, suggesting more rapid transmission. In this cohort, a tendency for higher ADG and weight of vaccinated pigs began at week 12, coinciding with this period of greater SIV challenge.

Results of both the SP-ratio and empty models suggested slower SIV transmission than had been previously recorded in the herd. Such atypical SIV transmission has been previously reported (3), characterized by either partial seroconversion or seroconversion without clinical signs. During the first 6 wk of the study period, more variation in the probability of seroconversion at the pen-level was observed than during the second 6 wk in both cohorts. This might be explained by clustering of SIV transmission by pen in the early phase of the epidemic, followed by more uniform transmission among pens in the later phase. Different microclimates associated with weather conditions and air-exchange might have existed in this barn during the study, with a subsequent impact on SIV transmission. Slow SIV transmission might also be associated with a larger number of resistant individuals in the population (herd immunity). We do not believe, however, that this is likely to have played a role at the room level in this study, as $< 25\%$ of the population was vaccinated. It is, however, intriguing to consider whether herd immunity might have played a role at the pen level. Although we initially blocked by pen, the population was redistributed into new pens once they entered the finisher barn, which might have resulted in an unequal ratio of vaccinated to non-vaccinated individuals by pen.

In addition to serological responses, development of coughing over time was used to assess respiratory disease dynamics in the barn. The clinical expression of coughing in this herd, at least in the second part of the finisher period, suggested involvement of SIV, and SIV was isolated from pigs that coughed. Because of presumed low specificity of cough for influenza classification we consider development of cough only as a supporting evidence for influenza spread in a barn. Fixed parameters in a model for coughing suggested a higher incidence in Cohort 1 pigs, and an increase in incidence with time. In addition, both covariance parameters and pen-specific estimates of intercept and slope suggested considerable variation in the initial pen incidence of coughing and its development over time. This variation might have been influenced by 2 pens in Cohort 1 that had a high initial incidence of coughing that decreased with time, a pattern opposite to that in all other pens. Swine influenza virus was first isolated from 2 coughing pigs in one of these 2 pens, suggesting that

Table IV. Risk of seroconversion[a] to swine influenza in 2 six-week intervals during a field trial of a swine influenza vaccine in two cohorts of pigs

		Week 0 to 6			Week 6 to 12		
Cohort	Parameter	Estimate	($S_{\bar{x}}$)	*P*	Estimate	($S_{\bar{x}}$)	*P*
1	Intercept	−1.39	0.52	0.03	−0.36	0.35	0.34
	Pen[b] level variance	0.66	—	—	0.00	—	—
	Probability[c] (%) (95% CI)	20.00	(7.0–45.3)		41.20	(23.9–61.0)	
	% at the pen level[c]	17.00			0.00		
2	Intercept	−1.01	0.45	0.05	−1.15	0.43	0.04
	Pen[b] level variance	0.43	—	—	0.14	—	—
	Probability[c] (%) (95% CI)	26.80	(11.6–50.4)		25.70	(11.3–48.3)	
	% at the pen level[c]	11.60			4.10		

$S_{\bar{x}}$ — standard error.

[a] Seroconversion was defined as a change from the serologically negative to serologically positive between 2 sequential testing in control treated pigs of 2 cohorts (0 to 6, and 6 to 12 weeks).

[b] Mean estimate of variance at the pen level. Residual level variance was held constant at 1. Standard errors and *P*-value for covariance estimates were not reported.

[c] Calculated from the model estimates.

it was the index pen for SIV infection. Although the cough model suggested an increase in the mean incidence of coughing over time, no pigs seroconverted between weeks 12 and 15. If the increase in coughing indeed indicated an increasing rate of SIV infection, this would probably not be evident on ELISA SP ratios because of the lag time between infection and seroconversion (18).

Atypical transmission of SIV may also be associated with maternally derived antibody (MDA) in pigs. An absence of typical clinical signs of SI, prolonged shedding time, and a tendency for a slower growth rate after exposure to SIV were identified in pigs with MDA, in a study by Loeffen et al (19). In addition, H1N1 vaccine efficacy was reduced after pigs were vaccinated in the presence of MDA and challenged with a heterologous H1N1 strain (20). Hence, the impact of MDA on clinical signs, transmission pattern, and on reduced vaccine efficacy in our study cannot be completely disregarded, but was not likely, since a maximum of 6% of pigs in a group were seropositive at the beginning of the study, and the mean SP ratio in each intervention group was below the cut-off recommended for vaccination (21). Moreover, the higher proportion of SIV-positive pigs was detected in Cohort 1, in which vaccine efficacy was higher than in Cohort 2.

Similarity between a vaccine strain and a field strain could also impact vaccine efficacy. Vaccine was matched at the subtype level as field isolate was classified as H1N1. However, previous research indicates that vaccine efficacy could be reduced after challenge with a virus with the identical HA, but low antigenic cross-reactivity to the vaccine strain. For example, an inactivated vaccine based on a H1N1 strain was not clinically efficacious after challenge with a heterologus H1N2 strain (22); and 3 different commercial vaccines containing cluster 1 H3N2 strains reduced clinical signs but not SIV shedding after challenge with a cluster 3 H3N2 strain (23). Assessment of genetic or antigenic similarity between vaccine and field strains in this study was not done, precluding us from further investigating this question. Given the epidemiological situation at the time study was done, involvement of other undetected subtypes was possible, but likely minimal because triple-reassortant H3N2 strain, that could contribute to further reassortments, emerged in Ontario approximately 1 y (in 2005) after this study ended (24,25), and pandemic H1N1 emerged in human population in 2009.

Detrimental effect of vaccination in the absence of sufficient challenge cannot be disregarded. This could occur through at least 2 different mechanisms, apart from unmatched vaccine and field strain. First, in an effort to match vaccine response to expected challenge the booster vaccination was scheduled before transport to the finisher barn. Such practice alone might have had detrimental effect on weight gain of animals. This stresses the importance of careful vaccination protocol design at times where pigs are exposed to other stressful events. Second, in other studies immune stimulation induced by vaccines, adjuvants, timing of vaccination, or age of animals at vaccination were suggested as factors that may contribute to expression of PCVAD (26–29), although this has not been consistently demonstrated under experimental conditions (30,31). In this study, there was no detectable difference in mortality or probability of treatment over time [data not shown; (15)] between groups vaccinated with different vaccines. This question could not be fully investigated due to the nature of experimental groups.

Of other infections that could influence vaccine efficacy, PRRS was likely the most important. In a recent study, Kitikoon et al (8) suggested that SI vaccination protected almost completely against SIV challenge in the absence of PRRSV infection, but only partially when pigs were infected with PRRSV, even in pigs with high SIV HI titers. The study herd was PRRSV-positive and nursery pigs were vaccinated with attenuated live PRRSV vaccine in the nursery. Thus, infection with PRRSV might have also lowered vaccine efficacy. Additionally, a recent study showed that PCV-2 infection resulted in higher severity of macroscopic lung lesions in pigs that were vaccinated with modified live PRRSv vaccine and subsequently challenged with PRRSv (32). Strict adherence to inclusion criteria,

blocking, and randomization likely helped to equalize these factors between the treatment groups, but could not eliminate overall detrimental effect of these factors on the performance of entire population as well as on vaccine efficacy.

In conclusion, the results of this study suggest that vaccinating for SIV is of questionable value in a herd experiencing relatively low exposure to H1N1 influenza and a PCVAD outbreak. Influenza vaccination protocols should be considered carefully, not only to target the decline in maternal immunity, but also to avoid coinciding with other stressful events or concurrent disease outbreaks. In this study, vaccination induced a high serological response. Transmission of SIV was relatively slow and clinical signs were subtle and easy to miss, in agreement with observations of other authors (2,4). It is possible that this was the typical spread of H1N1 SIV in Ontario pig herds at the time study was done, in contrast to the outbreak form with dramatic coughing and inapettence.

Acknowledgments

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Article

Distribution of genotypes of porcine reproductive and respiratory syndrome virus in Ontario during 2004–2007 and the association between genotype and clinical signs of disease

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Abstract

Restriction fragment length polymorphism (RFLP) was first proposed to classify porcine reproductive and respiratory syndrome virus (PRRSV) in 1998. The primary objective of this study was to identify associations between different PRRSV RFLP types in swine herds in southern Ontario and clinical signs of disease in those herds. Herds included in the study submitted samples to the Animal Health Laboratory at the University of Guelph between September 2004 and August 2007. Each farm owner was surveyed to describe the clinical disease in the herd and the RFLP pattern of an isolate of PRRSV was obtained from a diagnostic sample. The most frequent isolates were RFLP types 1_4 (25.1%), 252 (14.7%), 134 (12%), and 1_2 (7.7%). The distribution of RFLP types in this study was found to be different from a previous investigation in Ontario. Those RFLP types most associated with clinical disease in the farrowing phase of production were 1_4, 1_2, and 134. The only virus type to be significantly associated with disease in the finisher phase was RFLP type 262. During the study period RFLP type 184 emerged in the population in November 2005.

Résumé

En 1998 le polymorphisme de longueur des fragments de restriction (RFLP) a été proposé pour la première fois afin de classifier le virus du syndrome reproducteur et respiratoire porcin (PRRSV). L'objectif premier de la présente étude était d'identifier les associations entre les différents types de RFLP de PRRSV dans les troupeaux porcins dans le sud de l'Ontario et les signes cliniques de maladie dans ces troupeaux. Les troupeaux inclus dans l'étude ont soumis des échantillons au Animal Health Laboratory à l'University of Guelph durant la période de septembre 2004 à août 2007. Chaque propriétaire de ferme était questionné afin qu'il décrive les signes cliniques dans le troupeau et le profil RFLP d'un isolat de PRRSV était obtenu d'un échantillon diagnostique. Les isolats les plus fréquents avaient les types de RFLP 1_4 (25,1 %), 252 (14,7 %), 134 (12 %) et 1_2 (7,7 %). La distribution des types de RFLP dans cette étude était différente d'une enquête antérieure en Ontario. Dans cette dernière, les types de RFLP les plus fréquemment associés avec des signes cliniques dans la phase naisseur de cette production étaient 1_4, 1_2 et 134. Le seul type viral à être associé de manière significative avec la maladie dans la phase de finition était le type RFLP 262. Durant la période d'étude, le type RFLP 184 a émergé dans la population en novembre 2005.

(Traduit par Docteur Serge Messier)

Introduction

Porcine reproductive and respiratory syndrome virus (PRRSV) is responsible for clinical disease throughout every stage of production in a swine farm. The disease is primarily characterized by abortions in sows of more than 100 d gestation, respiratory disease and mortality in nursing and weaned pigs, as well as respiratory disease in older pigs. Porcine reproductive and respiratory syndrome (PRRS) can result in large production losses during a herd outbreak, and without intervention, may result in endemic disease (1). Clinical presentation of PRRS in the individual animal is known to be strain dependent when different viruses are administered to pigs in a controlled environment (2–5). Viruses that are reported to be associated with different clinical disease have also been shown to be genetically different (6,7). However, specific genetic differences in PRRSV associated with pathogenicity are not entirely clear and have only recently been investigated (5,7–10). A previous observational study investigated the association between PRRSV genotype of isolates from an Illinois diagnostic laboratory and clinical disease observed in the herd (11). The authors determined that herds with more similar PRRSV isolates had similar sow mortality, but they did not find associations with other clinical signs of PRRS (11).

Restriction fragment length polymorphism (RFLP) was first proposed to classify PRRSV in 1998 (12). This method employs 3 restriction enzymes, *Mlu*I, *Hinc*II, and *Sac*II, to cut open reading frame 5 (ORF5) of the PRRSV genome, and viruses are classified on the basis of the resulting cut pattern (12). The classification method proposed by Wesley et al (12) in 1998 was developed using a group of North American PRRSV isolates and is not optimized to differentiate viruses of European or Asian origin (12). Restriction fragment

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Table I. Association between clinical signs and wild-type PRRSV compared to vaccine-like PRRSV as measured by odds ratios (OR)

Clinical signs	Proportion of herds reporting clinical signs[a] (%)	Wild-type PRRSV RFLP patterns									
		114	124	132	134	144	182	184	1_2	1_4	262
Abortion	139/325 (42.8)	—	—	14.0[b]	8.6[b]	—	—	11.2[b]	10.2[b]	18.2[b]	—
Sows off-feed	153/329 (46.5)	—	—	13.0[b]	6.3[b]	—	—	9.3[c]	8.1[b]	7.4[b]	—
Stillborn pigs	131/315 (41.6)	—	—	—	—	—	—	—	—	3.3[b]	—
Weak-born pigs	145/319 (45.5)	—	—	5.2[c]	10.9[b]	6.4[c]	—	23.4[c]	8.3[b]	8.7[b]	—
Sow/boar mortality	83/322 (25.8)	—	—	6.1[c]	—	8.0[c]	—	—	—	11.2[b]	—
Pre-weaning mortality	148/319 (46.4)	—	—	—	6.4[b]	—	—	—	6.1[b]	4.4[b]	—
Nursery respiratory	187/309 (60.5)	—	—	—	—	—	—	—	—	—	—
Nursery mortality	202/318 (63.5)	—	—	—	—	—	—	—	—	—	—
Finisher respiratory	118/273 (43.2)	—	—	0.1[c]	—	—	—	—	—	—	—
Finisher mortality	123/274 (44.9)	—	—	0.1[c]	—	—	—	—	—	—	19.5[b]

[a] The number of herds in the denominator changes for each clinical sign depending on how many herds had the applicable stage of production.
[b] Clinical sign associated with wild-type virus ($P \leq 0.05$).
[c] Clinical sign tends to be associated with a wild-type virus ($0.05 < P \leq 0.1$).

length polymorphism analysis is currently the only standardized method for classifying PRRS viruses. It allows for comparison with previously published findings without access to a library of viruses or sequencing results. Cai et al (13) described the frequency of RFLP types of PRRSV in Ontario from 1998 to 2000. The objectives of the present study were to identify associations between different PRRSV RFLP types in swine herds in southern Ontario and clinical signs of disease in those herds; to describe the distribution of RFLP types in Ontario during the study period; and to determine if the distribution of RFLP types changed over the time of the study period.

Materials and methods

Diagnostic submissions from Ontario swine herds that tested positive for PRRSV by reverse transcription polymerase chain reaction (RT-PCR) at the Animal Health Laboratory (AHL) University of Guelph (Guelph, Ontario) during the period September 1, 2004 to August 31, 2007 were eligible for inclusion in this study. The RT-PCR was performed as described (13) to amplify 433 base pairs of open reading frame 7 (ORF 7) of the PRRSV for cases before December 4, 2006. Samples from cases submitted to the AHL after December 4, 2006 were tested by the Tetracore PRRSV multiplex real time RT-PCR (Tetracore, Rockville, Maryland, USA). The AHL database was searched on one occasion for all PRRSV-positive submissions, referred to as "cases" by AHL, from September 1, 2004 to January 14, 2006. These retrospective cases were eligible only if samples from the original case had been stored by the AHL. The AHL database was searched for PRRSV-positive prospective cases twice a week starting January 15, 2006. For the purposes of this study, the following definitions are applied to be in agreement with previous literature (14,15). The term ownership refers to any premises housing pigs under a single corporate or private ownership. The term premise refers to a single contiguous land parcel with 1 or multiple buildings housing pigs; an ownership may have 1 or multiple premises. The term herd refers to a group of pigs housed at the same premise at the same point in time. The unit of interest for the study was the herd. Herds were eligible for inclusion if the premise had not been included in the study during the previous 30 d. The veterinarian listed on the case report was contacted to determine which premise had been sampled and to obtain contact information for the owner. The owners were contacted and asked if they would like to participate in the study. Permission was obtained for sequencing PRRSV-positive samples in the AHL database. Participating owners or managers were surveyed by telephone. The case and premise information was then entered into a study database in Microsoft Access (Microsoft Corporation, Redmond, Washington, USA).

Table II. The distribution of PRRSV with specific restriction fragment length polymorphism (RFLP) patterns in Ontario 2004–2007

RFLP	Number of sequences identified	Proportion of all sequences (%)
1_4	111	25.12
252[a]	65	14.71
134	53	11.99
1_2	34	7.69
132	24	5.43
142[b]	22	4.98
182	21	4.75
184	16	3.62
144	15	3.39
262	12	2.71
124	11	2.49
114	10	2.26
Others	48	10.86
Total	442	100

[a] Boehringer-Ingelheim Ingelvac® PRRS MLV vaccine strain.
[b] Boehringer-Ingelheim Ingelvac® PRRS ATP vaccine strain.

The telephone survey was completed in approximately 30 minutes. Survey questions included herd demographics and perceptions of clinical signs of PRRS in the herd. The question regarding perceptions of clinical signs of disease was phrased as follows: "At the time

Table III. The percentage of herds reporting each clinical sign for each RFLP pattern and the rank of the RFLP pattern for frequency of the clinical sign is presented in brackets

Clinical signs	Vaccine types	114	124	132	134	144	182	184	1_2	1_4	262
Abortion	18 (9)	33 (8)	17 (10)	67 (3)	61 (4)	36 (6)	36 (6)	75 (1)	58 (5)	71 (2)	11 (11)
Sows off-feed	25 (11)	56 (6)	33 (9)	67 (2)	58 (4)	45 (7)	36 (8)	75 (1)	58 (4)	63 (3)	33 (9)
Stillborn pigs	40 (7)	44 (5)	17 (11)	61 (1)	58 (3)	36 (8)	27 (10)	50 (4)	42 (6)	61 (1)	33 (9)
Weak-born pigs	30 (11)	44 (8)	33 (9)	61 (4)	67 (2)	55 (6)	45 (7)	75 (1)	56 (5)	65 (3)	33 (9)
Sow/boar mortality	14 (10)	22 (8)	33 (5)	44 (3)	31 (6)	36 (4)	22 (8)	67 (1)	23 (7)	47 (2)	13 (11)
Pre-weaning mortality	40 (7)	33 (8)	33 (8)	56 (4)	67 (1)	45 (6)	55 (5)	25 (11)	58 (3)	60 (2)	33 (8)
Nursery respiratory	67 (6)	67 (6)	70 (5)	64 (9)	72 (4)	67 (6)	89 (2)	100 (1)	57 (11)	63 (10)	75 (3)
Nursery mortality	66 (9)	78 (5)	70 (8)	55 (11)	79 (4)	80 (3)	89 (2)	100 (1)	71 (7)	73 (6)	63 (10)
Finisher respiratory	59 (5)	33 (10)	50 (6)	33 (10)	37 (9)	75 (2)	60 (4)	75 (2)	42 (8)	44 (7)	78 (1)
Finisher mortality	51 (5)	17 (11)	50 (6)	27 (10)	47 (8)	63 (3)	58 (4)	75 (2)	50 (6)	45 (9)	89 (1)

of the sample submission to AHL, what clinical problems did you have in the barn? Please indicate with 'Yes,' 'No,' 'Don't know,' or 'N/A' for each clinical problem. If you do not know whether a clinical problem occurred, please respond 'Don't know.' If the problem is not applicable to the barn (for example, if you only have nursery pigs, then the clinical problem 'abortion' does not apply), please respond 'N/A.'" The interviewer then listed each of the clinical signs presented in Table I, and allowed the interviewee to respond to each one. The survey responses were recorded on paper and entered into the study database.

Reverse transcription polymerase chain reaction was used to amplify ORF5 of PRRSV as previously described (12). The product was sequenced at the Guelph Molecular Supercentre, University of Guelph. From the sequencing results, computer-predicted RFLP typing was performed using the *Mlu*I, *Hinc*II, and *Sac*II enzymes and Invitrogen Vector NTI Advance 10 software. The RFLP cut patterns were categorized using previously described nomenclature (12), and the information was added to the study database. The RFLP cut patterns are presented as 3 digits representing the results of the 3 enzyme digestions. An underscore (_) is used to represent a cut pattern for the given enzyme that is not consistent with the nomenclature presented by Wesley et al (12).

Sequences were classified on the basis of their RFLP pattern as either wild-type virus or, for those with RFLP patterns 252 and 142, vaccine-like virus (16). Clinical signs of disease in herds where wild-type PRRSV was isolated were compared to clinical signs in herds where vaccine-like virus was isolated. The RFLP patterns shared by < 10 isolates in the dataset were not included in these analyses. Data were analyzed using univariable logistic regression controlling for repeated measures at the premise level by including a random intercept variable at that level. Logistic regression was completed using maximum likelihood based on Gauss Hermite quadrature. A separate model was completed for each clinical sign listed in Table I. In each model, the clinical sign was the binary outcome. Herds where the interviewee responded "Don't know" or "N/A" for a clinical sign were not included in the model for that clinical sign. The RFLP pattern was tested as a categorical independent variable, with the vaccine-type viruses as the referent group. Pearson residuals and deviance residuals were calculated for each model to investigate outlier covariate patterns.

For each clinical sign listed in Table I, within each RFLP type, the proportion of herds reporting the clinical sign was calculated. The RFLP types were then ranked, from highest to lowest within each clinical sign, by the proportion of herds reporting the clinical sign.

The temporal distributions of all RFLP types were examined by plotting the 60-d moving average of the ratio of the number of cases in an RFLP type divided by the total number of cases in that period. The temporal scan statistic was used to assess temporal clustering within each RFLP type, using the Poisson model in SaTScan software version 7.0.3 (Kuldorff M, Harvard Medical School, Boston, Massachusetts, USA). For the temporal scan statistic, the number of cases per day of an RFLP type was used as the numerator or 'Case File,' and the total number of all virus types per day was used as the denominator or 'Population File.' Also, 999 Monte Carlo replications were used, time was aggregated into 30-day periods, and the maximum allowable cluster was set to be 50% of the study period. Data were aggregated by 30-day periods to reduce the chance of detecting false clusters of short duration. The analysis was also adjusted for the presence of a log linear trend over time, allowing the software to automatically calculate the trend.

