Articles

Farm-level prevalence and risk factors for detection of hepatitis E virus, porcine enteric calicivirus, and rotavirus in Canadian finisher pigs
Barbara Wilhelm, Danielle Leblanc, David Leger, Sheryl Gow, Anne Deckert, David L. Pearl, Robert Friendship, Andrijana Rajic, Alain Houde, Scott McEwen

Genetic diversity of Streptococcus suis serotype 2 isolated from pigs in Brazil
Daniela Sabatini Doto, Luisa Zanolli Moreno, Franco Ferraro Calderaro, Carlos Emilio Cabrera Matajira, Vasco Tulio de Moura Gomes, Thais Sebastiania Porfida Ferreira, Renan Elias Mesquita, Jorge Timenetsky, Marcelo Gottschalk, Andrea Micke Moreno

Comparison of 2 commercial single-dose Mycoplasma hyopneumoniae vaccines and porcine reproductive and respiratory syndrome virus (PRRSV) vaccines on pigs dually infected with M. hyopneumoniae and PRRSV
Changhoon Park, Ikjae Kang, Hwi Won Seo, Jiwoon Jeong, Kyuhyung Choi, Chanhee Chae

Safety and early onset of immunity with a novel European porcine reproductive and respiratory syndrome virus vaccine in young piglets
Michael Piontkowski, Jeremy Kroll, Christian Kraft, Teresa Coll

Development of a double-monoclonal antibody sandwich ELISA: Tool for chicken interferon-γ detection ex vivo
Hua Dai, Zheng-zhong Xu, Meiling Wang, Jun-hua Chen, Xiang Chen, Zhi-ming Pan, Xin-an Jiao

Effects of ketamine and lidocaine in combination on the sevoflurane minimum alveolar concentration in alpacas
Patricia Queiroz-Williams, Thomas J. Doherty, Anderson F. da Cunha, Claudia Leonardi

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Farm-level prevalence and risk factors for detection of hepatitis E virus, porcine enteric calicivirus, and rotavirus in Canadian finisher pigs

Barbara Wilhelm, Danielle Leblanc, David Leger, Sheryl Gow, Anne Deckert, David L. Pearl, Robert Friendship, Andrijana Raji´c, Alain Houde, Scott McEwen

Abstract

Hepatitis E virus (HEV), norovirus (NoV), and rotavirus (RV) are all hypothesized to infect humans zoonotically through swine and pork. Our study objectives were to estimate Canadian farm-level prevalence of HEV, NoV [specifically porcine enteric calicivirus (PEC)], and RV in finisher pigs, and to study risk factors for farm level viral detection. Farms were recruited using the Canadian Integrated Program for Antimicrobial Resistance Surveillance (CIPARS) and FoodNet Canada on-farm sampling platforms. Six pooled groups of fecal samples were collected from participating farms, and a questionnaire capturing farm management and biosecurity practices was completed. Samples were assayed using validated real-time polymerase chain reaction (RT-PCR). We modeled predictors for farm level viral RNA detection using logistic and exact logistic regression.

Seventy-two herds were sampled: 51 CIPARS herds (15 sampled twice) and 21 FoodNet Canada herds (one sampled twice). Hepatitis E virus was detected in 30/88 farms [34.1% (95% CI 25.0%, 44.5%)]; PEC in 18 [20.5% (95% CI: 13.4%, 30.0%)], and RV in 6 farms [6.8% (95% CI: 3.2%, 14.1%)]. Farm-level prevalence of viruses varied with province and sampling platform. Requiring shower-in and providing boots for visitors were significant predictors ($P < 0.05$) in single fixed effect mixed logistic regression analysis for detection of HEV and PEC, respectively. In contrast, all RV positive farms provided boots and coveralls, and 5 of 6 farms required shower-in. We hypothesized that these biosecurity measures delayed the mean age of RV infection, resulting in an association with RV detection in finishers. Obtaining feeder pigs from multiple sources was consistently associated with greater odds of detecting each virus.

Résumé

Le virus de l’hépatite E (VHE), le norovirus (NoV), et le rotavirus (RV) sont tous suspectés être des agents zoonotiques associés à une exposition aux porcs ou à la viande de porc. Les objectifs de la présente étude étaient d’estimer, dans des fermes canadiennes, la prévalence de VHE, NoV [spécifiquement le calicivirus entérique porcin (CEP)], et le RV chez des porcs en finition, et d’étudier les facteurs de risque pour la détection virale à la ferme. Les fermes ont été recrutées à l’aide des plateformes d’échantillonnage à la ferme du Programme intégré canadien de surveillance de la résistance aux antimicrobiens (PICRA) et de FoodNet Canada. Six groupes d’échantillons amalgamés de matières fécales ont été récoltés dans les fermes participantes, et un questionnaire relevant les pratiques de gestion à la ferme et les mesures de biosécurité a été complété. Les échantillons ont été analysés au moyen d’une méthode validée de réaction d’amplification en chaîne par la polymérase en temps réel (RT-PCR). Des prédicteurs de détection de l’ARN viral sur la ferme ont été modélisés à l’aide de régressions logistiques et de régressions logistiques exactes. Soixante-douze troupeaux ont été échantillonnés : 51 troupeaux du programme CIPARS (15 troupeaux échantillonnés deux fois) et 21 troupeau du programme FoodNet Canada (un troupeau échantillonné deux fois). Le VHE a été détecté dans 30/88 fermes [34,1 % (IC 95 %: 25,0 %, 44,5 %)], CEP dans 18 [20,5 % (IC 95 %: 13,4 %, 30,0 %)], et RV dans 6 fermes [6,8 % (IC 95 %: 3,2 %, 14,1 %)]. La prévalence des virus dans les fermes variait selon la province et la plate-forme d’échantillonnage. Une douche obligatoire avant l’entrée dans la porcherie et le fait de fournir des bottes aux visiteurs s’avéraient des prédicteurs significatifs ($P < 0.05$) pour la détection du VHE et du CEP, respectivement, dans une analyse par régression logistique mixte à effet fixe unique. Ceci contrastait avec le fait que toutes les fermes positives pour RV fournissaient des bottes et des couvre-tout, et 5 des 6 fermes exigéaient une douche à l’entrée. Nous émettons l’hypothèse que ces mesures de biosécurité ont retardé l’âge moyen d’une infection par le RV, ce qui résultait en une association entre la détection de RV et les animaux en finition. L’acquisition de porcs en croissance de sources multiples était constamment associée avec une probabilité plus grande de détecter chaque virus.
**Introduction**

The RNA viruses hepatitis E virus (HEV), norovirus (NoV), and rotavirus (RV), have all been hypothesized over the past decade to infect humans zoonotically (1,2,3). A recent scoping review identified a small number of zoonotic cases [HEV \( n = 3 \); NoV \( n = 0 \); RV \( n = 40 \) (zoonoses \( n = 3 \); human-animal re-assortants \( n = 37 \)] categorized as “likely” zoonoses (4). Several researchers have assayed Canadian pigs for detection of HEV: a survey of 70 Quebec farms reported 34% categorized as HEV-infected (5); the prevalence of HEV RNA shedding in 51 close-to-market pigs on one infected farm in Quebec was estimated at 42.2% (6). Hepatitis E virus isolates obtained from 2 locally acquired Canadian Hepatitis E cases in the province of Quebec demonstrated close (> 97% nt) sequence identity between case isolates and those collected from pigs in the same province over part of the ORF2 gene (7). Canadian pig farms have been sampled for NoV, which was detected in 30 of 120 samples from 10 Ontario farms (8), and in Quebec, in which NoV RNA was detected in samples from 4 of 20 farms (9). Rotavirus RNA was detected on 9 of 10 Ontario pig farms (10) and more recently in 9 of 10 Quebec finisher farms (11). A recent Canadian study reported detection of HEV on retail pork livers (12). However, a Canadian national survey of finisher pigs to estimate the prevalence of these viruses has not been conducted to date. Our study objectives, therefore, were to estimate farm-level prevalence of HEV, porcine enteric calicivirus (PEC), and RV in Canadian finisher pigs, and to investigate potential associations between farm level detection and farm management practices.

**Materials and methods**

**Recruitment and sample collection**

Expected farm-level and individual-level prevalence of HEV in finisher pigs were estimated by a meta-analysis (MA) of 12 published North American investigations of HEV detection in fecal samples in pigs aged 4 to 6 mo using the Comprehensive Meta-analysis 2 software (Biostat; Englewood, New Jersey, USA) and the method of moments (13). Hepatitis E virus was used in preference over PEC and RV for sample size calculations, as this was the virus for which we were able to obtain the most comprehensive dataset. Heterogeneity, or across study variation in viral prevalence, was investigated by calculation of Higgins’ \( I^2 \) (14) and \( T^2 \), an estimate of \( r^2 \) or true variance in prevalence across studies (15). Sample size calculations to estimate the number of pooled fecal samples to detect HEV RNA on-farm, using the MA summary estimates of within-farm and farm-level prevalence (desired precision +/- 10%), were conducted in EpiTools (16).

Two sampling platforms were used to recruit pig farms: the Canadian Integrated Program for Antimicrobial Resistance Surveillance (CIPARS) sampling platform for finisher pigs (17), and the FoodNet Canada (FC) sampling platform (8,10,18). The CIPARS maintains a national network of participating veterinarians, each of whom invite client farms to participate in the CIPARS program by completing annual questionnaires regarding management practices and herd performance, and allowing collection of pooled fecal samples from finisher pigs (17). For this project, each CIPARS veterinarian was sent an electronic invitation to participate. Those agreeing received a package containing invitation letters for the clients they recruited, and a consent form for the participating farmers. During the farm visit, the CIPARS farm program questionnaire capturing herd-level data on demographics, current management practices, biosecurity status, pig inventory, and herd performance, was completed by the producer with the help of the herd veterinarian. Data volunteered by the farmer were then validated by the veterinarian and entered on the questionnaire. Missing or incomplete data, or those entries falling outside pre-set logical limits, were further investigated by a telephone call or e-mail to the veterinarian when completed questionnaires were received by CIPARS.

Similarly, during a routine visit for FC sample collection, each pig farm participating in the FC sentinel pig farm program was invited to participate. The FC farms were a subset of 100 farms located in a single province, originally selected as a random sample of 5000 pig farms, stratified by size (18). During the farm visit, a questionnaire identical to the CIPARS farm program questionnaire with the exception of the CIPARS antimicrobial use questions, which were not administered to the FC farmers was completed by the producer with the help of the FC sample collection technician.

All fecal samples and questionnaires used farm herd codes as identifiers; the identities of the participating farms were known only to the CIPARS herd veterinarians or the FC program technician. This project received approval from the Research Ethics Board of the University of Guelph.

All sample collection, including those samples collected from FC farms, followed the CIPARS sampling protocol. Briefly, 5 fresh fecal samples were collected into one specimen jar and mixed with a stir stick. One jar was collected from each of 6 close-to-market pens, and shipped by courier to the Public Health Agency of Canada (PHAC) diagnostic laboratory in St. Hyacinthe, Quebec. Samples were then divided, and part of each pooled fecal sample originating from a farm participating in this project was forwarded to the Agriculture and Agri-Food Canada laboratory (AAFC) in St. Hyacinthe, where they were held frozen at -80°C prior to batch processing and testing. Samples from FoodNet Canada farms were stored in a freezer at -80°C until completion of sampling, when they were forwarded by courier to AAFC St. Hyacinthe.

**Viral RNA extraction from feces**

Fecal samples were thawed and a 1 g portion of sample was further diluted 1:5 (p/v) in PBS pH 7.4 (Life Technologies, Burlington, Ontario). After vigorous shaking, mixtures were centrifuged at 4000 × g for 20 min and supernatants were considered as viral suspensions. A volume of 13 μL of SDS 10%, 0.69 μL of proteinase K (20 mg/mL) and 4.8 × 10⁶ PFU of feline calicivirus used as sample process control were added to 130 μL of viral suspension. The mixture was incubated at 37°C for 1 h. The RNA was extracted from 140 μL of the treated viral suspension with QiAampViral RNA mini (Qiagen, Toronto, Ontario) and the robotic workstation system QIAcube for automated sample purification of RNA; the QIAcube manufacturer’s protocol was used. The extracted RNA was recovered in 60 μL volume of AVE solution and frozen at -80°C until further use.
Table I. Description of variables and participating farms

<table>
<thead>
<tr>
<th>Variable</th>
<th>Categories</th>
<th>CIPARS Number of samples (%)</th>
<th>FC Number of samples (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>General and Farm Descriptors</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Herd veterinarian</td>
<td>Unique identifier</td>
<td>n = 17 veterinarians</td>
<td>N = 1 technician</td>
</tr>
<tr>
<td></td>
<td>for each veterinarian</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Herd identifier</td>
<td></td>
<td>n = 51 herds</td>
<td>N = 21 herds</td>
</tr>
<tr>
<td>Number of days sampled</td>
<td></td>
<td>1 = 36 herds</td>
<td>1 = 20 herds</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2 = 15 herds</td>
<td>2 = 1 herd</td>
</tr>
<tr>
<td>Province</td>
<td></td>
<td>108 samples, 18 herds</td>
<td>132 samples, 22 herds</td>
</tr>
<tr>
<td></td>
<td>Province A</td>
<td>72 samples, 12 herds</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Province B</td>
<td>114 samples, 19 herds</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Province C</td>
<td>102 samples, 17 herds</td>
<td></td>
</tr>
<tr>
<td>Production system used</td>
<td>Continuous</td>
<td>192 (48.5%)</td>
<td>96 (72.8%)</td>
</tr>
<tr>
<td></td>
<td>All-in-all-out</td>
<td>204 (51.5%)</td>
<td>18 (13.6%)</td>
</tr>
<tr>
<td></td>
<td>Missing observations</td>
<td>18 (13.6%)</td>
<td></td>
</tr>
<tr>
<td>Month collected</td>
<td>January</td>
<td>18</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>February</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>March</td>
<td>48</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>April</td>
<td>12</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>May</td>
<td>12</td>
<td>0</td>
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<tr>
<td></td>
<td>June</td>
<td>12</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>July</td>
<td>18</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>August</td>
<td>48</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>September</td>
<td>42</td>
<td>36</td>
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<td></td>
<td>October</td>
<td>36</td>
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<td>November</td>
<td>96</td>
<td>54</td>
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<tr>
<td></td>
<td>December</td>
<td>48</td>
<td>12</td>
</tr>
<tr>
<td>Season</td>
<td>Winter</td>
<td>72 (18.2%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td></td>
<td>Spring</td>
<td>36 (9.1%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td></td>
<td>Summer</td>
<td>108 (27.3%)</td>
<td>36 (27.3%)</td>
</tr>
<tr>
<td></td>
<td>Fall</td>
<td>180 (45.5%)</td>
<td>96 (72.7%)</td>
</tr>
<tr>
<td>Finishers onsite</td>
<td>0–2000</td>
<td>240 (60.6%)</td>
<td>120 (90.9%)</td>
</tr>
<tr>
<td></td>
<td>2001–4000</td>
<td>150 (37.9%)</td>
<td>12 (9.1%)</td>
</tr>
<tr>
<td></td>
<td>$\geq$ 4000</td>
<td>6 (1.5%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td></td>
<td>Mean (SD)</td>
<td>1706 (SD 1172)</td>
<td>810 (SD 1175)</td>
</tr>
<tr>
<td>Finishers onsite, numerical value</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Biosecurity predictors</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Multiple source</td>
<td>No</td>
<td>342 (86.4%)</td>
<td>114 (86.4%)</td>
</tr>
<tr>
<td></td>
<td>Yes</td>
<td>54 (13.6%)</td>
<td>6 (54.5%)</td>
</tr>
<tr>
<td></td>
<td>Not captured</td>
<td>0 (0%)</td>
<td>12 (9.1%)</td>
</tr>
<tr>
<td>Boots provided</td>
<td>No</td>
<td>66 (16.7%)</td>
<td>6 (4.5%)</td>
</tr>
<tr>
<td></td>
<td>Yes</td>
<td>330 (72.3%)</td>
<td>114 (86.4%)</td>
</tr>
<tr>
<td></td>
<td>Not captured</td>
<td>0 (0%)</td>
<td>12 (9.1%)</td>
</tr>
<tr>
<td>Coveralls provided</td>
<td>No</td>
<td>72 (18.2%)</td>
<td>48 (36.4%)</td>
</tr>
<tr>
<td></td>
<td>Yes</td>
<td>324 (81.8%)</td>
<td>66 (16.7%)</td>
</tr>
<tr>
<td></td>
<td>Not captured</td>
<td>0 (0%)</td>
<td>18 (13.6%)</td>
</tr>
</tbody>
</table>
Viral RNA detection

Detection of HEV, PEC, and RV was done using the quantitative real-time polymerase chain reaction (qRT-PCR) detection systems previously described (12) adapted in Brilliant II RT-PCR Core reagent kit, 1-Step (Agilent Technologies Canada, Mississauga, Ontario). Briefly, a sample of 2.5 μL of RNA template was used in a final volume of 25 μL of core RT-PCR buffer containing 2.5 μL of MgCl₂, 1.0 μL of dNTP, 1.25 μL of bovine serum albumin at 20 mg/mL, each of the forward and reverse primers and the TaqMan probe, 1 μL of the reference dye diluted 1:500, 1 μL of reverse transcriptase diluted 1:10, and 1.25U of SureStart DNA polymerase (Qiagen). Porcine enteric calicivirus, NoV G II, and RV were detected in a monoplex assay and HEV/FCV in duplex assay.

Viral quantification

Extracted nucleic acids showing amplification inhibition in the first detection step were treated with OneStep PCR Inhibitor Removal kit (Zymo Research, Irvine, California, USA) before retesting. Addition of 2 μL of Hepatitis A virus RNA (3.98 × 10⁵ Tissue culture infectious dose) to each assay was used as amplification control. For each detection system, a calibration curve was generated using 10-fold serial dilutions of the plasmid constructs to the corresponding virus in a 5 ng/mL salmon sperm solution. Arithmetic mean of the forward and reverse primers and the TaqMan probe, 1 μL of the reference dye diluted 1:500, 1 μL of reverse transcriptase diluted 1:10, and 1.25U of SureStart TaqDNA polymerase (Qiagen). Porcine enteric calicivirus, NoV G II, and RV were detected in a monoplex assay and HEV/FCV in duplex assay.

Data storage, cleaning, and analysis

Data pertaining to detection of viral RNA in samples, and questionnaire responses from each participating farm were captured in Excel spreadsheets (Microsoft, Mississauga, Ontario) and forwarded to a central repository. Data were cleaned in Excel and exported to Stata IC 13 (StataCorp, College Station, Texas, USA) for descriptive statistics and modeling. Missing data received their own unique code. However, we found that a small group (n = 3) of FC respondents had not answered the entire group of biosecurity questions (Table I). Therefore, we deleted this subset of farms from the “biosecurity” variable analyses. We checked the correlation among predictor variables to avoid issues associated with collinearity. Where collinear variables were eligible for inclusion in the full model, the variable with the most complete set of observations was selected.

In assessing the effect of a predictor variable in univariable analysis, we included random intercepts for herd and sampling event using the “xtmelogit” command (19) in Stata 13 to account for the hierarchical data structure. We estimated the proportion of variance occurring at each level of the model, assuming that the lowest level variance on the logit scale was π²/3, based on use of the latent variable technique (20). All variables for which P < 0.20 in univariable analysis were considered for inclusion in the multivariable model. However, where full multivariable models failed to converge, precluding multivariable analysis, only single fixed effect mixed models are presented.

For sparse datasets exact logistic regression was employed, with farm-level detection of viral RNA as the outcome of interest, and a farm was categorized “positive” if viral RNA were detected in one or more of 6 pooled samples collected on-farm.