Results

Eight hundred and fifty-nine (859) cases were PRRSV-positive by RT-PCR between September 1, 2004 and January 14, 2006; of these, 116 were included in the study. One thousand and seven (1007) cases were PRRSV-positive by RT-PCR between January 15, 2006 and August 31, 2007; of these, 326 were included in the study. Three hundred and thirty-three (333) premises, distributed across southern Ontario, were included in the study. Four hundred and forty-two (442) surveys and sequences were collected from these premises: 256 premises were sampled once, 54 premises were sampled twice, 15 premises were sampled three times, 7 premises were sampled four times, and 1 premise was sampled five times. Telephone interviews were conducted between January 2006 and December 2007. The distribution of the RFLP patterns that were found in ≥ 10 isolates, is illustrated in Table II. The minor RFLP patterns (those found in < 10 isolates) were, in decreasing order of prevalence, 212, 122, 152, 112, 1_3, 162, 222, 1_1, 1__, 2_2, 113, 141, 164, 181, 183, 214, 251, and 264. The list of clinical signs, the proportion of herds where each

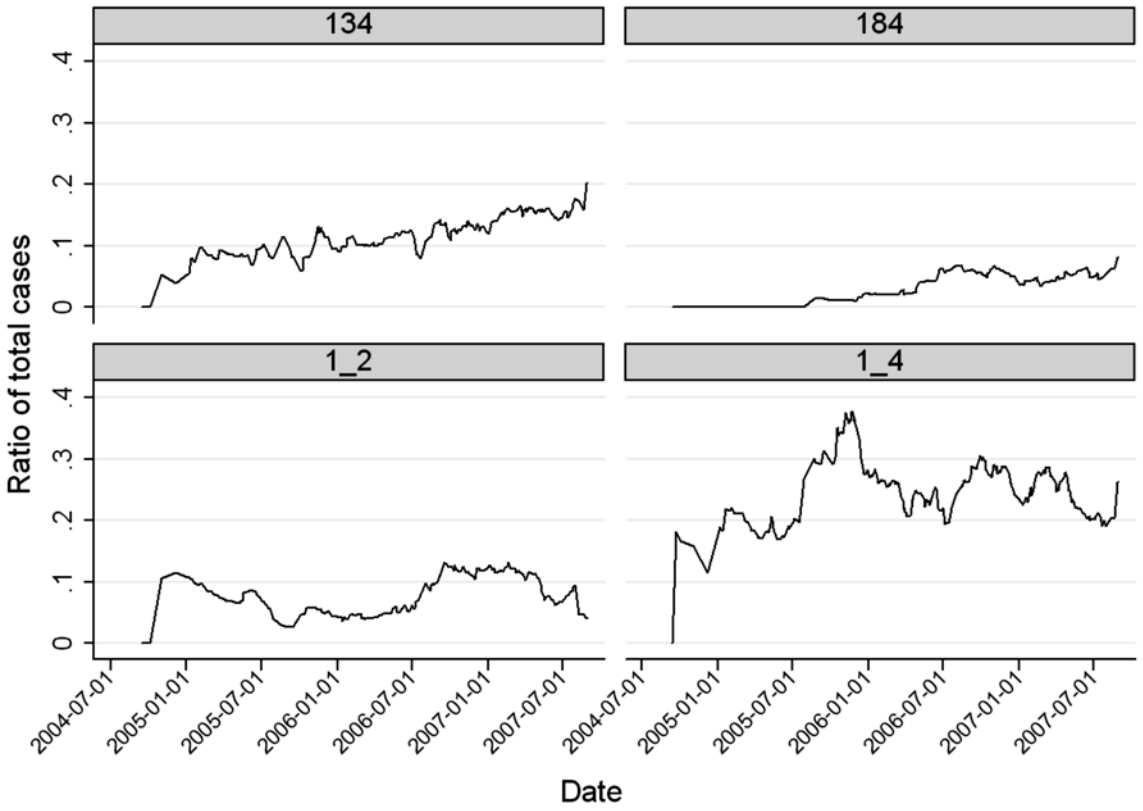

Figure 1. The temporal distribution of the ratio of the number of cases of the 3 most common types (1_4, 1_2, and 134) and RFLP type 184 over the total number of cases using a 60-day moving average.

clinical sign of PRRS was reported, and the associations between clinical signs and isolated RFLP types are illustrated in Table I. The Pearson and deviance residuals for each model were within the range of -2 and 2; because of this, the effects of individual observations on the models were not further investigated.

The percentage of herds reporting clinical signs within each RFLP pattern and the ranks of each RFLP pattern by clinical signs are illustrated in Table III. The frequencies of the 3 most common RFLP types (1_4, 134 and 1_2) compared to the total number of cases is illustrated in Figure 1. Temporal clustering was identified in each of the RFLP patterns tested. In RFLP pattern 1_4, a cluster was identified between September 2005 and July 2007 with a relative risk of 6.1 ($P = 0.001$). In RFLP pattern 134, a cluster was identified between September 2005 and August 2007 with a relative risk of 7.2 ($P = 0.001$). In RFLP pattern 1_2 a cluster was identified between September 2006 and April 2007 with a relative risk of 6.2 ($P = 0.001$). The first case of RFLP type 184 was identified in Nov 2005. This emergence and the temporal distribution of RFLP type 184 is illustrated in Figure 1.

Discussion

In this study, RFLP type 1_4 was the most frequently identified type and was associated with clinical signs specifically in the sow herd (Table I). This type also ranked high in clinical signs occurring in the farrowing phase of production (Table III). Type 1_4 was not reported in a 1998–2000 Ontario study (13), but was found in approximately 21% of PRRSV isolates in a 1998–2002 Quebec study (16). Because it was not previously reported in Ontario and is the most frequently isolated type in the current study, an outbreak of this virus type was suspected. An outbreak is defined as 2 or more occurrences of an RFLP type that are epidemiologically linked (17). In the current investigation, proximity in time of similar PRRSV isolates was used to suggest the possibility of epidemiological linkage and therefore an outbreak. In the current study, RFLP type 1_4 did not appear to change in relative frequency across the study period when compared with all virus types identified. The temporal scan statistic did identify a large cluster which included approximately 50% of the study period beginning in September 2005. This cluster was not interpreted as a discrete outbreak of the 1_4 RFLP type because in the 1-year period preceding the cluster, 20.7% of cases were RFLP type 1_4 versus 25.8% during the cluster. It was concluded that this small difference may have indicated an increase over time that was part of a larger outbreak not captured by the data. Because this RFLP type was not found in the 1998–2000 study it is hypothesized that it emerged between 2000 and September 2004 (13). The frequency of all cases of PRRS in the study database was used as a denominator for the temporal scan statistic for each RFLP type. This may have resulted in a lower sensitivity of outbreak detection than if a denominator from the primary population had been used. In the current approach, if there were concurrent outbreaks of several PRRS genotypes, one could mask the other. A recent extension of the RFLP classification system described by Wesley et al (12) is being widely used by North American diagnostic

laboratories (Russow K, University of Minnesota, personal communication, 2009). This system would further classify cut patterns of the HINCII enzyme which would create a subgroup within RFLP type 1_4. Further classification of the RFLP type 1_4 might change the results of the current investigation.

In the present study, type 1_2 was associated with the clinical signs of abortion, anorexia, weak-born pigs, and high pre-weaning mortality in the sow herd when compared to herds with vaccine-type virus. Type 1_2 was rarely reported in the Quebec study (16) and was not reported in a previous Ontario study (13). However, type 1_2 was ranked 3rd among wild type viruses only in pre-weaning mortality and was otherwise ranked in the middle range for other clinical signs. This low ranking may indicate that virus type 1_2 is not as clinically important as suggested by the statistical test against the vaccine group. It may be that this virus is associated with increased odds of clinical signs when compared to the vaccine group, but is not different from other wild-type viruses. The temporal distribution of RFLP type 1_2 did not indicate a clear outbreak pattern. The relative frequency of this type did not change greatly over the study period. Type 1_2 possibly emerged recently in Ontario, as it was not found in the 1998–2000 Ontario study (13) but did emerge before the period of the present study. Type 1_2 would be further subclassified by the recent changes to the standard RFLP classification system, and this alternate nomenclature might change the results of the current investigation (Russow K, University of Minnesota, personal communication, 2009).

Virus RFLP type 134 was identified very infrequently in the 1998–2002 Quebec study (16) and represented only 4% of virus isolates in the 1998–2000 Ontario study (13). In contrast, type 134 represented 12% of all viruses in the current study, and steadily increased in relative frequency over the study period. Between January and July 2005, 7.8% of cases were RFLP type 134, while 16.0% of cases were RFLP type 134 between January and July 2007. This increase was interpreted as an outbreak of type 134 because of the consistency of the pattern over time and the near doubling of the relative frequency over the study period.

Type 124 was the second most frequent RFLP type in the 1998–2000 Ontario study, representing 9.4% of isolates (13), and also represented 9% of PRRS viruses in the Quebec study (16). In contrast, type 124 represented only 2.5% of isolates in the present study.

Type 132 which was associated with clinical disease in the farrowing phase of production, in the current study, was rarely isolated in the Quebec study (16) but represented 6.3% in the previous Ontario study (13). In a study of 35 Japanese isolates (1991–1999), 54% were RFLP pattern 132, higher than in the present study, the Quebec study, and the previous Ontario study (18). However, RFLP type 132 was also the only virus type, in the current study, to tend to be associated with absence of clinical disease in any phase of production. Herds with this virus type tended to report less disease in the finisher phase of production than those with the vaccine types.

Type 184 was the most frequent RFLP type in the Quebec study, representing 28% of isolates (16), but was not isolated in the 1998–2000 Ontario study (13), and represented only 3.6% of viruses in the present study. Type 184 was associated with higher odds of abortion than in herds with vaccine-like virus, but was not significantly associated, at $P < 0.05$, with other clinical signs. Type 184 ranked 1st or 2nd among wild type viruses in all clinical signs except stillborn piglets and pre-weaning mortality. The discrepancy between the ranking and relative odds may be due to the small number of herds with this RFLP type, resulting in insufficient power to detect statistically significant differences. For example, of the 16 herds with RFLP type 184, only 8 had sows on site, 7 had nursery pigs, and 5 had finisher pigs. Only questions pertaining to the phases of production on-site would have been answered, resulting in only a small number of responses to questions concerning clinical signs of PRRS in the herd. Type 184 ranked higher for most clinical signs than did other wild type viruses, suggesting that type 184 may be clinically more important than can be shown statistically. Type 184 also appeared to emerge in the population during November 2005, which may have represented an outbreak of this virus type. However, cases from before September 2004 would have to be investigated to confirm that type 184 was not present between the end of the previous study, ending in 2000, and the beginning of our study (13).

Viruses with RFLP type 262 were not identified in the Quebec study (16) but were identified in 3% of isolates in the previous Ontario study (13). Type 262 was both highly ranked in clinical signs in the finisher phase and was associated with finisher pig mortality compared to herds with vaccine-like virus. Type 262 was the only RFLP type found to be associated with clinical signs in the finisher phase.

In a previous analytic observational study, differences in sow mortality was the only clinical sign of PRRS that was found to be associated with genetic differences (11). In the present study, sow and boar mortality was measured and was the least frequently reported clinical problem. Sow and boar mortality was also only significantly more frequently reported in those herds with type 1_4 than those with the vaccine type. Results of these studies are not directly comparable due to the following reasons. First, the measurements of clinical signs in the previous and present study were both done by interview and were both collected as binary variables. These strategies of data collection are crude and difficult to repeat. Second, the research question and analytical approach differed between the 2 studies. While the previous study investigated associations between differences, our study investigated associations between discrete outcomes and discrete genotypes and therefore comparisons between these studies are not interpretable.

There are several limitations to the present study. The use of a secondary-based study design limited the inclusion of cases that submitted to the participating laboratory. The laboratory was chosen because it receives the largest number of samples for PRRSV PCR-testing in Ontario, and was willing to allow us to access their submission database. A second limitation was the way in which data was collected about clinical disease in the herds. The questions were open to interpretation by the person being interviewed based on what they perceived to be a problem or not. This format was chosen in order to make it possible to conduct the survey over the telephone without the need for collection of production records of varied quality. Third, the use of the RFLP classification system designed by Wesley et al (12) limits the interpretation of the results to this virus classification system. This system was chosen because it is the most common classification system and was being used by the

participating laboratory to report PRRSV ORF 5 sequencing results to Ontario veterinarians.

In conclusion, the distribution of PRRSV RFLP types in Ontario has changed since the report from 1998–2000 (13) and showed some similarity to the 1998–2002 Quebec study (16). Furthermore, observed clinical signs in our study were associated with specific RFLP types. Epidemic patterns were observed for two RFLP types, 134 and 184 but could not be ruled out for any of the RFLP types. Data from before the study would have been required to determine whether these virus types had emerged recently.

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Article

Changes in lymphocyte function and subsets in dogs with naturally occurring chronic renal failure

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Abstract

Chronic renal failure (CRF) causes immunosuppresion in humans and is thought to be one of the causes of noninfectious secondary immunosuppression in dogs. Hematological, biochemical, and immunological examinations were performed on blood samples obtained from dogs in various stages of CRF. The number of dogs with lymphopenia increased with the progression of clinical signs. All main subsets of lymphocytes were decreased, but more considerable reduction was detected in B-cells, Tc-cells, and NK cells. Depressed lymphocyte response to concanavalin A and pokeweed mitogen was found in dogs with severe clinical signs and lymphopenia. Our results, showing impaired immunological functions, are similar to results obtained from uremic humans, suggesting that infection may be an important complication in dogs with CRF.

Résumé

La défaillance rénale chronique (CRF) entraîne une immunosuppression chez l'humain et on croit qu'elle serait une des causes de l'immunosuppression secondaire non-infectieuse chez les chiens. Des examens hématologiques, biochimiques et immunologiques ont été effectués sur des échantillons sanguins obtenus de chiens avec des stades différents de CRF. Le nombre de chiens avec lymphopénie a augmenté avec la progression des signes cliniques. Tous les différents sous-groupes principaux de lymphocytes étaient diminués, mais une réduction plus importante était détectée avec les cellules B, les cellules Tc et les cellules NK. Une réponse lymphocytaire diminuée à la concanavaline A et au mitogène de la phytolaque a été notée chez les chiens avec des signes cliniques sévères et une lymphopénie. Nos résultats, qui montrent une atteinte des fonctions immunologiques, sont similaires aux résultats obtenus d'humains urémiques, ce qui suggère qu'une infection pourrait être une complication importante chez les chiens souffrant de CRF.

(Traduit par Docteur Serge Messier)

Introduction

Chronic renal failure (CRF) is a common problem of aging dogs and cats. Renal failure is defined as a loss of 3/4 of functioning nephrons and it is associated with various clinical signs. The most common is polyuria and polydipsia due to loss of concentrating ability. Gastrointestinal complications (inapetence, anorexia, vomiting, diarrhea, weight loss) are very common, and they are usually the first signs that prompt owners to visit a veterinarian. Neurological abnormalities associated with CRF are very common and include dullness, lethargy, tremors, seizures, stupor, and coma; however, the severity of these clinical signs may vary. The patients can be presented at various stages of the disease, ranging from subclinical (detected by laboratory tests only — the presence of azotemia and inadequate urinary concentrating ability), mild (vomiting, weight loss, mild neurological signs) to severe azotemia (end stage, where the homeostasis is so disturbed, that it is incompatible with life). Chronic renal failure is associated with many changes in laboratory tests. The presence of azotemia and hyperphosphatemia is typical, anemia (nonregenerative, normochromic, and normocytic) is also commonly found (1).

Chronic renal failure causes secondary immunosuppression. This is well-documented in humans who have chronic kidney disease, where infection is a severe, life threatening complication (2–4). Chronic renal failure is often complicated by lymphopenia, and the $CD4^+/CD8^+$ ratio may be unchanged or diminished. The depressed proliferative responses to the T-cell mitogens phytohemaglutinin (PHA) and concanavalin A (ConA) may be, in part, responsible for the uremic immunodeficiency (5–8). Similar results have been found in uremic rats (9,10).

Lymphopenia is well-documented in dogs with CRF, but there is no other study of immunological changes in uremic dogs (11,12). Even veterinary textbooks of nephrology and urology dealing with chronic renal failure as the cause of secondary immunosuppresion have gleaned their information from human medicine (13). According to our knowledge there is no previous study of the changes in the immune response in dogs with CRF. The purpose of this study was to evaluate the occurrence and extent of immunosuppression in dogs with chronic renal failure and to find any important correlation between lymphopenia and the function of lymphocytes and other parameters.

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Table I. Characteristics of dogs, number in each group, average age [mean ± standard deviation *(s)*] and their clinical signs

	Control dogs	Group 1	Group 2	Group 3	Subgroup 3 (lymphopenic)
Number	15	10	15	20	10
Sex (M/F)	11/4	7/3	6/9	8/12	5/5
Average age (y)	9.6 ± 2.9	11.4 ± 2.5	9.2 ± 4.3	9.4 ± 4.3	9.9 ± 3.6
Clinical signs	no without azotemia	no with azotemia	mild	severe end-stage CRF	severe end-stage lymphopenia

Table II. Characteristics of dogs [creatinine, urea, and phosphorus levels, red blood (cell) counts expressed as a mean ± standard deviation *(s)*]

Parameter	Units	Control dogs	Group 1	Group 2	Group 3	Subgroup 3
Number		15	10	15	20	10
Clinical signs		no without azotemia	no with azotemia	mild	severe end-stage CRF	severe end-stage lymphopenia
Creatinine	μmol/L	92.5 ± 16.9	194.8 ± 39.3	334.2 ± 136.4	939.8 ± 604.4	972.3 ± 658.2
Urea	mmol/L	6.7 ± 2.4	20.0 ± 8.3	28.0 ± 13.8	61.6 ± 34.2	62.7 ± 36.5
Phosphorus	mmol/L	1.4 ± 0.5	1.8 ± 0.5	2.5 ± 1.2	5.6 ± 1.8	5.0 ± 1.8
Red blood cells	10^{12}/L	6.9 ± 0.9	6.0 ± 1.6	5.6 ± 1.3	4.8 ± 1.8	5.59 ± 1.9

Materials and methods

Animals

Forty-five dogs with diagnosed chronic renal failure were assessed in the study. These dogs were patients of the Clinic of Dog and Cat Diseases, Faculty of Veterinary Medicine, University of Veterinary and Pharmaceutical Sciences Brno, Czech Republic and were presented to the clinic from May 2005 to June 2007. Dogs were diagnosed with CRF by the presence of persistent azotemia (serum creatinine level above the laboratory reference range of 125 μmol/L) in conjunction with poor urinary concentrating ability. Hematological examinations, standard biochemistry profiling, and urinanalyses were performed in all dogs, while the ultrasonographic examination of kidneys was carried out in 36 dogs. In cases where a differentiation of acute and chronic renal failure was not possible, histopathological examination of renal tissue was performed. Animals with signs of significant extrarenal disease or prerenal/postrenal azotemia at the time of initial diagnosis were excluded from the study. Animals with CRF and another disease which could cause secondary immunosuppression (such as, hyperadrenocorticism, hypoadrenocorticism, diabetes mellitus, neoplasia, pyometra) were excluded as well.

The group comprised 24 females (5 neutered) and 21 males (1 neutered) of various breeds. Average age was 9.85 y (from 5 mo to 16 y).

Dogs were divided into 3 groups according to the extent of clinical signs at the time of diagnosis. Dogs without clinical signs of CRF were placed in the 1st group. Azotemia was found by routine serum biochemistry examination and a diagnosis of chronic renal failure was confirmed by other findings (urinanalysis, ultrasonographic examination). The 2nd group included dogs with clinical signs of uremia (vomiting, inapetence, anorexia) and which responded well to therapy. The 3rd group included dogs that were in the end-stage of renal failure; these animals were not able to deal with changes in homeostasis and died or were euthanized shortly after diagnosis because of a lack of response to therapy. The characteristics of each group are listed in Table I.

Forty-five dogs were diagnosed with CRF according to history, clinical findings, haematological, and biochemical examination and urinanalysis. The diagnosis was confirmed by ultrasonography and in some cases by histopathological examination of renal tissue. The more severe clinical signs occurred when levels of creatinine and urea increased. In addition, these signs strongly correlated with diminishing numbers of red blood cells. Anemia observed in these animals was typically nonregenerative. The main biochemical parameters (serum creatinine, urea, and phosphorus) and red blood cells are shown in Table II. A typical finding was isostenuria and we found both active and passive urine sediment. In ultrasonography, the kidneys were mostly hyperechogenic. Diffuse chronic membranous glomerulonephritis was the most frequent observation in histopathology.

Control dogs

Fifteen clinically normal dogs [11 females (4 neutered); 4 males (1 neutered)] of various breeds that came to our clinic for vaccinations were included in the control group. Of similar age to the CRF dogs (average age 9.6 y), the control dogs were considered to be healthy based on their history, physical examination findings, and normal results of hematological and serum biochemistry analyses.

Blood samples

Where possible, blood samples were taken prior to any treatment. Some dogs were presented as acute patients, so they received fluids and other therapy (antiemetics, antiulcerotic drugs) before

Table III. Differential leukocyte counts in dogs with CRF and control group [mean ± standard deviation *(s)*]

Parameter	Units	Control dogs	Group 1	Group 2	Group 3	Subgroup 3
Number		15	10	15	20	10
Clinical signs		no	no	mild	severe	severe
		without azotemia	with azotemia		end-stage CRF	end-stage lymphopenia
Leukocytes	10^6/L	8250 ± 1818	12023 ± 7523	9995 ± 2312	10311 ± 4004	8418 ± 2548
Neutrophils	10^6/L	5506 ± 1331	8387 ± 6904	7822 ± 2552*	7933 ± 3495**	6541 ± 2863
Lymphocytes	10^6/L	2075 ± 680	2567 ± 1271	1573 ± 694*	1413 ± 1211*	601 ± 237***
Monocytes	10^6/L	303 ± 177	688 ± 554	404 ± 256	526 ± 309*	524 ± 279
Eosinophils	10^6/L	345 ± 282	381 ± 439	196 ± 244**	125 ± 148***	124 ± 121**

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ when compared with control dogs.

Table IV. Lymphocyte subsets of dogs with CRF and healthy controls (mean ± standard deviation *(s)*]

Lymphocyte subset	CD molecules	Units	Control dogs	Group 1	Group 2	Group 3	Subgroup 3
Number			15	10	15	20	10
Clinical signs			no	no	mild	severe	severe
			without azotemia	with azotemia		end-stage CRF	end-stage lymphopenia
T-lymphocytes	CD3+	10^6/L	1722 ± 588	2207 ± 1087	1359 ± 604**	1204 ± 901**	563 ± 205***
T-helper	CD4+	10^6/L	889 ± 409	1199 ± 508	788 ± 370*	813 ± 785*	343 ± 133***
T-cytotoxic	CD3+4-8+	10^6/Ll	626 ± 199	564 ± 404	330 ± 198***	278 ± 265***	130 ± 67***
DP-cells +	CD4+8+	10^6/L	97 ± 174	55 ± 64	30 ± 14**	32 ± 37**	12 ± 7***
NK-cells	CD3-8+	10^6/L	64 ± 194	11 ± 11	12 ± 14	9 ± 7	6 ± 4
γδ-TCR	γδ TCR+	10^6/L	93 ± 99	191 ± 430	44 ± 50	34 ± 29*	15 ± 15**
B-lymphocytes	CD21+	10^6/L	374 ± 223	683 ± 525	285 ± 148	239 ± 198*	97 ± 68***

+ Double positive cells.

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ when compared with control dogs.

blood samples were taken. The blood samples in these dogs were taken immediately. The dogs that had received corticosteroids were excluded from this study. The blood samples were taken by venipuncture from *v. jugularis* or *v. cephalica antebrachii*. The blood samples were collected into different tubes: EDTA tubes for hematological examination, standard biochemistry tubes and heparinised tubes for immunological examination. All examinations were carried out within 24 hours after collection.