Results

Meta-analysis of 12 published North American surveys of finisher swine yielded a summary estimate of within-farm HEV prevalence in finisher pigs of 23.0% [95% confidence interval (95% CI): 13.2%, 35.8%] with Higgins’ I² value of 89.3% and T² of 1.18, suggesting marked heterogeneity across studies. Calculation of the number of pooled samples (consisting of 5 samples from individual animals) required to demonstrate freedom from viral presence, using the meta-analysis estimate of 23.0% prevalence on-farm, confirmed that only 3 pooled samples would be sufficient to identify freedom from disease. The standard operating procedure of the CIPARS on-farm swine surveillance system (i.e., collection of 6 pooled samples per farm), was adopted for the purposes of this study, on all participating farms including the FC farms, given the heterogeneity in the dataset supporting the MA estimate of prevalence. Meta-analysis of 5 published North American surveys of finisher pigs yielded a summary estimate of farm-level HEV prevalence in finisher pigs of 30.0% (95% CI: 14.7%, 51.6%) with Higgins’ I² value of 71.9% and T²
Table II. Prevalence and mean load of hepatitis E virus, porcine enteric calicivirus, and rotavirus in Canadian finisher pigs

<table>
<thead>
<tr>
<th></th>
<th>Hepatitis E virus</th>
<th>Porcine enteric calicivirus</th>
<th>Rotavirus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Farms</td>
<td>Samples</td>
<td>Farms</td>
<td>Samples</td>
</tr>
<tr>
<td>Number positive</td>
<td>Number positive</td>
<td>/number positive</td>
<td>/number</td>
</tr>
<tr>
<td>/number sampled (%)</td>
<td></td>
<td></td>
<td>sampled (%)</td>
</tr>
<tr>
<td>National</td>
<td>30/88 (34.1%)</td>
<td>17/528 (13.8%)</td>
<td>18/88 (20.5%)</td>
</tr>
<tr>
<td>Province A</td>
<td>3/18 (16.7%)</td>
<td>3/108 (2.8%)</td>
<td>2/18 (11.1%)</td>
</tr>
<tr>
<td>Province B</td>
<td>6/12 (50.0%)</td>
<td>14/72 (19.4%)</td>
<td>3/12 (25.0%)</td>
</tr>
<tr>
<td>Province C</td>
<td>14/41 (34.1%)</td>
<td>42/246 (17.1%)</td>
<td>6/41 (14.6%)</td>
</tr>
<tr>
<td>Province D</td>
<td>7/17 (41.2%)</td>
<td>14/102 (13.7%)</td>
<td>7/17 (41.2%)</td>
</tr>
<tr>
<td>Month</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>January</td>
<td>1/3 (33.3%)</td>
<td>1/18 (5.6%)</td>
<td>1/3 (33.3%)</td>
</tr>
<tr>
<td>February</td>
<td>0/1 (0%)</td>
<td>0/6</td>
<td>0/1 (0%)</td>
</tr>
<tr>
<td>March</td>
<td>2/8 (25.0%)</td>
<td>7/48 (14.6%)</td>
<td>1/8 (12.5%)</td>
</tr>
<tr>
<td>April</td>
<td>1/2 (50.0%)</td>
<td>2/12 (16.7%)</td>
<td>0/2 (0%)</td>
</tr>
<tr>
<td>May</td>
<td>0/2 (0%)</td>
<td>0/12</td>
<td>0/2 (0%)</td>
</tr>
<tr>
<td>June</td>
<td>1/2 (50.0%)</td>
<td>2/12 (16.7%)</td>
<td>1/2 (50.0%)</td>
</tr>
<tr>
<td>July</td>
<td>0/3 (0%)</td>
<td>0/18 (0%)</td>
<td>1/3 (33.3%)</td>
</tr>
<tr>
<td>August</td>
<td>1/8 (12.5%)</td>
<td>1/48 (2.1%)</td>
<td>1/8 (12.5%)</td>
</tr>
<tr>
<td>September</td>
<td>3/13 (23.1%)</td>
<td>3/78 (3.8%)</td>
<td>1/13 (7.7%)</td>
</tr>
<tr>
<td>October</td>
<td>8/11 (72.7%)</td>
<td>20/66 (30.3%)</td>
<td>2/11 (18.2%)</td>
</tr>
<tr>
<td>November</td>
<td>8/25 (32.0%)</td>
<td>26/150 (17.3%)</td>
<td>5/25 (20.0%)</td>
</tr>
<tr>
<td>December</td>
<td>5/10 (50.0%)</td>
<td>11/60 (18.3%)</td>
<td>5/10 (50.0%)</td>
</tr>
<tr>
<td>GM/g (all samples)</td>
<td>619 401 gc/g</td>
<td>31 644 gc/g</td>
<td>275 277 gc/g</td>
</tr>
<tr>
<td>GM/g (‘positive’ samples)</td>
<td>4 360 583 gc/g</td>
<td>4 360 583 gc/g</td>
<td>4 360 583 gc/g</td>
</tr>
<tr>
<td>SD</td>
<td>5 819 952 gc/g</td>
<td>442 402 gc/g</td>
<td>6 051 227 gc/g</td>
</tr>
</tbody>
</table>

a gc/g = genomic copies per gram of sample.
GM — geometric mean; HEV — hepatitis E virus; PEC — porcine enteric calicivirus; RV — rotavirus; SD — standard deviation.

of 0.92, suggesting marked heterogeneity across studies. Calculation of the number of farms required to estimate a prevalence of 30% with desired precision yielded a target of 75 farms participating.

**Farm recruitment and sample collection**

All 26 CIPARS veterinarians were approached to participate in this study in the summer of 2011. Nine veterinarians enrolled and they recruited 17 finisher herds in total. In the fall of 2011 the FC farms were invited by the FC program technician to participate, and 20 of 28 enrolled with one farm being tested twice. At the end of 2011, CIPARS veterinarians were again invited to participate. In 2012, 15 veterinarians participated, enrolling 34 new farms, and re-testing 15 farms.

A total of 528 samples from 72 herds from 4 provinces (A, B, C, and D) with 6 pooled samples per herd were collected over a 17-month period and stored at −80°C until further use. A summary of the characteristics of participating farms in both sampling platforms is presented in Table I. Overall, relative to CIPARS farms, FC farms fed fewer finisher pigs; all were located in province C, and most practiced continuous pig flow (72.7%) as opposed to all-in-all-out flow. Compared with CIPARS farms, more FC farms provided boots (86.4% versus 72.3%), but fewer provided coveralls prior to entry (16.7% versus 81.8%); fewer required shower-in (18.2% versus 45.5%), and fewer required downtime after visiting another pig farm, prior to entry (9.1% versus 69.7%). Sampling was only conducted September to December, for FC farms. “Retest” was included as a categorical variable in the dataset as 16 farms were sampled twice.

Data were missing from a sub-group of FC farms for the following biosecurity variables: provision of boots or coveralls, use of a boot dip, posting a biosecurity sign, requirement for shower-in, or downtime, and obtaining pigs from multiple sources.

**Hepatitis E virus**

Hepatitis E virus RNA was detected from one or more samples collected from 19 of 66 CIPARS farms (estimated herd-level prevalence 28.8%; 95% CI: 19.3%, 40.6%), and 11 of 22 FC farms (estimated herd-level prevalence 50.0%, 95% CI: 30.7%, 69.3%). Hepatitis E virus RNA was detected from 35/396 individual pooled samples collected from CIPARS farms (estimated sample-level prevalence 8.8%; 95% CI: 6.4%, 12.2%), and 38/132 pooled samples from FC farms (estimated sample-level prevalence 28.8%; 95% CI: 21.8%, 37.0%). Hepatitis E virus prevalence by province is presented in Table II.
Table III. Single fixed effect logistic regression modeling predictors of viral detection in Canadian finisher pigs: general and farm descriptors

<table>
<thead>
<tr>
<th>Variable</th>
<th>Hepatitis E virus HEV</th>
<th>Porcine enteric calicivirus PEC</th>
<th>Rotavirus RV</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>OR (95% CI)</td>
<td>OR (95% CI)</td>
<td>OR (95% CI)</td>
</tr>
<tr>
<td>Sampling platform</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Referent</td>
<td>0.01</td>
<td>0.29</td>
<td>0.0007</td>
</tr>
<tr>
<td>CIPARS</td>
<td>Referent</td>
<td>Referent</td>
<td>Referent</td>
</tr>
<tr>
<td>FC</td>
<td>14.58 (1.82, 116.93)</td>
<td>0.12 (0.003, 5.89)</td>
<td>0.05 (0, 0.24)</td>
</tr>
<tr>
<td>Province</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>Referent</td>
<td>Referent</td>
<td>Referent</td>
</tr>
<tr>
<td>B</td>
<td>16.65 (0.62, 447.63)</td>
<td>3.50 (0.06, 188.94)</td>
<td>0.73 (0.31, 1.34)</td>
</tr>
<tr>
<td>C</td>
<td>8.92 (0.73, 109.04)</td>
<td>1.58 (0.06, 44.15)</td>
<td>0.63 (0.28, 1.50)</td>
</tr>
<tr>
<td>D</td>
<td>10.82 (0.50, 232.98)</td>
<td>10.82 (0.24, 485.40)</td>
<td>0.06 (0.35)</td>
</tr>
<tr>
<td>Year of sampling</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Year 1</td>
<td>Referent</td>
<td>Referent</td>
<td>Referent</td>
</tr>
<tr>
<td>Year 2</td>
<td>0.19 (0.03, 1.19)</td>
<td>1.95 (0.18, 21.05)</td>
<td>48.35 (8.26, Inf)</td>
</tr>
<tr>
<td>Re-test</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>Referent</td>
<td>Referent</td>
<td>Referent</td>
</tr>
<tr>
<td>Yes</td>
<td>2.06 (0.07, 58.72)</td>
<td>8.45 (0.45, 158.54)</td>
<td>0.08 (0.46)</td>
</tr>
<tr>
<td>Season</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Winter</td>
<td>Referent</td>
<td>Referent</td>
<td>Referent</td>
</tr>
<tr>
<td>Spring</td>
<td>1.58 (0.03, 74.37)</td>
<td>1.42 (0.005, 402.51)</td>
<td>1.0 (0, Inf)</td>
</tr>
<tr>
<td>Summer</td>
<td>0.31 (0.02, 5.47)</td>
<td>1.05 (0.02, 70.60)</td>
<td>14.34 (2.41, Inf)</td>
</tr>
<tr>
<td>Fall</td>
<td>5.45 (0.42, 72.20)</td>
<td>2.37 (0.05, 102.61)</td>
<td>7.04 (1.19, Inf)</td>
</tr>
<tr>
<td>Production type</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Continuous</td>
<td>Referent</td>
<td>No convergence^d</td>
<td>Referent</td>
</tr>
<tr>
<td>All-in-all-out</td>
<td>2.29 (.31, 16.80)</td>
<td>2.87 (1.30, 6.26)</td>
<td>0.007</td>
</tr>
<tr>
<td>Not captured</td>
<td>184.26 (0.09, 3.87e + 5)</td>
<td>1.41 (0, 9.48)</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Finishers on-farm</td>
<td>0.99 (0.99, 1.00)</td>
<td>1.00 (0.99, 1.00)</td>
<td>No convergence</td>
</tr>
<tr>
<td>Farm size</td>
<td>No convergence</td>
<td>No convergence</td>
<td>Referent</td>
</tr>
<tr>
<td>0 to 2000 finishers</td>
<td>Referent</td>
<td></td>
<td>0.0003</td>
</tr>
<tr>
<td>2 to 4000 finishers</td>
<td></td>
<td></td>
<td>0.34</td>
</tr>
<tr>
<td>&gt; 4000 finishers</td>
<td></td>
<td></td>
<td>6.93 (1.96, 22.18)</td>
</tr>
</tbody>
</table>

^a Mixed logistic regression with random intercepts for “farm” and “sampling event” with “sample positive” as the outcome measure.

^b Exact logistic regression with “farm positive” as the outcome measure.

^c Mean unbiased estimate. ^d Maximum memory allocated in Stata = 2 Megabytes.

CIPARS — Canadian Integrated Program for Antimicrobial Resistance Surveillance; FC — FoodNet Canada; HEV — hepatitis E virus; Inf — infinity; OR — odds ratio; PEC — porcine enteric calicivirus; RV — rotavirus; 95% CI — 95% confidence interval.
Mean viral load in positive samples was 4,360,583 genomic copies/g (SD = 15,000,000 gc/g).

We performed mixed logistic regression modeling HEV RNA detection in a sample as the outcome measure, with a single fixed effect and random intercepts for farm and sampling event (Tables III, IV). The following predictors were eligible for inclusion in the full multivariable model (i.e., \( P < 0.20 \)): sampling platform — with FC farms having significantly (\( P < 0.05 \)) greater odds of HEV RNA detection on-farm relative to CIPARS farms; province — with province A farms having reduced odds of HEV detection relative to the other provinces; year of sampling, with farms sampled in year 2 having reduced odds of HEV detection relative to year 1 sampling; season — with samples collected in spring or fall having greater odds of HEV detection relative to winter; and the number of finisher pigs on-farm — with larger farms having reduced odds of HEV detection. Requiring shower-in was associated with significantly (\( P < 0.05 \)) reduced odds of HEV detection.

In an intercept-only model, with random intercepts for herd and sampling event, 45.1% of the variance occurred at the individual sample level (i.e., the lowest hierarchical level of the data), with 32.6% of the variance occurring at the herd level, and 22.3% at the sampling event. Similar proportions were observed for each single fixed effect model.

The full multivariable model failed to converge, as did most models incorporating a subset of the eligible variables, precluding investigation of interaction or confounding.

**Porcine enteric calicivirus**

Porcine enteric calicivirus RNA was detected from one or more samples collected from 17 of 66 CIPARS farms (estimated farm-level prevalence 25.8%; 95% CI: 16.8%, 37.4%), and 1 of 22 FC farms (farm-level prevalence 4.6%; 95% CI: 0.80%, 21.8%). Porcine enteric calicivirus RNA was detected from 36 of 396 individual pooled samples collected from CIPARS farms (estimated sample-level prevalence...
9.1%; 95% CI: 6.6%, 12.3%), and 2 of 132 pooled samples from FC farms (estimated sample level prevalence 1.5%; 95% CI: 0.4%, 5.9%). Porcine enteric calicivirus prevalence by province is presented in Table II. Mean viral load in positive samples was 388,556 gc/g (SD = 1,521,130 gc/g). In mixed logistic regression with single fixed effect and random intercepts for farm and sampling event, providing boots on entry to the unit was associated with significantly (P<0.05) reduced odds of PEC detection. Samples collected during re-test of a farm were associated with increased (P=0.15) odds of PEC detection. However, a multivariable model including both of these predictors failed to converge.

In an intercept-only model, with random intercepts for herd and sampling event, 53.9% of the variance occurred at the individual sample level (i.e., the lowest hierarchical level of the data), with negligible variance occurring at the herd level, and 46.1% at the sampling event. Similar proportions were observed for each single fixed effect model.

Rotavirus

Rotavirus RNA was detected from one or more samples collected from 6/66 CIPARS farms (estimated sample level prevalence 1.5%; 95% CI: 0.4%, 5.9%). Porcine enteric calicivirus prevalence by province is presented in Table II. Mean viral load in positive samples was 388,556 gc/g (SD = 1,521,130 gc/g). In mixed logistic regression with single fixed effect and random intercepts for farm and sampling event, providing boots on entry to the unit was associated with significantly (P<0.05) reduced odds of PEC detection. Samples collected during re-test of a farm were associated with increased (P=0.15) odds of PEC detection. However, a multivariable model including both of these predictors failed to converge.

In an intercept-only model, with random intercepts for herd and sampling event, 53.9% of the variance occurred at the individual sample level (i.e., the lowest hierarchical level of the data), with negligible variance occurring at the herd level, and 46.1% at the sampling event. Similar proportions were observed for each single fixed effect model.

Rotavirus RNA was detected from one or more samples collected from 6/66 CIPARS farms (estimated farm-level prevalence 9.1%, 95% CI: 4.2%, 18.5%), and 0 of 22 FC farms. Rotavirus RNA was detected from 8 of 396 individual pooled samples collected from CIPARS farms (estimated sample level prevalence 2.0%, 95% CI: 1.0%, 3.9%), and 0 of 132 pooled samples from FC farms. Rotavirus prevalence by province is presented in Table II. Mean viral load in positive samples was 18,200,000 gc/g (SD = 48,800,000 gc/g). In univariable exact logistic regression with "farm positive" as the outcome measure (Table III), both sampling platform and province were significant (P<0.05) predictors for RV detection, with farms from province A having greatest odds of RV detection, and FC farms reduced odds of RV detection as compared with CIPARS farms (OR = 0.05, 95% CI: 0.0, 0.24). Production type was a significant predictor for RV detection, with farms practicing all-in-all-out pig flow having significantly increased odds relative to farms using continuous flow (OR = 2.87, 95% CI: 1.30, 6.26). Farms obtaining grow-finish pigs from multiple sources had greater odds of RV detection (OR = 4.42, 95% CI: 1.89, 9.89) as compared with those only using one source. Provision of boots and coveralls for visitors, requiring a shower prior to entering the unit, and posting a biosecurity sign at the entry to the site, all were significantly (P<0.05) associated with increased odds of RV detection in finisher pigs.

Overall, FC farms had greater odds of HEV RNA relative to CIPARS farms, and reduced odds of RV RNA detection. Province A had lower odds of HEV relative to the other provinces, and province D had lower odds of RV RNA detection relative to province A.

Discussion

The overall farm-level prevalence of HEV in finishers in our survey (34.1%, 95% CI: 25.0%, 44.5%) is comparable with published North American estimates ranging from 25% to 68% (6,21). The FC farms tend to be smaller multi-species farms as compared with CIPARS farms (Deckert & Friendship, 2015, University of Guelph, personal communication). Other species, including cats, dogs, cattle, sheep, goats, and rabbits have been reported to be capable of infecting pigs with HEV. The fact that HEV prevalence in finisher pigs was lower in FC farms than in CIPARS farms suggests that FC farms may have better biosecurity practices than CIPARS farms. However, further research is needed to confirm this hypothesis.

In conclusion, this study provides valuable information on the prevalence of HEV and PEC in finisher pigs in Canada. The results indicate that HEV is a common virus in finisher pigs in Canada, and that PEC is less common. The results also suggest that biosecurity practices may be important in preventing the spread of these viruses. Further research is needed to determine the impact of these viruses on pig health and productivity, and to develop effective management strategies to control these viruses.
of HEV sero-conversion and/or shedding (3), and it is possible that HEV shedding of other species on-farm may have contributed to the introduction of HEV to the pig herd. The significant association between requiring shower-in and reduced odds of HEV RNA detection on-farm (OR = 0.16, 95% CI: 0.03, 0.96) is consistent with the possibility that asymptomatic human shedders could be a potential exposure source of HEV for pigs, as has been hypothesized for pork (7). Conditional estimates of effect would be required to further investigate this finding, which was not possible in this dataset.

Overall farm-level prevalence of PEC was 20.5% (95% CI: 13.4%, 30.0%). In comparison, a Dutch study reported PEC RNA detected in 3- to 9-month-old pigs on only 2 of 100 farms sampled (22) and a US investigation detected PEC RNA in 9 of 9 farms sampled (23). The significant association estimated between provision of boots upon entry to the unit, and reduced odds of PEC RNA detection on-farm (OR = 0.08, 95% CI: 0.007, 0.83), is consistent with an agent transmitted via the fecal-oral route.

Rotavirus had the lowest farm-level prevalence of the 3 viruses of interest in this survey. A recent Vietnamese survey reported a farm-level RV prevalence of 74% (77/104) (24). However, with a smaller sample size, an Italian survey reported a numerically lower prevalence (3 of 8 herds) surveyed (25). In comparison with HEV and PEC, RV had a different geographic distribution, with province “A” having greater odds of RV detection on-farm, and no RV detected in province “D” farms.

While few of the predictor variables from this study, with the exception of geographic region, have been investigated in other published research, we generally expected that the “biosecurity” variables (providing boots/boot dip/coveralls, requiring shower-in/downtime) would be associated with reduced odds of detection, and sampling platform and province could be significant predictors, for viral RNA detection on-farm. For example, investigators in France have reported a significant association between lack of hygiene measures such as failure to provide boots, and detection of HEV in the livers of slaughter pigs (26). In our study, obtaining finishers from multiple sources was the one predictor variable consistently associated with increased odds of viral RNA detection. This is similar to other research, which reported that obtaining pigs from more than one source was a significant \( P < 0.05 \) predictor for the presence of *Salmonella* spp. on-farm (27,28) in cross-sectional studies of 109 and 359 farms, respectively.

However, the measures of association for some predictor variables differed in magnitude and, in some cases, direction, across the 3 viruses studied, although the primary transmission route for all 3 is the fecal-oral route. The varying relationships between predictor variables and outcome across the 3 viruses may reflect differences in viral biology and relative importance of various possible methods of introduction/transmission. On an infected farm, incident HEV infection occurs most frequently in the nursery (21), RV infection occurs most frequently in suckling pigs, and mean age of PEC infection has varied across studies (29,30). In our study, RV detection in finishers was significantly associated with adoption of widely accepted biosecurity measures such as provision of boots and coveralls (Table III). Possibly, given the tendency for RV infection to occur early in the pig’s life, the application of these biosecurity measures may functionally delay the mean age of RV infection. This could result in biosecurity measures such as provision of boots being significantly associated with RV detection in finishers, particularly in farrow-to-finish herds.

The fact that sampling platform was a significant predictor for HEV and RV RNA was noteworthy. However, it highlights the potential importance of stratified sampling in surveillance programs; some farms (e.g., small scale/mixed species), although perhaps not producing a large proportion of market animals, may be important biologically in pathogen transmission and control.

Our study has several limitations. The farmers who participated in this survey are volunteers, suggesting the potential for selection bias, by functionally using an incomplete definition to define the eligible population (31). There is no national sampling frame of finisher farms with which our study group might be compared with regards to important demographic or management parameters. However, CIPARS also operates an abattoir-level sampling platform that includes cecal sampling of pigs. The close similarity between CIPARS farm level antimicrobial resistance estimates, and those obtained from the national abattoir component of CIPARS (which randomly samples slaughter pigs and is therefore unaffected by producer or veterinarian willingness to participate), indicates that the convenience sample of farms obtained by the CIPARS on-farm sampling platform is likely representative of Canadian pig production (17). Unfortunately, however, no similar mechanism exists for estimating the similarity of the FC farms to the national herd.

Additionally, participants self-reported farm management and biosecurity practices. A minority (10 of 20) of respondents did not complete some survey questions, including those pertaining to farm biosecurity practices, with 3 FC respondents not answering any of these questions. We hypothesize that the missing biosecurity data could be missing not at random, perhaps reflecting respondents’ reluctance to report that fairly widely endorsed biosecurity measures (32) were not practiced. If the 3 farms for which data were missing were, in fact, not using these measures, their inclusion in the analysis would have increased the observed protective effect of these biosecurity measures.

While the diagnostic sensitivity and specificity of the RT-PCR assays used in this study have been investigated in fecal samples collected from other populations (e.g., humans, younger pigs), the diagnostic sensitivity and specificity of these assays in fecal samples from finisher pigs are not known. Additionally, the RV assay targets the NSP3 gene of group A RVs, and its performance in detecting RVs of other serotypes such as group C RVs, which are also reported in finisher pigs (33) is also unknown. A Brazilian survey of diarrheic pigs 1 to 4 weeks old identified 144 fecal samples inconclusively categorized using polycrylamide gel assay. Of these, 19 and 5 were identified as group B and C RVs, respectively, using group-specific RT-PCR, highlighting both the potential for their detection on-farm, and the requirement for appropriate assays (34).

Biosecurity practices in pig farms, and farmers’ attitudes towards them, have been increasingly investigated in North America and Europe, with sow replacement policy, and protocols around trucking of livestock being consistently recognized as important components of a farm biosecurity protocol (35). These practices were not captured by our questionnaire, which was originally designed to capture data.
pertinent to development of antimicrobial resistance. The extent to which missing predictors/confounders may have affected our estimates is therefore unknown.

Our recruitment and ultimately our sample size was limited by both the lack of a complete sampling frame of pig farms in Canada, and the relatively recent swine flu pandemic of 2009, which participating veterinarians reported decreased farmer willingness to participate in this project. It is possible that a larger sample size, and therefore increased study power, would have resulted in more of the predictor variables being significantly associated with viral detection. Additionally, we sampled the finisher pigs at only one point in their lives. It is possible that some of the predictors investigated (e.g., all-in-all-out pig flow) might be significantly associated with delaying, but not preventing, viral infection. Longitudinal studies would be helpful in understanding viral kinetics, and the effects of predictors (i.e., biosecurity/management practices) on mean age of infection. We identified knowledge gaps in addition to the lack of a Canadian sampling frame. Detection of HEV, PEC, and RV on Canadian finisher farms suggests the potential for occupational exposure to these viruses amongst swine workers. Field surveys and research synthesis have supported the hypothesis that swine workers and veterinarians have increased odds of exposure to HEV (36,37). However, the extent of Canadian illness attributable to occupational exposure (if any) remains unknown. This reflects both the challenges in data collection (HEV infection is not federally notifiable in Canada) and possibly also incomplete understanding of the causal complement, or combination of risk factors, required to produce clinical disease after viral exposure.

In conclusion, all 3 viruses were detected in finishing pigs, suggesting occupational exposure to feeder pigs is a potential source of human exposure to these viruses. Overall, HEV had the greatest farm-level prevalence of the 3 viruses studied; sampling platform was a significant predictor of HEV and RV viral RNA detection in finishers in this dataset. The significance, effectiveness, and direction of effect of the biosecurity practices captured (provision of boots, or coveralls, on entry; requiring shower-in prior to entry; use of boot dip; posting biosecurity sign; requiring downtime prior to entry) varied across the 3 viruses studied. The management procedure consistently associated with greater odds of viral RNA detection for all 3 viruses, was obtaining feeder pigs from multiple sources.

Acknowledgments

We gratefully acknowledge the assistance of Bryan Bloomfield, Danielle Daignault, Louise Bellai, Karen Richardson, the CIPARS veterinarians, and the farmers participating in this study, as well as the funding support of OMAFRA — University of Guelph Partnership Research Program (Grant # 027118).

References

Genetic diversity of *Streptococcus suis* serotype 2 isolated from pigs in Brazil

Daniela Sabatini Doto, Luisa Zanolli Moreno, Franco Ferraro Calderaro, Carlos Emilio Cabrera Matajira, Vasco Tulio de Moura Gomes, Thais Sebastiana Porfida Ferreira, Renan Elias Mesquita, Jorge Timenetsky, Marcelo Gottschalk, Andrea Micke Moreno

**Abstract**

*Streptococcus suis* is an emerging zoonotic pathogen that causes septicemia, meningitis, arthritis, and pneumonia in swine and humans. The present study aimed to characterize the genetic diversity of *S. suis* serotype 2 isolated from pigs showing signs of illness in Brazil using pulsed-field gel electrophoresis (PFGE), single-enzyme amplified fragment length polymorphism (SE-AFLP), and profiling of virulence-associated markers. A total of 110 isolates were studied, 62.7% of which were isolated from the central nervous system and 19.1% from the respiratory tract. Eight genotypes were obtained from the combination of virulence genes, with 43.6% and 5.5% frequencies for the *mrp/epf/sly* genotypes, respectively. The presence of isolates with *epf* gene variation with higher molecular weight also appears to be a characteristic of Brazilian *S. suis* serotype 2. The PFGE and SE-AFLP were able to type all isolates and, although they presented a slight tendency to cluster according to state and year of isolation, it was also evident the grouping of different herds in the same PFGE subtype and the existence of isolates originated from the same herd classified into distinct subtypes. No further correlation between the isolation sites and *mrp/epf/sly* genotypes was observed.

**Résumé**

*Streptococcus suis* est un agent pathogène zoonotique en émergence responsable de septicémies, des méningites, d’arthrites, et de pneumonies chez les porcs et les humains. La présente étude visait à caractériser la diversité génétique de souches de *S. suis* sérotype 2 isolées au Brésil de porcs montrant des signes de maladie à l’aide des techniques suivantes : électrophorèse en champs pulsés (PFGE), polymorphisme des fragments amplifiés par un enzyme unique (SE-AFLP), et profilage des marqueurs de virulence. Un total de 110 isolats a été étudié, 62,7 % isolats provenant du système nerveux central et 19,1 % du tractus respiratoire. Huit génotypes furent obtenus de la combinaison de gènes de virulence, avec des fréquences de 43,6 % et 5,5 % pour les génotypes *mrp/epf/sly* et *mrp/epf/sly*, respectivement. La présence d’isolats avec la variation du gène *epf* et un poids moléculaire plus élevé semble être également une caractéristique de *S. suis* sérotype 2 d’origine brésilienne. Les méthodes PFGE et SE-AFLP ont été en mesure de permettre le typage de tous les isolats et, bien qu’il y ait une légère tendance à se regrouper selon l’état et l’année d’isolement, il était également évident qu’il y avait du regroupement d’isolats provenant de différents troupeaux dans le même sous-type de PFGE et de l’existence d’isolats provenant du même troupeau classifiés dans des sous-types différents. Aucune autre corrélation entre le site d’isolement et les génotypes de *mrp/epf/sly* ne fut observée.

(Traduit par Docteur Serge Messier)

**Introduction**

*Streptococcus suis* infection is one of most important diseases in the swine industry worldwide. *Streptococcus suis* is also considered an emerging zoonotic pathogen that causes septicemia and meningitis in humans (1). From the 35 serotypes described thus far, *S. suis* serotype 2 is most commonly associated with disease in both pigs and humans in most countries (2).

The most common virulence markers associated with serotype 2 strains are suilysin (SLY; *sly*), muramidase-released protein (MRP; *mrp*) and extracellular factor (EF; *epf*) (3,4). A correlation between the *mrp/epf/sly* genotype and clinical human and porcine isolates has already been suggested (5,6). Although the *mrp/epf/sly* genotype is the most prevalent worldwide, the *mrp/epf* genotype appears to be increasing among human and porcine *S. suis* isolates in Asia, Europe, and North America (2).
Few data are available regarding the epidemiology and molecular characteristics of \textit{S. suis} serotype 2 isolated from pigs showing signs of illness in South America (7). Therefore, the aim of this study was to characterize the genetic diversity of \textit{S. suis} serotype 2 isolated from diseased pigs in Brazil by pulsed-field gel electrophoresis (PFGE), single-enzyme amplified fragment length polymorphism (SE-AFLP) and profiling of virulence-associated genes.

### Materials and methods

#### Bacterial strains and phenotypic identification

A total of 133 \textit{S. suis} strains were isolated from 103 pigs with clinical signs of meningitis, septicaemia, arthritis, and/or pneumonia that originated from 88 herds in different Brazilian states (São Paulo, Santa Catarina, Paraná, Pernambuco Bahia, Minas Gerais, and Rio Grande do Sul) during the period of 2001 to 2003. Animal samples were plated onto Columbia blood agar base containing 5% sheep blood supplemented with SR-126 (Oxoid, Cambridge, United Kingdom). The presumptive bacteriological identification was based on the presence of \( \alpha \)-hemolysis on blood agar; the production of amylase; the absence of growth in brain-heart-infusion (BHI) medium supplemented with 6.5% NaCl, inulin, and trehalose fermentation; and a lack of mannitol and sorbitol fermentation, as well as a negative Voges-Proskauer test. From each animal examined, 1 to 6 different \textit{S. suis} colonies were selected isolated from different body sites (CNS, lungs, thoracic cavity, and peritoneum).

#### Species identification and profiling of virulence-associated markers

Genomic DNA was purified according to Boom et al (8) protocol, and \textit{S. suis} identification was confirmed through \textit{gdh} amplification according to Okwumabua et al. (9). The \textit{epf} and \textit{sly} virulence genes were amplified through conventional PCR as previously described by Wisselink et al (10) and King et al (11), respectively. For partial amplification of the \textit{mrp} gene, primers \textit{mrp-Fw} (TGGTTCAATAGGCCAACAC) and \textit{mrp-Rv} (GAGAAATTTCATGTCCAG) (binding sites 311-1189 bp; accession no. X64450.1) were applied (M. Gottschalk, University of Montreal, personal communication). Amplicons were stained using BlueGreen (LGC Biotecnologia, Sao Paulo, Brazil) and separated by electrophoresis using 1.5% agarose gels and 100 bp DNA ladder (New England Biolabs, Ipswich, Massachusetts, USA) for the amplicons size estimation.

#### Serotyping

Serotype 2 was identified by using the coagglutination test as described by Gottschalk, et al (12).

#### Molecular typing by SE-AFLP and PFGE

Isolates were also genotyped through SE-AFLP and PFGE. The SE-AFLP was done according to McLauchlin et al (13) protocol. DNA fragments were detected by electrophoresis at 24 V for 26 h using a 2% agarose gel stained using BlueGreen (LGC Biotecnologia). The PFGE culture conditions, plug preparation, and DNA extraction followed the protocol described previously by Vela et al (14).

### Table I. Classification of \textit{Streptococcus suis} serotype 2 isolates among virulence genotypes and isolation sites

<table>
<thead>
<tr>
<th>Genotype</th>
<th>CNS N (%)</th>
<th>RT N (%)</th>
<th>Others N (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{mrp} \textit{epf} \textit{sly}\textsuperscript{1}</td>
<td>1 (0.9)</td>
<td>0</td>
<td>1 (4.8)</td>
</tr>
<tr>
<td>\textit{mrp} \textit{epf} \textit{sly}\textsuperscript{1}</td>
<td>1 (0.9)</td>
<td>0</td>
<td>1 (4.8)</td>
</tr>
<tr>
<td>\textit{mrp} \textit{epf} \textit{sly}\textsuperscript{2}</td>
<td>4 (3.6)</td>
<td>3 (4.4)</td>
<td>0</td>
</tr>
<tr>
<td>\textit{mrp} \textit{epf} \textit{sly}\textsuperscript{2}</td>
<td>6 (5.5)</td>
<td>4 (5.8)</td>
<td>1 (4.8)</td>
</tr>
<tr>
<td>\textit{mrp} \textit{epf} \textit{sly}\textsuperscript{2}</td>
<td>14 (12.7)</td>
<td>10 (14.5)</td>
<td>3 (14.2)</td>
</tr>
<tr>
<td>\textit{mrp} \textit{epf} \textit{sly}\textsuperscript{1}</td>
<td>17 (15.5)</td>
<td>12 (17.4)</td>
<td>0</td>
</tr>
<tr>
<td>\textit{mrp} \textit{epf} \textit{sly}\textsuperscript{1}</td>
<td>19 (17.3)</td>
<td>7 (10.1)</td>
<td>6 (28.6)</td>
</tr>
<tr>
<td>\textit{mrp} \textit{epf} \textit{sly}\textsuperscript{1}</td>
<td>48 (43.6)</td>
<td>33 (47.8)</td>
<td>9 (42.8)</td>
</tr>
</tbody>
</table>

CNS — central nervous system; RT — respiratory tract; Others — joints, blood, peritoneum, heart or pericardium; \textit{epf} — EF variation.

The restriction enzyme \textit{SmaI} (New England BioLabs) was used for DNA digestion at 30°C for 24 h. Electrophoresis was done using 1% Seakem Gold agarose (Cambrex Bio Science Rockland, New Jersey, USA) and a CHEF-DR III System (Bio-Rad Laboratories, California, USA) with 0.5× TBE at 14°C. DNA fragments were separated at 6 V/cm at a 120° fixed angle, with pulse times from 0.5 to 40 s for 18 h. Gels were stained with 1× SYBR Safe (Invitrogen Corporation, Carlsbad, California, USA) for 30 min and imaged under UV trans-illumination. Lambda DNA-PFGE marker (New England BioLabs) was used for fragment size determination.

The SE-AFLP and PFGE fingerprint patterns were analyzed by a comprehensive pairwise comparison of restriction fragment sizes using the Dice coefficient. The mean values obtained from the Dice coefficient were applied in UPGMA (BioNumerics 7.5; Applied Maths NV, Sint-Martens-Latem, Belgium) to generate dendrograms. A similarity cut-off value of 90% was used to analyze SE-AFLP clusters. For the PFGE analysis, the isolates were characterized in different pulsotypes when they differed by $\geq 4$ bands and assigned into subtypes when they differed by 1 to 4 bands (15). Discriminatory indexes were calculated according to Hunter and Gaston (16).

### Results

#### Virulence-associated gene profiling

One hundred and ten of the 133 \textit{S. suis} strains isolated in this study were characterized as serotype 2. Specifically, 62.7% were isolated from the central nervous system; 19.1% from the respiratory tract; and 18.2% from joint, peritoneum, pericardium/heart, and blood samples. From the 21 \textit{S. suis} serotype 2 strains isolated from the respiratory tract, only 2 originated from nasal swabs and, therefore, were considered as non-invasive isolates. The remaining strains were obtained from lungs and thoracic cavity samples of animals with pneumonia.

The presence of \textit{mrp}, \textit{epf}, and \textit{sly} genes was characterized by the visualization of fragments of 879, 626, and 1818 bp, respectively. The \textit{epf} gene can present fragment size variations (EF variation — \textit{epfv}) with possible amplicons of 1278, 1505, 2313, 2537, and 2993 bp. Of
Figure 1. Pulsed-field gel electrophoresis (PFGE) dendrogram showing the relationship among Streptococcus suis serotype 2 pulsotypes.
Figure 2. Dendrogram showing the relationship among the *Streptococcus suis* serotype 2 isolates single-enzyme amplified fragment length polymorphism (SE-AFLP) patterns.
the isolates, 15.5% presented EF variation (higher molecular weight). Eight genotypes were obtained from the combined presence of mrp, epf, and sly genes (Table I). Only 5.5% of serotype 2 isolates were negative for all of the studied genes.

**Molecular typing**

The PFGE analysis resulted in 14 pulsotypes (A — N), which were divided into 2 main clusters with over 55% of genetic similarity (Figure 1). One of these clusters comprised exclusively pulsotype A, which consists of 86 isolates that were further classified into subtypes A1 to A31. The other main cluster comprised the remaining 23 S. suis isolates divided among 13 pulsotypes and respective subtypes. Among the A1 to A31 subtypes, there is a tendency to cluster according to isolates state and year of origin, although it is also evident that some isolates from different herds were grouped into the same subtype (e.g., A3 and A12) and that some isolates from the same herd were classified into distinct subtypes (e.g., H103 and H108). However, no correlation between the isolation sites and mrp/epf/sly genotypes was observed.

The SE-AFLP analysis resulted in 20 patterns (P1 to P20) distributed among 3 primary clusters with 65% similarity (Figure 2). It exposes pattern P11, which grouped 61 S. suis isolates from different origin, isolation site, and virulence genotypes. With the exception of this pattern, the isolates tended to group according to state and year of origin, but no further correlation with mrp/epf/sly genotypes was observed. Interestingly, mrp+/epf+/sly+ isolates presented high genetic similarity and appeared to cluster closely in both techniques.

The discriminatory indexes for PFGE using SmaI and SE-AFLP using HindIII were 0.97 and 0.67, respectively. Even though both techniques enabled the isolates state and year of origin, although it is also evident that some isolates from different herds were grouped into the same subtype (e.g., A3 and A12) and that some isolates from the same herd were classified into distinct subtypes (e.g., H103 and H108). However, no correlation between the isolation sites and mrp/epf/sly genotypes was observed.

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The only 2 S. suis non-invasive isolates studied (SS166 and SS167) presented the genotypes mrp+/epf+/sly- and mrp+/epf-/sly+, respectively; nevertheless, they grouped together with mrp+/epf+/sly+ isolates in both PFGE and SE-AFLP. The presence of isolates that presented epf gene variation with higher molecular weight appears to be a characteristic of Brazilian S. suis serotype 2 as previously described by Martinez et al (7). Even though we detected a lower incidence than Martinez et al (7), the mrp+/epf+/sly+ genotype was also obtained from diseased animals contradicting the statement that EF variant strains are weakly virulent (4).

We also observed the presence of mrp+/epf+/sly+ genotype in invasive strains, which is unprecedented in Brazil; however, they also grouped together with mrp+/epf+/sly+ isolates in both PFGE and SE-AFLP. Differing from the European and Asian scenario, the MRPl+/EF+/SLY- and MRPl+/EF+/SLY- phenotypes appear to be common in virulent North American S. suis serotype 2 strains (2,18,19). Therefore, further studies are necessary to understand the distribution and pathogenicity of mrp+/epf+/sly+ isolates in the continent.

Although both PFGE and SE-AFLP had been previously applied for S. suis typing, their clustering patterns were highly associated with mrp/epf/sly genotypes and isolate origins (14,20—22). The greater genetic heterogeneity observed in this study could be related to the distinct origin and year of isolation, and it stands out in the existence of isolates originating from the same herd classified into distinct PFGE subtypes and even different patterns in both techniques, such as the isolates from herd 108.

The greater genetic heterogeneity observed for S. suis serotype 2 originated from the same herd and could be related to the introduction of infected animals and the herd’s high turnover. Therefore, the high variation of S. suis virulence genotypes and genotypic patterns in the Brazil demand attention to quality of the sanitary status of the traded animals and the implications for their treatment and the pathogen control among respective herds.

Nevertheless, serotype 2 isolated from diseased pigs also appears to be more variable than human strains (23). Therefore, although the molecular epidemiology of S. suis infection continues to improve worldwide, further studies are necessary to enhance the knowledge regarding virulence genotype relevance and distribution in diseased animals and carriers, and its geographical variability.

**Discussion**

The high frequency of S. suis serotype 2 isolation from the central nervous system corroborates previous studies of swine infection (14,17), as well as the characterization of this pathogen as one of the most common causes of meningitis in Asia (2). The high frequencies of mrp+/epf+/sly- and mrp+/epf+/sly+ genotypes among serotype 2 isolates from diseased pigs also corroborate previous reports of S. suis infection in Europe and China (5,6).

The only 2 S. suis non-invasive isolates studied (SS166 and SS167) presented the genotypes mrp+/epf+/sly- and mrp+/epf+/sly+, respectively; nevertheless, they grouped together with mrp+/epf+/sly+ isolates in both PFGE and SE-AFLP. The presence of isolates that presented epf gene variation with higher molecular weight appears to be a characteristic of Brazilian S. suis serotype 2 as previously described by Martinez et al (7). Even though we detected a lower incidence than Martinez et al (7), the mrp+/epf+/sly+ genotype was also obtained from diseased animals contradicting the statement that EF variant strains are weakly virulent (4).