Immunological tests

Total and differential leukocyte counts

Total leukocyte counts were determined using the Digicell 500 cell counter (Contraves AG, Zurich, Switzerland); differential leukocyte counts were enumerated from blood smears stained with May-Grünwald, Giemsa-Romanovski.

Flow cytometry

Cells were stained by the indirect double color technique. Fifty microliters of blood were incubated with monoclonal antibodies for 15 min at laboratory temperature. Erythrocytes were lysed by 3 mL of a hemolytic solution (8.36 g NH_4Cl, 1 g $KHCO_3$, and 0.037 g EDTA per 1 L of distilled water). Mouse anti-dog monoclonal antibodies CD3 (Ca17.2A12-IgG1), CD4 (CA13.1E14-IgG1), CD8 (CA9.JD3-IgG2a), CD21 (CA2.1D6-IgG1), γδTCR (CA20.8H1-IgG2a), CD45 (CA12.10C12-IgG1), CD45RA (CA4.1D3-IgG1) provided by P.F. Moore and secondary goat anti mouse IgG1-FITC or IgG2a-R-PE antibodies (SouthernBiotech, Birmingham, Alabama, USA) were used. Propidium iodide was used to stain the DNA of dead and damaged cells and to exclude such events from analysis. Data were acquired on a standard FACSCalibur flow cytometer (Becton Dickinson, Mountain View, Alberta) operated by the CELLQuest software. Gating of the lymphocyte population was based on forward angle and right angle scatter signals. In each sample 10 000 cells were measured.

Lymphocyte transformation test

The proliferation activity of lymphocytes was determined using the mitogen-driven lymphocyte transformation test (LTT). Mononuclear cells were isolated on a cell separating medium of density 1.077 (Histopaque 1077; Sigma-Aldrich Chemie, Munich, Germany). The density of the cell suspension was adjusted to 10^6/mL with RPMI 1640 medium with 10% of fetal calf serum. Twenty μL of mitogens — concanavalin A (ConA, 10 μg/mL; Pharmacia Biotech, AB, Sweden), pokeweed mitogen (PWM, 10 μg/mL; Sigma-Aldrich Chemie) were pipetted in triplicates into wells of microtiter plates (Gama Group, Ceské Budejovice, Czech Republic). Cell suspension (200 μL) was then added to each well including triplicate wells without mitogen. The microplates were incubated for 3 d at 37°C and with 5% CO_2. ^{3}H-thymidine (50 μL) was added 20 h before the end of incubation. The incorporation of ^{3}H-thymidine was measured with

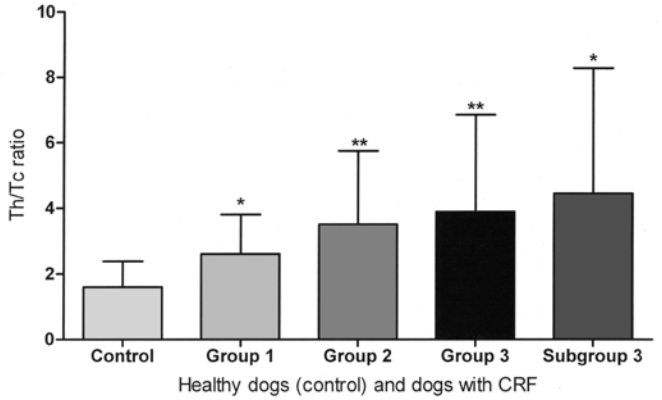

Figure 1. Th/Tc ratio in dogs with CRF and in healthy controls. * $P < 0.05$, ** $P < 0.01$ when compared with control dogs.

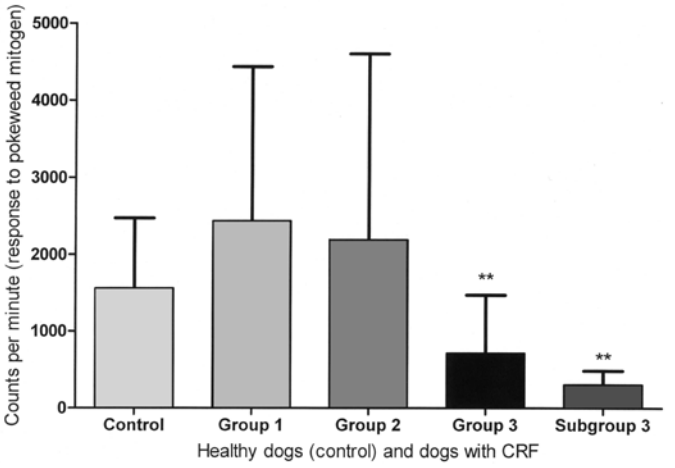

Figure 2. Lymphocyte response to pokeweed mitogen stimulation expressed as counts per minute in dogs with CRF and healthy controls. ** $P < 0.01$ when compared with control dogs.

a liquid scintillation counter (Top Count NXT; Packard Bioscience Instrument Company, Meriden, Connecticut, USA). The results were expressed as counts per minute (CPM) in stimulated samples versus CPM in nonstimulated controls.

Data analysis

Statistics were calculated with MS-Excel 6.0 [mean ± standard deviation *(s)*] and Graph Pad prism software (inter-group differences). Statistical differences between groups were estimated with the unpaired nonparametric Mann-Whitney test. Differences with $P < 0.05$ and $P < 0.01$ were interpreted as significant and highly significant, respectively. Correlations between parameters were calculated according the Spearman test.

Results

Total and differential leukocyte counts

Total and differential leukocyte counts are summarized in Table III. We found statistically important lymphopenia and observed decreases in the number of lymphocytes with increasing clinical signs and azotemia.

Lymphocyte subsets

Corresponding to the decreased count of lymphocytes in groups of dogs with clinical signs, the counts of all main subsets also decreased. Nevertheless, a more considerable reduction was detected in $CD21^+$ (B-cells), $CD3^+4^-8^+$ (Tc-cells), and $CD3^-8^+$ (NK subset). Lymphocyte subsets are presented in Table IV. A nonproportional decreased count of lymphocyte subsets was demonstrated by a statistically significant increase Th/Tc ratio (Figure 1). The T/B ratio tended to increase but not in statistically significant manner.

Activity of lymphocytes

A significantly lower response to mitogens was observed in the group with severe clinical signs, especially in the lymphopenic dogs. The number of animals with depressed lymphocyte activity increased with the extent of clinical signs. We found a statistically very significant depression of response to pokeweed mitogen stimulation (Figure 2) and a statistically significant depression of response to concanavalin stimulation (data not shown).

Correlation between parameters

There was a statistically significant negative correlation between the number of lymphocytes and the number of erythrocytes ($r = -0.3007$, $P = 0.0047$, *). There was a statistically significant positive correlation between the number of lymphocytes and their activity after stimulation with concanavalin A ($r = 0.4313$, $P = 0.0031$, **) and a very significant positive correlation between the number of lymphocytes and their activity after stimulation with pokeweed mitogen ($r = 0.5360$, $P = 0.0003$, ***).

Discussion

Various abnormalities of the immune system have been demonstrated in humans with end-stage renal failure (2). Impaired polymorphonuclear functions, lymphopenia, and a lower response of lymphocytes to mitogens are well-described phenomena in such patients. Both cellular and humoral immunity are impaired (14,15). The clinical expression includes a high susceptibility to bacterial infection, prolonged allograft survival, cutaneous anergy, abnormal antibody responses to T-dependent antigens and to viral infection, and an increased incidence of *Mycobacterium tuberculosis* infection (5,16). Bacterial infection and sepsis are important causes of morbidity and mortality in people in the end-stage of renal disease (3).

The mechanisms of immunosuppression in uremia are only partially understood. Metabolic and toxic consequences of CRF and/or the compounding effects of malnutrition, vitamin deficiency, and drug therapy may each alone, or in any combination, contribute to the genesis of the deranged immune system in CRF. In most cases, the determination of the underlying cause of chronic renal failure is not possible. At the time of diagnosis, the organism is usually influenced by uremia per se, not by the primary disease.

Chronic renal failure is a very common disease especially in aging dogs and cats. Polyuria and polydipsia are usually the first clinical signs and are a consequence of a disturbed concentrating

ability of the renal tissue. Patients with polyuria are at risk of water-soluble vitamin deficiency (especially vitamin B and vitamin C). The excessive losses of these vitamins may contribute to poor immune responses in dogs with CRF. A deficiency in vitamins B6 and B12 adversely affects the cytotoxic activity of Tc-lymphocytes, decreases the number of T-cells (B12) and hampers the response of lymphocytes to mitogen (B6). Vitamin C deficiency leads to an increased susceptibility to bacterial or viral infections due to altered chemotaxis, a bactericidal activity of polymorphonuclears. It also lowers the lymphocyte response to mitogens (17).

Our results did not confirm this speculation, because all patients with CRF had polyuria (regardless of extent of clinical signs or serum creatinine levels) and we didn´t find any statistically important evidence of immunosuppression when all CRF dogs were compared with healthy controls. In addition, polyuria occurs before azotemia — in the phase of renal insuficiency (when 2/3 of the renal mass is lost).

A typical finding in patients with CRF is lymphopenia, both in humans and in dogs. Our study confirmed lymphopenia in dogs associated with CRF. The lymphopenia was more pronounced in dogs with the most severe clinical signs. Studies in uremic humans have shown that peripheral blood lymphocytes undergo accelerated apoptosis when cultured in vitro (6). Accelerated apoptosis may result from a higher expression of Fas (CD95) or a lower expression of Bcl-2 (6,7). Fas (CD95) is a widely expressed 45 kD membrane protein member of the tumor necrosis factor of cell surface molecules. The Fas molecule mediates apoptosis of T-lymphocytes following interaction with its natural ligand FasL (18). An increased expression of the Fas molecule has frequently been observed in uremic lymphocytes. Bcl-2 is a member of the Bcl-2 family with antiapoptogenic effects and is capable of protecting lymphocytes against a variety of apoptotic signals. A significantly decreased expression of Bcl-2 has been observed in CD4+, CD8+ and CD19+ lymphocytes obtained from uremic patients (19,20). CD69 antigen is a phosphorylated cell surface protein and a member of the C-type lectin family. Meier et al (7) described elevated levels of the CD69 antigen in chronically hemodialyzed patients. The induction of CD69 is an early biochemical event preceding T-cell proliferation in subjects with normal kidney function. But it is generally agreed that chronic hemodialysis patients show low T-cell proliferative activity (21). It has been discovered that levels of CD69 were significantly higher even in nondialyzed patients with CRF than in controls and a significantly high percentage of T-cells ultimately do not proliferate but become apoptotic (7).

Finally, apoptosis has been shown to be one of the mechanisms responsible for lymphopenia associated with aging. Because of the fact that CRF is very common in aging dogs and cats, all the patients and controls employed in the present study were age matched (22).

These observations suggest that the reduction in the numbers of T-cells in uremic patients may be due to a heightened susceptibility to apoptosis. Potential factors that may contribute to increased apoptosis include uremic toxins and reactive oxygen species; however, the exact mechanisms are still unclear. Lymphopenia per se does not explain the immune dysfunction described in uremic patients. The cytokine profile produced by T lymphocytes determines the immune response.

A decrease in lymphocytes was found in all main subsets in our study, but it was more evident in B- and Tc-cells; so the T/B and Th/Tc ratio increased with the extent of clinical signs. The depression in total T-cell counts (CD3+) is, in most studies, proportionally distributed between the Th (CD4+) and Tc (CD8+) and leads to no significant change in the Th/Tc ratio. A small number of studies have described a decrease in this ratio (23,24).

A significantly lower response to mitogens (concanavalin A, pokeweed mitogen) was found in the group with the most severe clinical signs, especially in lymphopenic dogs. Peripheral blood mononuclear cells obtained from humans with chronic renal failure display depressed proliferative responses to phytohemagglutinin and concanavalin A (25,26). Abnormal PHA induced proliferation does not necessarily signify T-cell abnormalities, since stimulation of proliferation by PHA requires the action of intact accessory cells (monocyte/macrophage). Proliferative hyporesponsiveness may be largely down to monocyte defects. Phytohemagglutinin and concanavalin A are T-cell mitogens, whereas pokeweed mitogen acts on both T- and B-cells. The most significant decrease in lymphocyte response occurred after stimulation by pokeweed mitogen. Thus, the observed decrease in lymphocyte response may be associated with a more considerable fall in numbers of B-cells than of T-cells.

Normochromic, normocytic anemia is the most frequently noted hematological change in patients with chronic renal failure. The origin of this anemia is multifactorial and is not only caused by a lack of erythropoietin. Decreased erythrocyte lifespan, uremic inhibitors of erythropoiesis (such as spermine, spermidine, and parathyroid hormone), and bone marrow fibrosis due to secondary renal hyperparathyroidism may contribute to renal failure anemia. The number of lymphocytes was in a negative correlation with the number of erythrocytes in our study, so some of these factors might be associated with the altered immune response in CRF patients and may contribute not only to anemia, but also to lymphopenia.

Chronic renal failure is often complicated by secondary renal hyperparathyroidism with elevated levels of parathyroid hormone (PTH). It has been postulated that PTH may affect the immune system by increasing levels of cytosolic calcium and thereby stimulating T lymphocytes (27). High concentrations of intracellular calcium are responsible for many cellular dysfunctions. Previous data obtained from the incubation of normal lymphocytes with PTH were controversial. Human lymphocytes incubated with increasing amounts of PTH showed a considerable decrease in lymphocyte transformation and significant decrease in helpers to suppressors ratio (28). The T-cell proliferative response to PHA stimulation was significantly higher in lymphocyte cultures obtained from rats with high PTH levels than from normal rats (29). The proliferation of lymphocytes from uremic people incubated with PTH was significantly decreased. It suggests that the uremic state changes the response of T-cells to PTH (30).

Our finding of lymphopenia in uremic dogs is consistent with results reported in uremic humans. The causes of lymphopenia are likely the same as in humans suffering from CRF. Although we lack corresponding data from dogs with CRF, our findings of immunosuppression suggest that bacterial infection in uremic patients should be considered as a severe complication. Our finding of a correlation between lymphopenia and depressed lymphocyte activity

might have an practical conclusion: a diagnosis of lymphopenia by a routine hematological test in CRF dogs could signal an altered immune response; however, complicated immunological tests are not provided in practice.

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Article

Effect of parity on lymphocytes in peripheral blood and colostrum of healthy Holstein dairy cows

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Abstract

Investigation of the bovine systemic and mammary gland immune cells at calving might provide crucial information about the susceptibility of the mammary gland to infection. This study investigated the leukocyte population and cytokine mRNA levels in peripheral blood mononuclear cells (PBMCs) and colostrum mononuclear cells (CCs) obtained from healthy cows soon after calving. Fifty dairy cows that did not show clinical diseases were divided into 4 groups on the basis of parity: heifer (group 1, $n = 10$), 2nd calving (group 2, $n = 11$), 3rd calving (group 3, $n = 14$), and more than 3rd calving (group 4, $n = 15$). In the peripheral blood the numbers of $CD3^+TcR1\text{-}N12^+$, $CD3^+$, $CD4^+$, and major histocompatibility complex class II^+CD14^- lymphocytes were significantly higher in group 1 than in group 4, whereas in the colostrum the percentages of $CD4^+$ and $CD4^+CD26^+$ lymphocytes and the $CD4^+/CD8^+$ ratio were significantly lower in group 1 than in group 4. There were no significant differences in the cytokine mRNA levels of PBMCs among the 4 groups; however, in the CCs the ratio of interferon gamma to interleukin 4 was significantly lower in group 1 than in group 3. These results suggest that the cellular immune function of PBMCs is lower, whereas mammary gland immune cells are more active, in cows with higher parity compared with heifers at calving.

Résumé

L'étude des cellules immunitaires au niveau systémique et de la glande mammaire au moment du vêlage pourrait fournir des informations cruciales sur la susceptibilité de la glande mammaire à l'infection. Une étude a été réalisée sur les populations leucocytaires et les niveaux d'ARNm de cytokines dans les cellules mononucléaires du sang périphérique (PBMCs) et des cellules mononucléaires du colostrum (CCs) obtenues de vaches en santé peu de temps après le vêlage. Cinquante vaches laitières ne démontrant aucun signe clinique ont été réparties en 4 groupes basés sur la parité : taures (groupe 1, n = 10), 2e vêlage (groupe 2, n = 11), 3e vêlage (groupe 3, n = 14) et plus de 3 vêlages (groupe 4, n = 15). Le nombre de lymphocytes $CD3^+TcR1\text{-}N12^+$, $CD3^+$, $CD4^+$ et du complexe majeur d'histocompatibilité de classe II^+CD14^- retrouvé dans le sang périphérique était significativement plus élevé dans le groupe 1 comparativement au groupe 4, alors que dans le colostrum les pourcentages de lymphocytes $CD4^+$ et $CD4^+CD26^+$ et le ratio $CD4^+/CD8^+$ étaient significativement plus faibles dans le groupe 1 comparativement au groupe 4. Il n'y avait aucune différence significative dans les niveaux d'ARNm des cytokines dans les PBMCs entre les 4 groupes; toutefois, dans les CCs le ratio d'interféron gamma par rapport à l'interleukine 4 était significativement plus faible dans le groupe 1 comparativement au groupe 3. Ces résultats suggèrent que la fonction immunitaire à médiation cellulaire des PBMCs est inférieure, alors que les cellules immunitaires de la glande mammaire sont plus actives chez les vaches avec une parité plus élevée comparativement aux taures au moment de la mise-bas.

(Traduit par Docteur Serge Messier)

Introduction

Mastitis is one of the most economically significant diseases in the dairy industry worldwide (1). Its incidence is higher immediately after calving. Drastic changes of systemic immune function in dairy cows during the periparturient period are thought to render cows more susceptible to mastitis. The number of T-cells and the production of interferon gamma (IFN-γ) in the bovine peripheral blood mononuclear cells (PBMCs) decrease during the periparturient period (2,3). Although the exact mechanism and factors have not been clearly defined, the decrease in T-cell population and function is thought to be responsible for the increased susceptibility of dairy cows to infection during the periparturient period.

Drastic changes in the milk T-cell population during the periparturient period have been reported for dairy cattle. In the dried, nonlactating mammary glands 45% to 55% of lymphocytes are $CD4^+$ T-cells (4). The percentage of $CD4^+$ T-cells decreases after calving, and the level remains below 20% throughout the lactation period;

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Table I. Monoclonal antibodies used for the immunostaining of mononuclear leukocytes

Antigen	MAb clone	Isotype	Specificity	Source[a,b]
CD2	BAQ95A	IgG1	T-cell	VMRD
CD3	MM1A	IgG1	Pan T-cell	VMRD
CD4	CACT138A	IgG1	Helper/inducer T-cell	VMRD
CD8	CACT80C	IgG1	Cytotoxic T-cell	VMRD
CD8	BAQ111A	IgM	Cytotoxic T-cell	VMRD
CD21	GB25A	IgM	B-cell	VMRD
CD26	CACT114A	IgM	Activated T-cell	VMRD
TcR1-N12	CACT61A	IgM	γδT-cell receptor	VMRD
MHC class II	CAT82A	IgG1	Monocyte/B-cell	VMRD
A2	CACT26A	IgG1	Activated T-cell	VMRD
CD14	MY4	IgG1	Monocyte	Coulter

[a] VMRD = VMRD (Pullman, Washington, USA).

[b] Coulter = Coulter Immunology (Hailed, Florida, USA).

MAb — monoclonal antibody; MHC — major histocompatibility complex.

the predominant T-cells in the mammary glands of lactating cows are $CD8^+$ and γδ (5). In addition to lymphocytes the colostrum contains very important immune components, and the transfer of numerous cytokines and growth factors via colostrum activates the human infant's immune system (6,7). Bovine colostrum contains high levels of proinflammatory cytokines, such as interleukin (IL) 1 and 6 and tumour necrosis factor alpha (TNF-α), compared with milk in the lactation period (8). Colostrum is important not only to provide immune components to the newborn calf but also to protect from infectious diseases of the mammary gland.

Clinical and subclinical mastitis are more frequent in older cows (9). Previous studies investigating the effect of aging on immune function in humans have indicated a shift toward an increased role of type 2 cytokines, such as IL-4 and IL-5, which help antibody production, and a diminished role of type 1 cytokines, such as IFN-γ and IL-2, which promote cell-mediated immunity (10). It is possible that changes in the lymphocyte subpopulations and cytokine production in older cows increase the susceptibility to mastitis during the periparturient period. Age-dependent changes in the peripheral blood lymphocyte subpopulations of T-cells were reported in Holstein cattle (11). Younger calves have a higher percentage of γδ T-cells and a lower percentage of B-cells; the proportions are reversed around 6 to 9 wk after birth (12). But the characteristics of systemic and mammary gland immune cells in cows at calving and their association with aging have not been clarified. Comparison of cell-mediated immunity in the mammary gland and the peripheral blood during the periparturient period would help our understanding of the susceptibility of the mammary gland to infection during this period. We analyzed the lymphocyte subpopulations and mRNA expression of cytokines in lymphocytes obtained from the peripheral blood and colostrum of clinically healthy cows at calving and examined the effect of parity on these parameters.

Materials and methods

Fifty dairy cows from 10 dairy farms in Aomori, Japan that did not have either infectious or metabolic diseases during the periparturient period were divided into 4 groups on the basis of parity: heifer (group 1, $n = 10$), 2nd calving (group 2, $n = 11$), 3rd calving (group 3, $n = 14$), and more than 3rd calving (group 4, $n = 15$). The mean ages and standard errors were 2.11 ± 0.05, 3.05 ± 0.08, 4.05 ± 0.07, and 6.46 ± 0.28 y.

Peripheral blood and colostrum were obtained within 4 h after calving. Blood samples were collected from the tail vein into tubes containing dipotassium–ethylene diamine tetraacetic acid or heparin. The total leukocyte count was determined with a blood cell counter (Celltacα MEK-6358, NIHON KOHDEN, Tokyo, Japan). Colostrum samples were obtained from a front quarter into three 50-mL tubes per animal. Examination of all colostrum samples with a California Mastitis Test confirmed the absence of clinical or subclinical mastitis.

To lyse the erythrocytes and isolate the leukocytes, 2 mL of each blood sample was mixed with 4 mL of 0.83% ammonium chloride solution. The colostrum samples were centrifuged at $400 \times g$ for 10 min. Precipitated cells were suspended with phosphate-buffered saline (PBS), pH 7.2, and layered on a lymphocyte separating solution (specific gravity 1.077). After 15 min of centrifugation at $400 \times g$, colostrum mononuclear cells (CCs) were isolated and used for the flow cytometric analysis as previously described (13).