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**Acknowledgments**

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**References**


Comparison of 2 commercial single-dose *Mycoplasma hyopneumoniae* vaccines and porcine reproductive and respiratory syndrome virus (PRRSV) vaccines on pigs dually infected with *M. hyopneumoniae* and PRRSV

Changhoon Park, Ikjae Kang, Hwi Won Seo, Jiwoon Jeong, Kyuhyung Choi, Chanhee Chae

**Abstract**

The objective of this study was to compare the efficacy of 2 different commercial *Mycoplasma hyopneumoniae* vaccines and porcine reproductive and respiratory syndrome virus (PRRSV) vaccines in regard to growth performance, microbiological and immunological analyses, and pathological observation from wean to finish (175 d of age). Pigs were administered *M. hyopneumoniae* and PRRSV vaccines at 7 and 21 d of age, respectively, or both at 21 d old and then challenged with both *M. hyopneumoniae* and PRRSV at 49 d old. Significant (*P* < 0.05) differences were observed between the 2 vaccinated challenged groups in average daily weight gain, nasal shedding of *M. hyopneumoniae*, *M. hyopneumoniae*-specific interferon-γ secreting cells, and macroscopic and microscopic lung lesions. Induction of interleukin-10 following PRRSV vaccination does not interfere with the immune responses induced by *M. hyopneumoniae* vaccine. The present study demonstrated that the single-dose vaccination regimen for *M. hyopneumoniae* and PRRSV vaccine is efficacious for controlling coinfection with *M. hyopneumoniae* and PRRSV based on clinical, microbiological, immunological, and pathological evaluation.

**Introduction**

*Mycoplasma hyopneumoniae* is the etiological pathogen of enzootic pneumonia, which is characterized by a chronic, nonproductive cough (1). Infection of *M. hyopneumoniae* causes considerable economic losses, due to decreased growth rates, high feed conversion ratios, increased medication costs, and the susceptibility of sick pigs to infection by other organisms (1,2). Porcine reproductive and respiratory syndrome (PRRS) virus (PRRSV) is an enveloped, single-stranded, positive-sense RNA virus belonging to the *Arteriviridae* family in the order *Nidovirales* (3) that can cause reproductive problems in sows and respiratory problems in growing pigs (4).

In pigs, respiratory disease is multifactorial and complex and is caused by sequential or concurrent infections with several viral or bacterial pathogens; therefore, the name porcine respiratory disease complex (PRDC) is used to describe this disease (5,6). The economic impact of PRDC is tremendous, mainly due to decreased fattening performance and the cost of medication (7,8). Currently, the use of antibiotics for controlling PRDC is limited due to increased risk of antimicrobial resistance and residue in carcasses (9). Therefore, vaccinations are of prime importance and are routinely applied worldwide.

Since coinfection with *M. hyopneumoniae* and PRRSV is one of the most economically important situations in PRDC (10), vaccination of pigs with both *M. hyopneumoniae* and PRRSV is necessary to control PRDC efficiently. The commercial modified live PRRSV vaccine (Ingelvac PRRS MLV; Boehringer Ingelheim Vetmedica, St. Joseph, Missouri, USA) was first licensed for worldwide use in...
1994. In 2012, another new commercial modified live PRRSV vaccine (Fostera PRRS; Zoetis, Florham Park, New Jersey, USA) was introduced to the international market to control respiratory disease in growing pigs. A comparison of both single-dose M. hyopneumoniae and PRRSV vaccines together, therefore, is more practical and mirrors field conditions, rather than a comparison of each single dose M. hyopneumoniae and PRRSV vaccines by themselves. The objective of the present study was to compare the efficacy of 2 commercial single-dose M. hyopneumoniae vaccines and PRRSV vaccines in regard to virological and immunological analysis, pathological observation, and growth performance from wean to finish using a challenge model.

**Materials and methods**

**Commercial vaccines**

Two types of commercial M. hyopneumoniae vaccines were used in this study: A — the inactivated M. hyopneumoniae bacterin (RespiSure-One; Zoetis) given as one 2.0-mL dose at 7 d of age and B — the inactivated M. hyopneumoniae bacterin (Ingelvac MycoFLEX; Boehringer Ingelheim Vetmedica) given as one 1.0-mL dose at 21 d of age. Two types of commercial PRRSV vaccines were used in this study: A — the modified live PRRSV vaccine (Fostera PRRS; Zoetis) given as one 2.0-mL dose at 21 d of age, and B — the modified live PRRSV vaccine (Ingelvac PRRS MLV; Boehringer Ingelheim Vetmedica) given as one 2.0-mL dose at 21 d of age. All vaccines used in this study were administered according to the manufacturer’s label claims with regards to timing and route of injection (intramuscularly).

**Inocula**

*Mycoplasma hyopneumoniae* strain SNU98703, used as inoculum, was isolated from lung samples from postweaned pigs with severe enzootic pneumonia (11). *Mycoplasma hyopneumoniae* strain SNU98703 caused typical lesions consisting of peribronchial, peribronchiolar, and perivascular lymphoid hyperplasia in experimentally infected pigs (11). The PRRSV strain SNUVR90851 (type 2 genotype, lineage 1, GenBank no. JN315685), used as inoculum, was isolated from lung samples from different newly weaned pigs in Chungcheong Providence in 2010 (12). This virus strain caused interstitial pneumonia characterized by thickened alveolar septa with increased numbers of interstitial macrophages and lymphocytes and by type II pneumocyte hyperplasia in experimentally infected pigs (12).

**Animals**

A total of 60 colostrum-fed, cross-bred, conventional piglets were purchased at 5 d of age from an M. hyopneumoniae- and PRRSV-free commercial farm, based on serological testing of breeding herd and long-term clinical and slaughter history. All piglets were negative for M. hyopneumoniae, PRRSV, and porcine circovirus type 2 (PCV2) according to routine serological testing. *Mycoplasma hyopneumoniae* was not detected in the nasal samples by the real-time polymerase chain reaction (PCR) (13). The PRRSV and PCV2 were not detected in the serum and nasal samples by the real-time polymerase chain reaction (PCR) (14,15).

**Experimental design**

A total of 60 pigs were randomly divided into 4 groups: Zoetis-Vac (n = 20 pigs), BI-Vac (n = 20 pigs), positive control (n = 10), and negative control (n = 10) groups using the random number generation function (Excel; Microsoft Corporation, Redmond, Washington, USA). In the Zoetis-Vac group, pigs were immunized with the M. hyopneumoniae A bacterin and the PRRSV A vaccines at 7 and 21 d of age, respectively. In BI-Vac group, pigs were immunized with both the M. hyopneumoniae B bacterin (left side of the neck) and PRRSV B vaccine (right side of the neck) at 21 d of age. At 49 d of age [0 days post challenge (dpc)], pigs in the Zoetis-Vac, BI-Vac, and positive control groups were intratracheally administered a 10 mL dose of a lung homogenate of *M. hyopneumoniae* strain SNU98703 (1:100 dilution in Friis medium) at a final concentration of 10⁴ to 10⁵ color-changing units (CCU)/mL in the morning, as previously described (16). In the afternoon of the same day, same pigs were intranasally administered 3 mL of type 2 PRRSV (strain SNUVR90851; 2nd passage in MARC-145 cells) containing 1.2 × 10⁸ TCID₅₀/mL. Blood samples from each pig were collected by jugular venipuncture at −42, −28, 0, 14, 28, 63, 91, and 126 dpc. Sterile polyester swabs (Fisher Scientific, Pittsburgh, Pennsylvania, USA) were used to swab the nasal mucosa of both nostrils, reaching deeply into the turbinates at −42, −28, 0, 14, 28, 63, 91, and 126 dpc. Swabs were stored in 5 mL plastic tubes (Fisher Scientific) containing 1 mL of sterile saline solution. Pigs were sedated with an intravenous injection of azaperon (Stresnil; Janssen Pharmaceutica, Beerse, Belgium) and then euthanized by electrocution for necropsy at 175 d of age (126 dpc). Tissues were collected from each pig at necropsy. All of the methods were approved by the Seoul National University Institutional Animal Care and Use Committee.

**Clinical evaluation**

Following *M. hyopneumoniae* and PRRSV inoculation, the pigs were monitored daily for physical condition and scored weekly for clinical respiratory disease severity using scores ranging from 0 (normal) to 6 (severe dyspnea and abdominal breathing) (17). Observers were blinded to vaccination and challenge status. Pigs were observed daily at the same time of day. Rectal body temperature was recorded daily from 0 to 21 dpc.

**Assessment of growth performance**

The live weight of each pig was measured at 3 (−28 dpc), 7 (0 dpc), 10 (21 dpc), 16 (63 dpc), and 25 (126 dpc) weeks of age. The average daily weight gain (ADWG, grams/pig per day) was analyzed over 4 time periods: between 3 and 7; 7 and 10; 10 and 16; and 16 and 25 weeks of age. The ADWG during these various production stages was calculated as the difference between the starting and final weights divided by the duration of the stage. Data from dead pigs were included in the calculation.

**Serology**

The serum samples were tested using the commercially available *M. hyopneumoniae* and PRRSV enzyme-linked immunosorbent assay (ELISA; IDEXX M.hyo Ab Test and PRRS X3 Ab Test, IDEXX Laboratories, Westbrook, Maine, USA). Serum virus neutralization
Figure 1. Mean values of the clinical sign scores (A) and the rectal body temperature (B) in the different groups: Zoetis-Vac (□), BI-Vac (○), positive control (▲), and negative control (●) groups.

**Significant \( P < 0.05 \) difference between groups.
tests for PRRSV were also done using the challenge strain, as previously described (18,19). Serum samples were considered to be positive for neutralizing antibodies (NA) if the titer was greater than 2.0 (log₂) (20).

**Quantification of M. hyopneumoniae DNA**

DNA was extracted from the nasal swabs using a kit (QIAamp DNA Mini Kit; QIAGEN, Crawley, United Kingdom). The DNA extracts were then used to quantify the *M. hyopneumoniae* genomic DNA copy numbers by real-time PCR, as previously described (13).

**Quantification of PRRSV RNA**

RNA was extracted from serum samples and nasal swabs using a kit (QIAamp Viral RNA Mini Kit; QIAGEN) to quantify type 2 PRRSV genomic cDNA copy numbers by real-time PCR, as previously described (14).

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**Table I. Average daily weight gain (ADWG, g/pig per day), proportion of viremic pig and nasal shedder, pathology, in-situ hybridization of Mycoplasma hyopneumoniae (Mhp) and immunohistochemistry of porcine reproductive and respiratory syndrome virus (PRRSV) among 4 groups at different days post challenge (dpc)**

<table>
<thead>
<tr>
<th>Number of pigs</th>
<th>Zoetis-Vac*</th>
<th>BI-Vac†</th>
<th>Positive control</th>
<th>Negative control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of dead pigs</td>
<td>20</td>
<td>20</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>ADWG (wk old)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3–7</td>
<td>328 ± 28</td>
<td>324 ± 24</td>
<td>327 ± 19</td>
<td>330 ± 18</td>
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<tr>
<td>7–10</td>
<td>613 ± 26</td>
<td>601 ± 30</td>
<td>603 ± 28</td>
<td>615 ± 26</td>
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<tr>
<td>10–16</td>
<td>806 ± 34a</td>
<td>786 ± 33a</td>
<td>729 ± 31b</td>
<td>802 ± 35a</td>
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<tr>
<td>16–25</td>
<td>746 ± 30a</td>
<td>698 ± 25b</td>
<td>623 ± 27c</td>
<td>750 ± 31a</td>
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<tr>
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<td>669 ± 25a</td>
<td>640 ± 23b</td>
<td>595 ± 20c</td>
<td>670 ± 24a</td>
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<td>Mhp nasal shedders (dpc)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
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<td>11/20a</td>
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<td>0/10</td>
</tr>
<tr>
<td>28</td>
<td>11/20a</td>
<td>12/19a</td>
<td>9/9b</td>
<td>0/10</td>
</tr>
<tr>
<td>63</td>
<td>9/20a</td>
<td>11/19a</td>
<td>9/9b</td>
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</tr>
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<td>91</td>
<td>7/20a</td>
<td>8/19a</td>
<td>6/8a</td>
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<td>PRRSV viremic pigs (dpc)</td>
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<tr>
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<td>11/20a</td>
<td>12/20a</td>
<td>10/10b</td>
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<td>6/19a</td>
<td>9/9b</td>
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<td>1/19a</td>
<td>3/9a</td>
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<td>PRRSV nasal shedders (dpc)</td>
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<td>10/20a</td>
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<td>10/10b</td>
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<td>28</td>
<td>6/20a</td>
<td>7/19a</td>
<td>8/9b</td>
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</tr>
<tr>
<td>63</td>
<td>1/20a</td>
<td>1/19a</td>
<td>3/9a</td>
<td>0/10</td>
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<td>1/20a</td>
<td>1/19a</td>
<td>1/8a</td>
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<td>0/10</td>
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<td>Macroscopic lung lesion score</td>
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<td>3.48 ± 0.65b</td>
<td>10.75 ± 5.83c</td>
<td>0.53 ± 0.38d</td>
</tr>
<tr>
<td>Microscopic lung lesion score</td>
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<td>3.10 ± 0.36b</td>
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<td>0.87 ± 0.35d</td>
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<tr>
<td>Mhp DNA score</td>
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<td>1.50 ± 0.52b</td>
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<tr>
<td>PRRSV antigen score</td>
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<td>2.05 ± 3.25</td>
<td>2.70 ± 3.02</td>
<td>0</td>
</tr>
</tbody>
</table>

* Pigs that had received RespiSure-One bacterin at 7 days old and Fostera PRRS vaccine at 21 days old followed by a dual challenge at 49 days old.
† Pigs that had received Ingelvac MycoFLEX bacterin and Ingelvac PRRS vaccine at 21 days old followed by a dual challenge at 49 days old.
Different letters (a, b, and c) indicate significant (P < 0.05) difference among groups.
Enzyme-linked immunospot assay

*Mycoplasma hyopneumoniae* and PRRSV antigens were prepared as previously described (21,22). The numbers of *M. hyopneumoniae* - and PRRSV-specific interferon-γ secreting cells (IFN-γ-SC) stimulated by challenging *M. hyopneumoniae* and PRRSV were determined in peripheral blood mononuclear cells (PBMC) as previously described (23–25). The results were expressed as the numbers of IFN-γ-SC per million PBMC.

Quantification of interleukin-10 secretion

The interleukin-10 (IL-10) protein levels were quantified in the supernatants of PBMC cultures (2 × 10⁶ cells per well; 250 μL) *in vitro* for 20 h with challenging type 2 PRRSV (0.01 of MOI) or PHA (10 μg/mL) by using a commercial ELISA (Pig Interleukin-10 ELISA kit; Cusabio Biotech, Wuhan, China) according to the manufacturer’s instructions. Detection limits for IL-10 were 1.5 pg/mL.

Macroscopic lung lesion scores

The total extent of macroscopic lung lesions was estimated and calculated as previously described (26). The frequency distribution of the lung lesion scores for each lung lobe was calculated by treatment (16,26).

Morphometric analysis

For the morphometric analysis of pulmonary histopathological changes, 3 lung sections were taken from ventromedial part of the right and left caudal lobe, and right middle lobe. Lung sections were examined blindly and given an estimated severity score for the measures of pulmonary pathology (i.e., atelectasia, epithelial necrosis, hemorrhage, airway plugging, epithelial hyperplasia, interstitial change, and leukocyte infiltration) (26). Each individual measure was given a score ranging from 0 (no lesion visible) to 3 (severe lesions). These scores were added to obtain an overall score for each section.

In-situ hybridization and immunohistochemistry

*In-situ* hybridization (ISH) for *M. hyopneumoniae* was done as previously described (27). Immunohistochemistry (IHC) for PRRSV was done as previously described (28). The results of ISH and IHC were analyzed morphometrically as previously described (29,30).

Statistical analysis

Continuous data (rectal body temperatures, ADWG, quantified *M. hyopneumoniae* DNA and PRRSV RNA, *M. hyopneumoniae* and PRRSV serology, *M. hyopneumoniae* - and PRRSV-specific IFN-γ-SC, PRRSV-specific IL-10, macroscopic lung lesion scores, and PRRSV...
antigen scores) were analyzed using a repeated measures analysis of variance (ANOVA). If the repeated measures ANOVA showed a significant effect, Tukey’s multiple comparison test was done at each time point. Discrete data (clinical respiratory scores, mortality rate, microscopic lung lesion scores, and *M. hyopneumoniae* DNA scores) were analyzed using the Mann-Whitney test. The correlation between the ADWG and lung lesions was assessed by Spearman’s correlation. A value of *P* < 0.05 was considered significant.

Figure 3. Mean values of the genomic copy numbers of PRRSV RNA in the serum samples (A) and nasal swabs (B) in the different groups: Zoetis-Vac (□), BI-Vac (○), and positive control (△) groups.

*a, b, c* Significant (*P* < 0.05) difference between groups.
Figure 4. Immunological responses against *Mycoplasma hyopneumoniae* (Mhp). Mean values of the anti-*M. hyopneumoniae* antibody levels. Mean number of *M. hyopneumoniae*-specific interferon-γ secreting cells (IFN-γ-SC) in the different groups: Zoetis-Vac (□), BI-Vac (○), and positive control (△) groups. 

*a,b,c* Significant (*P* < 0.05) difference between groups.

Figure 5. Immunological responses against *Mycoplasma hyopneumoniae* (Mhp). Mean number of *M. hyopneumoniae*-specific interferon-γ secreting cells (IFN-γ-SC) in the different groups: Zoetis-Vac (■), BI-Vac (●), and positive control (□) groups. 

*a,b,c* Significant (*P* < 0.05) difference between groups.
Figure 6. Immunological responses against porcine reproductive and respiratory syndrome virus (PRRSV). A — Mean values of the anti-PRRSV antibody levels. B — Mean values of PRRSV-specific neutralizing antibodies (NA). Zoetis-Vac (□), Bi-Vac (○), and positive control (△) groups.

a,b,cSignificant (P < 0.05) difference between groups.
Figure 7. Immunological responses against porcine reproductive and respiratory syndrome virus (PRRSV). Mean number of PRRSV-specific interferon-γ secreting cells (IFN-γ-SC) in the different groups: Zoetis-Vac (■), BI-Vac (▲), and positive control (□) groups.

a,b,cSignificant (P < 0.05) difference between groups.

Figure 8. Level of challenging type 2 porcine reproductive and respiratory syndrome virus (PRRSV)-specific interleukin (IL)-10 in the different groups; Zoetis-Vac (■), BI-Vac (▲), and positive control (△) groups.

a,bSignificant (P < 0.05) difference between groups.
Results

Clinical evaluation

Pigs in the Zoetis-Vac and BI-Vac groups remained normal throughout the study, as measured by their respiratory scores and rectal temperatures, whereas moderate to severe respiratory signs were observed in the positive controls (Figures 1A and B). The mean rectal temperature (ranging from 39.8°C to 40.2°C) was significantly higher ($P < 0.05$) in the positive control groups than in the Zoetis-Vac and BI-Vac groups at 3, 4, 5, 6, and 7 dpc (Figure 1B). The mean respiratory scores were significantly higher ($P < 0.05$) in the positive control group than in the Zoetis-Vac and BI-Vac groups from 14 to 63 dpc. No genomic copies of \textit{M. hyopneumoniae} were detected in any of the nasal samples from negative controls throughout the experimental period. The overall mortality rate was significantly lower ($P < 0.05$) for the vaccinated challenged pigs (0%, 0/20 in the Zoetis-Vac group; 5%, 1/20 in the BI-Vac group) than the positive control group (20%; 2/10) (Table I). Diagnostic results indicated that mortality was primarily related to severe pneumonia.

Growth performance

No significant difference in the ADWG was observed between Zoetis-Vac and BI-Vac group during weeks 3 to 10. However, during weeks 10 ot 16, the ADWG of Zoetis-Vac group was significantly higher ($P < 0.05$) than that of BI-Vac group. The overall growth performance (from 3 to 25 wk of age) in pigs from the Zoetis-Vac group was significantly higher ($P < 0.05$) than that of pigs from the BI-Vac group (Table I). The other significant results are summarized in Table I.

Quantification of \textit{M. hyopneumoniae} DNA in nasal swabs

Prevalence rates of \textit{M. hyopneumoniae} positive pigs are summarized in Table I. At the time of the challenges, no genomic copies of \textit{M. hyopneumoniae} were detected in any of the nasal samples from any of the 4 groups. Pigs in the Zoetis-Vac and BI-Vac groups had a significantly lower ($P < 0.05$) number of genomic copies of \textit{M. hyopneumoniae} in their nasal swabs than the positive controls throughout the experimental period (Figure 2). Pigs in the Zoetis-Vac group had a significantly lower ($P < 0.05$) number of genomic copies of \textit{M. hyopneumoniae} in their nasal swabs than pigs in the BI-Vac group from 14 to 63 dpc. No genomic copies of \textit{M. hyopneumoniae} were detected in any of the nasal samples from negative controls throughout the experimental period.

Quantification of PRRSV RNA in sera and nasal swabs

Prevalence rates of PRRSV positive pigs were summarized in Table I. At the time of the challenges, no genomic copies of PRRSV were detected in any of the serum samples or nasal swabs from any of the 4 groups. Pigs in the Zoetis-Vac and BI-Vac groups had a significantly lower ($P < 0.05$) number of genomic copies of PRRSV in their sera and nasal swabs than pigs in positive controls at 14 and 28 dpc (Figure 3). No genomic copies of PRRSV were detected in any of the serum samples or nasal swabs from the negative controls throughout the experimental period.

Immunological responses of \textit{M. hyopneumoniae}

Pigs in the Zoetis-Vac group had significantly higher ($P < 0.05$) anti-\textit{M. hyopneumoniae} antibody levels (Figure 4) and numbers of \textit{M. hyopneumoniae}-specific IFN-γ-SC (Figure 5) at various dpc compared to pigs in the BI-Vac and negative control groups. The other significant results are summarized in Figure 4. No anti-\textit{M. hyopneumoniae} antibodies or \textit{M. hyopneumoniae}-specific IFN-γ-SC were detected in the negative controls.

Immunological responses of PRRSV

Pigs in the Zoetis-Vac and BI-Vac groups had significantly higher ($P < 0.05$) anti-PRRSV antibody levels from 0 to 28 dpc compared to positive control group (Figure 6A). The PRRSV-specific NA was detected in pigs in the Zoetis-Vac and BI-Vac groups at 91 and 126 dpc only (Figure 6B). Pigs in the Zoetis-Vac and BI-Vac groups had significantly higher ($P < 0.05$) numbers of PRRSV-specific IFN-γ-SC from 0 to 28 dpc compared to positive control group (Figure 7). No anti-PRRSV antibodies, PRRSV-specific NA, or PRRSV-specific IFN-γ-SC were detected in the negative controls.

The PRRSV-specific IL-10

After stimulation with challenging type 2 PRRSV, IL-10 gradually increased until 0 dpc and thereafter it decreased until 63 dpc in pigs in the Zoetis-Vac and BI-Vac groups (Figure 8). In positive controls, IL-10 had gradually increased at 28 dpc, after which it decreased until 63 dpc. Pigs in the Zoetis-Vac group had significantly lower ($P < 0.05$) levels of IL-10 than pigs in the BI-Vac group at 0 dpc. Pigs in the Zoetis-Vac and BI-Vac groups had significantly lower ($P < 0.05$) levels of IL-10 than pigs in the positive group at 28 dpc. Interleukin-10 was not detected in negative controls.

Lung lesion scores

Pigs in the Zoetis-Vac group had significantly lower ($P < 0.05$) scores for macroscopic and microscopic pulmonary lesions than pigs in the BI-Vac group at 126 dpc. The other significant results of scores for pulmonary lesions are summarized in Table I. Additionally, a negative correlation was found between ADWG and macroscopic lung lesion ($r = -0.578$, $P < 0.01$), and between ADWG and microscopic lung lesion ($r = -0.538$, $P < 0.01$).

\textit{In-situ} hybridization of \textit{M. hyopneumoniae}

Pigs in the Zoetis-Vac and BI-Vac groups had significantly lower ($P < 0.05$) lower amounts of \textit{M. hyopneumoniae} DNA in their lungs than positive controls (Table I).

Immunohistochemistry for PRRSV

There was no significantly different number of PRRSV-positive cells per unit in the lungs from pigs in the Zoetis-Vac, BI-Vac, or positive control groups (Table I).

Discussion

The present study demonstrated that the single-dose vaccination regimen for \textit{M. hyopneumoniae} bacterin and PRRS modified live vaccine is efficacious for controlling coinfection with \textit{M. hyopneumoniae}.
and PRRSV from wean to finish based on clinical, microbiological, immunological, and pathological evaluation. Vaccination of pigs with *M. hyopneumoniae* and PRRSV is able to reduce the levels of mycoplasmal nasal shedding, PRRSV viremia, and severity of lung lesions compared to unvaccinated challenged pigs. In contrast with earlier findings that vaccination with the PRRSV vaccine reduced the efficacy of the *M. hyopneumoniae* bacterin (31), sequential or concurrent single-dose vaccination with *M. hyopneumoniae* and PRRSV could not reduce the efficacy of the *M. hyopneumoniae* bacterin or the PRRSV vaccine in this study. Our results agree with previous findings in which no negative influence of the PRRSV vaccination on *M. hyopneumoniae* vaccine efficacy was observed (32,33). Interestingly, high levels of IL-10 were detected in pigs during the first 4 to 5 wk following PRRSV vaccination. Because IL-10 is a known potent immunosuppressive cytokine (34), induction of IL-10 following PRRSV vaccination may cause interference of the mycoplasmal vaccine’s efficacy. However, *M. hyopneumoniae*-specific IFN-γ-SCs gradually increased at the same time as the gradual increase of IL-10, indicating that levels of IL-10 following PRRSV vaccination are not enough to interfere with immune responses induced by the mycoplasmal vaccine.

Selection of an appropriate challenge virus is critical when comparing 2 PRRSV vaccines, which are only 91.7% homologous to each other. The Ingelvac PRRS MLV vaccine virus is from lineage 5 and the Fostera PRRS vaccine virus is from lineage 8. A challenge strain was chosen that was not closely related to either vaccine virus and had a similar level of homology to the 2 vaccine viruses, based on open reading frame 5 (ORF5) nucleotide sequences. Strain SNUVR090851 is from lineage 1 with homologies of 87.2% and 85.9% to Fostera PRRS and Ingelvac PRRS MLV, respectively. However, our results should be interpreted carefully because only ORF5 of the genome is used for the comparison. Therefore, additional studies are needed to confirm heterogenicity using full genome of challenge and vaccine viruses.

Pathological observation is also important parameter to evaluate the mycoplasmal and PRRSV vaccine because a reduction in *M. hyopneumoniae*- and PRRSV-induced lesions is correlated with improved weight gain (35–38). In the present study, coinfection with *M. hyopneumoniae* and PRRSV induces severe lung lesions, which are similar to typical PRDC. The most striking and consistent microscopic lesions were severe interstitial pneumonia with some degree of peribronchial and peribronchiolar fibrosis and lymphoid tissue hyperplasia. A reduction in lung lesions was correlated with improved weight gain, as previous studies have shown (35–38). Comparison of 2 vaccines is more practical and may be reflected field conditions because *M. hyopneumoniae* and PRRSV are 2 major contributors to PRDC (10). The 2 commercial vaccines used in this study were shown to be efficacious in controlling coinfection with *M. hyopneumoniae* and PRRSV based on clinical, microbiological, immunological, and pathological evaluation under experimental conditions.

### Acknowledgments

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### References

Safety and early onset of immunity with a novel European porcine reproductive and respiratory syndrome virus vaccine in young piglets

Michael Piontkowski, Jeremy Kroll, Christian Kraft, Teresa Coll

Abstract

Porcine reproductive and respiratory syndrome virus (PRRSV) can be difficult to manage in commercial settings. A novel type I PRRSV vaccinal strain (94881) was evaluated for safety and efficacy/onset of immunity (OOI) in piglets. In 2 experiments, groups of piglets were vaccinated intramuscularly (IM) at approximately 14 d of age with a maximum-range commercial dose, an overdose, or a placebo in experiment 1 and either a minimum-range commercial dose or a placebo in experiment 2. The piglets in experiment 1 were evaluated for local and systemic reactions from days −2 through 14 after vaccination. The piglets in experiment 2 were challenged with a virulent heterologous type I PRRSV isolate 14 d after vaccination and observed once daily for general health from days −1 through 12 after vaccination and once daily for clinical signs associated with challenge from days 13 through 24 after vaccination. The average daily weight gain (ADWG) and the results of serologic and viremia testing were evaluated in experiments 1 and 2. Lung lesion scores and results of testing for PRRSV in lung tissue were evaluated in experiment 2. In experiment 1 the vaccine was shown to be safe, as there were no relevant differences between the vaccinated piglets and the piglets given a placebo. In experiment 2 the vaccine’s efficacy, with an OOI of 14 d after vaccination, was established, as the vaccinated and challenged piglets exhibited significantly lower lung lesion scores, viremia, viral load in lung tissue, and total clinical sign scores, along with a significantly greater ADWG, compared with the placebo-vaccinated and challenged piglets.

Résumé

La gestion du virus du syndrome reproducteur et respiratoire porcin (VSRRP) peut être difficile dans un environnement de production commerciale. Une nouvelle souche vaccinale du VSRRP de type 1 (94881) a été évaluée d’un point de vue sécurité et efficacité/début de l’immunité (DDI) chez des porcelets. Dans deux expériences, des groupes de porcelets ont été vaccinés par voie intramusculaire (IM) à l’âge d’environ 14 j avec une dose commerciale maximale, une surdose, ou un placebo dans l’expérience 1 et une dose commerciale minimale ou un placebo dans l’expérience 2. Les porcelets dans l’expérience 1 furent évalués pour des réactions locale et systémique à compter du jour −2 jusqu’au jour 14 post-vaccination. Les porcelets dans l’expérience 2 furent soumis à une infection défi avec un isolat virulent hétérologue de VSRRP de type 1 14 j après la vaccination et observés une fois par jour pour leur état de santé général du jour −1 jusqu’au jour 12 après la vaccination et une fois par jour pour des signes cliniques associés avec l’infection du jour 13 au jour 24 après l’infection. Le gain moyen quotidien (GMQ) et les résultats des analyses sérologiques et de virémie ont été évalués dans les expériences 1 et 2. Les pointages de lésions pulmonaires et les résultats de détection du VSRRP dans le tissu pulmonaire ont été évalués dans l’expérience 2. Dans l’expérience 1, le vaccin s’est montré sécuritaire étant donné qu’il n’y avait aucune différence significative entre les porcelets vaccinés et les porcelets ayant reçu un placebo. Dans l’expérience 2, l’efficacité du vaccin, avec un DDI de 14 j après la vaccination, a été établie, étant donné que les porcelets vaccinés, et soumis à une infection défi avaient des valeurs significativement moins élevées de pointage de lésions pulmonaires, de virémie, de charge virale dans le tissu pulmonaire, et des pointages de signes cliniques totaux, avec un GMQ significativement plus élevé, comparativement au porcs vaccinés avec un placebo et soumis à une infection défi.

(Traduit par Docteur Serge Messier)
studies have determined that the level of genetic similarity between the vaccine strain and the challenge strain was not necessarily an accurate predictor of vaccine efficacy (4,13), whereas others have concluded that the level of protection a vaccine provides against PRRSV infection may depend on the degree of relatedness between the vaccine and challenge strains (7,10).

It has been reported that MLV vaccines against PRRSV are at least partially effective at reducing symptoms and viremia, as well as virus shedding (14). Furthermore, MLV vaccines based on a single virus strain offer consistently good protection against homologous challenge; however, protection against heterologous challenge is neither as consistent nor as effective (2,6,14). In an attempt to broaden vaccine protection, a vaccine containing 5 type II strains of attenuated PRRSV was developed; this vaccine, however, was found to offer no more effective protection against heterologous challenge than a single-strain vaccine (15). Geldhof et al (5) summarized the difficulties facing development of an effective PRRSV vaccine by reporting that infection with different strains leads to diverse virologic and immunologic effects (i.e., various degrees of homologous and heterologous protection) and that the susceptibility of PRRSV strains to antibody neutralization likewise differs. Since new strains, including highly pathogenic ones (3,16), continue to be found, it is important to continue to search for a single-strain vaccine that is effective in the face of heterologous challenge. One important factor to consider in this search is the virulence of the challenge strain: it should reflect what is found currently in the field. In the past, clinical signs of PRRSV infection have been difficult to reproduce with a single-strain type I PRRSV challenge, through either virus administration or contact with infected pigs (1,4,7,12,17,18). In addition to efficacy concerns, the safety of PRRS MLV vaccines has been questioned. It has been reported that these vaccines may cause clinical signs of respiratory disease and decreased growth performance, as well as viremia, and that the virus may subsequently spread to other naïve animals when administered to piglets (2,5,9,14,19). Additionally, PRRS MLV vaccines have been found to initiate protection in a relatively delayed manner, at around 3 to 4 wk after administration (2,17,18).

The present study was conducted to evaluate the safety and efficacy of a new European-derived PRRS MLV vaccine based on a novel type I strain (1) [European Collection of Cell Cultures accession numbers 11012501 (parent strain) and 11012502 (attenuated strain)] when administered to young piglets in the face of a disease-inducing heterologous PRRSV challenge.

Materials and methods

Animals

The protocols were reviewed and approved by the Contract Research Organization’s Institutional Animal Care and Use Committee before the study was started. Two experiments were conducted to establish safety (experiment 1) and efficacy/onset of immunity (OOI) (experiment 2) with 32 and 50, respectively, commercial mixed-breed castrated male and female piglets. The piglets in experiment 1 were blocked by weight and randomly assigned to group A (1A, n = 11), B (1B, n = 11), or C (1C, n = 10), except for the last block, which was assigned to either 1A or 1B. The piglets in experiment 2 were assigned to 1 of 3 treatment groups in the following manner: the piglets were blocked by weight, assigned a random number by means of the random number function in Microsoft Excel (Microsoft Corporation, Redmond, Washington, USA), and ranked in numerically ascending order by block; then the 2 lowest numbers of each block were assigned to group A (2A, n = 20), the next 2 numbers were assigned to group B (2B, n = 20), and the highest number was assigned to group C (2C, n = 10).

The piglets in experiment 2 were administered cefiofur crystalline-free acid (Excede; Pharmacia & Upjohn Company, Division of Pfizer, New York, New York, USA) intramuscularly (IM) in the right ham, according to label directions, at the time of arrival. The piglets in groups 1A, 1B, and 2A received the test vaccine (Ingelvac PRRSFLEX EU; Boehringer Ingelheim Vetmedica, St. Joseph, Missouri, USA) (1) at the maximum-range commercial dose, at an overdose, and at the minimum-range commercial dose, respectively, whereas the piglets in groups 1C, 2B, and 2C were administered a placebo. All the piglets were 14 ± 3 d of age at the time of treatment and were clinically healthy. The piglets in groups 1A, 1B, and 1C were monitored for local and systemic reactions to determine vaccine safety. The piglets in groups 2A and 2B were challenged with a heterologous strain of type I PRRSV, and those in group 2C were not challenged, serving as negative controls, to evaluate vaccine efficacy and OOI.

All the piglets were housed in multiple raised pens (5 or 6 pigs per pen) equipped with a nipple waterer, a feeder, and plastic slatted flooring. Each group was housed in separate but similar rooms for the duration of the experiments. The rooms were biosafety level 2-compliant, thermostat-controlled, and mechanically ventilated, with high-efficiency particulate air filtering, to ensure biosecurity. Additionally, appropriate measures were taken to prevent accidental cross-contamination from vaccinates and/or challenged animals. All the piglets were fed an age-appropriate commercially available ration medicated with tiamulin, 35 g/tonne, and chlortetracycline, 400 g/tonne (Lean Metrics Infant; Purina Mills, St. Louis, Missouri, USA). Feed and water were available ad libitum.

Serologic and viremia testing before vaccination

Blood was collected from all the piglets before vaccination. Briefly, venous blood was collected into serum separator tubes and allowed to clot at room temperature. Aliquots of serum were dispensed into appropriate tubes and held at either 2°C to 8°C or −70°C ± 10°C before testing. The samples held at 2°C to 8°C were tested for PRRSV antibodies at the Boehringer Ingelheim Vetmedica Health Management Center, Ames, Iowa, USA, with a commercially available enzyme-linked immunosorbent assay (ELISA) kit (IDEXX HerdChek PRRS X3 ELISA; IDEXX Laboratories, Westbrook, Maine, USA). Results were reported as negative [sample to positive (S/P) ratio of < 0.4] or positive (S/P ratio ≥ 0.4). The samples held at −70°C ± 10°C were tested for PRRSV RNA by quantitative polymerase chain reaction (qPCR; bioScreen GmbH, Münster, Germany) as described by Revilla-Fernandez et al (20) with use of the 2× TaqMan Universal PCR Kit (Applied Biosystems, Foster City, California, USA) with AmpErase uracil N-glycosylase (Applied Biosystems), the EU6-MGB.
(TGAGGCTCCGAAGYCCW; antisense). A PCR run was considered valid when the plasmid standard curve was linear, its \( R^2 \) value was greater than or equal to 0.95, and the non-template control did not cross the threshold line. Test samples were evaluated for the presence or absence of a signal crossing the threshold, shown as the cycle threshold (CT) value. For reporting purposes the samples were designated as positive or negative according to the point at which the PCR signal crossed the threshold in all replicates. Genomic equivalence (GE) was determined with use of the plasmid standard. The standard curve was generated and then used to calculate the initial concentration of an unknown sample by comparing its Ct value with the standard curve. The resulting initial concentration of the reaction (GE per reaction) was extrapolated to the amount of GE per milliliter.

Vaccination

All piglets in both experiments were administered the test vaccine or placebo at 14 ± 3 d of age. Those in groups 1A and 1B were vaccinated IM once with 1 mL of the vaccine (according to the manufacturer’s label instructions), at the maximum-range commercial dose (the maximum release dose of the vaccine at the time of production) and at an overdose (10 times the release dose), respectively. The piglets in group 1C served as negative controls and were administered 1 mL of sterile phosphate-buffered saline IM. The piglets in group 2A were vaccinated IM with 1 mL of the vaccine at the minimum-range commercial dose (minimum dose that provides protection). The piglets in groups 2B and 2C were injected IM with 1 mL of a lyophilized placebo product containing the inert vaccinal material without the PRRS MLV fraction.

Observations after vaccination

The piglets in experiment 1 were observed once daily for clinical signs of disease in terms of behavior (recumbency, shivering, lethargy, unconsciousness, or death), respiration (mild to severe coughing, sneezing, abdominal breathing, or rapid respiration), and digestion (vomiting, diarrhea, change in appetite), as well as for other relevant clinical signs (e.g., hernia, thinness, lameness, and edema around the eyes) on days −2 and −1 and from days 1 to 14 after vaccination, as well as twice on day 0 (just before and at 4 h after vaccination), and in the piglets in experiment 2 from days 13 to 24 after vaccination (days −1 to 10 after challenge). A normal physiological range was considered to be 38.7°C to 39.9°C, and fever was defined as an increase in temperature to 40.0°C or beyond.

In experiment 1 the individual body weight was measured in all the piglets on days −2, 0, and 14 after vaccination, and the average daily weight gain (ADWG) was determined from days 0 to 14 after vaccination. In experiment 2 the individual body weight was measured in all the piglets on days 0, 14, and 24 after vaccination, and the ADWG was calculated from days 0 to 14 and days 14 to 24 after vaccination.

Serologic and viremia testing for PRRS

Blood was collected via jugular venipuncture from the piglets in experiment 1 on days 7 and 14 after vaccination and from the piglets in experiment 2 on days 7, 14, 17, 21, and 24 after vaccination. Blood was processed for serum and used to determine the S/P ratio of antibodies against PRRSV by ELISA and PRRSV viremia (log_{10} GE/mL) by qPCR as described.

Postmortem examination and lung studies

All the piglets in experiment 1 were euthanized by sedation and then electrocution and underwent necropsy on day 14 after vaccination. Each injection site was palpated, incised, and evaluated for gross lesions. Additionally, the thoracic and abdominal cavities were exposed and examined for gross lesions. Two lung tissue samples containing lesions were collected from 1 piglet (in group 1A). One sample was placed in a Whirlpak (United States Plastic Corporation, Lima, Ohio, USA) and the other in a container with 10% formalin and submitted to the Iowa State University Veterinary Diagnostic Laboratory, Ames, Iowa, USA. The fresh sample was cultured for bacteria and the formalin-fixed sample examined histopathologically and by immunohistochemistry testing for PRRSV, porcine circovirus 2 (PCV-2), and Mycoplasma hyopneumoniae antigens.

All the piglets in experiment 2 were similarly euthanized and underwent necropsy on day 24 after vaccination (day 10 after infection).

Serologic and viremia testing for PRRS

Blood was collected via jugular venipuncture from the piglets in experiment 1 on days 7 and 14 after vaccination and from the piglets in experiment 2 on days 7, 14, 17, 21, and 24 after vaccination. Blood was processed for serum and used to determine the S/P ratio of antibodies against PRRSV by ELISA and PRRSV viremia (log_{10} GE/mL) by qPCR as described.

Postmortem examination and lung studies

All the piglets in experiment 1 were euthanized by sedation and then electrocution and underwent necropsy on day 14 after vaccination. Each injection site was palpated, incised, and evaluated for gross lesions. Additionally, the thoracic and abdominal cavities were exposed and examined for gross lesions. Two lung tissue samples containing lesions were collected from 1 piglet (in group 1A). One sample was placed in a Whirlpak (United States Plastic Corporation, Lima, Ohio, USA) and the other in a container with 10% formalin and submitted to the Iowa State University Veterinary Diagnostic Laboratory, Ames, Iowa, USA. The fresh sample was cultured for bacteria and the formalin-fixed sample examined histopathologically and by immunohistochemistry testing for PRRSV, porcine circovirus 2 (PCV-2), and Mycoplasma hyopneumoniae antigens.