For the flow cytometry, cells were washed and resuspended with PBS. About 2×10^6 cells/mL were incubated at 4°C for 60 min with monoclonal antibodies against the bovine cell surface markers. To differentiate lymphocyte subpopulations, we used monoclonal antibodies against CD2 (expressed on all T-cells except γδ), CD3 (total T-cells), CD4 (T helper cells), CD8 (T-cytotoxic cells), CD21 (B-cells), CD14 (monocytes), CD26 (activated T-cells), A2 (activated T-cells), TcR1-N12 (γδ T-cells), and major histocompatibility complex (MHC) class II (monocytes/B lymphocytes). The list of primary antibodies and a description of the working solutions (original concentration 1 mg/mL) are provided in Table I. After incubation with the primary antibodies, cells were washed twice with PBS and incubated at 4°C for 30 min with antibodies, fluorescein isothiocyanate-conjugated goat anti-mouse IgM and phycoerythrin-labelled goat anti-mouse IgG (ICN Biomedicals, Costa Mesa, California, USA). Samples were washed twice and resuspended with PBS. Data were acquired with a FACScan flow cytometer (Becton Dickinson, Bedford, Massachusetts, USA) at 10 000 to 20 000 events per sample. Data analysis for the

Table II. Numbers of peripheral blood leukocytes in 4 groups of dairy cows at calving

	Mean ± standard error (cells/μL)			
Cell type	Group 1	Group 2	Group 3	Group 4
Leukocyte	13 760.0 ± 932.5	13 584.6 ± 1314.2	13 757.1 ± 1330.6	12 194.7 ± 863.1
PBMC	3669.5 ± 254.3	2934.8 ± 249.4	2551.4 ± 231.5	2604.1 ± 339.1
Granulocyte	10 510.5 ± 860.3	10 326.7 ± 1307.8	11 205.7 ± 1337.5	9590.7 ± 880.6
$CD3^+TcR1\text{-}N12^-$	771.8 ± 118.8	512.2 ± 87.7	470.9 ± 56.4	499.8 ± 76.7
$CD3^+TcR1\text{-}N12^+$	364.8 ± 51.8[a]	158.0 ± 36.5[a,b]	109.2 ± 18.2[a,b]	73.3 ± 10.9[b]
$CD3^+$	1136.6 ± 156.4[a]	670.1 ± 116.8[a,b]	580.1 ± 66.3[b]	573.1 ± 86.7[b]
$CD4^+$	346.0 ± 39.5[a]	246.0 ± 40.0[a,b]	222.8 ± 20.6[b]	245.2 ± 30.9[b]
$CD8^+$	161.0 ± 26.8	128.2 ± 26.5	104.5 ± 15.7	103.6 ± 13.1
	Mean ± standard error (rate)			
MHC class-II $CD14^-$	905.7 ± 67.5[a]	761.1 ± 119.0[a,b]	612.2 ± 54.8[b]	575.8 ± 66.2[b]
MHC class-II $^+CD14^+$	359.8 ± 73.4	301.7 ± 43.9	287.3 ± 35.6	359.7 ± 61.2
MHC class-II $^-CD14^+$	671.2 ± 100.0	583.4 ± 71.2	724.9 ± 136.7	664.9 ± 122.3
$CD4^+/CD8^+$	2.48 ± 0.24	2.27 ± 0.45	2.51 ± 0.20	2.65 ± 0.31

PBMC — peripheral blood mononuclear cell.

Different superscript letters indicate a significant difference ($P < 0.05$).

Table III. Percentages of PMBCs in the 4 groups

	Mean ± standard error (%)			
Cell Type	Group 1	Group 2	Group 3	Group 4
$CD3^+TcR1\text{-}N12^-$	20.5 ± 1.8	17.9 ± 2.1	18.6 ± 1.2	21.1 ± 1.9
$CD3^+TcR1\text{-}N12^+$	9.7 ± 1.5[a]	5.1 ± 1.0[b]	4.4 ± 0.7[b]	3.3 ± 0.4[b]
$CD3^+$	30.3 ± 2.8	23.0 ± 2.6	23.0 ± 1.5	24.4 ± 2.1
$CD4^+$	9.9 ± 0.7	8.4 ± 0.9	9.2 ± 0.4	10.8 ± 0.6
$CD8^+$	4.2 ± 0.6	4.5 ± 0.8	4.0 ± 0.4	4.4 ± 0.6
MHC class-II $^+CD14^-$	25.6 ± 1.9	27.7 ± 119.0	25.0 ± 2.0	23.3 ± 2.2
MHC class-II $^+CD14^+$	10.4 ± 1.9	10.8 ± 1.4	11.8 ± 1.4	12.6 ± 1.9
MHC class-II $^-CD14^+$	19.7 ± 3.3	22.9 ± 2.8	26.5 ± 2.4	25.1 ± 2.7

PBMC — peripheral blood mononuclear cell.

Different superscript letters indicate a significant difference ($P < 0.05$).

mononuclear cell population was performed with CellQuest software (Becton Dickinson). Data analysis for the colostrum samples was performed as previously described (14).

For mRNA analysis, 2 million PBMCs or CCs in 1 mL of 10% FCS-RPMI were placed in a 24-well plate and stimulated with phytohemagglutinin (Sigma-Aldrich, St. Louis, Missouri, USA), 5 μg/mL, for 12 h at 37°C. Next, the PBMCs were washed and resuspended with TRIzol reagent (Invitrogen, Carlsbad, California, USA) to collect RNA from the cells: 2 mg of total RNA from each sample was used for the synthesis of 1st-strand cDNA with the use of oligo-dT primers (Invitrogen) and Superscript II Reverse Transcripts (Invitrogen) according to the manufacturer's protocols. Real-time polymerase chain reaction (PCR) was performed with SYBR Green Master Mix on an ABI prism 7700 Sequence Detector (Applied Biosystems, Foster City, California, USA). The target DNA sequence was specifically amplified by means of the primers previously designed for IL-2, IL-4, IL-8, IL-10, IL-12, IFN-γ, and TNF-α (14). The analyzed cytokines were categorized as type 1 (IL-2, IL-12, and IFN-γ), type 2 (IL-4), immunoregulatory (IL-10), or proinflammatory (IL-8 and TNF-α). The melting curve was determined for each PCR product. The method of comparative threshold cycle number (2-ΔΔCt) was used after a validation experiment, which demonstrated that the efficiencies of the target and reference (β-actin) were approximately equal. The results were presented as ΔCt values, where ΔCt is the difference in threshold cycles for the target and β-actin as an internal control. Fold changes in expression for the 2 groups (ΔΔCt) were calculated by means of the formula 2-ΔΔCt as previously described for ΔCt methods (15).

Statistical analysis was performed with use of a Steel test to determine the differences among the 4 groups for each parameter. A P-value of < 0.05 was considered significant. Data were expressed as mean ± standard error. Since there were no significant differences among the herds, data for the cows in all the herds were combined for the statistical analysis.

Results

In the peripheral blood there were no significant differences in the numbers of leukocytes, PBMCs, or granulocytes between the 4 groups (Table II). The numbers of T- and B-cells in group 1 were higher than

Table IV. Percentages of colostrum mononuclear cells (CCs) in the 4 groups

	Mean ± standard error (%)			
Cell type	Group 1	Group 2	Group 3	Group 4
$CD2^+TcR1\text{-}N12^-$	65.2 ± 4.0	74.0 ± 2.6	68.5 ± 2.2	70.8 ± 3.6
$CD2^+TcR1\text{-}N12^+$	17.3 ± 2.0	12.0 ± 1.4	13.0 ± 1.8	11.5 ± 1.8
$CD3^+$	66.9 ± 4.4	70.7 ± 3.03	66.6 ± 1.7	65.2 ± 6.5
$CD4^+$	24.6 ± 4.4[a]	28.9 ± 4.5[a,b]	35.0 ± 2.6[a,b]	43.6 ± 3.7[b]
$CD8^+$	26.0 ± 2.9	23.2 ± 3.2	24.0 ± 2.9	19.2 ± 1.8
	Mean ± standard error (ratio)			
$CD4^+CD26^+$	16.2 ± 5.9[a]	26.3 ± 3.3[a,b]	22.1 ± 3.1[a,b]	35.5 ± 5.0[b]
$CD8^+A2^+$ (%)	5.5 ± 1.6	4.0 ± 0.6	4.8 ± 0.9	4.1 ± 0.8
MHC class-II $^+CD14^-$	11.5 ± 2.8	15.3 ± 2.5	11.5 ± 2.0	16.4 ± 3.0
MHC class-II $^+CD14^+$	5.8 ± 1.5	5.8 ± 1.2	9.0 ± 3.2	8.0 ± 1.3
MHC class-II $^-CD14^+$	6.9 ± 2.9	8.2 ± 2.9	5.3 ± 1.2	6.3 ± 1.9
$CD4^+/CD8^+$	1.11 ± 0.14[a]	1.73 ± 0.3[a,b]	1.88 ± 0.20[a,b]	2.40 ± 0.33[b]

Different superscript letters indicate a significant difference ($P < 0.05$).

Table V. Cytokine mRNA levels in the PBMCs of the 4 groups

	Mean ± standard error (ΔCt)			
Cytokine	Group 1	Group 2	Group 3	Group 4
IL-2	1.03 ± 0.29	0.82 ± 0.19	1.39 ± 0.35	2.04 ± 1.03
IL-4	1.07 ± 0.68	0.49 ± 0.15	0.52 ± 0.23	1.29 ± 0.83
IL-8	0.86 ± 0.19	1.99 ± 0.62	1.32 ± 0.21	2.60 ± 0.90
IL-10	1.24 ± 0.16	1.42 ± 0.36	1.01 ± 0.14	1.48 ± 0.24
IL-12	0.78 ± 0.18	1.08 ± 0.27	0.91 ± 0.21	1.01 ± 0.18
IFN-γ	1.75 ± 0.54	1.42 ± 0.43	1.32 ± 0.26	4.10 ± 2.25
TNF-α	0.97 ± 0.11	1.24 ± 0.39	0.97 ± 0.11	0.83 ± 0.08
	Mean ± standard error (ratio)			
IFN-γ/IL-4	4.33 ± 2.33	5.35 ± 1.71	15.19 ± 5.89	6.95 ± 1.49

IFN — interferon; TNF — tumor necrosis factor.

those in the other groups. The number of $CD3^+TcR1\text{-}N12^+$ T-cells in group 4 was significantly lower than that in group 1. Groups 3 and 4 cows had significantly lower numbers of $CD3^+$, $CD4^+$, and MHC class II^+CD14^- cells compared with group 1 cows. The percentage of $CD3^+TcR1\text{-}N12^+$ T-cells was significantly lower in groups 2, 3, and 4 than in group 1 (Table III).

In the colostrum the percentage of $CD2^+TcR1\text{-}N12^+$ T-cells tended to be lower in groups 2, 3, and 4 than in group 1 (Table IV). The percentage of $CD4^+$ T-cells and the $CD4^+/CD8^+$ ratio gradually increased with age, and a significantly higher percentage of $CD4^+$ T-cells was observed in group 4 compared with group 1. The percentage of $CD4^+CD26^+$ T-cells was highest in group 4, and there was a significant difference between groups 1 and 4.

In the PBMCs there were no significant differences between the 4 groups in levels of cytokine gene expression, but the mRNA levels of IL-2, IL-8, and IFN-γ were highest in group 4 (Table V). In the CCs the ratio of IFN-γ/IL-4 was significantly lower in group 1 than in group 3 (Table VI). Group 1 had the highest levels of IL-4 and the lowest levels of IL-12, but the differences between the groups were not significant.

Discussion

This study demonstrated some differences in the immune cells of peripheral blood and colostrum among cows with different parities at the time of calving. The numbers of $CD3^+TcR1\text{-}N12^+$, $CD3^+$, and $CD4^+$ T-cells and MHC class II^+CD14^- monocytes and B-cells in the peripheral blood were significantly lower in the oldest group compared with the heifers. In addition, the percentage of $CD3^+TcR1\text{-}N12^+$ T-cells (γδ T-cells) was significantly lower in the 3 older groups of cows compared with the youngest. In humans, the lymphocyte population tends to decline with age, and there are significant age effects on total lymphocytes, $CD3^+$ and $CD4^+$ T-cells, and $CD19^+$ B-cells in the blood (16). We found similar results in the present study. The level of peripheral T- and B-cells was low around the time of calving and increased after parturition (3,13). The stable immune cell population numbers in the systemic circulation of periparturient dairy cows probably helps prevent infectious disease, since a systemic immune response can be elicited (13). These findings suggest that older cows may have increased susceptibility to infection around the time of calving.

Table VI. Cytokine mRNA levels in the CCs of the 4 groups

	Mean ± standard error (ΔCt)			
Cytokine	Group 1	Group 2	Group 3	Group 4
IL-2	0.58 ± 0.17	0.91 ± 0.25	0.42 ± 0.13	0.35 ± 0.12
IL-4	1.56 ± 0.70	0.70 ± 0.33	0.28 ± 0.10	0.77 ± 0.56
IL-8	1.11 ± 0.18	0.91 ± 0.08	1.22 ± 0.30	0.83 ± 0.12
IL-10	0.83 ± 0.16	1.16 ± 0.19	1.35 ± 0.41	0.92 ± 0.14
IL-12	0.76 ± 0.16	2.31 ± 0.93	9.66 ± 4.76	1.30 ± 0.51
IFN-γ	0.92 ± 0.21	0.70 ± 0.12	0.96 ± 0.20	1.05 ± 0.60
TNF-α	1.15 ± 0.17	0.80 ± 0.09	0.98 ± 0.11	0.85 ± 0.07
		Mean ± standard error (ratio)		
IFN-γ/IL-4 ratio	1.65 ± 0.73[a]	3.23 ± 0.87[a,b]	9.93 ± 3.03[b]	6.22 ± 1.82[a,b]

Different superscript letters indicate a significant difference ($P < 0.05$).

The number of peripheral γδ T-cells is higher in calves than in adult cows (17–19). Ayoub and Yang (11) observed age-dependent changes in the percentages of peripheral blood lymphocytes in cattle between the ages of 1 to 2 mo and 2 to 2.5 y. The absolute counts of $CD2^+$, $CD4^+$, $CD8^+$, and $WC1^+$ γδ T-cells did not significantly differ with age. They concluded that the age-dependent changes were due to the high absolute count of B-cells in the animals 2 to 2.5 y old. In humans, the γδ T-cell reduction is related to a lower number of peripheral lymphocytes, a markedly lower number of γδ T-cells being observed in elderly people (20). A significant decrease in both number and percentage of γδ T-cells in the blood of cows with high parity may cause significant changes in immune function of these cattle. Rogers et al (21) indicated that IFN-γ production is correlated with the number of $WC1^+$ γδ T-cells, and thus γδ T-cells appear to be important for cell-mediated immunity. The lower number of γδ T-cells in the cattle with higher parity suggests that the peripheral cellular immune response may be functionally impaired in these cattle. This may be associated with the higher incidence of mastitis in older cows during the periparturient period.

In the colostrum the ratio of $CD4^+/CD8^+$ T-cells increased with parity in the present study. In previous reports, the proportion of $CD4^+$ T-cells was 45% to 55% of the lymphocytes in the dry mammary gland secretion in healthy cattle (4), a proportion similar to that in group 4, but the percentage of $CD4^+$ cells was lower in the cows with lower parity. These cells play an important role in activating all immune cells, such as B-cells, T-cells, and macrophages. Our study suggests that T-cell function in milk is probably influenced by an increase in parity. Mucosal lymphocytes, such as bronchoalveolar and ileal intraepithelial lymphocytes, are predominantly $CD8^+$ T-cells (18,22). A high count of $CD8^+$ T-cells in milk was observed in healthy lactating cows (23), suggesting that these cells predominate in the lymphocyte population of the mammary mucosa. On the other hand, the percentage of $CD4^+$ T-cells in mammary gland secretions was higher than that of $CD8^+$ T-cells during the dry period (5). The mammary glands of dairy cows are continually exposed to pathogens. Defence mechanisms in the glands successfully protect against bacterial infection; thus, clinical or subclinical mastitis does not develop in most dairy cows. If mastitis develops in lactating cows, the percentage of $CD4^+$ T-cells in milk increases (14). In the present study the percentages of $CD4^+$ T-cells and $CD4^+CD26^+$ T-cells (activated $CD4^+$ T-cells) and the $CD4^+/CD8^+$ ratio in the colostrum were significantly lower in the heifers than in the oldest group, whereas the percentages of $CD8^+$ T-cells and $CD8^+A2^+$ T-cells (activated $CD8^+$ cells) showed no significant difference among the groups. An increased proportion of $CD4^+$ T-cells in the mammary glands of cows with higher parity suggests that more $CD4^+$ T-cells were being recruited to restore the tissue damage caused by repeated lactation. The lower percentage of colostral $CD4^+$ T-cells in heifers is probably due to a lack of prior exposure to pathogens during lactation.

Decreased IFN-γ mRNA production by peripheral lymphocytes has been observed from 3 wk before to 2 wk after calving (2). High IL-4 and low IFN-γ production of peripheral lymphocytes has also been reported in the first 3 d after calving compared with mid-lactation (24). Additionally, $CD4^+$ T-cells shift from type 1 to type 2 during the periparturient period. A lower type 1/type 2 cytokine ratio is needed during pregnancy because type 2 cytokines are important for maintaining the pregnancy (25). Data from a previous study that showed decreased cytokine mRNA expression by PBMCs in dairy cows around the time of calving (13) were similar to the data from the current study.

Our data indicate 2 contradictory phenomena: activation of lymphocytes in the mammary gland for production of colostrum and suppression of the systemic type 1 reaction in periparturient healthy cows. Several cytokines, including IL-2, IL-4, IL-12, and IFN-γ, were detected in the colostrum of women who delivered at term (26). In our study, a higher ratio of IFN-γ/IL-4 was observed in the CCs of cows with higher parity. Since IFN-γ is important in activating cellular immune function and it inhibits the production of IL-4, an increased IFN-γ/IL-4 ratio suggests a shift of the immune system from cell-mediated to humoral immunity. In cows with mastitis, several proinflammatory and regulatory cytokines, including IL-1, IL-6, TNF, and IL-12, were synthesized in the infected mammary glands, whereas no IL-2 and IL-4 mRNA was detected (14). The higher ratio of IFN-γ/IL-4 and percentage of $CD4^+$ T-cells in the colostrum of the cows with higher parity seemed to cause the activation of cellular immune function, which might have derived from the previous inflammatory responses in the mammary glands. Although there was no mastitis among the animals in this study, the colostrum of cows with higher parity may have a higher percentage

of activated/memory $CD4^+$ T-cells owing to the previous mastitis. The drastic change in the immune system around the time of calving likely affects the ratio of type 1/type 2 cytokines in the colostrum immune cells in healthy cows, especially those of higher parity.

We believe that a low level of peripheral blood lymphocytes is one of the possible reasons for a higher incidence of infectious disease in older cows. It is possible that the immune composition of the colostrum is influenced by the previous health of the mammary gland. Therefore, studies are necessary to clarify the characteristics of the mammary immune mechanism of older cows in the periparturient period in order to prevent mastitis in early lactation.

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Article

Effect of systematic parturition induction of long gestation Holstein dairy cows on calf survival, cow health, production, and reproduction on a commercial farm

Aurora Villarroel, V. Michael Lane

Abstract

The objective of this study was to evaluate the effect of parturition induction on dairy cattle with long gestation (past due-date) single pregnancies on calf survivability, cow health, production, and reproduction. There was an induction period during which all cows and heifers reaching 282 days of gestation were induced with dexamethasone (n = 614). Control cows calved the year after, had a gestation length > 282 d and were not induced (n = 508). As the induced and non-induced groups were not contemporaneous, data were standardized using the ratio between the herd baselines for each period. Multivariate analyses of the data showed that induced cows were 1.41 times more likely (P = 0.020) to become pregnant in the lactation following the studied calving than non-induced cows with long gestation. There was no difference in the risk of difficult calvings, stillbirths, culling due to reproductive reasons, average milk production, average days open or risk of abortion in the following lactation between induced and non-induced cows. There seemed to be a relationship between parturition induction and a lower risk of post-partum death, although this was not statistically significant (P = 0.162), because including induction as a factor in the model markedly improved the fit of the data. There was no information on incidence of retained placenta (RP) for the non-induced group. In conclusion, parturition induction resulted in more cows becoming pregnant and a seemingly lower risk of post-partum death without affecting calving difficulty, calf viability, or milk production.

Résumé

L'objectif de la présente étude était d'évaluer l'effet de l'induction de la parturition chez des bovins laitiers avec une gestation unique prolongée (dépassée la date de parturition) sur la survie des veaux, la santé des vaches, la production et la reproduction. Il y avait une période d'induction durant laquelle toutes les vaches et les taures atteignant 282 jours de gestation ont été induites avec de la dexaméthasone (n = *614). Les vaches témoins ont mis bas l'année suivante, ont une durée de gestation de > 282 j et n'ont pas été induites* (n = *508). Étant donné que les groupes de vaches induites et non-induites n'étaient pas contemporains, les données ont été standardisées en utilisant les ratios entre les niveaux de base des troupeaux pour chaque période. Les analyses multivariées des données ont montré que les vaches induites étaient 1,41 fois plus sujettes* (P = *0,020) à devenir gestante au cours de la lactation suivant le vêlage étudié que les vaches non-induites avec une gestation prolongée. Il n'y avait aucune différence en ce qui regarde le risque de vêlages difficiles, de mortinatalités, la réforme pour des raisons de reproduction, la production laitière moyenne, la moyenne de jours ouverts ou le risque d'avortement durant la lactation suivante entre les vaches induites et non-induites. Il semblait y avoir une relation entre l'induction de la parturition et un risque plus faible de mortalité post-partum, malgré que la différence n'était pas statistiquement significative* (P = *0,162), étant donné qu'en incluant l'induction comme facteur dans le modèle on améliorait de façon marquée l'ajustement des données. Il n'y avait aucune information sur la fréquence de rétention placentaire (RP) pour le groupe de vaches non-induites. En conclusion, l'induction de la parturition s'est soldée par plus de vaches devenant gestantes et l'apparence d'un risque plus faible de mortalité post-partum sans qu'on affecte les difficultés au vêlage, la viabilité des veaux ou la production laitière.*

(Traduit par Docteur Serge Messier)

Introduction

Although lactation can be hormonally induced (1), parturition is still considered a necessary event to initiate milk production in most cows. However, parturition can be a stressful event and many dairy cows are culled or die shortly after calving (2), mostly attributable to difficult deliveries, injuries during calving, or metabolic disease. Cows that experience stillbirths have been reported having higher risk for periparturient diseases such as retained placenta (RP), downer cow syndrome and metabolic diseases (3), low milk production (4), decreased fertility and a higher risk of dying or being culled (4,5).

Stillbirths and dystocia are associated with delivery of large calves (6,7), which in turn are associated with longer gestations

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(6–8). In fact, it has been established that the growth rate and weight gain of the bovine fetus is maximum during the last few weeks of the pregnancy (9). Therefore, shortening gestation length can be hypothesized to reduce the size of calves and therefore reduce the incidence of dystocia. Gestation length can be reduced by parturition induction (10–16) through administration of different salts of corticosteroids alone (10,11), or in combination with prostaglandins (12,13), estrogens (14), or relaxin (16).