All the piglets in experiment 2 were similarly euthanized and underwent necropsy on day 24 after vaccination (day 10 after infection).
challenge). The thoracic cavity was exposed, and the heart and lungs were removed. The lungs were examined for gross lesions, and the percentage of each lobe that was abnormal was recorded. Total lung lesion scores were determined as a percentage of lung involvement, calculated according to a weighting formula that accounts for the relative weight of each of the 7 lobes. The assessed percentage of lung lobe area with typical lesions was multiplied by the lobe factor (i.e., left apical = 0.05, left cardiac = 0.06, left diaphragmatic = 0.29, right apical = 0.11, right cardiac = 0.10, right diaphragmatic = 0.34, and intermediate = 0.05), and the total weighted lung lesion score was determined.

Lung samples were collected from all the piglets in experiment 2 for quantitation of PRRSV in the lung tissue. Samples in Whirlpaks were stored at −70°C ± 10°C until thawed and liquefied into a homogenate, subjected to RNA extraction procedures, and then tested for PRRSV RNA by qPCR as described. The results were reported as log_{10} GE/mL for left and right/intermediate lung samples.

Statistical analyses

The statistical analyses were conducted and data summaries prepared by Dr. Martin Vanselow (Biometrie & Statistik, Hannover, Germany) using SAS software release 8.2 or later (SAS Institute, Cary, North Carolina, USA).

For experiment 1, all data listings and summary statistics by treatment group, including mean, median, standard deviation, and/or frequency distribution, were generated for all primary variables, including local (injection-site) reactions, systemic (behavioral, respiratory, digestive, and other, as well as fever) reactions, and ADWG. Specifically, the proportions of piglets in each group with any abnormal clinical observation, with a specific clinical observation (behavioral, respiratory, digestive, or other score greater than 0), with an increase in rectal temperature greater than 1.5°C when compared with baseline (day 0), with any injection-site reaction score greater than 0, or with a specific injection-site reaction score greater than 0 for at least 1 d from 0 + 4 h to 14 d after vaccination were evaluated by Fisher’s exact test; the numbers of days per animal with any abnormal clinical observation and with any injection-site reaction from 0 + 4 h to 14 d after vaccination were evaluated by the Wilcoxon Mann–Whitney test; the mean daily rectal temperature for each group from 0 + 4 h to 14 d after vaccination and the initial mean body weight for each group on day 0 were evaluated by analysis of variance (ANOVA); and the mean ADWG for each group from 0 to 14 d after vaccination was evaluated by the t-test.

For experiment 2, data were analyzed with the assumption of a randomized block design, and all tests on differences were designed as 2-sided at an α-value of 5%. Frequency tables of animals with at least 1 positive clinical finding between days 1 and 12 after vaccination, animals with at least 1 positive clinical finding from days 15 to 24 after vaccination (days 1 to 10 after challenge), and animals with positive ELISA results were generated, and differences between groups were compared by Fisher’s exact test. Differences in mean daily rectal temperature between treatment groups, as compared with baseline, were tested by ANOVA and t-tests, and least-squares (LS) means of groups and differences between LS means with 95% confidence intervals were calculated from the ANOVA. Comparisons between treatment groups for total lung lesion scores, maximum and mean clinical scores (for coughing and abnormalities of respiration and behavior, as well as for all 3 added together) per animal for days 15 to 24 after vaccination, the PRRSV qPCR (viremia) data (evaluated separately for each day), as well as the area under the curve (AUC) for individual responses between days 14 and 24 and between days 17 and 24 after vaccination were analyzed with the Wilcoxon Mann–Whitney test.

Results

After vaccination no piglets in groups 1A and 1B (single-dose and overdose vaccination, respectively) exhibited abnormal behavior, whereas 1 piglet in group 1C (placebo) was lethargic on days 8 and 9. There was no significant difference in behavior (P = 0.4762) between the vaccinated groups and the nonvaccinated group. Conversely, at least 1 piglet in each group exhibited abnormal respiration for a minimum of 1 d after vaccination: 1 piglet in group 1A, 2 piglets in group 1B, and 3 piglets in group 1C exhibited mild coughing; sneezing was noted for 3 piglets in group 1C; and severe coughing was noted for 1 piglet. There was no significant difference in respiration (P ≥ 0.1486) between the vaccinated groups and the nonvaccinated group. No piglets exhibited any abnormal digestive findings after vaccination. Several abnormal clinical findings not related to PRRSV vaccination (designated as “other”) were also noted: 1 piglet in group 1A was noted as thin on days 7 to 10 after vaccination (likely a result of decreased feed intake due to competition at the feeder) but was otherwise normal; 1 piglet in group 1B was found to have rectal prolapse on days 6 to 12; and 1 piglet in group 1B was noted as having a scrotal hernia on days 10 to 14. There was no significant difference in “other” clinical signs (P ≥ 0.4762) between the vaccinated groups and the nonvaccinated group. Likewise, no significant differences in clinical observations were noted between either of the vaccinated groups and the nonvaccinated group when all categories of clinical observation were combined (P ≥ 0.3615) or in the number of days piglets exhibited any clinical abnormalities (P = 0.2662 and 0.7726 for groups 1A and 1B, respectively) (Table 1). No clinical abnormalities related to PRRSV were noted in any piglets in experiment 2 after vaccination and before challenge (i.e., on days −1 to 12 after vaccination); however, 1 piglet in group 2B had a lesion anterior to the right front leg beginning 9 d after vaccination.

At the injection site, redness, heat, and pain were not noted in any piglet in experiment 1, but 1 piglet each in groups 1A (single dose) and 1B (overdose) exhibited minimal swelling (palpable only) for at

Table 1. Number of days on which piglets exhibited any abnormal clinical signs after vaccination against porcine reproductive and respiratory syndrome virus (PRRSV)

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of piglets</th>
<th>Number of days</th>
<th>P-valuea</th>
</tr>
</thead>
<tbody>
<tr>
<td>1A (maximum dose)</td>
<td>11</td>
<td>4</td>
<td>0.2662</td>
</tr>
<tr>
<td>1B (overdose)</td>
<td>11</td>
<td>7</td>
<td>0.7726</td>
</tr>
<tr>
<td>1C (placebo)</td>
<td>10</td>
<td>8</td>
<td>NA</td>
</tr>
</tbody>
</table>

a In comparison with group 1C.
NA — not applicable (no analysis conducted).
compared with group 2A on days −1, 0, and 2 after challenge. St.
test vaccine (Ingelvac PRRSFLEX EU; Boehringer Ingelheim Vetmedica,
drome virus (PRRSV) (groups 2A and 2B) 14 d after administration of the
heterologous strain of type I porcine reproductive and respiratory syn-
Figure 1. Mean rectal temperatures of piglets after challenge with a
significant difference ($P < 0.0001$) in the temperatures for group 2B
compared with group 2A on days −1, 0, and 2 after challenge.

least 1 d after vaccination; no piglets in group 1C (placebo) exhibited
swelling after vaccination. These differences were not significant
($P = 1.0000$) (Table II).

After challenge, abnormal respiration was observed in 2 (10%) of
the 20 vaccines (group 2A) and 6 (30%) of the 20 challenge con-
trols (group 2B); however, these proportions were not significantly
different ($P = 0.2351$). Groups 2A and 2B had maximum abnormal
respiration scores of 1 (panting/rapid respiration) and 2 (dyspnea),
respectively, a difference that was not significant ($P = 0.1872$); both
groups had a median maximum respiration score of 0. The mean
respiration score for group 2A was lower than that for group 2B
(0.02 versus 0.07; $P = 0.1394$). Similarly, although coughing was
observed in more of the group 2B piglets than in the group 2A piglets
(11 of 20 (55%) versus 6 of 20 (30%)), the difference was not significant
($P = 0.2003$). Groups 2A and 2B had maximum scores for coughing of
1 (soft or intermittent; median score of 0) and 2 (harsh or severe and
repetitive; median score of 1), respectively; these differences were
not significant ($P = 0.1129$). The mean coughing score was lower for
group 2A than for group 2B (0.07 versus 0.17; $P = 0.0535$). Abnormal
behavior was observed in significantly fewer piglets ($P = 0.0012$) in
group 2A than in group 2B: 0 of 20 versus 9 of 20 (45%). Group 2A
had a significantly lower ($P = 0.0012$) maximum behavior score than
group 2B: 0 (normal) 1 (mild to moderate lethargy). The mean
behavior score for group 2A was significantly lower ($P = 0.0012$) than
that for group 2B: 0.00 versus 0.12. When combined, the percentages
of piglets with total clinical scores greater than 0 were 30% (6 of 20)
in group 2A and 65% (13 of 20) in group 2B and were not significa-
cantly different ($P = 0.0562$). Group 2A had a significantly lower
maximum total score than group 2B: 1 versus 4 ($P = 0.0072$). The mean
total scores for group 2A were significantly lower ($P = 0.0103$) than
those for group 2B: 0.08 versus 0.35. No clinical signs were observed
in the negative controls (group 2C) at any time after challenge, and
the group had a score of 0 for each parameter.

For the piglets in experiment 1, all the mean rectal temperatures
were within the normal physiological range, and no piglets exhibited
an increase in rectal temperature of 1.5°C or more above baseline on
any day after vaccination. In experiment 2, the mean and LS mean
rectal temperatures for the piglets in group 2A (vaccinated and
challenged) were 39.77°C on the day before challenge (day 13 after
vaccination) and ranged from 39.69°C to 40.68°C after challenge
(on days 1 and 2 after challenge, respectively), for the piglets in
group 2B (nonvaccinated and challenged) they were 39.39°C on the
day before challenge and ranged from 39.77°C to 40.61°C after chal-
lenge (on days 2 and 6, respectively), and for the piglets in group 2C
(nonvaccinated and not challenged) they remained at 39.68°C or
less throughout the study. The LS means were significantly lower
than group 2B: 0.0528) between the 2 groups on any day, as the rectal temperatures for
both groups were at or above 40°C from days 4 (group 2B) and 5
(group 2A) through day 10 after challenge (Figure 1).

In experiment 1 there were no significant differences in mean
body weight between the groups on days 0 and 14 after vaccina-
tion ($P = 0.7582$ and $P = 0.2273$, respectively) and no significant
differences ($P = 0.1562$ and 0.1628, respectively) in ADWG from
0 to 14 d after vaccination (Table III). In experiment 2 there were no significant differences in LS mean body weight between the piglets in
groups 2A and 2B on days 0 ($P = 0.8743$) and 14 ($P = 0.4297$) after
vaccination. However, on day 24 after vaccination (day 10 after chal-
lenge) the LS mean body weight of the piglets in group 2A (10.26 kg)

<table>
<thead>
<tr>
<th>Number of days after vaccination</th>
<th>Number of piglets</th>
<th>Minimum</th>
<th>Maximum</th>
<th>Median</th>
<th>95% CI</th>
<th>Mean</th>
<th>P-value a</th>
</tr>
</thead>
<tbody>
<tr>
<td>1A (maximum dose)</td>
<td>11</td>
<td>0</td>
<td>4</td>
<td>0</td>
<td>0</td>
<td>0.4</td>
<td>1.0000</td>
</tr>
<tr>
<td>1B (overdose)</td>
<td>11</td>
<td>0</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>0.3</td>
<td>1.0000</td>
</tr>
<tr>
<td>1C</td>
<td>10</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.0</td>
<td>NA</td>
</tr>
</tbody>
</table>

| a In comparison with group 1C.  
CI — confidence interval; NA — not available.

<table>
<thead>
<tr>
<th>Group</th>
<th>Day 0</th>
<th>Day 14</th>
<th>P-value a</th>
<th>Weight (kg)</th>
<th>ADWG (kg/d)</th>
<th>P-value a</th>
</tr>
</thead>
<tbody>
<tr>
<td>1A</td>
<td>4.5</td>
<td>8.1</td>
<td>0.7582</td>
<td>0.3</td>
<td>0.1562</td>
<td></td>
</tr>
<tr>
<td>1B</td>
<td>4.3</td>
<td>8.0</td>
<td>≥ 0.2273</td>
<td>0.3</td>
<td>0.1628</td>
<td></td>
</tr>
<tr>
<td>1C</td>
<td>4.4</td>
<td>8.6</td>
<td>NA</td>
<td>0.3</td>
<td>NA</td>
<td></td>
</tr>
</tbody>
</table>

| a In comparison with group 1C.  
NA — not available.
Table IV. Least-squares mean weight and ADWG on days 0 to 14 and 14 to 24 after vaccination and days 0 to 10 after challenge with a virulent heterologous type I PRRSV isolate 14 d after vaccination in experiment 2

<table>
<thead>
<tr>
<th>Group</th>
<th>Weight (kg)</th>
<th>ADWG (kg/d)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 0</td>
<td>Day 14(a)</td>
</tr>
<tr>
<td>2A (20 vaccinates)</td>
<td>4.14</td>
<td>7.64</td>
</tr>
<tr>
<td>2B (20 controls)</td>
<td>4.17</td>
<td>7.39</td>
</tr>
</tbody>
</table>

\(a\) Day 0 after challenge.
\(b\) Day 10 after challenge.
\(c\) For the difference between groups 2A and 2B on day 24 after vaccination. On days 0 and 14 after vaccination there were no significant differences between the 2 groups (P = 0.8743 and 0.4297, respectively).
\(d\) For differences between groups 2A and 2B.

was significantly higher (P = 0.0063) than that of group 2B (8.87 kg). Likewise, the difference in LS mean ADWG between groups 2A and 2B was not significant (P = 0.1889) from days 0 to 14 after vaccination; however, from days 14 to 24 (days 0 to 10 after challenge) the LS mean ADWG was significantly higher (P = 0.0003) for the piglets in group 2A compared with those in group 2B (Table IV). Therefore, the piglets vaccinated 14 d before challenge had an ADWG significantly higher than the piglets that were not vaccinated before challenge.

All the piglets in experiment 1 were PRRSV-seronegative on day 0 after vaccination. On day 14 all 11 piglets in group 1A and 10 (91%) of the 11 piglets in group 1B were seropositive, whereas the 10 piglets in group 1C remained seronegative. These results indicate that the single dose and overdose of vaccine were effective at causing an immune response. All the piglets in experiment 2 were PRRSV-seronegative on days 0 and 7 after vaccination. On day 14 the proportion of seropositive piglets was significantly higher (P 0.0001) in group 2A than in group 2B: 17 of 20 (85%) versus 0 of 20. Three days after challenge (17 d after vaccination) the proportion of seropositive piglets in group 2A had increased to 19 of 20 (95%); the piglets in group 2B remained seronegative. On day 7 after challenge (day 21 after vaccination) 11 of 20 (55%) of the piglets in group 2B were seropositive, a significant difference (P 0.0012) from the 20 of 20 in group 2A. On day 10 after challenge (day 24 after vaccination), however, 20 (100%) and 19 (95%) of the piglets in groups 2A and 2B, respectively, were PRRSV-seropositive. All the piglets in group 2C remained seronegative throughout the experiment.

No PRRSV RNA was detected in the serum of any piglets in experiment 2 on day 0. On days 7 and 14 after vaccination the piglets in group 2A had median values of 3.00 and 3.32 log10 GE/mL, respectively, whereas group 2B (nonvaccinated) remained free of PRRSV RNA; however, on day 17 (day 3 after challenge) the piglets in group 2B had significantly higher values (P 0.0001) than those in group 2A: 8.18 versus 6.72 log10 GE/mL. There were no significant differences (P 0.0565) between the 2 groups on days 21 and 24 after vaccination (days 7 and 10 after challenge), the values for group 2A being 7.38 and 7.15 log10 GE/mL, respectively, and those for group 2B being 7.87 and 7.27 log10 GE/mL, respectively. However, on days 17 to 24 after vaccination (days 3 to 10 after challenge) the daily AUC for group 2A was significantly lower (P = 0.0039) than the AUC for group 2B: 49.52 GE/mL versus 54.35 GE/mL. No PRRSV RNA was detected in the serum of any piglet in group 2C (Figure 2).

No injection-site abnormalities or gross lesions in the abdominal and thoracic cavities were noted at necropsy for any of the piglets in experiment 1, except for 1 piglet in group 1A that had exhibited mild coughing for 1 d and was found to have minor lung lesions. After sampling and testing, the lesions were determined to be a result of infection with *Bordetella bronchiseptica*. The piglet was negative for PRRSV, PCV-2, and *M. hyopneumoniae* in IHC testing. Interestingly, the 4 group 1C piglets that exhibited sneezing, mild coughing, or severe coughing did not have gross lesions at necropsy. Additionally, at the time of necropsy the weight of the group 1A piglet that had been observed as thin for 4 d was found to be within the range of the other piglets in the group, and no gross lesions were noted. Thus, the sneezing, coughing, and temporary thinness noted for the piglets were not associated with vaccination. In experiment 2, the mean total lung lesion score was significantly lower (P = 0.0002) for group 2A than for group 2B: 27.36% versus 54.84% (Table V).

Although PRRSV RNA was detected in the lung tissue of all piglets in groups 2A and 2B, the mean qPCR values were significantly lower (P = 0.0003) for the piglets in group 2A than in group 2B. On days 0 to 14 after challenge there were no significant differences between groups 2A and 2B (Table IV). On days 0 and 14 after vaccination there were no significant differences between the 2 groups (P = 0.8743 and 0.4297, respectively).

For the difference between groups 2A and 2B on day 24 after vaccination. On days 0 and 14 after vaccination there were no significant differences between the 2 groups (P = 0.8743 and 0.4297, respectively).

For differences between groups 2A and 2B.
lower (P = 0.0101) in group 2A than in group 2B at the time of necropsy (day 24 after vaccination; day 10 after challenge): 7.47 log_{10} GE/mL versus 7.88 log_{10} GE/mL.

### Discussion

Vaccinating weaned piglets with a PRRS MLV vaccine is a beneficial practice for a variety of reasons, such as increased ADWG and decreased frequency of clinical signs in piglets exposed to PRRSV (1,5,21). The effectiveness of a PRRS MLV vaccine in protecting piglets from clinical signs is important because clinical disease generally leads to decreased health and growth. In addition to being effective, a PRRS MLV vaccine must be safe to administer (i.e., not cause local or systemic reactions and result in minimal viremia and virus shedding). Safety was evaluated in this study by monitoring for local and systemic reactions (including through clinical assessment) and calculating the ADWG; effectiveness was determined primarily from the total lung lesion scores, with supportive parameters including the results of clinical assessment, serologic study, viremia testing after vaccination, ADWG, and viral load in lung tissues. According to these criteria the data clearly demonstrated that vaccination with the novel type I PRRSV 94881-based MLV vaccine (Ingelvac PRRSFLEX EU) is a safe option for reducing the incidence of disease associated with PRRSV. Specifically, in experiment 1 young piglets (16 to 17 d of age) that had been vaccinated once with either the maximum-range commercial dose or an overdose of the test vaccine, whereas the other 2 groups received a placebo. The 20 group 2B piglets were challenged with the same strain as group 2A, whereas the 10 group 2C piglets were not challenged.

IQR — interquartile range; NI — not included in the statistical analysis; CI — confidence interval.

A single dose of the vaccine at the minimum commercial range induces immunity 14 d after vaccination when administered to very young piglets (14 ± 3 d of age). Moreover, when compared with the nonvaccinated piglets the vaccinated piglets in experiment 2 had significantly lower lung lesion scores, viremia, viremia over time (AUC), viral load in lung tissue, behavior scores, and total clinical scores, and they had a significantly higher ADWG after challenge (i.e., 14 to 24 d after vaccination).