Parturition induction is a management tool used in Australia and New Zealand to maximize pasture utilization (10,15,17). In this situation, premature parturition has been reportedly induced independent of gestation length, including cows that were pregnant for only 3 mo (resulting in a non-viable fetus) (10). Induced parturition has been associated with high incidence of RP (13,17) and lower milk production in the subsequent lactation (18,19). The incidence of RP in induced cows apparently varies in direct relationship with the amount of time between induction date and due date; cows induced 2 wk or more prior to due date had a higher incidence of RP (13,16,20), while cows induced within a week of due date had no difference in RP incidence compared with non-induced cows (12,20).

We hypothesize that a single dose of dexamethasone given to induce parturition in cows that are past the average due date (282 d) will 1) decrease incidence of difficult calvings and thus improve perinatal calf viability, 2) not increase reproductive and health problems in the cow, and 3) not decrease milk production in the subsequent lactation when compared with non-induced cows allowed extended gestation length beyond the recorded due date.

Materials and methods

Study herd

The study was conducted on a closed commercial 1500-milking cow dairy in northeast Spain. The voluntary period for the herd was 60 d. Heat detection was performed via pedometers (S.A.E. Afimilk, Kibbutz Afikim, Israel) and artificial insemination (AI) was the only breeding method practiced on the farm. Pregnancy was diagnosed via rectal palpation at 40 to 47 d after insemination. The combination of these methods ensured the accuracy of breeding dates. Cows included in the study were to have their 1st to 8th parturition. Mean gestation length was 279.3 ± 4.9 d (median = 280.0 d), calculated during the control period when there was no direct intervention that would affect this parameter.

On this farm, cows that show signs of imminent parturition (stage 1) were moved from a dry lot corral to individual calving pens to be closely monitored. Personnel were scheduled in the maternity area for 24 h/d. Each calving is assigned a "calving-ease" code: normal, for deliveries in which cows are not helped or are helped slightly by only one person; assistance needed, for deliveries where more than one person was needed to assist during delivery; posterior presentation; and dystocia, for deliveries needing repositioning of the calf or veterinary assistance (including cesarean sections). For the purpose of this study, deliveries coded as "assistance needed" or "dystocia" were classified as "difficult" calvings.

Parturition induction was started on this herd in October 1998 after 2 very difficult calvings in cows that had long gestation periods (297 and 299 d, respectively). Induction was stopped in May 2000 when a new herd manager was hired. Breeding management remained equal throughout the study length (October 1998 – May 2001), using the same pedometers to detect estrus, and the same voluntary waiting period and criteria for coding cows as "do not breed" (a combination of milk production, lactation number, conformation, and number of breedings). In May 2001 there was a major change in the administration and management of the herd introducing new criteria for breeding and culling decisions; therefore, data for this study includes only that collected until May 2001.

Trial design

This was a retrospective study performed by evaluating computerized and handwritten records. All cows and heifers that reached 282 d of gestation from October 1998 through April 2000, received a single IM administration of 0.1 mg/kg of sodium phosphate dexamethasone (Dexametasona CAG; Corporació Alimentària Guissona, Lérida, Spain). Parturition induction day was set systematically at 282 d of gestation because the custom herd management software identified this day as the nominal due date. Because all cows and heifers that reached 282 d during the induction period were induced, no control cows were available during this period. The control group for comparison, therefore, was formed by all cows and heifers that reached 282 d of gestation during the following year (May 2000 through May 2001) and were not induced. Due to the time difference between measurement of parameters in the induced group and the non-induced group, parameters for the non-induced group were standardized according to the baseline difference between periods (ratio of $CHF_{induced}$ to $CHF_{non\text{-}induced}$) as follows:

$$\text{Standardized value}_{non\text{-}induced} = \frac{CHF_{induced}}{CHF_{non\text{-}induced}} \times actual\ value_{non\text{-}induced}$$

Baseline parameters were calculated for the rest of the cows of the herd that delivered singleton calves (gestation of ≤ 282 d) during each period. This portion of the herd was designated the complimentary herd fraction (CHF). Milk production data were standardized within parity. The standardized values for the control period were used as reference categories for the parameters of interest for the induced period. The CHF represented 77.1% of the singleton calvings during the induced period and 71.8% during the non-induced period.

Data collection

Production records were obtained and stored automatically via online milk meters (S.A.E. Afimilk) at each milking. Health, management, and production records for every animal in the herd were maintained electronically in custom software (Tauste Ganadera, S.A., Zaragoza, Spain). Data on RP were available only for the induction period from handwritten records of the maternity area. Data collected during the study included cow ID, lactation number, insemination date, calving date, "calving-ease" code, stillbirths (calves that were born dead or died within 24 h), incidence of RP (presence of fetal membranes for more than 24 h after calving), total milk production for the subsequent lactation, lactation length (days in milk or DIM), cows conceiving again (declared pregnant in the lactation following the studied calving), how long it took them to conceive

again (days open), and which cows left the herd due to culling or death, and the reasons for leaving. Only deaths due to post-partum problems and culling due to reproductive reasons were used as a factor for the statistical analyses. All reasons related to reproductive impairment were lumped together to improve statistical power and clinical interpretation. The values for total milk production for the subsequent lactation and lactation length were used to calculate the average milk production per day present in the herd.

Data analyses

Sample size calculation to determine the difference between 10% of difficult calvings in control cows (non-induced) and an expected 8.5% in the induced group (assuming similar figures to the rest of the herd or CHF) at a level of significance of 5%, 80% power and continuity correction for sampling more than 10% of the herd yielded a requirement of 2849 cows in each group (induced and non-induced). Given that this would be impossible with a closed steady-state herd size of 1500 milking cows, statistical significance for this comparison will most likely not be achieved. Sample size calculation to determine a difference of 2 kg/cow/d in milk production at a level of significance of 5% and 80% power yielded 258 animals in each group (induced and non-induced). All animals that reached 282 d of gestation during the study were included in the analyses. Statistical analyses were performed using statistical software (Minitab 15; Minitab, State College, Pennsylvania, USA).

Continuous data are presented as mean ± standard deviation *(s)*, while categorical data are presented as percentages (absolute frequency/population size × 100). Baseline comparisons were performed between each study group (induced and non-induced) and their corresponding CHF. A standard Z-test was used to evaluate difference in proportions, and a Student's *t*-test for 2 samples with unequal variances was used for comparison of continuous variables between each study group and their corresponding CHF. Multivariate regression was used to evaluate if parturition induction was a risk factor for production and health differences in cows with a gestation length > 282 d (induced versus non-induced cows). Continuous outcome variables such as average milk production and average days open were evaluated using multiple stepwise regression (forward selection), using an alpha value of 15% to include a variable in the model. "Induction" (yes/no) was forced into every model to evaluate its significance as a risk factor for each studied outcome.

Dichotomous outcomes such as difficult calving (yes/no), stillbirth (yes/no), post-partum death (yes/no), culling due to reproductive reasons (yes/no), becoming pregnant in the lactation following the studied calving (yes/no), or abortion of this new pregnancy (yes/no) were studied by use of binary logistic regression using the logit link function (inverse of the cumulative logistic distribution function). "Induction" (yes/no) was forced into every model to evaluate its significance as a risk factor. The best fitting model was selected as that with the maximum log-likelihood and maximum *P*-value for the Hosmer-Lemeshow's goodness-of-fit test. Statistical significance for the test that all slopes of the predictive equation were zero was established at a level of 5%. Results with *P*-values > 5% but < 10% were deemed to be statistical tendencies (statistical significance was not achieved probably due to limited sample size).

Table I. Summary of results for a study of systematic parturition induction of long gestation Holstein cows. Induced and non-induced cows had a gestation length of at least 282 d. The complimentary herd fraction (CHF) for the corresponding period included only cows that delivered a single calf and had 282 days of gestation length or less. Data presented as mean ± s

	$CHF_{induced}$	*n*	Induced	*n*	*P*-value	$CHF_{non\text{-}induced}$	*n*	Non-induced	*n*	*P*-value
	≤ **282 d**		> **282 d**			≤ **282 d**		> **282 d**		
Gestation length (days)	277.1 ± 3.8	*2070*	284.3 ± 1.2	*614*	< **0.001**	277.3 ± 3.8	*1294*	284.6 ± 2.3	*508*	< **0.001**
Range	260–282		283–287			260–282		283–306		
Lactation number	2.4 ± 1.7	*2070*	3.0 ± 1.7	*614*	< **0.001**	2.4 ± 1.7	*1294*	2.7 ± 1.6	*508*	< **0.001**
Average milk production (kg)	28.0 ± 6.5	*2020*	27.8 ± 7.1	*596*	0.518	28.6 ± 6.3	*1153*	28.7 ± 7.0	*442*	0.783
Average days open	108.7 ± 61.5	*1307*	117.8 ± 70.6	*375*	**0.015**	100.9 ± 55.7	*847*	104.3 ± 61.0	*287*	0.383
Difficult calvings	8.7%	*2001*	9.4%	*593*	0.328	7.1%	*353*	10.3%	*126*	0.171
Stillbirths	9.2%	*2070*	11.6%	*614*	**0.046**	10.0%	*1294*	10.6%	*508*	0.385
Cows conceiving again	62.8%	*1837*	61.1%	*528*	0.255	65.5%	*1169*	56.5%	*446*	< **0.001**
Abortions in following pregnancy	3.7%	*1307*	5.6%	*375*	0.069	4.5%	*847*	7.3%	*287*	**0.045**
Deaths due to post-partum problems	0.6%	*2070*	0.8%	*614*	0.399	0.7%	*1294*	2.2%	*508*	**0.007**
Culling due to reproductive reasons	7.8%	*2070*	8.1%	*614*	0.438	5.4%	*1294*	11.0%	*508*	< **0.001**
Retained placenta	4.3%	*2070*	8.6%	*614*	< **0.001**	No data	—	No data	—	

s — standard deviation; d — day.

Results

During the induction period there were 614 animals (cows and heifers) with singleton pregnancies that were induced, and 2070 completed records used for the herd average during this period. During the control period there were 508 animals non-induced (naturally long gestations), while 1294 animals contributed to the $CHF_{non\text{-}induced}$ average. Due to the retrospective nature of the study some cows were included in both groups; first year as induced and second year as non-induced (n = 127).

Inducing parturition at 282 d reduced not only the mean gestation length, but also the standard deviation of gestation length compared with the non-induced group ($P < 0.001$). The difference in gestation length does not appear biologically significant, although it masks the large range in non-induced cows (Table I) because most cows calved within a few days after day 282 of gestation. The difference in gestation length between induced and non-induced animals is more evident in Figure 1. Table I summarizes the results for the evaluated variables for the induced and non-induced groups, the CHF for each corresponding period, and the standardized values for the non-induced group. Tables II and III show results of multivariate analyses.

Despite the fact that gestation length for twins was on average almost 5 d shorter than for singleton calvings (274.1 ± 6.0 versus 278.8 ± 5.2, $P < 0.001$), there were 6 twin calvings in the induced group and 9 in the non-induced group during this study. Because twin calvings have been reported as a risk factor for post-partum disease and culling, only singleton calvings were included in the statistical analyses. Lactation number also was a factor that influenced gestation length. Cows in their 1st calving had a shorter gestation than cows with 2 or more calvings (277.8 ± 4.8 versus 279.4 ± 5.3, $P < 0.001$). A total of 30.1% of multiparous cows had long gestations (> 282 d), compared with only 12.0% of primiparous cows.

Calving difficulty and perinatal viability

Parturition induction did not influence the risk of difficult calvings [odds ratio (OR) = 1.07, P = 0.844] or the risk of stillbirths (OR = 0.74, P = 0.578) when compared with non-induced cows (Table II). Difficult calvings were associated with 47.0% stillbirths, which could explain why these 2 factors showed confounding throughout the statistical analyses. Therefore, only the factor with the most significant association was kept in the models. Calvings that resulted in a stillborn calf were 6.94 times more likely to be difficult than calvings resulting in a live birth ($P < 0.001$).

First lactation cows were 3.33 times more likely ($P < 0.001$) to have a difficult calving than non-induced cows, and 2.46 times more likely to deliver a stillborn calf (P = 0.001). Difficult calvings were associated with 47.0% stillbirths, compared with only 6.4% stillbirths in normal (unassisted) calvings ($P < 0.001$). This difference between difficult and normal calvings was consistent across lactation strata and most pronounced in primiparous cows (52.0% of stillbirths in difficult calvings and 10.0% in normal calvings, $P < 0.001$). Therefore, primiparous cows had higher proportion of stillbirths than 2nd and 3+ lactation cows (16.3% versus 5.3% and 6.8%, respectively, $P < 0.001$). Overall, dystocia was associated with 80.4% stillbirths, which was significantly higher ($P < 0.001$) than the 40.1% stillbirths in calvings needing assistance, 21.9% in posterior presentation calvings and 5.4% in normal calvings. Difficult calvings (those needing assistance, including dystocias) occurred mostly in primiparous cows (15.5%, $P < 0.001$).

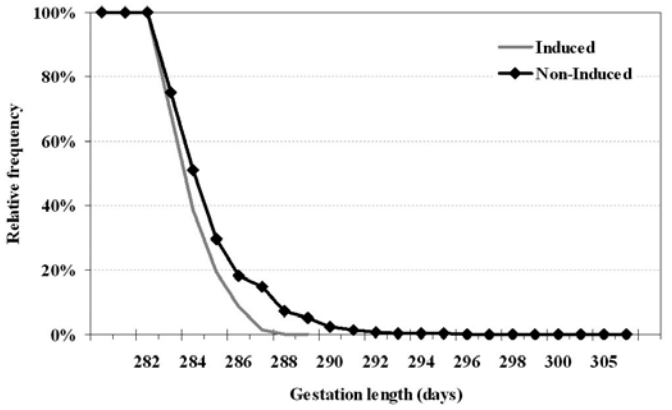

Figure 1. Survival curves of gestation length for a study of parturition induction in long gestation Holstein dairy cows (> 282 d of gestation). Comparison of gestation length in induced cows (n = 614) and non-induced cows (n = 508).

Cow death and culling

Parturition induction was not significantly associated with a difference in post-partum deaths of cows when compared with non-induced cows (OR = 0.35, P = 0.162). Although there was no statistically significant association, including parturition induction as a risk factor in the model markedly increased the log-likelihood of the model (from −124.5 to −40.78) and the fit of the model that included only difficult calvings (from no convergence of the model to a Hosmer-Lemeshow's Goodness-of-Fit test with a P-value of 0.891 and a significant test of all slopes being zero — P = 0.042). According to the final model (Table II), cows were 5.80 times more likely to die if they had a difficult calving than if they had a normal calving (P = 0.018).

Parturition induction was not associated with culling due to reproductive reasons (OR = 1.11, P = 0.663). Risk factors for increased reproductive culling were not becoming pregnant in the lactation following the studied calving (OR = 12.50 inverse of OR = 0.08 for pregnant cows, $P < 0.001$) and being a multiparous cow (OR = 2.70 inverse of OR = 0.37 for primiparous cows, P = 0.006). There was also a tendency for increased culling due to reproductive reasons among cows that calved in the fall (September, October, and November) (OR = 1.73, P = 0.054).

Throughout the study, culling due to reproductive reasons was significantly lower in 1st lactation cows (5.3%) than in 2nd lactation cows (8.3%, P = 0.002) and 3rd + lactation cows (9.7%, $P < 0.001$). Reproductive culling was more likely associated with stillbirths (13.7%) than with live calf deliveries (7.1%, $P < 0.001$). Deaths due to post-partum problems were significantly higher among 3+ lactation cows (1.2%) compared with 0.5% in 2nd lactation cows (P = 0.037) but not compared with 1st lactation cows (0.7%, P = 0.123). Post-partum cow deaths were more likely associated with stillbirths (3.6%) than with live calf deliveries (0.5%, $P < 0.001$).

Table II. Summary of results of multivariate logistic regression analyses for a systematic parturition induction program in long gestation Holstein cows (> 282 days of gestation). Data expressed as OR, (95% CI) and *P*-value. *P*-values highlighted in gray are > 5% level of significance tested, but risk factors were kept because they improved the fit of the model

	Risk factors								
							Season		
Outcome variable	Induction	Primiparous cows	Difficult calving	Stillbirth	Conceiving again	Culling (reproductive)	2	3	4
Difficult calving	**1.07** (0.54, 2.14) *0.844*	**3.33** (1.92, 5.77) *< 0.001*		**6.95** (3.93, 12.30) *< 0.001*					
Stillbirth	**0.8** (0.44, 1.45) *0.457*	**2.46** (1.47, 4.11) *< 0.001*	**6.95** (3.93, 12.30) *< 0.001*						
Cows conceiving again	**1.41** (1.05, 1.88) *0.020*					**0.08** (0.05, 0.14) *< 0.001*			
Deaths due to post-partum problems	**0.35** (0.08, 1.52) *0.162*		**5.8** (1.35, 24.98) *0.018*						
Culling due to reproductive reasons	**1.11** (0.70, 1.75) *0.663*	**0.37** (0.18, 0.75) *0.006*			**0.08** (0.05, 0.14) *< 0.001*		**0.7** (0.33, 1.47) *0.342*	**1.33** (0.69, 2.56) *0.397*	**1.73** (0.99, 3.03) *0.054*

OR — odds ratio; 95% CI — 95% confidence interval

Seasons: (reference) 1 = Dec, Jan, and Feb
2 = Mar, Apr, and Jun
3 = Jul, Aug, and Sep
4 = Oct, Nov, and Dec

Table III. Summary of results of multivariate regression analyses for continuous outcomes for a systematic parturition induction program in long gestation Holstein cows (> 282 days of gestation)

	Average milk production (kg/cow/day)			Average days in milk (DIM)		
Risk factors	Coeficient	$S_{\bar{x}}$ Coef	*P*-value	Coeficient	$S_{\bar{x}}$ Coef	*P*-value
Constant	24.340	0.5795	< **0.001**	285.946	6.789	< **0.001**
Induction	−0.703	0.4007	**0.080**	15.046	5.63	**0.008**
Lactation length (DIM)	0.018	0.0017	< **0.001**	—	—	—
Primiparous cows	−3.393	0.4829	< **0.001**	25.493	6.803	< **0.001**
Death (post-partum disease)	−8.894	2.1750	< **0.001**	—	—	—
Culling (reproductive reasons)	−4.096	0.6351	< **0.001**	61.865	9.76	< **0.001**
Conceiving again	—	—	—	16.277	6.587	**0.014**

$S_{\bar{x}}$ standard error.

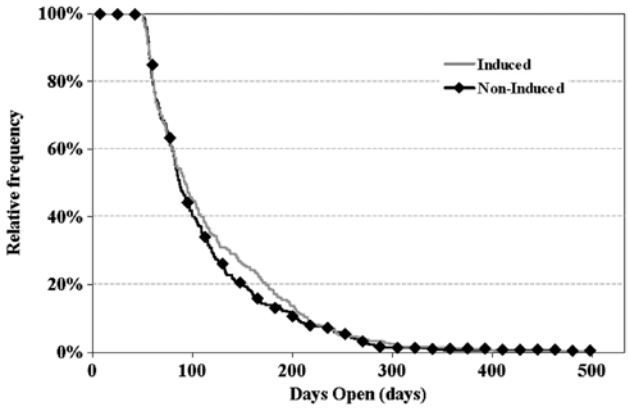

Figure 2. Survival curves of days to conception (days open) for cows that conceived again in a study of parturition induction in long gestation Holstein dairy cows (> 282 d of gestation). Comparison of days open in induced cows (*n* = 614) and non-induced cows (*n* = 508).

Reproduction and cow health

Induced cows were 1.41 times more likely to become pregnant again than non-induced cows ($P = 0.020$). The only other risk factor associated with risk of pregnancy was reproductive culls, which were 12.5 times less likely to be pregnant than cows that cows that were not culled or were culled due to other reasons (inverse of OR = 0.08 for pregnancy, $P < 0.001$). Risk of abortion and average days open (Figure 2) were not significantly associated to any of the studied variables.

Incidence of RP in induced cows was 8.6%, which could only be compared to the incidence of the $CHF_{induced}$ (4.3%, $P < 0.001$). Multivariate analysis of the risk of RP during the induction period (1998–2000) showed that induced cows were twice as likely to have RP than cows in the $CHF_{induced}$ group (singleton calvings from gestations < 282 days long). Additionally, primiparous cows were 1.61 times less likely to develop RP (OR = 0.62, $P = 0.020$). Also, there was a higher risk for RP in all seasons compared to winter (December, January, and February).

Retained placenta rates could not be evaluated in the control period ($CHF_{non\text{-}induced}$ and the non-induced group) or within lactation strata because data from the maternity handwritten records were not available. Of all RP, 59.42% happened in 3+ lactation cows ($P < 0.001$). Retained placenta was associated with longer days open compared to cows that did not have RP (132.2 ± 71.3 versus 110.1 ± 63.4, $P = 0.005$). However, there was no effect of RP on the proportion of cows conceiving again (52.8% — RP versus 61.8% — no RP, $P = 0.254$). There was also no effect of RP on proportion of cows culled due to reproductive reasons, cows that died due to post-partum problems, stillbirths or difficult calvings. There was a significant association between RP and a posterior presentation (10.1% versus 4.6% of cows with posterior presentation and no RP, $P = 0.003$).

Milk production

Average milk production was significantly higher during the control period than the induction period ($P = 0.007$) (Table I). After standardizing the non-induced group for the ratio between both CHF baselines (induced and non-induced), there was no difference in milk production between induced and non-induced cows ($P = 0.648$). This result was consistent across lactation strata. Average milk production did not change in the non-induced group after standardizing the distribution across lactation strata observed in the induced group. This standardization was performed to eliminate possible influence of the higher proportion of multiparous cows in the induced group compared to the non-induced group (multiparous cows have higher production than primiparous cows).

Multivariate analysis of average milk production per cow per day showed DIM as the only factor positively associated with milk production (higher DIM were associated with higher average milk production, $P < 0.001$) (Table III). Factors negatively associated with average milk production ($P < 0.001$) were primiparous cows (versus multiparous cows), cows culled due to reproductive reasons, and cows that died due to post-partum problems. Cows that were induced tended to produce on average 0.70 kg/cow/d less than non-induced cows ($P = 0.080$). Induced cows, however, produced milk for an additional 15 d compared with non-induced cows ($P = 0.008$). Other factors that increased average DIM for the lactation following the studied calving were primiparous cows and cows culled for reproductive reasons ($P < 0.001$) as well as cows that had conceived again ($P = 0.014$). The models explained only a small portion of the variability in milk production and DIM ($R^2 = 17.3\%$ and 5.8%, respectively).

The average lactation number was higher in the long gestation groups (induced and non-induced) compared with their respective CHF ($P < 0.001$) (Table I). This was mainly due to a difference in the proportion of primiparous cows: 19.9% in the induced group, 41.9% in the $CHF_{induced}$, 24.6% in the non-induced group and 39.8% in the $CHF_{non\text{-}induced}$.

Cows that delivered a live calf produced 2.1 kg/d more milk than cows that had a stillbirth (28.2 ± 8.2 versus 26.1 ± 6.3, $P < 0.001$). Cows with normal deliveries (not difficult) had a similar advantage in production over difficult calvings (28.0 ± 8.3 versus 26.5 ± 6.7, $P < 0.001$). These results were not confounded by many difficult calvings resulting in stillbirths. For example, cows with difficult calvings that produced a live calf produced 2.5 kg/day more milk than cows with difficult calvings that had stillbirths (27.6 ± 5.9 versus 25.2 ± 7.1, $P = 0.002$). Cows with normal calvings and a live calf also produced more milk than cows with normal calvings with stillbirths (28.1 ± 6.3 versus 26.7 ± 7.9, $P = 0.010$). Thus, independent of each other, stillbirths and difficult calvings were associated with lower milk production than live calves and normal calvings.

Discussion

The findings of this study were that systematic parturition induction of Holstein cows at 282 d of gestation did not affect the risk of difficult calvings or stillbirths, nor did it affect culling due to reproductive reasons during the following lactation. However, parturition induction increased the probability of pregnancy. Our result contrasts with that of a previous study (20) in which induced cows had lower pregnancy rates in the subsequent lactation. However that study induced cows 12 d earlier than the study herein. Other studies show no difference in the proportion of cows conceiving again (12,21). In spite of the higher probability in induced cows to conceive again in the next lactation compared with non-induced cows, induction was not a risk factor for increased days open. Previous studies (14,21) also show no difference in average days open between induced and non-induced cows.

Incidence of stillbirths was very similar in both groups and similar to the corresponding CHF; therefore, we conclude that inducing parturition in cows did not affect neonatal survival (positively or negatively). Our results are in agreement with other studies (15,19,22), although some studies have found a detrimental effect of parturition induction in neonatal viability when inducing parturition in cows with < 8 mo of gestation (10,11). It is possible that our results could have been influenced by arbitrary induction precisely at day 282 of gestation, which has been associated with the lowest perinatal mortality in a previous study (6). Inducing parturition with corticosteroids mimics the natural hormonal profile in the cow and the calf, allowing for physiological development such as the surfactant action in the calf's lungs (23). In this study, the chemical formulation used was a dexamethasone sodium phosphate, which has a much shorter duration of action and reaches higher serum concentrations (24) than long-acting formulations such as those used in other parturition induction studies: dexamethasone trimethyl acetate (12,15,21) and dexamethasone isonicotinate (21). No other induction studies using the sodium phosphate formulation of dexamethasone have been found. It is possible that the different chemical formulation of short-acting dexamethasone used in this study compared with other studies could account for some of differences between the results from this study and those others.

There was a similar incidence of assisted calvings in both groups. Personnel were available 24 h/d to observe calvings and intervene, if necessary, to improve neonatal survival. In dairies where calving observation is not constant, reducing the size of the calf through parturition induction may be effective in reducing neonatal mortality.

Comparison of induced cows with the $CHF_{induced}$ group (gestation length < 282 d) only showed differences in a larger average lactation number, a higher incidence of RP, and longer days open (Table I). The larger average lactation number in the induced group resulted because cows in this study were induced if they had not calved on their due date. It has been previously reported that heifers have shorter gestations than multiparous cows (6,8). The higher average lactation number, therefore, is explained by the higher proportion of multiparous cows reaching induction day because they have longer gestations. The result on incidence of RP is similar to that reported in other studies in which cows were induced within a week before their due date (12,20). However the incidence in our study was much smaller than the incidence of 38% to 100% reported in studies where cows were induced ≥ 2 wk before their due date (13,16,20). The increase in average days open in the induced group compared with the $CHF_{induced}$, although statistically significant, is not considered biologically significant because of its relatively small magnitude and the fact that it remains within the range of standards for production goals (between 95 and 125 d open) (25). All other studied parameters did not differ between the induced group and the $CHF_{induced}$ average. The fact that there was no difference in reproductive culling rates between induced cows and the $CHF_{induced}$ suggests that inducing parturition in cows close to their due date does not negatively affect reproduction in the subsequent lactation. This was confirmed by the finding that more induced cows conceived again compared with non-induced cows.

The 3 main reasons of involuntary culling on dairy farms have been reported as poor production, mastitis, and reproductive problems (26,27). In this study, cows allowed natural termination of gestation (non-induced) had significantly higher mortality due to post-partum problems, and more culling due to reproductive problems (involuntary culling), than cows with gestations < 282 d long ($CHF_{non\text{-}induced}$). It is reasonable to assume that culling due to reproductive reasons (repeat breeders, uterine scars, adhesions, and other uterine abnormalities) could be directly related to difficult calvings or calving accidents. Twice as many cows were culled in the non-induced group compared with the $CHF_{non\text{-}induced}$ baseline (Table I). However, the incidence of difficult calvings was not significantly different ($P = 0.123$) in non-induced cows and the $CHF_{non\text{-}induced}$. Although the association was not statistically significant due to small sample size ($n = 8$ dead cows), inclusion of induction as a risk factor for death due to post-partum problems was important in convergence of the model. Other studies have reported no difference in total culling or death of induced cows versus non-induced cows when assessing health effects of parturition induction on the dam (10,28,29). The discrepancy between our results and these studies may be due to our focus in reproductive culling instead of total culling.

The higher than average milk production during the control period (2000–2001) compared with the induction period (1998–2000) could be explained by better feed rations or better cow comfort. Another explanation could be the different proportions of 1st, 2nd, and later lactation cows in each group. Average milk production did not differ between each study group and the corresponding CHF average (gestation length < 282 d). This finding contrasts with that of other studies of parturition induction (18,19) that show a decrease in milk production in induced cows. Because the induced group had a higher percentage of multiparous cows compared to the non-induced group (favoring milk production towards the induced group), milk production in the non-induced group was adjusted for the proportion of 1st, 2nd, and 3+ lactation cows that were present in the induced group. The finding in our study that induced cows tended to produce 0.70 kg/cow/d less milk that non-induced cows could be confounded by the fact that induced cows had longer lactations than non-induced cows (extending the part of the lactation curve with the lowest daily milk production). Additionally, the increase in the proportion of induced cows that conceived again compared to non-induced cows could be considered a beneficial trade-off for the tendency to lower milk production. Our finding that higher DIM were associated with higher average milk production per cow per day could be explained by the fact that cows that make a longer lactation are kept because they produce enough milk to be profitable and those cows that are not profitable would be culled earlier.

Parturition induction has been reported to increase herd profitability (30,31). The economic impact of systematic parturition induction can be estimated by adding losses due to stillbirths, RP incidence, milk production, cow deaths, and culling because of reproductive reasons in induced and non-induced cows relative to their corresponding CHF averages. However, in this study it was not possible to evaluate the economic impact of parturition induction due to a lack of data on RP in the non-induced group and the impossibility of estimating actual losses in milk production in cows that died or were culled at different times during lactation. Economic evaluation of systematic parturition induction would depend largely on current values for cull cows and replacement heifers. However, these evaluations could not estimate losses in genetic value or those due to potential biosecurity risks when introducing new replacement animals. It would also be difficult to accurately estimate losses in milk production because of cows lost (death or involuntary culling) at different points in time during the lactation period. Primiparous cows usually have the best genetic potential and the largest residual cost of the herd because they have not had enough time to offset heifer-rearing costs. Therefore, losses due to death or involuntary culling in this group of animals generally result in the largest losses in the herd. In this study none of the 122 induced primiparous cows died due to postpartum problems versus a 3.0% death loss in the non-induced primiparous cows.

In conclusion, we found that inducing parturition in cows that were past the selected due date (282 d of gestation) was not detrimental to perinatal viability, was associated with a higher probability of the dam to become pregnant again, and could possibly be associated with a lower risk of death of the dam due to post-partum problems. Systematic parturition induction should be considered as an effective management tool in dairies.

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Low minimum inhibitory concentrations associated with the tetracycline-resistance gene *tet*(C) in *Escherichia coli*

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Abstract

Twenty-eight *Escherichia coli* isolates from various animal and environmental sources with defined tetracycline-resistance genotypes for *tet*(A), *tet*(B), and *tet*(C) were tested for their susceptibility to tetracycline by means of both broth microdilution and Etest. All *tet*(C)-positive isolates had tetracycline minimum inhibitory concentrations clustering around an intermediate susceptibility range of 2 to 16 μg/mL. Detecting *tet*(C)-positive isolates by means of susceptibility testing may therefore be difficult with use of the current breakpoint for tetracycline of the Clinical and Laboratory Standards Institute guidelines.

Résumé

*Vingt-huit isolats d'*Escherichia coli *provenant de diverses espèces animales et de l'environnement possédant les génotypes définis de résistance à la tétracycline pour* tet*(A),* tet*(B) et* tet*(C) ont été testés pour leur sensibilité à la tétracycline par micro-dilution en bouillon et Etest. Tous les isolats* tet*(C) positifs avaient des concentrations minimales inhibitrices regroupées autour des valeurs de sensibilité intermédiaire variant de 2 à 16 μg/mL. La détection des isolats* tet*(C) positifs à l'aide d'épreuve de sensibilité pourrait ainsi s'avérer difficile en utilisant les valeurs seuils actuelles pour la tétracycline telles qu'indiquées dans les recommandations du «Clinical and Laboratory Standards Institute».*

(Traduit par Docteur Serge Messier)

Tetracycline is a broad-spectrum bacteriostatic agent that binds to the 30S ribosomal subunit and inhibits protein synthesis in bacteria (1). Because of its extensive use, resistance to this antimicrobial agent is one of the most frequently observed in bacteria from animals, including *Salmonella enterica* and indicator bacteria such as *Escherichia coli* (2). A variety of mechanisms cause resistance to tetracycline, but in *Enterobacteriaceae* the main cause is tetracycline-specific efflux pumps that reduce the intracellular concentration of the antibiotic (1). From work with DNA–DNA hybridizations (3) and, more recently, amino acid sequences (4), many tetracycline-efflux pumps in bacteria have been described (1,5). However, previous studies have shown that 3 types of efflux pumps, encoded by the genes *tet*(A), *tet*(B), and *tet*(C), are responsible for most of the tetracycline resistance observed in *E. coli* from animals (6–8).

While investigating *E. coli* isolates in collaboration with the Canadian Integrated Program for Antimicrobial Resistance Surveillance (CIPARS), we recently noticed that despite being classified as susceptible to tetracycline (2,9) a number of isolates were positive for the *tet*(C) gene. We therefore selected a larger sample from our collection of *E. coli* isolates, which came from a variety of sources, to investigate further the relationship between *tet* gene variants and tetracycline minimum inhibitory concentrations (MICs). We wanted in particular to assess whether *tet*(C) is consistently associated with a low tetracycline MIC and thus to determine the association's general relevance.

Twenty-eight *E. coli* isolates were investigated. They were obtained between 2003 and 2008 from various sources in Canada, including cattle (n = 8), swine (n = 9), raccoons (n = 4), a house mouse (n = 1), a field mouse (n = 1), and surface waters (n = 5) (Table I). These isolates, previously tested by polymerase chain reaction (PCR) for the presence of tetracycline-resistance genes (10), were selected on the basis of their genotypes to represent the major resistance genes *tet*(A) (n = 4), *tet*(B) (n = 3), and *tet*(C) (n = 16), as well as fully susceptible isolates without any of these genes (n = 5). For a representative sample, isolates with each *tet* gene were selected randomly within each source, without regard to their tetracycline MIC.

Susceptibility to 15 antimicrobial agents was tested with the use of broth microdilution, according to CIPARS protocols (2). Tetracycline MICs were confirmed for each isolate through broth microdilution with extended dilution series ranging from 0.125 to 512 μg/mL, according to the standards of the Clinical and Laboratory Standards Institute (CLSI) (9). In addition, tetracycline MICs were determined by means of Etest strips, according to the instructions of the manufacturer (AB Biodisk, Solna, Sweden), in parallel with the broth microdilution.

The original tetracycline-resistance genotypes of the 28 isolates were confirmed with a 2nd *tet* PCR (11), and the identity of 4 representative *tet*(C) amplicons was confirmed by DNA sequencing. For this purpose, additional primers were designed on the basis of the *tet*(C) sequence of pAPEC-O1-R (GenBank accession no. DQ517526; www.ncbi.nlm.nih.gov) to obtain a set of overlapping PCR products and to sequence the entire *tet*(C) gene of all 4 isolates. The *tetR* gene and the region between *tet*(C) and *tetR* containing the *tet*(C) promoter were also sequenced in 3 of the 4 isolates (isolates 1, 4, and 23) with a

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Table I. Origins, resistance genes (determined by polymerase chain reaction) and tetracycline susceptibility [minimum inhibitory concentration (MIC) as determined by broth dilution and Etest] of wild-type isolates and transformants of *Escherichia coli*

						Bacteria; susceptibility test; MIC (μg/mL)		
						Wild-type isolate		
		Resistance gene			*tet*(C) GenBank	Broth		Transformant
Isolate	Source	*tet*(A)	*tet*(B)	*tet*(C)	accession no.	dilution	Etest	Broth dilution
1[a]	Swine	−	−	+	EU751610	8	6	8
2	Swine	−	−	+		8	4	
3[a]	Swine	−	−	+		8	8	16
4[a]	Swine	−	−	+		8	6	8
5[a]	Swine	−	−	+		8	10	16
6	Swine	−	−	+		8	4	
7	Swine	+	−	−		32	24	
8	Swine	−	+	−		128	128	
9	Swine	−	−	−		1	1.5	
10	Raccoon	−	−	+	EU751611	8	4	
11	Raccoon	+	−	−		64	24	
12	Raccoon	−	+	−		128	128	
13	Raccoon	−	−	−		0.5	1.5	
14	House mouse	−	−	+		8	6	
15	Field mouse	−	−	+		8	6	
16[a]	Surface water	−	−	+		4	4	8
17	Surface water	−	+	−		256	128	
18	Surface water	+	−	−		32	64	
19	Surface water	−	−	−		1	1.5	
20	Surface water	−	−	−		0.5	1	
21	Cattle	−	−	+		4	4	
22	Cattle	−	−	+		4	4	
23[a]	Cattle	−	−	+	EU751612	2	3	8
24[a]	Cattle	−	−	+	EU751613	16	12	16
25	Cattle	−	−	+		8	8	
26	Cattle	−	−	+		4	4	
27	Cattle	+	−	−		128	64	
28	Cattle	−	−	−		1	1.5	

[a] Plasmid preparations and transformants were obtained for these isolates.

combination of PCR and primer walking with plasmid preparations. The resulting sequences were compared with other sequences available in GenBank by means of the Basic Logical Alignment Search Tool (BLASTn) (12).

Plasmid preparations from the 16 *tet*(C)-positive isolates were obtained with use of a QIAGEN Plasmid Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. To confirm the plasmid location of *tet*(C), a *tet*(C)-specific probe was synthesized by means of the PCR DIG Probe Synthesis Kit (Roche Diagnostics, Mannheim, Germany), a *tet*(C) PCR product being used as the template (10). The probe was subsequently used on Southern blots of the plasmid preparations according to standard protocols (13). Seven of the plasmid preparations (from isolates 1, 3, 4, 5, 16, 23, and 24), from isolates with broth MICs between 2 and 16 μg/mL, were electroporated into *E. coli* DH10B (Invitrogen, Carlsbad, California, USA) according to standard protocols (Bio-Rad Laboratories, Hercules, California, USA). Transformants were selected on Mueller–Hinton agar with 2 μg/mL of tetracycline (Becton Dickinson Microbiology Systems, Sparks, Maryland, USA). Successful transformation of the *tet*(C) gene was confirmed by PCR (11), and the plasmids from these transformants were sized by gel electrophoresis, with use of the BAC-Tracker Supercoiled DNA Ladder (EPICENTRE Biotechnologies, Madison, Wisconsin, USA) as a molecular weight marker.

The tetracycline MICs obtained by broth microdilution for the 16 *tet*(C)-positive isolates clearly clustered between 2 and 16 (mode 8) μg/mL and were distinct from the MICs for the *tet*(A)-positive, *tet*(B)-positive, and *tet*-negative isolates, irrespective of origin (Figure 1). The tetracycline MICs obtained by Etest for the *tet*(C)-positive isolates ranged from 3 to 12 (average 5.8) μg/mL. The MICs obtained by the 2 methods were highly correlated across all genotypes (Figure 2). The MICs for the *tet*(C) reference sequence of pBR322 (4) (GenBank accession no. J01749) in *E. coli* K12 TB1 (New England Biolabs, Ipswich, Massachusetts, USA) were slightly

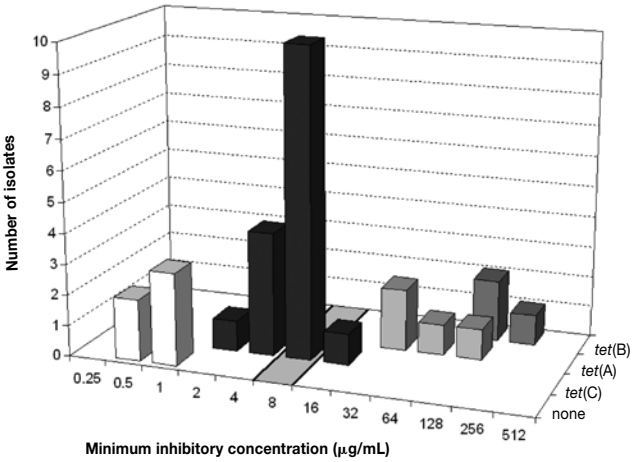

Figure 1. Distribution of minimum inhibitory concentrations (MICs) of tetracycline, determined by broth microdilution, in 28 isolates of *Escherichia coli* and the association with *tet* genes. Tetracycline breakpoints (9) are shown; *tet*(C)-positive isolates are currently classified as "intermediate".

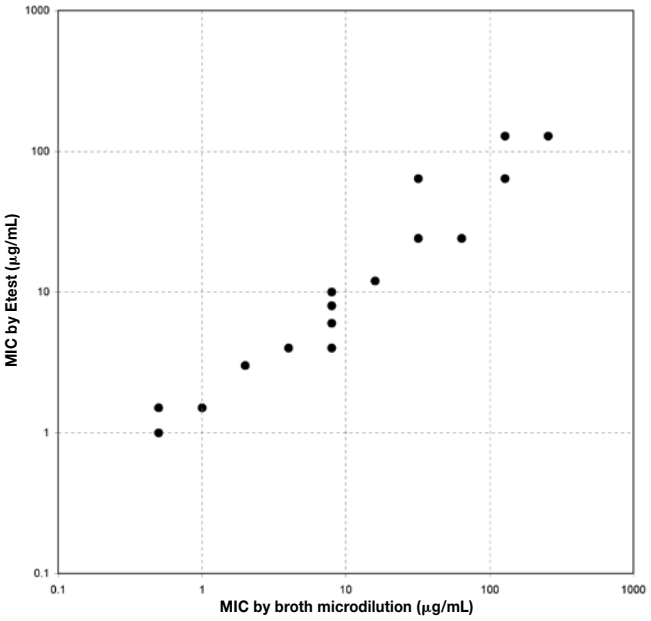

Figure 2. Correlation of tetracycline MICs obtained by broth microdilution and Etest among 28 *E. coli* isolates of diverse origin. A single point may represent several isolates with an identical MIC.

higher than those for the field isolates (64 μg/mL by broth microdilution and 14 μg/mL by Etest). However, this plasmid lacks the *tetR* repressor gene.

Three of the 4 sequenced *tet*(C) genes (GenBank accession nos. EU751610 to EU751612) were identical to pBR322 except for a single substitution at position 1049, resulting in an amino acid change at position 384 of the Tet C protein. However, these 3 sequences were identical to numerous other *tet*(C) sequences in GenBank, including both cloning vectors and sequences from wild-type bacteria (data not shown). The 4th *tet*(C) sequence (EU751613) from our study showed a frameshift deletion of 15 base pairs (bp) near its 3′-end, resulting in truncation of 5 amino acids. This particular *tet*(C) allele was associated with the highest MIC among the *tet*(C)-positive wild-type isolates. Therefore, this truncation seems to have no deleterious effect on the tetracycline-resistance function of the Tet C protein. Sequencing of the *tetR* gene and the promoter region between *tet*(C) and *tetR* in 3 of our isolates (1, 4, and 23) showed sequences (EU751614, EU751615) identical to previous GenBank entries (DQ517526, AJ639924, and others).

Southern blots showed that *tet*(C) was located on the plasmids prepared from all 16 isolates tested (data not shown). The size of the plasmids carrying *tet*(C) in the 7 *tet*(C) transformants ranged from 10 to 100 kbp. The tetracycline MICs of these transformants obtained by broth microdilution ranged from 8 to 16 μg/mL (Table I). Susceptibility testing showed cotransfer of sulfonamide and streptomycin resistance in 2 of the 7 transformants; PCR (14) demonstrated *sul1* and *aadA* in these 2 transformants, whose parent strains were from pigs of unrelated origin. No cotransfer of resistance to antimicrobial agents other than tetracycline was observed with the other 5 transformants.

These results contrast strongly with those obtained in another study, in which an average MIC of 143.9 μg/mL was obtained by Etest in *tet*(C)-positive *E. coli* isolates from Scottish swine (15). However, others had already observed lower MICs (8 to 64 μg/mL) for *tet*(C)-positive coliform bacteria from swine (16). The potential reasons for these discrepancies between studies of *tet*(C)-positive isolates may be numerous, but the diversity of sources for the isolates in this study suggests that the low MICs we observed in association with *tet*(C) represent a potentially widespread phenomenon in animal and environmental *E. coli* isolates in Canada. Although different testing procedures were used in the other studies (15,16), our results showing a close correlation between MICs determined by broth microdilution and Etest suggest that the reasons for these discrepancies are not related to methodologic issues in susceptibility testing. Since the MICs for our *tet*(A)- and *tet*(B)-positive isolates were in the same range as those found by Blake et al (15), the discrepancies observed seem to affect only *tet*(C). In contrast to the present study, Blake et al used selective media containing tetracycline (4 μg/mL) for the isolation of porcine fecal *E. coli* from intensive farms. This may have eliminated most of the *tet*(C)-positive isolates with low tetracycline MICs and selected for a subset of *tet*(C)-positive isolates with particularly high MICs, which were not detected in our study on nonselective media.

Although further testing is needed, the single-substitution difference between the reference *tet*(C) from pBR322 and the *tet*(C) from our isolates is unlikely to be the reason for the low observed MICs. The regulation of *tet*(C) expression may have differed between our isolates and those of Blake et al (15). Nevertheless, the identity of the regulatory and promoter regions in the isolates in the present study and sequences published by others suggests that the findings of this study are of general relevance. The *tet*(C) determinant associated with low-level resistance to tetracycline in the present study was found on a variety of plasmids, which suggests horizontal spread across *E. coli* populations. Similarly, the isolates in the study of Lee et al (16) all carried *tet*(C) on plasmids, but it is unclear whether the *tet*(C) genes from the isolates in the study of Blake et al (15) were chromosomal or located on plasmids. Unfortunately, no sequence data are available from these 2 earlier studies. Thus,

further hypotheses based on the potential effects of *tet*(C) location, gene variants, and regulation mechanisms to explain the differences between tetracycline MICs in these studies are purely speculative.