Several previously reported studies examined the clinical effectiveness of MLV PRRS vaccines. Martelli et al (4) found that a single IM or intradermal (ID) dose of an MLV vaccine (based on the type I PRRSV vaccinal strain DV) administered to 5-week-old piglets offered approximately 70% protection against clinical signs of disease when the piglets were challenged with a heterologous type I Italian wild-type strain (05R1421) through natural exposure 45 d after vaccination. The challenge strain was shown to be virulent, as 100% of the controls had increased rectal temperatures, increased lethargy, and decreased appetite throughout the study compared with 90% of the vaccinated animals, which showed consistently less severe signs of disease. However, the frequency and severity of clinical signs in all the groups may have been complicated by the presence of other pathogens within the herd (i.e., PCV-2, M. hyopneumoniae, Pasteurella multocida, and Streptococcus sp.). Various reports have indicated that PRRSV may have an immunosuppressive effect, thus making pigs more susceptible to secondary infections (5,12,14). Additionally, the exposure levels of the field challenge will vary among herds and between individual pigs, such that it is difficult to determine the occurrence of a vaccinal effect or if some animals were less exposed to the challenge virus. Another study evaluated a single-strain MLV vaccine (based on the EU vaccinal strain ALL-183) (19) administered on 2 occasions, 3 wk apart, to 5-month-old gilts that were challenged with a heterologous Lelystad strain 28 d after initial vaccination (18). In this trial it was determined that the vaccine had no significant effect on clinical signs, rectal temperatures, and ADWG; however, the challenge strain was not considered virulent. On the other hand, there were significant differences between the vaccinated and nonvaccinated pigs in level of viremia and viral load in lungs and tonsils after heterologous challenge, as in the present study. In contrast, the vaccine did not induce a serologic response to PRRSV until 21 d after initial vaccination; the ELISA S/P ratio was negative (< 0.4) on day 14 after vaccination, whereas in the present study there was a positive strong serologic response, an S/P ratio ≥ 0.4, in 17 out of 20 piglets on day 14. Likewise, another study found that pigs vaccinated once at 7 wk of age with a commercially available attenuated vaccine based on the type I strain DV experienced a
shortened duration of viremia compared with pigs vaccinated twice, at 5 and 9 wk of age, with a commercially available inactivated vaccine based on the type I strain P120 or with either of 2 experimental inactivated vaccines based on the type I field isolates 07 V063 and 08 V194 in the face of fever-inducing heterologous challenge with either 07 V063 or 08 V194 (resulting in temperatures > 39.5°C and ≤ 40.6°C, respectively) at 13 wk of age (5). Trus et al (11) reported a significant reduction in severity and duration of fever as well as in the AUC for viremia in 4- and 7-week-old pigs vaccinated with an MLV PRRS vaccine (type I strain DV) and challenged with a highly pathogenic PRRSV strain (Lena, isolated from a farm in the Republic of Belarus) 8 and 6 wk, respectively, after vaccination when compared with nonvaccinated controls. However, differences in gross lung lesions between the groups were not significant. This trial used older pigs that were challenged much later after vaccination, compared with the present study, in which the younger piglets were challenged 14 d after vaccination. Another study using an MLV PRRS vaccine based on the European DV strain found that vaccinated 4-week-old pigs exhibited significantly reduced viremia levels and shorter duration of viremia, compared with nonvaccinated controls, when challenged with a heterologous strain (3267, isolated from a Portuguese farm) by contact with inoculated pigs 37 d after vaccination (12). The challenge strain was not known to induce noteworthy clinical signs outside of mild respiratory symptoms, which is characteristic of type I PRRSV strains; however, PRRSV was detected by means of qPCR in the lung tissue of all vaccinated and nonvaccinated pigs that were subsequently euthanized (n = 5 and 4, respectively). In contrast, in the present study the vaccinated piglets had significantly lower scores for total clinical signs, reduced PRRS viral load in the lung tissue, and, most importantly, significant reductions in scores for PRRSV-specific lung lesions compared with the nonvaccinated piglets after challenge. Roca et al (22) found that a commercially available MLV vaccine based on type I PRRSV strain VP046 BIS administered to 4-week-old pigs offered partial protection against challenge with a highly virulent type II strain (HP-PRRS21) 6 wk after vaccination. In that trial the vaccinated piglets showed a significant reduction in fever on 2 d (days 11 and 12 after challenge), significantly increased weight gain, decreased lethargy and/or anorexia, and decreased viremia (which was detected only on day 7 after challenge); however, the vaccine did not significantly reduce the score for lung lesions. In comparison, in the present study the vaccinated piglets had significantly lower LS mean rectal temperatures 1 d after challenge, significantly increased ADWG in the postchallenge period, a significantly lower incidence of abnormal behavior (lethargy), and significantly reduced viremia (on day 3 after challenge and AUC on days 3 to 10 after challenge) and lung abnormalities compared with the nonvaccinates after challenge. Prieto et al (13) evaluated the efficacy of multiple doses of vaccine before challenge: 4-week-old pigs were vaccinated on 3 occasions, 21 d apart, with the type I strain DV, beginning at 28 d of age, and were challenged with the type I PRRSV strain 5710 (isolated in Spain) 4 wk after the final vaccination. There were no significant differences between the vaccinated and nonvaccinated pigs in clinical signs or virus titer in all tissues collected at necropsy. However, the clinical signs were less severe in the vaccinated pigs, and the virus was found less frequently in their tissues, compared with the nonvaccinated pigs. Another study using the MLV PRRS European DV strain determined (by virus titration in bronchoalveolar lavage fluids and serum by immunoperoxidase monolayer assay) that pigs vaccinated at 5 wk of age either IM or ID were fully protected from homologous wild-type challenge but only partially protected from heterologous challenge (with an Italian strain) at 49 d after vaccination, as evidenced by significantly lower mean virus titers in serum samples from the vaccinated pigs as compared with the nonvaccinated controls (4). Antibodies to PRRSV began developing around 7 d after vaccination; however, 100% seroconversion did not occur until 35 d after vaccination, whereas in the present study the pigs in groups 1A, 1B, and 2A were 100%, 91%, and 85% seropositive, respectively, on day 14 after vaccination. Likewise, the data from the present study show that the novel PRRSV vaccine strain 94881 was effective against viremia after challenge, the vaccines having significantly lower mean log_{10} GE/mL values on day 3 after challenge and a significantly lower AUC for 3 to 10 d after challenge compared with the controls.

Effectiveness of vaccination was shown in the above instances in a manner similar to that in the present study (i.e., through decreased clinical signs, viremia, and viral load in lung tissue, as well as increased ADWG). However, no other vaccine was shown to positively affect all these parameters in a single trial involving challenge with a heterologous European-derived, type I PRRSV that induced clinical signs and lung lesions. In contrast to the previously reported studies, the present study showed significant reductions in lung abnormalities in young pigs vaccinated with the PRRS 94881 MLV vaccine. Moreover, to our knowledge this is the first time a 14-d OOI has been reported in piglets vaccinated before 3 wk of age.

Most PRRS MLV vaccines are considered safe if there are no significant local and/or systemic reactions, as well as differences in clinical signs, after vaccination (5,7,9,13,17); however, little is known about the likelihood of European-derived PRRS MLV vaccine strains to cause viremia as well as the shedding of vaccine virus and subsequent transmission to naïve pigs. One study aimed to alleviate this knowledge gap by evaluating the safety of 3 vaccines based on type I strains that are commercially available in Europe (9): 4-week-old pigs were vaccinated with 3 PRRS MLV vaccines based on the strains VP046 BIS, ALL-183, and DV, and unvaccinated sentinel pigs were introduced at day 3 after vaccination. As with the present study, the vaccines were all deemed clinically safe, as no significant differences were noted in clinical signs between vaccinated and nonvaccinated pigs, and vaccination did not negatively affect ADWG. In contrast, the authors found that the vaccinated groups had varied mild lung lesion scores on 1 or more necropsy days (i.e., 7, 14, and 21 d after vaccination) that decreased over time. Additionally, all the vaccine strains induced viremia (in 57.3% to 88% of the pigs), and PRRSV was found in lung tissues in all the groups (in 20% to 66.7%). These results are contrary to the data from the present study, as no pigs in experiment 1 had lung lesions associated with vaccination, and PRRSV was not isolated from lung tissues at the time of necropsy even after the pig had received an overdose of the vaccine. Although the vaccinated piglets in experiment 2 also exhibited viremia, the level was significantly less than in the nonvaccinated group after challenge. Moreover, the vaccinated groups in the safety trial (9) shed virus in oropharyngeal and nasal secretions as well as feces (4.89%
to 6.67% of all samples tested positive); thus, the sentinel pigs for each group became viremic and subsequently shed virus (2.22% to 5.71% of all samples tested positive). Although the vaccinated pigs in the present study did exhibit viremia, there was no evidence that the vaccine virus infected their lungs even after an overdose. Additionally, good biosecurity practices ensured that all negative control animals remained negative for all parameters tested. Other studies have also reported the presence of vaccine virus in the blood after vaccination (4,9,13,17).

These studies, as well as the present data, support the knowledge that the variability of PRRSV continues to confound the development of a single-strain vaccine that may be effective against the virus in the field. Although it is difficult to compare these data in a side-by-side manner, similarities and trends do exist. Generally, vaccination with an MLV vaccine has been found to offer significant protection against certain aspects of PRRSV infection (viremia, clinical signs, decreased productivity, etc.). The present study offers evidence that a vaccine based on the novel type I PRRSV strain 94881 administered to very young piglets may offer similar, if not improved, protection against the negative clinical and economic effects of PRRSV infection.

In conclusion, a vaccine that is safe and effective in preventing the detrimental effects of type I PRRSV in a timely manner is an essential part of swine production practices. The results from this study support the clinical safety and efficacy of Ingelvac PRRSFLEX EU when administered to piglets approximately 14 d of age. When the vaccine was administered once at the maximum-range commercial dose or as an overdose, there were no significant differences in clinical signs attributable to the vaccine; only transient, minimal swelling was noted at the injection site of 1 piglet, and ADWG was not affected. Through the significant reduction in lung lesions, viremia, viral load in lung tissue, and clinical signs, as well as the improvement in ADWG, it has been shown that administration of a single IM dose of the novel PRRS 94881 MLV vaccine to piglets induced the response required to build protective immunity by 14 d after vaccination. Consequently, a vaccine formulated from the type I PRRS viral strain 94881 has been proven to be a safe and effective method of protection against the detrimental effects of virulent PRRSV infection in young piglets.

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References


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Development of a double-monoclonal antibody sandwich ELISA: Tool for chicken interferon-γ detection ex vivo

Hua Dai, Zheng-zhong Xu, Meiling Wang, Jun-hua Chen, Xiang Chen, Zhi-ming Pan, Xin-an Jiao

Abstract

The aim of the present work was to develop reagents to set up a chicken interferon-γ (ChIFN-γ) assay. Four monoclonal antibodies (mAbs) specific for ChIFN-γ were generated to establish sandwich ELISA based on 2 different mAbs. To improve the detection sensitivity of ChIFN-γ, a double-monoclonal antibody sandwich ELISA was developed using mAb 3E5 as capture antibody and biotinylated mAb 3E3 as a detection reagent. The results revealed that this ELISA has high sensitivity, allowing for the detection of 125 to 500 pg/mL of recombinant ChIFN-γ, and also has an excellent capacity for detecting native ChIFN-γ. This ELISA was then used to detect ChIFN-γ level in chickens immunized with a Newcastle disease virus (NDV) vaccine, the immunized chicken splenocytes were stimulated by NDV F protein as recall antigen. From our results, it appears that the sensitivity range of this sandwich ELISA test is adequate to measure the ex vivo release of ChIFN-γ.

Résumé

L’objectif de la présente étude était de développer des réactifs afin de mettre au point une épreuve de détection de l’interféron-γ de poulet (ChIFN-γ). Quatre anticorps monoclonaux (AcMo) spécifiques pour ChIFN-γ ont été produits afin d’avoir un ELISA sandwich reposant sur deux AcMo différents. Afin d’améliorer la sensibilité de détection de ChIFN-γ, un test ELISA sandwich a deux anticorps monoclonaux a été développé utilisant l’AcMo 3E5 comme anticorps de capture et l’AcMo 3E3 biotinylé comme réactif de détection. Les résultats ont démontré que ce test ELISA possède une sensibilité élevée, permettant la détection de 125 à 500 pg/mL de ChIFN-γ recombinant, et ayant également une excellente capacité à détecter le ChIFN-γ original. Ce test ELISA a par la suite été utilisé pour détecter les quantités de ChIFN-γ chez des poulets immunisés avec un vaccin contre la maladie de Newcastle (NDV), les cellules de la rate des poulets immunisés ont été stimulées par la protéine F du NDV comme antigène de rappel. À partir de nos résultats, il semble que la plage de sensibilité de ce test ELISA sandwich est adéquate pour mesurer la libération ex vivo de ChIFN-γ.

Introduction

Interferon-γ (IFN-γ; also called type II interferon), a cytokine produced predominantly by T-helper type 1 (TH1) cells and Natural Killer cells in response to antigenic or mitogenic stimulation (1,2), plays a critical role in initiating and regulating cell mediated immunity, which is a central player in initiating the TH1 response against intracellular pathogens (3,4).

Chicken provides an important animal model of a number of intracellular infections. Like its mammalian counterpart, chicken IFN-γ (ChIFN-γ) strongly upregulates the expression of class II major histocompatibility complex (MHC) proteins (5–7) so that antimicrobial and antiviral activities of chickens are improved (5,7–10). The ChIFN-γ also enhances immunity against tumors and parasites (11–15). Previous studies showed that the level of IFN-γ following antigenic/mitogenic stimulation allows for accurate evaluation of cell-mediated immunity (CMI) (16,17). Unfortunately, methods of detecting ChIFN-γ are limited. So far, ChIFN-γ is commonly detected based on its ability to inhibit viral replication in vitro or activate the HD11 macrophages. Both of these assays are labor-intensive, time-consuming, and nonspecific methods that exhibit low sensitivity and are difficult to standardize. Although real-time PCR (RT-PCR) or Northern blot can detect very low levels of ChIFN-γ, these methods can be used to analyze ChIFN-γ only at the mRNA, but not the protein level.

Therefore, a qualitative and quantitative assay to accurately and efficiently determine ChIFN-γ levels in biological samples is extremely urgent, especially to study response to infections induced by intracellular bacteria, parasites, and viruses. Until now, there were 2 kinds of assays reported that could successfully evaluate ChIFN-γ in the protein level (18,19). One is a monoclonal antibody (mAb)-based direct binding enzyme-linked immunosorbent assay (ELISA), the other is a quantitative ELISA based on the combination of a rabbit anti-ChIFN-γ serum with a mAb. Both of them could measure ChIFN-γ in a variety of formats and the latter is more sensitive than the former. But these assays are still limited and need to be improved in detecting trace amounts of ChIFN-γ. To address this problem, this study was designed to develop a ChIFN-γ-specific ELISA. We have used recombinant ChIFN-γ, which was generated before (20) to develop mAbs against ChIFN-γ. Using these antibodies we have developed a capture ELISA system for the detection of both recombinant and native ChIFN-γ.
Materials and methods

Chickens
Four-week-old, white Laihang, specific pathogen free (SPF) chickens and 8-week-old BALB/C mice used in this study were provided by the Comparative Medical Center of Yangzhou University (Yangzhou, China), the animals were housed and handled at the Animal Biosafety Facilities and all procedures were approved by the Institutional Animal Experimental Committee.

Vaccines, plasmids, and recombinant proteins
The Newcastle disease mild, living (La Sota strain) vaccine (100992007, Wuhan Chopper Biology, Wuhan, Hubei, China) was used to vaccinate chickens. Chickens were immunized via the oculo-nasal route according to the manufacturer’s instructions. Recombinant plasmid pVAX1-ChIFN-γ was provided by Jiangsu Key Lab of Zoonosis (Jiangsu, China). Newcastle disease virus recall antigen (recombinant protein of NDV F protein) (21), bovine IFN-γ (BovIFN-γ), cervine IFN-γ (CerIFN-γ), chicken IFN-γ (ChIFN-α), and chicken interleukin 4 (ChIL-4), all provided by Jiangsu Key Lab of Zoonosis.

Production of recombinant ChIFN-γ proteins and native ChIFN-γ
The production and purification of Escherichia coli-derived recombinants, histidine (His)-tagged ChIFN-γ (His-ChIFN-γ), 330 µg/mL, glutathione S-transferase(GST)-tagged ChIFN-γ (GST-ChIFN-γ), 1500 µg/mL and the baculovirus-derived recombinant Bac-ChIFN-γ (the supernatant of recombinant virus infected S9) were described previously (20,21).

Natural ChIFN-γ was produced by concanavalin A (Con A)-stimulation of spleen cells from SPF chickens. Splenocyte suspensions were prepared as described (22) and adjusted to 10⁷ cells/mL in the growth medium RPMI 1640 (Invitrogen, Thermo Fisher Scientific, Waltham, Massachusetts, USA) containing 10% inactivated fetal bovine serum (Hyclone; Thermo Fisher Scientific), 100 U penicillin/mL, 100 µg streptomycin/mL. Then, 2.5 × 10⁶ cells (250 µL) per well were transferred into flat-bottomed 24-well plates. Equal volumes (250 µL) of medium containing 6, 12, or 24 µg/mL of final concentration Con A were added in triplicate, and cultures were incubated for 4 d. Negative controls received 250 µL RPMI 1640 medium only. After 4 d of incubation at 41°C, 5% CO₂ supernatant was harvested from each well for the measurement of ChIFN-γ production.

Production of monoclonal antibodies
The 8-week-old BALB/c mice were immunized at an interval of 2 wk by subcutaneous injections with 100 µg of recombinant His-ChIFN-γ emulsified in Freund’s adjuvant (Sigma-Aldrich, St. Louis, Missouri, USA), and boosted intravenously with 100 µg of His-ChIFN-γ without adjuvant 3 d prior to fusion. Hybridomas were tested for the presence of antibodies against GST-ChIFN-γ by using an ELISA (see below). Positive cells were cloned 3 times by limiting dilution. The immunoglobulin (Ig) sub-class of mAbs was determined using a mouse mAb isotyping kit (Sigma-Aldrich, according to the manufacturer’s instructions. BALB/c mice were injected intraperitoneally using positive hybridoma cell line secreting anti-ChIFN-γ mAb. Ascites fluids containing abundant anti-ChIFN-γ mAb were produced by these immunized mice and purified by protein A chromatography (GE Healthcare, Wauwatosa, Wisconsin, USA). Purified mAbs were biotinylated using standard methods (GenScript Biotechnique Company, Nanjing, Jiangsu, China).

The ChIFN-γ ELISA for screening of ChIFN-γ antibodies
The 96-well plates (XiaMen YunPeng Biotechnique Company, Xiamen, Fujian, China) were coated with purified GST-ChIFN-γ diluted at 1.5 µg/mL in 0.05 M/L carbonate-bicarbonate buffer (pH 9.6) for 24 h at 4°C, 50 µL each well. Subsequently, the plates were washed 3 times using phosphate-buffered saline (PBS) containing 0.05% Tween-20 (PBS-T). The plates were then blocked with PBS-T containing 10% (v/v) newborn bovine serum (Lanzhou National Hyclone Bio-engineering Corp., Lanzhou, Gansu, China) for 4 h at 37°C. After washing, 50 µL of hybridoma supernatants were added for 1 h at 37°C and then washed as above. Binding of antibodies was revealed using horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (Sigma-Aldrich), incubated for 30 min at 37°C. The HRP activity was revealed by addition of 3,3’-diaminobenzidine (DAB) for 15 min at 37°C. The reaction was stopped by addition of 2 M H₂SO₄, 490 nm wavelength was used to detect the color development due to HRP reacting with OPD, OD₄₉₀ value indicates the presence of mAbs against ChIFN-γ.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and western-blotting
Proteins were separated by SDS-PAGE on a 12% polyacrylamide gel (pH 8.8) with a 5% stacking gel (pH 6.8), in Tris-glycine running buffer (pH 8.7), and transferred onto Polyvinylidene Fluoride (PVDF) membrane. The membranes were blocked with 5% milk protein in PBS, then incubated with anti-ChIFN-γ mAbs in blocking buffer for 1 h. After washing, the membranes were incubated with HRP-conjugated goat anti-mouse IgG (Sigma-Aldrich) in blocking buffer for 0.5 h. Washing 5 times, the membranes were developed using western blotting detection reagent [PBS containing 0.05% 3, 3’-diaminobenzidine (DAB) and 0.03% H₂O₂]. This DAB substrate solution deposits a brown specific stain in the presence of HRP, then the reaction was quickly stopped by distilled water. After air drying the chromogenic membrane was scanned by the scanner (2580, Epson China Co., Beijing, China).

(COS)-7 cells, Cercopithecus aethiops kidney cells transformed by SV40, were seeded in 24-well-plates and cultured in Dulbecco’s Modified Eagle’s Medium (DMEM; Invitrogen, Thermo Fisher Scientific) containing 10% fetal bovine serum (Hyclone) and incubated overnight to 50% cell confluent at 37°C, 5% CO₂. Recombinant plasmid pVAX1-ChIFN-γ and control plasmid pVAX1 were transduced into COS-7 cells, respectively, using lipofectin reagent (Invitrogen; Thermo Fisher Scientific) following the manufacturer’s instruction. After an additional 48 h incubation at 37°C, 5% CO₂ the supernatant of each well was discarded, plates were washed 3 times using ice cold PBS (pH 7.2), and directly fixed by ice cold methanol for 5 min at room temperature. Then methanol was removed and
allow to air dry. After PBS washing, an optimal concentration of mAb was added and incubated for 1 h at room temperature in a shaker, 50 times/min (TS-2, Kylin-Bell Lab Instrument Co., Haimen, Jiangsu, China). After washing, the second antibody fluoresceine isothiocyanate (FITC)-conjugated goat anti-mouse IgG (H + L) (R&D systems, Minneapolis, Minnesota, USA) was added into each well for 30 min incubation at room temperature in dark. After the washing steps, fluorescence of each well was observed and photos were taken by fluorescence microscope (TS 100, Nikon, Japan).