In conclusion, our results show that *tet*(C)-positive isolates recovered in Canada may often be considered intermediate in resistance or even fully susceptible according to the CLSI guidelines (9). A dilution series extended beyond that used by CIPARS and the National Antimicrobial Resistance Monitoring System in the United States (17), down to 0.25 μg/mL, may be appropriate in a full epidemiologic examination of tetracycline resistance. In addition, epidemiologic breakpoints lower than those used for clinical purposes should be considered for tetracycline-resistance surveillance. Many *tet*(C)-positive isolates may otherwise be misclassified as susceptible and may not be tested further for the presence of tetracycline-resistance genes. As suggested by Blake et al (15), such isolates with low tetracycline MICs may behave differently from those carrying *tet*(A) or *tet*(B) when exposed to tetracycline. The linkage of *tet*(C) with integrons (*sul1* and *aadA*) in some of these isolates also suggests that tetracycline use at levels currently considered subinhibitory may still select for multiresistance through unnoticed tetracycline-resistance determinants.

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Genomic analysis of porcine circovirus type-2 isolates in Alberta pigs demonstrating clinical porcine circovirus associated disease (PCVAD)

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Abstract

Nineteen pigs with clinical signs of porcine circovirus associated diseases (PCVAD) on 5 Alberta pig farms were examined pathologically, including gross pathology, histopathology, and immunohistochemistry. Polymerase chain reaction (PCR) for porcine circovirus type-2 (PCV-2) and sequence analysis was performed on tissue samples of 12 animals. Results showed that new strains of porcine circovirus type-2 genogroup b were present in most pigs that were positive for PCV-2. Furthermore, a mixed infection with PCV-2a and PCV-2b occurred in the liver and lungs of 1 pig. Comparison of whole genome sequences representing known viruses and the newly discovered Alberta viruses revealed mutations distributed over the entire genome of PCV-2; however, sequences encoding for immunodominant epitopes of PCV-2 appear to be unaffected by these mutations.

Résumé

Dix-neuf porcs présentant des signes cliniques de maladie associée au circovirus (PCVAD) dans 5 fermes de l'Alberta ont été soumis à un examen pathologique, incluant un examen macroscopique, histopathologique et immuno-histochimique. Une réaction d'amplification en chaîne par la polymérase (PCR) pour le circovirus porcin de type 2 (PCV-2) et une analyse de séquence ont été effectuées sur des échantillons de tissus provenant de 12 animaux. Les résultats ont montré que de nouvelles souches de PCV-2 appartenant au génogroupe b étaient présentes chez la majorité des porcs qui étaient positifs pour PCV-2. De plus, une infection mixte avec PCV-2a et PCV-2b s'est produite dans le foie et les poumons de 1 porc. Une comparaison des séquences du génome entier représentant des virus connus et les nouveaux virus découverts en Alberta ont révélé des mutations distribuées sur l'ensemble du génome de PCV-2; toutefois, les séquences codant pour des épitopes immunodominants de PCV-2 ne semblaient pas être affectées par ces mutations.

(Traduit par Docteur Serge Messier)

Porcine circovirus type-2 (PCV-2) was discovered in the 1990s in association with a new wasting syndrome in growing pigs (1). This post-weaning multisystemic wasting syndrome (PMWS) and other porcine circovirus associated diseases (PCVAD) have caused severe economical losses for swine farmers and the pig industry worldwide. The exact mechanisms of how PCV-2 contributes to the clinical manifestation of PCVAD are poorly understood and might include host and viral genetic determinants (2,3), the presence of other infectious agents (4), or environmental factors.

Analysis of genome sequences led to the classification of 2 subgroups within PCV-2, based on a sequence motif in the capsid protein: PCV-2a and PCV-2b (5,6). Porcine circovirus type-2a (PCV-2a) was the predominant strain in North America (7) until recent studies revealed that PCV-2b is now present on a large number of pig farms throughout Canada (5,8,9).

Identification and characterization of PCV-2 in Alberta, was accomplished by analyzing PCV-2 genomes obtained from pigs with clinical signs consistent with PCVAD. Tissue samples of 19 pigs from 5 Alberta farms affected by PCVAD were collected during necropsy. Organs sampled included tonsils, inguinal, mesenteric, and bronchial lymph nodes, spleen, lung, liver, small intestine (jejunum and/or ileum), kidney, and skin. For histopathological and immunohistochemical (IHC) analysis, the samples were fixed in 10% neutral buffered formalin (NBF); for PCR analysis they were stored at −20°C until use. After fixation in 10% NBF, tissue samples were embedded in paraffin for routine hematoxylin & eosin staining and IHC processing. Immunohistochemical detection of PCV-2 antigens was performed using a streptavidin-biotin complex technique adapted for an automated slide stainer (CodeOn Histomatic slide stainer; Fisher Scientific, Edmonton, Alberta) as previously described (1), with the exception that the primary antibody was a chicken anti-PCV-2 antiserum (from Dr. G. Allan, Belfast, N. Ireland) used at a 1:1000 dilution. All sections were examined by light microscopy in a non-blinded manner applying the scoring system described by Opriessing et al (6) (Table I).

Amplification and cloning of viral genomic sequences, was accomplished by isolating DNA from tissue samples using QIAamp DNA Mini Kit (Qiagen) according to the manufacturer's protocol. Muscle tissue from a healthy pig mixed with distilled water was used as negative control and a synthesized PCV-2 genome from DNA2.0 (GenBank accession number EF394779) served as a positive control. Viral DNA was amplified through PCR using 4 different

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Table I. Severity and distribution of gross and histological lesions and PCV-2 staining intensity in PCVAD affected pigs from 5 farms

		Presence of clinical signs: — wasting — weight loss — dyspnea — enteritis	Presence of histopathological lesions in lymphoid tissues	Percentage of lymphoid follicles that have cells with staining for PCV-2 antigen (determined by immunohistochemistry)
Farm A	Pig 1	+	1	< 10%
	Pig 2	+	3	> 50%
	Pig 3	+	3	10% to 50%
Farm B	Pig 1	+	2	10% to 50%
	Pig 2	+	1	< 10%
	Pig 3	+	2	> 50%
	Pig 4	+	2	> 50%
	Pig 5	+	2	> 50%
Farm C	Pig 1	+	2	10% to 50%
	Pig 2	+	3	> 50%
	Pig 3	+	3	> 50%
	Pig 4	+	3	> 50%
Farm D	Pig 1	+	3	> 50%
	Pig 2	+	1	< 10%
	Pig 3	+	0	0
Farm E	Pig 1	+	3	> 50%

+ — present; 0 — not present; 1 — mild lymphoid depletion with loss of overall cellularity and mild histiocytic/granulomatous inflammation; 2 — moderate lymphoid depletion and moderate histiocytic/granulomatous inflammation; 3 — severe lymphoid depletion with loss of lymphoid follicle structure/replacement of follicles by histiocytic-granulomatous infiltration. Diagnosis of PCVAD based on the definition by Sorden (14) and on a scoring system by Opriessnig et al (6).

primer pairs as previously described (10,11), with 2 modifications: 1) A new primer with an identical nucleotide position was based on the genomic sequence of a strain with GenBank accession number AY094619 instead of AF201897; and 2) the nucleotide position of primer no. P6 was changed to 1569–1588 to cover a more conserved sequence among PCV-2 strains. Amplification of complete PCV genomes from tissue samples, was accomplished by cloning amplicons into pEGFP-N1 vector (Clontech). At least 10 positive clones were selected for plasmid DNA isolation from DH5α competent cells (Invitrogen), using QIAprep Spin Miniprep Kit (Qiagen) and were sent for sequencing (Genome Quebec, McGill University, Montreal, Quebec). Complete genome sequences were submitted to NCBI under GenBank accession numbers FJ233905 and FJ233908.

Using Blast, ClustalW2 (EMBL-EBI), and GeneDoc, fragments resulting from the same PCR primer pairs were aligned and genogroups were verified by the signature motif sequence in the cap gene as described by Cheung et al (12).

From all selected animals (2 to 5 pigs per farm with typical clinical signs of PCVAD), necropsies revealed the following gross pathological findings typical for PCVAD: the lungs failed to collapse and showed interstitial edemas which led to puffy, sponge-like consistency of the organ. Bronchial and mediastinal lymph nodes were enlarged. The gastro-intestinal system showed thickened intestinal walls with prominent Peyer's patches, enlarged mesenteric lymph nodes and an edematous mesentery. In some animals, livers and kidneys exhibited alterations consistent with hepatitis and focal glomerulonephritis, respectively. The inguinal lymph nodes were enlarged in most pigs that were examined. One animal showed multifocal cutaneous lesions, which are characteristic of the porcine dermatitis and nephropathy syndrome (PDNS) (13). Histopathological lesions included depletion of lymph follicles in the ileal Peyer's patches, tonsils, spleen, and lymph nodes with infiltration of histiocytic cells and occasional giant cell formation. The lung showed diffuse granulomatous pneumonia. Multifocal granulomatous nephritis was observed in the kidneys. The liver was affected by non-suppurative inflammation of the triads. The small intestine showed granulomatous enteritis and the mesenteric arteries were affected by non-suppurative arteritis. Not all animals clinically affected by PCVAD appeared to exhibit overt histological changes and/or positive immunostaining for PCV-2 (Table I).

Results from PCR analyses indicated that PCV-2b was present on farms A, C, D, and E; PCV-2a on farms A and B (Table II). One pig on farm A showed an infection with both PCV-2a and PCV-2b in the liver and lungs. Tissue samples from 3 pigs (farm C: pigs 5, 6; farm E: pig 2) were found PCR-negative for PCV-2. For further characterization, full genome sequencing was performed on a subset

Table II. Genotype of PCV-2 DNA detected by PCR and sequencing in different pig tissues

		Organ	Genogroup
Farm A	Pig 1	Lymph node	PCV-2b
		Liver	PCV-2b/PCV-2a
		Lung	PCV-2b/PCV-2a
		Small intestine	PCV-2b
	Pig 2	Tonsil	PCV-2b
		Lymph node	PCV-2b
		Liver	PCV-2b
		Lung	PCV-2b
		Small intestine	PCV-2b
Farm B	Pig 1	Tonsil	PCV-2a
		Lymph node	PCV-2a
	Pig 2	Lymph node	PCV-2a
Farm C	Pig 1	Lung	PCV-2b
		Lymph node	PCV-2b
	Pig 2	Tonsil	PCV-2b
		Lymph node	PCV-2b
	Pig 3	Tonsil	PCV-2b
		Lung	PCV-2b
	Pig 4	Tonsil	PCV-2b
		Lymph node	PCV-2b
Farm D	Pig 1	Lymph node	PCV-2b
		Kidney	PCV-2b
		Tonsil	PCV-2b
		Heart	PCV-2b
	Pig 2	Lymph node	PCV-2b
		Kidney	PCV-2b
		Spleen	PCV-2b
		Lung	PCV-2b
	Pig 3	Lymph node	PCV-2b
		Kidney	PCV-2b
		Lung	PCV-2b
		Skin	—
Farm E	Pig 1	Lymph node	PCV-2b
		Kidney	PCV-2b
		Lung	PCV-2b

of isolates. Two different PCV-2 strains were found in pig 1 on farm A: a PCV-2a strain, which is closely related to a South African strain (GenBank accession number AY325495.1) but displaying differences in 3 bases (nucleotide [nt] position 536, 1355, and 1369). The first mutation (A > G) at nt 536 is located within the overlapping portion of the genome which encodes for both open reading frames (ORF) 1 and 3. The mutation would result in 1 amino acid substitution (phenylalanine to leucine) in the ORF 3 protein. Both mutations at nt 1355 and nt 1369 are located within ORF 2 which encodes for the capsid protein (Cap). While the substitution of A > G at nt 1355 did not result in a change of an amino acid, replacing C > T at nt 1369 led to a substitution of valine by isoleucine. The second PCV-2 strain found in pig 1 on farm A is a PCV-2b strain, which is — with 99% sequence identity — closest related to the recently emergent PCV-2b strains from the USA (GenBank accession numbers EU594440, EU594438, and DQ629115) and to 1 strain from Quebec (GenBank accession number EF394777). This PCV-2b strain found in Alberta differs from the above-mentioned strains in 2 nucleotides: T > A (nt 236) which does not result in an amino acid change in the replication associated protein Rep; and A > C (nt 1735) which is located in a non-coding region immediately before the translation start of Cap.

The PCV-2b strain found on farm C showed 1 mutation compared to the reference strains: C > T (nt 1706), which leads to an amino acid substitution (arginine to lysine) in the nuclear localization signal region of Cap. Three mutations were found in the PCV-2b strain on farm D, 2 of which were silent (C > T): 1 occurred immediately before the translational start of the Rep protein (nt 50); the other was located in the encoding region of the Cap protein (nt 1132). The 3rd mutation led to an amino acid mutation (arginine to lysine) in the nuclear localization signal (NLS) of Cap (nt 1703).

In summary, we found mostly PCV-2 strains belonging to the previously described PCV-2b subtype (8). Before 2004, PCV-2b was only found in animals in Europe and Asia and the dominant genogroup in North America was PCV-2a (7). The severity of the PCVAD outbreaks caused by the PCV-2b subtype, as well as the distinct clinical and histopathological lesions of these cases, lead to the hypothesis that the newly recognized PCV-2b strains display a higher virulence compared with those of genogroup 2a (5). However, this assumption still needs to be verified, as a recent study showed that PCV-2b was also present in healthy or unaffected animals, as well as in diseased pigs (9).

The capsid protein is the immunodominant component of all PCV-2 piglet-vaccines. The mutations in Cap detected here are located in the NLS region, which is not expected to contain immunodominant epitopes. Hence, these mutations are unlikely to influence the immunity acquired through current PCV-2 vaccines.

One of the detected PCV-2b strains had a mutation located in the ORF 3 which led to an amino acid exchange in the ORF 3-protein. Whether or not this protein is expressed in PCV-2-infected pigs and plays a role in the pathogenesis remains to be elucidated.

One pig had a mixed infection of the liver and lungs, where strains of both PCV-2 genogroups were present. A co-infection of pig tissue with both genotypes of PCV-2 has been reported before (7); however, no data are available to test whether co-infection would lead to an increase of virulence.

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Molecular genealogy tools for white-tailed deer with chronic wasting disease

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Abstract

Molecular genetic data provide powerful tools for genealogy reconstruction to reveal mechanisms underlying disease ecology. White-tailed deer *(Odocoileus virginianus)* congregate in matriarchal groups; kin-related close social spacing may be a factor in the spread of infectious diseases. Spread of chronic wasting disease (CWD), a prion disorder of deer and their cervid relatives, is presumed to be associated with direct contact between individuals and by exposure to shared food and water sources contaminated with prions shed by infected deer. Key aspects of disease ecology are yet unknown. DNA tools for pedigree reconstruction were developed to fill knowledge gaps in disease dynamics in prion-infected wild animals. Kinship indices using data from microsatellite loci and sequence haplotypes of mitochondrial DNA were employed to assemble genealogies. Molecular genealogy tools will be useful for landscape-level population genetic research and monitoring, in addition to epidemiologic studies examining transmission of CWD in captive and free-ranging cervids.

Résumé

Les données de génétique moléculaire sont des outils puissants pour la reconstruction généalogique afin de révéler les mécanismes sous-jacents à l'écologie des maladies. Les cerfs de Virginie (Odocoileus virginianus) *se rassemblent en groupes matriarcaux; le rapprochement social d'animaux apparentés dans un espace restreint pourrait être un facteur dans la transmission de maladies infectieuses. La transmission de la maladie débilitante chronique (CWD), une maladie à prion des cerfs et des cervidés apparentés, est présumée être associée à des contacts directs entre les individus et par exposition à des aliments partagés et des sources d'eau contaminées par des prions excrétés par des cerfs infectés. Les aspects importants de l'écologie de la maladie sont encore inconnus. Les outils génétiques pour la reconstruction des pedigrees ont été développés afin de combler les manques de connaissance dans la dynamique de l'infection chez les animaux sauvages infectés par les prions. Les indices de parenté utilisant les données des loci des microsatellites et la séquence des haplotypes de l'ADN mitochondrial ont été utilisés pour former les généalogies. Les outils moléculaires en généalogie seront utiles pour la recherche et le suivi de la génétique des populations sur le terrain, en plus de permettre les études épidémiologiques examinant la transmission de CWD chez les cervidés sauvages et en captivité.*

(Traduit par Docteur Serge Messier)

Molecular genetics and computational tools now allow visualization of relationships in wildlife systems that was not previously possible. One exciting application is genealogy reconstruction to reveal patterns and mechanisms underlying disease ecology in individuals of unknown ancestry. Emerging statistical methods for kinship analysis will provide effective new tools for the study of wildlife epidemiology (1). Analyses of genotypic DNA data from highly polymorphic markers provide the means to disclose not only parent-offspring and sibling relationships, but also secondary and tertiary family relationships such as half siblings, niece-aunt, and grandparent-grand offspring (2).

Chronic wasting disease (CWD) is a transmissible spongiform encephalopathy (TSE) or prion accumulation disorder of cervid ruminants in the United States and Canada (3). The TSEs are a novel group of disorders, in which the transmissible agent is a misfolded isoform of the host cellular prion protein (4). Although the routes of transmission under natural conditions are not known, a transmissible agent is detected in saliva of experimentally infected deer (5) and there is evidence for indirect transmission and persistent environmental contamination by the infectious agent (6). Relative disease susceptibility and/or incubation period have been correlated with prion gene *(PRNP)* polymorphisms encoding amino acid

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Table I. Dyads of deer qualified as first order relatives using microsatellite and mitochondrial DNA analyses. Dyads with both members infected in parentheses. Fawns include both sexes. Chronic wasting disease prevalence of individuals tested in this study (*n* = 135) was 0.75 for adult females, 0.40 for adult males, and 0.13 for fawns

Relationship	Total	Dyads of adult female adult female	Dyads of adult female adult male	Dyads of adult male adult male	Dyads of adult female fawn	Dyads of adult male fawn	Dyads of fawn fawn
Parent-offspring	24 (9)	5 (4)	6 (2)	0 (0)	12 (3)	1 (0)	0 (0)
Full sibling	23 (5)	6 (4)	6 (0)	2 (0)	2 (0)	4 (1)	3 (0)
First order relative	47 (14)	11 (8)	12 (2)	2 (0)	14 (3)	5 (1)	3 (0)

substitutions at residue 96 for white-tailed deer (7). Dams dying of CWD on elk farms posed an increased CWD risk factor to calves (8). Because deer congregate in matriarchal groups with dispersal of males at adolescence (9), CWD may follow transmission patterns aligning within kin lines. These relationships require clarification to explain the dynamics of CWD in natural populations.

We developed a set of molecular genealogy reconstruction tools to analyze family relationships in deer from areas of high CWD prevalence. Genotypes from 38 nuclear microsatellite loci and mitochondrial sequences from 135 white-tailed deer were analyzed using statistical genetic algorithms to reconstruct genealogical relationships. Samples included 133 deer from a previously described CWD-infected semi-free-ranging herd (7) and 2 additional deer not previously described from the herd. Kin groups inferred from DNA analyses were assessed for association of CWD pathology and relatedness.

Sequence analysis of 418 bp of the 5′-peripheral domain of the mitochondrial control region extending from the *tRNA^{Pro}* gene through *TAS1* bordering the central conserved region (10) was performed. Sequences were confirmed using Sequencher software (Gene Codes Corporation, Ver. 4.7; Ann Arbor, Michigan, USA) then aligned. Haplotypes were characterized with TCS (Version 1.21). Variation at 48 single nucleotide sites and 1 insertion site of the mitochondrial control region revealed 19 haplotypes (GenBank accession numbers EF644627-EF644645), including 11 haplotypes each containing at least 2 individuals. There was no evidence of nuclear copies of the control region: replicate sequences from DNA yielded identical sequences. There was a single base pair insertion at position 21 in Haplotypes 2 and 16.

Thirty-eight microsatellite loci were polymerase chain reaction (PCR) amplified and included a panel of 18 loci routinely used for white-tailed deer parentage analysis *(ADCYC, AGLA226, BL42, BM203, BM4107, BM4208, BM6438, BM6506, CERVID1, CERVID2, CP026, ETH152, FCB193, JP15, JP38, B9, SRCRSP1,* and *TGLA94)* (11,12–18). These loci were augmented by 21 loci previously developed for mule deer (19), designated as *A, B, C, D, E, F, G, H, J, K, L, M, N, O, P, Q, R, S, T, V,* and *D1* and optimized in our laboratory for white-tailed deer. Products from PCR were electrophoretically separated with a 3730 DNA Analyzer (Applied Biosystems, Foster City, California, USA), then visualized and scored. Of the 38 microsatellite loci tested, 28 amplified consistently, conformed to Hardy-Weinberg and linkage equilibrium expectations, and displayed $< 10\%$ probability of null alleles (*ADCYC, AGLA226, BL42, BM4107, CERVID1, CERVID2, ETH152, FCB193, GNZ106, SRCRSP1, TGLA94, A, C, E, F, H, J, K, L, M, O, P, Q, R, S, T V,* and *D1)*. The *PRNP* locus did not deviate from expectations of linkage equilibrium with the microsatellite loci. Deer did not exhibit evidence of inbreeding or population substructure: $H_E = 0.71$, $F_{IS} = -0.0007$, average relatedness (standard deviation), R = 0.03 (0.06).

Of all possible pairs of deer, 47 dyads qualified as first order relatives using a maximum likelihood relatedness analysis of microsatellite and mitochondrial DNA data and yielded 24 parent-offspring and 23 full sib pairs (Table I). Four kin-groups containing at least 3 first-order relatives were identified and matched with qualifying second-order relatives of the same mitochondrial haplotype (Figure 1A–D; note that only a subset of the parent-offspring and full sib dyads of Table I are shown in the figure). Of the 47 dyads of first-order relatives, 14 were co-infected with CWD (9 parent-offspring dyads and 5 full sibling dyads; Table I). Four of the 19 mitochondrial haplotype groups contained at least 4 adult females; 1 of these 4 haplotype groups displayed a greater than expected proportion of CWD-positive adult females (91%; versus prevalence of 75% for female adults tested in this study). The 2 mitochondrial haplotype groups that contained at least 4 adult males had CWD proportions of 47% and 50% (as compared with 40% for all male adults in this study). No haplotypes contained at least 4 fawns.

Spatial pattern of kinship, likely a key factor influencing CWD dynamics in free-ranging deer populations, is critically important to understanding the ecology of CWD. These molecular tools will aid in establishing control programs to reduce risk of CWD transmission. Future molecular genetic work to address these factors across scales of population densities will clarify the patterns and mechanisms of chronic wasting disease in deer.

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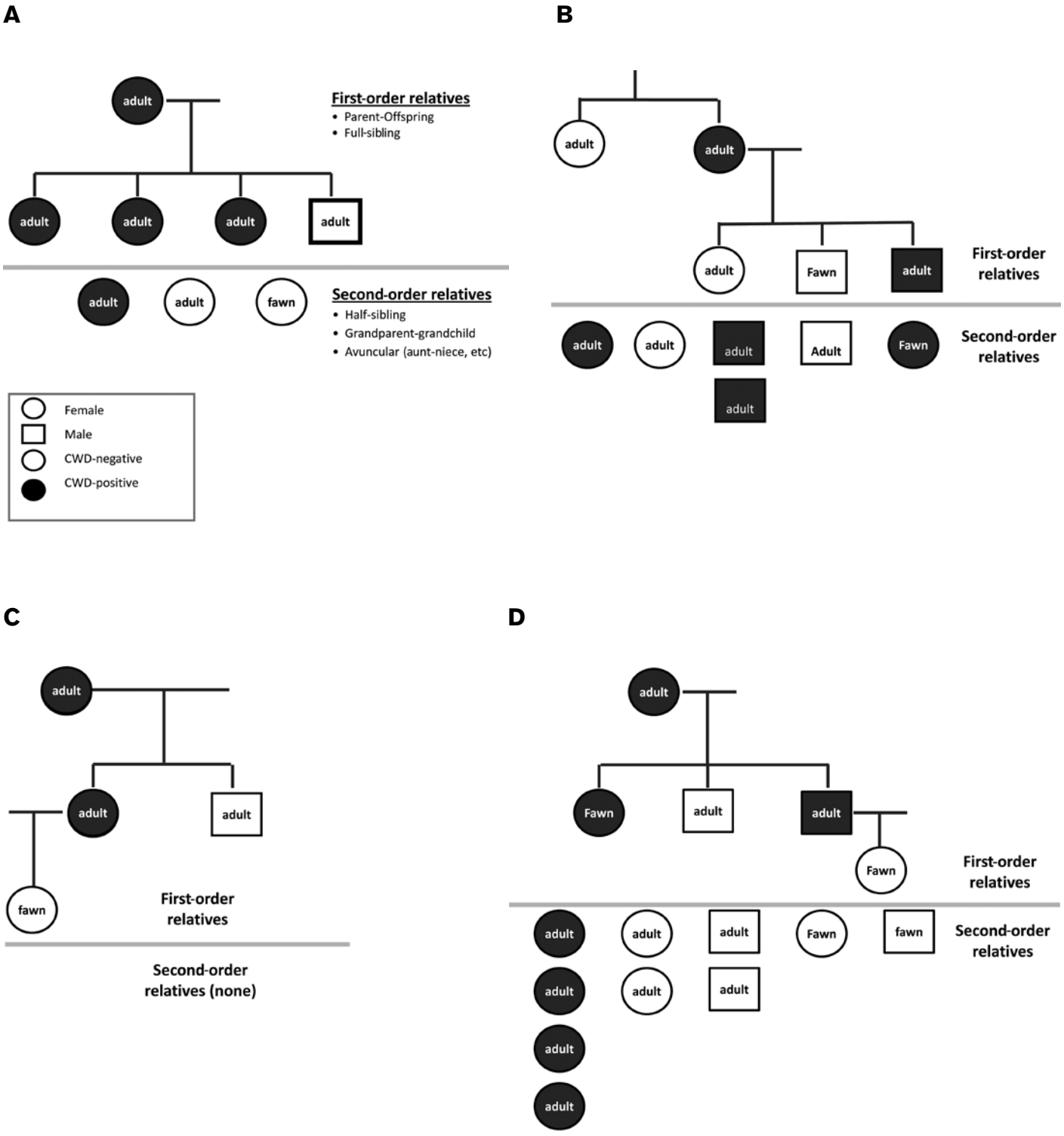

Figure 1. A, B, C, and D. Four reconstructed pedigrees with at least 3 first-order relatives (parent-offspring or full siblings). Individuals above the gray line represent first-order relatives. Parent-offspring relationships depicted by vertical lines. Full siblings are represented by individuals connected by horizontal lines. Individuals below the gray line correspond to matrilineal second-order relatives (half siblings, aunt-niece, etc.) as determined by shared mitochondrial haplotypes and kinship analysis of genotypes from 28 microsatellites. Filled symbols represent individuals testing CWD positive; empty symbols are CWD-negative individuals. Circles are females and squares are males. Note that not all dyads listed in Table I are represented in the figure.

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An experimental model to evaluate *Mycoplasma hyopneumoniae* transmission from asymptomatic carriers to unvaccinated and vaccinated sentinel pigs

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Abstract

The objective of this study was to determine the effect of vaccinating susceptible animals on the transmission of *Mycoplasma hyopneumoniae* from experimentally infected pigs during the chronic phase of infection. Thirty-six seeder pigs were experimentally infected with *M. hyopneumoniae*. Eighty and 200 d post-infection (dpi) 18 seeder pigs were placed in direct contact with 15 vaccinated and 15 unvaccinated age-matched naïve animals. Direct animal contact occurred over 14 d. Pigs were euthanized at the end of the contact period and bronchial swabs were collected and lung tissue examined. At 94 dpi, 15 out of 15 unvaccinated sentinels and 14 out of 15 vaccinated sentinels tested positive for *M. hyopneumoniae* by nested polymerase chain reaction (N-PCR). At 214 dpi, *M. hyopneumoniae* DNA was detected by PCR in 8 out of 15 unvaccinated and 6 out of 15 vaccinated sentinels. Vaccination against *M. hyopneumoniae* did not prevent colonization of sentinels in contact with infected animals. Transmission of *M. hyopneumoniae* from asymptomatic carriers to unvaccinated and vaccinated sentinels was not different.

Résumé

L'objectif de la présente étude était de déterminer l'effet de la vaccination d'animaux susceptibles sur la transmission de Mycoplasma hyopneumoniae *durant la phase chronique de l'infection à partir d'animaux infectés expérimentalement. Trente-six porcs excréteurs ont été infectés expérimentalement avec* M. hyopneumoniae. *Quatre-vingts et 200 j post-infection (dpi), 18 porcs excréteurs ont été mis en contact direct avec des animaux naïfs d'âge correspondant, soit 15 porcs vaccinés et 15 porcs non-vaccinés. Les contacts directs entre animaux se sont produits sur une période de 14 j. Les porcs ont été euthanasiés à la fin de la période de contact et des écouvillons bronchiaux ont été prélevés et les poumons examinés. Au jour 94 dpi, les 15 animaux sentinelles non-vaccinés et 14 des 15 animaux vaccinés se sont révélés positifs à* M. hyopneumoniae *par réaction d'amplification en chaîne par la polymérase (PCR). Au jour 214 dpi, l'ADN de* M. hyopneumoniae *a été détecté par PCR chez 8 des 15 porcs non-vaccinés et chez 6 des 15 porcs vaccinés. La vaccination contre* M. hyopneumoniae *n'a pas empêché la colonisation des animaux sentinelles en contact avec les animaux infectés. Il n'y avait pas de différence dans la transmission de* M. hyopneumoniae *des porteurs asymptomatiques à des animaux vaccinés ou non-vaccinés.*

(Traduit par Docteur Serge Messier)

Mycoplasma hyopneumoniae is the causative agent of Enzootic pneumonia (1–2), a disease that is prevalent in every country where pigs are raised (3–5). The economic impact of *M. hyopneumoniae* infections on the swine industry is a composite of the direct effect of infection and a high incidence of secondary respiratory infections (6).

Mycoplasma hyopneumoniae causes chronic respiratory disease characterized by growth retardation and a dry non-productive cough when uncomplicated by other agents (7). The cough initiates approximately 10 to 16 d after experimental infection and ceases within 2 mo (8). Pig-to-pig transmission by direct contact is one of the most common routes of *M. hyopneumoniae* infection (9). Pigs infected with *M. hyopneumoniae* transmit the bacteria to susceptible animals during the acute (10) and chronic phases of infection (11). One of the most important features of *M. hyopneumoniae* infection is the persistence of bacteria within the respiratory tract of the pig for long periods of time and that persistently infected animals become asymptomatic carriers capable of infecting susceptible pigs (11–12).

Control of *M. hyopneumoniae* infections involves the application of 1 or several strategies (6). Intervention strategies are used in order to avoid direct contact between different groups, to decrease the amount of bacteria shed by infected animals, or to protect susceptible animals. Commercial *M. hyopneumoniae* bacterins are often used as means of protection against disease for uninfected animals, although vaccination has not been reported to prevent colonization (10,13). A comparison between the transmission of *M. hyopneumoniae* from infected to susceptible unvaccinated or vaccinated pigs during the acute phase of infection demonstrated that the vaccine did not change the transmission rate of the bacteria among the 2 groups (14).

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Table I. Experimental design for the evaluation *Mycoplasma hyopneumoniae* transmission from asymptomatic carriers to unvaccinated and vaccinated populations

Days post-infection	Study activity	Experimental group
0	Experimental infection	Seeders[a] (n = 36)
80 to 94	Transmission assessment 1	Seeders (n = 9) + Unvaccinated sentinels[b] (n = 15)
		Seeders (n = 9) + Vaccinated sentinels[c] (n = 15)
200 to 214	Transmission assessment 2	Seeders (n = 9) + Unvaccinated sentinels (n = 15)
		Seeders (n = 9) + Vaccinated sentinels (n = 15)

[a] Gilts experimentally infected with *M. hyopneumoniae* strain 232.

[b] *M. hyopneumoniae* negative and unvaccinated gilts.

[c] *M. hyopneumoniae* negative gilts, vaccinated with a commercial bacterin 15 and 30 d prior to the first day of the transmission experiment.

Sentinels were age-matched with seeders.

However, the effect of vaccination of susceptible pigs on transmission during the chronic phase of infection has not been investigated. Our hypothesis is that vaccination of replacement gilts does not prevent *M. hyopneumoniae* colonization of susceptible pigs after contact with asymptomatic carriers. Therefore, the objective of this study was to determine the effect of vaccination of susceptible animals on the transmission of *M. hyopneumoniae* from experimentally infected pigs during the chronic phase of infection.

To test our hypothesis, pigs were obtained from a source known to be negative to *M. hyopneumoniae* and porcine respiratory and reproductive syndrome virus. The experiments were performed at the University of Minnesota Swine Disease Eradication Center Research Farm (Appleton, Minnesota, USA) and animals were cared for according to the guidelines of the Institutional Animal Care and Committee, University of Minnesota. A total of 96 pigs were used in this study. Animals were distributed into 3 experimental groups. Group 1 were seeder pigs (n = 36), 15-week-old female pigs experimentally infected with *M. hyopneumoniae*. Group 2 were unvaccinated sentinels (n = 30), age and sex-matched with seeders, naïve to *M. hyopneumoniae*. Group 3 were vaccinated sentinels (n = 30), age and sex-matched with seeders.

The experimental design for this research is presented in Table I. The study consisted of the experimental infection of seeders with *M. hyopneumoniae* on day 0. Pigs were intra-tracheally inoculated with a lung homogenate suspension containing 1×10^5 color-changing units of *M. hyopneumoniae* strain 232 (obtained from Iowa State University, Ames, Iowa USA). Vaccinated sentinels were injected twice (15 and 30 d prior to contact with seeders) with 2 mL of a commercial bacterin for *M. hyopneumoniae* (RespiSure; Pfizer, New York, New York, USA).

At 80 and 200 d post-infection (dpi) a subset of 18 seeders was moved to a different barn, where they were placed in direct contact with 15 vaccinated and 15 unvaccinated sentinels. Direct contact between seeders and vaccinated and unvaccinated sentinels was only allowed during the 2 14-day transmission assessments. The first transmission assessment went from 80 to 94 dpi and a second one from 200 to 214 dpi. Each transmission assessment group was divided into 2 subgroups. Subgroup 1 was made up of 9 seeders and 15 unvaccinated sentinels and subgroup 2 was made up of 9 seeders and 15 vaccinated sentinels. Subgroups were physically separated. Group size was estimated based on a previously described algorithm (15).

A subgroup of seeders was monitored for confirmation of infection during the acute phase of infection. Nasal swabs were collected at 0, 4, 7, 11, 13, 18, 25, and 36 dpi for this purpose. Clinical signs in the groups were evaluated by daily observation.

Serum samples were collected from all seeders at 0, 34, and 60 dpi, and from seeders and sentinels at the beginning and at the end of the transmission assessments (80 to 94 and 200 to 214 dpi). Serum samples were analyzed for *M. hyopneumoniae* antibodies using a commercially available enzyme-linked immunosorbent assay (ELISA) kit (DAKO; DakoCytomation California, Carpinteria, California, USA), following manufacturer's instructions.

Bronchial swabs were collected from seeders and from all sentinels at the end of the transmission assessments (94 and 214 dpi). Swabs were evaluated for the presence of a region of the *M. hyopneumoniae* 16S gene using a nested-polymerase chain reaction (PCR) assay (16).

Gross and microscopic lung lesions were evaluated in seeders and sentinels at the end of the transmission assessments (94 and 214 dpi). Gross lesions suggestive of *M. hyopneumoniae* infection were scored based on the proportion of lung tissue involved in the pneumonic process (17). Lung samples were obtained from macroscopic lesions and from a consistent location in pigs without visible lesions. Tissues were fixed, stained with hematoxylin and eosin, and processed for microscopic examination.

All animals used for this study were negative to *M. hyopneumoniae* upon arrival at the facilities, as confirmed by nested-PCR from nasal swabs and the lack of clinical signs and antibodies to *M. hyopneumoniae*. The successful infection of seeders was confirmed by detection of *M. hyopneumoniae* DNA, antibodies, and clinical signs (such as coughing in the group). Thirty-nine percent of the experimentally infected animals seroconverted to *M. hyopneumoniae* by 34 dpi, while the entire group was positive by 60 dpi. *Mycoplasma hyopneumoniae* DNA from nasal swabs was detected at 13, 18, 25, and 34 dpi in a subset of seeders. A non-productive cough was detected in the seeder pigs' barn from 11 until 68 dpi (data not shown).

Detection of *M. hyopneumoniae* in all experimental groups during the transmission assessments is presented in Table II. During the first transmission assessment (80 to 94 dpi) 17 out of 18 seeders had detectable antibodies to *M. hyopneumoniae*, as evaluated at 80 and 94 dpi. *Mycoplasma hyopneumoniae* DNA was detected in bronchial swabs of 18 out of 18 animals at 94 dpi. No unvaccinated sentinels

Table II. Detection of *Mycoplasma hyopneumoniae* in experimental pigs during the transmission assessments

	Transmission assessment 1				Transmission assessment 2			
Experimental groups	Antibody detection[a] 80 dpi	Antibody detection 94 dpi	DNA detection[b] 94 dpi	Lung lesions[c] 94 dpi	Antibody detection 200 dpi	Antibody detection 214 dpi	DNA detection 214 dpi	Lung lesions 214 dpi
Seeder pigs	17/18[d]	17/18	18/18	7/18	1/18	4/18	11/18	0/18
Unvaccinated sentinels	0/15	0/15	15/15	6/15	0/15	0/15	8/15	0/15
Vaccinated sentinels	15/15	15/15	14/15	8/15	15/15	15/15	6/15	1/15

[a] DAKO ELISA.

[b] Nested-PCR from bronchial swabs.

[c] Gross and microscopic lesions.

[d] Number of pigs positive/number of pigs tested.

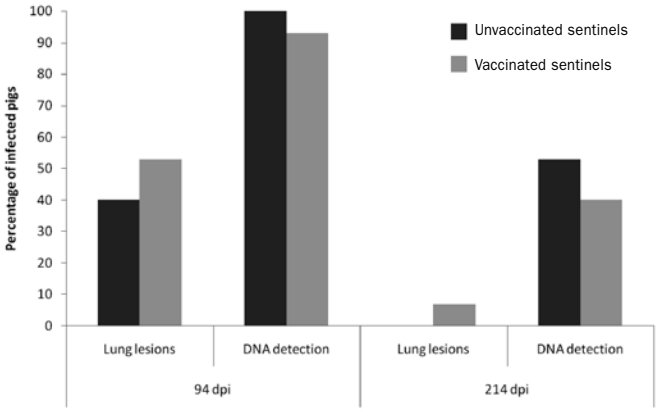

Figure 1. Proportion of *Mycoplasma hyopneumoniae* infected sentinels at 94 and 214 dpi (transmission assessments 1 and 2). *Mycoplasma hyopneumoniae* DNA was detected by nested-PCR from bronchial swabs. Gross lung lesions associated with *M. hyopneumoniae* infection were confirmed by histopathology. The proportion of unvaccinated and vaccinated sentinels in which *M. hyopneumoniae* DNA was detected was not statistically different ($P > 0.05$) at either 94 or 214 dpi. The proportion of unvaccinated and vaccinated sentinels in which lung lesions were detected at either 94 or 214 dpi was similar ($P > 0.05$).

(0 out of 15) had antibodies to *M. hyopneumoniae* at 80 and 94 dpi, while *M. hyopneumoniae* DNA was detected in 15 out of 15 bronchial swabs and gross lesions were observed in 6 out of 15 of those animals. All 15 vaccinated sentinels had detectable antibodies to *M. hyopneumoniae* at 80 dpi, while 10 out of 15 had antibodies at 94 dpi. *Mycoplasma hyopneumoniae* DNA was detected in bronchial swabs and lung lesions in 14 out of 15 and 8 out of 15 vaccinated sentinels, respectively, as evaluated at 94 dpi. The proportion of unvaccinated and vaccinated sentinels infected with *M. hyopneumoniae* at 94 dpi by nested-PCR and the proportion of lung lesions is shown in Figure 1. Statistical comparison of the proportion of infected animals, obtained by using the Fisher's exact test, did not show a significant difference ($P > 0.05$). The proportion of lung lesions in animals was similar ($P > 0.05$).

At the second transmission assessment, 1 out of the 18 seeders had antibodies to *M. hyopneumoniae* at 200 dpi, while 4 out of 18 seeders had antibodies at 214 dpi. *Mycoplasma hyopneumoniae* DNA was detected in 11 out of 18 animals using bronchial swabs at 214 dpi. Unvaccinated sentinels did not have detectable antibodies to *M. hyopneumoniae* at 200 or 214 dpi. *Mycoplasma hyopneumoniae* DNA was detected in 8 out of 15 unvaccinated sentinels using bronchial swabs at 214 dpi. Lung lesions were not observed in the group. All vaccinated sentinels had detectable antibodies to *M. hyopneumoniae* at 200 dpi and 214 dpi. *Mycoplasma hyopneumoniae* DNA was detected in 6 out of 15 vaccinated pigs using bronchial swabs at 214 dpi. One animal presented gross lesions suggestive of *M. hyopneumoniae* infection at 214 dpi. A comparison of the proportion of infected sentinels (unvaccinated and vaccinated) and proportions of lung lesions demonstrated no significant differences, $P > 0.05$. The proportion of infected animals and lung lesions are shown in Figure 1.

Vaccination of replacement gilts with *M. hyopneumoniae* bacterins is a common practice in *M. hyopneumoniae* infected herds. The purpose of the vaccination is to decrease gilt mortality, lung lesions, and clinical signs in the incoming animals, and, ultimately, to decrease the transmission of the pathogen from infected to naïve animals. However, *M. hyopneumoniae* vaccination fails to protect naïve animals from colonization (13,14). It should be noted that experimental studies supporting these results have been performed during the acute phase of *M. hyopneumoniae* infection. Therefore, the objective of this study was to determine the effect of vaccination of susceptible animals on the transmission of *M. hyopneumoniae* from experimentally infected pigs during the chronic phase of infection.

In this study, *M. hyopneumoniae* transmission from asymptomatic carriers to sentinels was examined at either 94 or 214 dpi. This is the first time that a comparison of transmission between unvaccinated and vaccinated populations was performed using asymptomatic carriers as seeder pigs. Asymptomatic carriers have been demonstrated to be infected with *M. hyopneumoniae* for periods of up to 214 dpi (11).

Under the conditions of this study, the proportion of vaccinated replacement gilts infected from the seeder pigs differed numerically, but not statistically, from the proportion of unvaccinated gilts that became infected. These results were similar during the 2 transmission assessments. Another investigation, in which transmission of *M. hyopneumoniae* from experimentally infected pigs to vaccinated and unvaccinated populations was quantified, failed to demonstrate significant differences in the transmission rates (14). However, the study only looked at the transmission rate during the acute phase of infection.

The proportion of lungs with lesions suggestive of *M. hyopneumoniae* infection was numerically higher in the vaccinated sentinels than in the unvaccinated ones at the conclusion

of the 2 transmission assessments. These results are contrary to reports that *M. hyopneumoniae* vaccination reduces lung lesions (6,13). Nevertheless, vaccine efficacy and lung lesion reduction is often evaluated after extended periods of time following vaccination (approximately 3.5 mo). Here, lung lesions were evaluated 14 d after the first contact with the infective animals. This short time period may be a factor in conflicting results. It could be hypothesized that vaccination exacerbates the inflammatory response in animals after a very recent infection, but lung lesions in vaccinated animals heal sooner than in the unvaccinated ones. It is important to note that this study did not evaluate production parameters, such as average daily gain and feed conversion ratio, which are usually improved in infected vaccinated animals when compared with unvaccinated ones (6,13,18,19).

The original design for this research included the quantification of *M. hyopneumoniae* transmission by calculating the basic reproductive ratio (R_0) (20) and comparing it between 2 populations. However, R_0 could not be estimated for the 2 populations in this study, since certain assumptions for the model did not apply to the study population. Namely, at least 1 animal (sentinel) would have to be negative at the end of the contact period, which did not occur in the 1st transmission assessment and all infected animals would have to remain infective during the contact period, which did not occur during the 2nd transmission assessment.

One limitation of this study was the fact that the length of the transmission assessments (14 d each) did not allow for either seroconversion of the infected animals (unvaccinated) or for onset of clinical signs in any newly infected pigs. Therefore, animals were classified as infected using the parallel interpretation, based on detection of *M. hyopneumoniae* DNA or lung lesions due to *M. hyopneumoniae.* In all cases, animals with mycoplasmal lesions contained *M. hyopneumoniae* DNA, as detected by nested-PCR.

In conclusion, vaccination of replacement gilts did not prevent colonization with *M. hyopneumoniae* of sentinels in contact with asymptomatically infected animals. Moreover, transmission of *M. hyopneumoniae* did not differ between the unvaccinated and vaccinated pigs, demonstrating that vaccination with bacterins does not protect incoming animals from becoming infected. Therefore, different control strategies or a combination of several interventions need to be evaluated in order to develop more effective approaches for protecting naïve animals that are incorporated into herds infected with *M. hyopneumoniae.* Ultimately, new vaccine technologies should be used to develop commercial products that provide better protection against *M. hyopneumoniae.*

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