Antigen-capture ELISA

To determine the ability of mAbs to capture ChIFN-γ in various biological test samples, 96-well ELISA plates were precoated with serial dilutions of purified mAbs in 0.01 M PBS at 4°C for 24 h, 100 µL each well. Precoated wells were washed 3 times in PBS-T, then blocked with 2% BSA (in PBS-T, Sangon Bio-engineering Corporation, Shanghai, China) overnight at 4°C. After blocking, 100 µL of each test sample diluted in 1% BSA (in PBS) was added to individual wells and incubated for 2 h at 37°C, followed by a washing step, 100 µL of an optimized dilution of the biotin-labeled mAbs were added and incubation was continued for 1 h at 37°C. The plates were washed 5 times in PBS-T, then incubated with 100 µL of avidin-HRP (diluted in 1: 2500 in PBS-T containing 1% BSA, 20% inactivated newborn bovine serum) for 30 min at 37°C, then exposed to 3,3’,5,5’-tetramethylbenzidine (TMB) peroxidase substrate solution (eBioscience, San Diego, California, USA). The reaction was stopped by addition of 2 M H2SO4; OD490 of each sample was measured.

Establishment of a double-mAb sandwich ELISA for the detection of ChIFN-γ

In a checker-board analysis, all 4 mAbs were used as capturing or revealing antibodies, and His-ChIFN-γ was used to identify compatible mAb sandwich partners as well as to determine the optimal concentration of coating antibody. The specificity of this sandwich ELISA was verified using Bac-ChIFN-γ and native ChIFN-γ secreted from splenocytes activated by Con A.

The ChIFN-γ stimulated by specific NDV F protein recall antigen

Four-week-old SPF chickens were vaccinated with the Newcastle disease mild vaccine. Three weeks after vaccination, splenocytes from each chicken were isolated for stimulation ex vivo. The NDV F protein was used as a recall antigen to test the response of spleno-
immunofluorescence analysis. All the results showed that 4 mAbs provided positive identification of recombinant ChIFN-γ without cross-reaction with other control proteins (Table I, Figures 1 and 2).

**The establishment of a double-mAb sandwich ELISA for recombinant ChIFN-γ**

In order to detect native ChIFN-γ, an antigen-capture ELISA system was set up using purified and biotinylated mAbs as capture and detection antibodies, respectively. Checker-board analysis revealed that even very low levels of recombinant ChIFN-γ could be detected when the biotin-labeled 3E3 was used as the detection antibody in combination with any of the other 4 mAbs used as capturing antibody (Figure 3). A double-mAb sandwich ELISA allowing the detection of 125–500 pg/mL of His-ChIFN-γ with low background was achieved using mAb 3E5 as the capture antibody (85 µg/mL) and biotinylated mAb 3E3 as the detection antibody (0.67 µg/mL) (Figures 4a, 4b, 5b).

**Sandwich ELISA for detection of native ChIFN-γ**

After establishment of the double-mAb sandwich ELISA shown in Figure 3, the specificity of this ELISA was evaluated by detecting recombinant ChIFN-γ (Bac-ChIFN-γ, His-ChIFN-γ) as well as native ChIFN-γ which is in the supernatant secreted from Con A activated chicken splenocytes. The ELISA could detect not only the recombinant ChIFN-γ but also the native ChIFN-γ (Figure 5a), and is not affected by recombinant ChIL-4 protein (Figure 5b). When the concentration of Con A in the cultured medium was increased from 0 to 12 µg/mL, the level of ChIFN-γ detected also increased. Concentrations of Con A greater than 12 µg/mL did not further increase the level of ChIFN-γ detected (Figure 6). Furthermore, extending the time over which splenocytes were incubated with Con A also led to an increase in the levels of ChIFN-γ detected, although the degree of increase was less pronounced after 48 h.

In addition, we found that increasing levels of ChIFN-γ were detected when the number of splenocytes was increased from 1 × 10^6 cell/mL to 5 × 10^6 cell/mL (Figure 7). These results indicate that this double-mAb sandwich ELISA is suitable to measure ChIFN-γ in a variety of settings.

**Sandwich ELISA for detection of native ChIFN-γ stimulated by NDV recall antigen**

Splenocytes from 4-week-old NDV immunized chickens were examined 3 wk after live vaccination for their ability to produce ChIFN-γ upon ex vivo stimulation. We found that splenocytes from vaccinated chickens produced ChIFN-γ after NDV-specific antigen recall stimulation with NDV F proteins. The ELISA could detect the highest production of ChIFN-γ after 96 h of exposure to 20–40 µg/mL of NDV F proteins (Figure 8). There was no significant difference in ChIFN-γ production from the antigen-stimulated cultures of unimmunized birds (data not show). These results suggest that this capture ELISA is able to detect native ChIFN-γ released ex vivo.

**Discussion**

Interferon-γ is a significant regulatory cytokine in animals’ and birds’ immune systems, and is therefore an important indicator of immune function. Due to the lack of efficient methods, the study of this important cytokine was hindered. Therefore, it is urgent to establish a convenient assay to detect this important cytokine especially on the protein level.

Previous studies have reported that mAbs with high biological activity could be developed using recombinant antigens derived from *E. coli*, and could be used to establish a potent sandwich ELISA (24–26). In this paper, 4 specific mAbs against recombinant ChIFN-γ were generated, and the specificity of the 4 mAbs was confirmed by ELISA, Western blot, and immunofluorescence analysis. In order to
find the optimal mAb combinations, the 4 mAbs were purified and used in different combinations, with the most sensitive mAb pair (3E5, 3E3) subsequently selected as capture and detecting reagents to develop a capture ELISA with high-sensitivity and low-background.

Our results demonstrated that this capture ELISA based on mAbs could detect both recombinant and native forms of ChIFN-γ. Increasing culture time, mitogen concentration, and numbers of spleenocytes activated led to increased detection of ChIFN-γ, indicating that this assay is adequate to measure in vitro release of ChIFN-γ. Furthermore, this capture ELISA was successfully used to detect limited native ChIFN-γ production stimulated ex vivo by NDV recall antigen, suggesting that this ELISA is likely to be useful for measuring native IFN-γ in biological samples and for studying the function of native IFN-γ in vivo.

This sandwich ELISA will be particularly useful in examining the role of ChIFN-γ in the induction of the immune response to several
ChIFN-γ production of splenocytes from immune chickens. Additionally, this assay could be used to detect ChIFN-γ as a surrogate for a robust Th1 response in several chicken models of infectious diseases. We are currently investigating whether the mAbs reported here can be applied to intracellular cytokine staining by flow cytometry or to ELISPOT assays to enumerate IFN-γ-secreting cells. At the very least, these antibodies can serve as useful reagents to develop additional specific IFN-γ immunoassays.

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**References**

Effects of ketamine and lidocaine in combination on the sevoflurane minimum alveolar concentration in alpacas

Patricia Queiroz-Williams, Thomas J. Doherty, Anderson F. da Cunha, Claudia Leonardi

Abstract

This study investigated the effects of ketamine and lidocaine in combination on the minimum alveolar concentration of sevoflurane ($MAC_{SEVO}$) in alpacas. Eight healthy, intact male, adult alpacas were studied on 2 separate occasions. Anesthesia was induced with SEVO, and baseline MAC ($MAC_b$) determination began 45 min after induction. After $MAC_b$ determination, alpacas were randomly given either an intravenous (IV) loading dose (LD) and infusion of saline or a loading dose [ketamine = 0.5 mg/kg body weight (BW); lidocaine = 2 mg/kg BW] and an infusion of ketamine (25 μg/kg BW per minute) in combination with lidocaine (50 μg/kg BW per minute), and $MAC_{SEVO}$ was re-determined ($MAC_t$). Quality of recovery, time-to-extubation, and time-to-standing, were also evaluated. Mean $MAC_b$ was 1.88% ± 0.13% and 1.89% ± 0.14% for the saline and ketamine + lidocaine groups, respectively. Ketamine and lidocaine administration decreased ($P < 0.05$) $MAC_b$ by 57% and mean $MAC_t$ was 0.83% ± 0.10%. Saline administration did not change $MAC_b$. Time to determine $MAC_b$ and $MAC_t$ was not significantly different between the treatments. The quality of recovery, time-to-extubation, and time-to-standing, were not different between groups. The infusion of ketamine combined with lidocaine significantly decreased $MAC_{SEVO}$ by 57% and did not adversely affect time-to-standing or quality of recovery.

Résumé

La présente étude visait à examiner les effets d’une combinaison de kétamine et de lidocaïne sur la concentration alvéolaire minimale de sevoflurane ($CAM_{SEVO}$) chez des alpagas. Huit alpagas mâles entiers et en santé ont été étudiés en deux occasions distinctes. L’anesthésie a été induite avec SEVO, et la détermination de la CAM de base ($CAM_b$) débutée 45 min après l’induction. Après détermination de la $CAM_b$, les alpagas ont reçu par voie intraveineuse (IV), sur une base aléatoire, une dose de charge (DC) et une infusion de saline ou une dose de [kétamine = 0,5 mg/kg de poids corporel (PC); lidocaïne = 2 mg/kg PC] et une infusion de kétamine (25 μg/kg PC par minute) en combinaison avec de la lidocaïne (50 μg/kg PC par minute), et la $CAM_{SEVO}$ re-déterminée ($CAM_t$). La qualité de la récupération, le temps pour extuber, et le temps pour se tenir debout ont également été évalués. La $CAM_b$ moyenne était de 1,88 % ± 0,13 % et de 1,89 % ± 0,14 % pour les groupes saline et kétamine + lidocaïne, respectivement. L’administration de kétamine et de lidocaïne a diminué une diminution ($P < 0,05$) de 57 % de $CAM_b$ et la $CAM_t$ moyenne était de 0,83 % ± 0,10 %. L’administration de saline n’a pas changé la $CAM_b$. Le temps pour déterminer la $CAM_b$ et la $CAM_t$ n’était pas significativement différent entre les groupes de traitement. La qualité de la récupération, le temps pour extuber, et le temps pour se tenir debout n’étaient pas significativement différents entre les groupes. L’infusion de kétamine combinée à la lidocaïne a diminué significativement la $CAM_{SEVO}$ de 57 % et n’affecta pas négativement le temps pour se tenir debout ou la qualité de la récupération.

(Traduit par Docteur Serge Messier)

Introduction

The use of multidrug regimens to maintain general anesthesia allows a decrease in the minimum alveolar concentration (MAC) of volatile anesthetics, and is generally associated with a lesser decrease in arterial blood pressure than when inhalational anesthesia alone is used (1). Ketamine and lidocaine infusions are commonly used in combination with inhalational anesthetics, such as sevoflurane (SEVO), for the maintenance of anesthesia.

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were aware of the selected treatments. For SEVO were measured for each anesthetic occasion. Investigators the MAC constant rate infusion of lidocaine and ketamine in combination on the MAC of volatile anesthetics in camelids. It was hypothesized that the combination would significantly decrease the MACSEVO.

Materials and methods

Animals

Eight healthy, intact male, adult (2.5- to 10-year-old) alpacas, weighing between 42.3 kg and 60.5 kg, were used in the study. Alpacas were determined to be healthy on the basis of history, physical examination, complete blood cell count, and serum biochemical profile. Food was withheld for 18 h prior to anesthesia but water was permitted. The study was approved by the Institutional Animal Care and Use Committee of the Louisiana State University School of Veterinary Medicine.

Experimental design

Alpacas were used in a randomized $8 \times 2$ crossover design (Wichman-Hill procedure for pseudo-random number generation). Briefly, each alpaca was studied on 2 different occasions, getting each treatment once, with a washout period of at least 10 d between treatments. Baseline MAC (MAC_B) and post-treatment MAC (MAC_T) for SEVO were measured for each anesthetic occasion. Investigators were aware of the selected treatments.

Anesthesia

Anesthesia was induced with SEVO at 6% (SevoFlo; Abbott Laboratories, Illinois, USA) in oxygen (5 L/min) delivered by face mask from a circle system attached to a small animal anesthetic machine (SurgiVet Small Animal Veterinary Anesthesia Machine; Smiths Medical, Ohio, USA). After endotracheal intubation, alpacas were placed in left lateral recumbency and anesthesia was maintained with SEVO in oxygen (3 L/min). Monitoring (Passport 2 Datascope Gas Module SE; Datascope, New Jersey, USA) consisted of electrocardiogram (ECG), indirect blood pressure, pulse oximetry, and continuous gas analysis for capnography (PE’CO₂), end-tidal oxygen (PE’O₂), and end-tidal sevoflurane (FE’SEVO), and body temperature. Gas sample was drawn from the proximal portion of the endotracheal tube at a rate of 150 mL/min. The gas analyzer was calibrated, according to the manufacturer’s instructions at the start of each experiment. Indirect blood pressure was monitored using an oscillometric technique, and a suitably sized cuff (width approximately 40% of limb circumference) placed between the elbow and carpal joint. Ventilation was controlled (SAV 2500 Anesthesia Ventilator; SurgiVet, Wisconsin, USA) to achieve PE’CO₂ between 30 and 45 mmHg. Body temperature was monitored continuously via an esophageal probe and maintained within normal limits (37.5°C to 38.6°C) using a forced warm air device (Bair Hugger; 3M, Minnesota, USA). Hemoglobin oxygen saturation was estimated using a probe placed on the tongue. A 16-gauge (51 mm) IV catheter (Abbocath-T; Venisystems, Illinois, USA) was placed into the right jugular vein for administration of a Normosol-R solution (Hospira, Illinois, USA) at 3 mL/kg BW per hour, and ketamine (Ketathesia; Butler Health Supply, Ohio, USA) and lidocaine (Lidocaine Injectable; Sparhawk Laboratories, Kansas, USA) administration. An 18-gauge (51 mm) IV catheter (Abbocath-T; Venisystems, Illinois, USA) was placed into the saphenous vein at the medial aspect of the left hind limb for blood sampling for drug analysis. Catheters were placed percutaneously and aseptically after animals were anesthetized and recumbent on the surgical table.

Determination of baseline minimum alveolar concentration

Approximately 45 min after induction of anesthesia, and with FE’SEVO held constant at 2.3 vol% for at least 20 min, determination of the MAC_B for SEVO was initiated. A noxious stimulus, which consisted of clamping a claw between the jaws of a 25.4 cm Vulsellum forceps, was delivered. The forceps were closed tightly to the first or second ratchet, depending on the claw size, just below the coronary band, and the claw was moved continuously for 1 min, or until purposeful movement occurred. Purposeful movement was defined as gross movement of the head or extremities. Coughing, stiffening of the neck or limbs, or chewing was not considered purposeful movement. If purposeful movement occurred, the FE’SEVO was increased by 0.1 vol%, otherwise it was decreased by 0.1 vol%, and the stimulus was re-applied after a 20-minute equilibration period. Claws were clamped in a rotating manner to prevent overuse of individual claws. The MAC_B for SEVO was defined as the mean of the highest concentration that resulted in gross purposeful movement and the lowest concentration that prevented that movement. The MAC_B was determined in duplicate for each anesthetic occasion and the mean of the mean values was used for statistical analysis. Time-to-MAC_B was recorded as the time from intubation to the completion of MAC_B determination, in duplicate.

Drug administration

After MAC_B determination, alpacas were randomly given one of the following 2 IV treatments as a loading dose (LD) and constant rate infusion (CRI), as follows:

- Saline: 0.9% NaCl solution (LD of 10 mL followed by a CRI at the volume of ketamine + lidocaine calculated for that specific animal)
- Ketamine + lidocaine: ketamine (LD of 0.5 mg/kg BW followed by CRI of 25 μg/kg BW per minute) combined with lidocaine (LD of 2 mg/kg BW followed by CRI of 50 μg/kg BW per minute)

Loading doses were made up to a final volume of 10 mL in normal saline (0.9% NaCl) and given over 3 min. The CRI was started immediately after the loading dose was administered. For each anesthetic occasion, the LD and CRI of ketamine + lidocaine were gently shaken several times into the syringes for homogeneous mixture of both drugs, ketamine and lidocaine. The LD and CRI were delivered using a syringe pump (Medfusion; Medex, Duluth, Georgia, USA).

Post-treatment MAC (MAC_T) determination began 30 min after the start of the LD with the FE’SEVO held constant for at least 20 min at each alpaca’s MAC_B value. MAC_T was determined in duplicate.
Table I. Effect of IV saline (0.9% NaCl) solution (control group) and ketamine (LD: 0.5 mg/kg BW IV; CRI: 25 μg/kg BW per minute) combined with lidocaine (LD: 2 mg/kg BW, IV; CRI: 50 μg/kg BW per minute) on sevoflurane MAC in alpacas (mean ± SEM)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>MACB</th>
<th>MACT</th>
<th>Change (%)a</th>
<th>Time MACB,b</th>
<th>Time MACT,b</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>1.88 ± 0.13c</td>
<td>1.84 ± 0.10c</td>
<td>-0.61 ± 4.6c</td>
<td>276 ± 31c</td>
<td>145 ± 25c</td>
</tr>
<tr>
<td>K + L</td>
<td>1.89 ± 0.13c</td>
<td>0.83 ± 0.10c</td>
<td>-56.9 ± 4.6e</td>
<td>230 ± 31c</td>
<td>169 ± 25c</td>
</tr>
</tbody>
</table>

a Percentage change from baseline MAC = [(MACT - MACB)/MACB] × 100.

b Time (min) to complete MACB and MACT determination in duplicate.
c,d,e Values in the same columns with different letters are significantly different (P < 0.05).
f Value significantly different from 0.

Recovery evaluation

Alpacas were placed in sternal recumbency for recovery with their heads elevated on a pad. A quality recovery score system was made and used to subjectively evaluate (by the same investigator, PQW) alpacas’ recovery using a 3-point scale as follows:

- **Score 1** = no drooping of eyelids; alert and able to hold head up and move head around while in sternal recumbency; no struggling or paddling; and standing on first attempt.
- **Score 2** = mild drooping of eyelids; partially able to hold head up and to move head around while in sternal recumbency; mild struggling and paddling; more than 2 attempts to stand.
- **Score 3** = moderate drooping of eyelids; unable to hold head up or to move head around while in sternal recumbency; moderate paddling movements and unwillingness to remain in sternal recumbency; more than 2 attempts to stand.

Extubation was performed after chewing motions were present. Time-to-extubation was defined as time (min), from discontinuing ketamine and SEVO to extubation. Time-to-standing (min), was defined as the time from discontinuing ketamine + lidocaine and SEVO to standing. Although animals were allowed to recovery freely, they were encouraged to stand up with noise stimulation (hand clapping and calling alpacas’ names).

Drug analysis

Samples were prepared for analysis by a protein precipitation method. A standard curve of ketamine, lidocaine, and lidocaine metabolites, monoethylglycinexylidide (MEGX), and glycinexylidide (GX) was prepared from 0, 50, 100, 500, 1000, 5000, and 10 000 ng/mL.

A TSQ Vantage triple quadrupole mass spectrometer with a Transend turboflow LC system (Thermo Scientific) was used for the positive ion electrospray (ESP+) analysis. An Eclipse Plus C18 column, 3.0 × 100 mm; 3.5 μm particle size (Agilent) was used for the analysis with an injection size of 10 μL. The mobile phases used were: A — 0.1% formic acid in H2O, and B — 0.1% formic acid in acetonitrile with a flow rate of 300 μL/min. The gradient was as follows: 0 to 1.5 min — 90% A: 10% B; 1.5 to 5 min — 2% A: 98% B; 5.5 to 10 min — 90% A: 10% B.

Multiple reaction monitoring analysis was performed for all compounds. Ion transitions used for quantization were 292 > 152 m/z for the internal standard (Morphine-d6); 235.16 > 86.15 m/z for lidocaine; 238.08 > 125.05 m/z for ketamine; 207.12 > 58.16 m/z for MEGX, and 179.13 > 122.15 m/z for GX. Dwell for each transition was 0.10 s and collision energy was optimized according to each compound. Mass spectrometric conditions were optimized for lidocaine by infusion of a pure standard. Although, the tube lens optimization was according to each compound.

Statistical analysis

Treatment effect on MACB, MACT, percent change in MAC, time to MACB, time to MACT, time-to-extubation, and time-to-standing were determined using the MIXED procedure of SAS (SAS system; SAS Institute, Cary, North Carolina, USA). Treatment (saline or ketamine), period (1 or 2) and sequence (1: saline first and ketamine + lidocaine second or 2: ketamine + lidocaine first and saline second) were included in the model as fixed effects. Plasma concentration of ketamine, lidocaine, and lidocaine metabolites (MEGX and GX) were analyzed including sequence and time of MACB determination (1 or 2) in the model as fixed effect. Alpaca within sequence was included as a random effect in all the above models. Recovery scores were analyzed using the GLIMMIX procedure of SAS using the model previously described for the non-plasma variables. The multinomial distribution with a cumulative logit link was implemented. Results are reported as least squares means ± SEM, unless stated otherwise. Significance was declared at P < 0.05.

Results

The least-squares mean of SEVO MACB was 1.88 ± 0.13% and 1.89% ± 0.14% for the saline and K + L groups, respectively, and did not significantly differ between treatments (Table I). Ketamine combined with lidocaine significantly (P = 0.0001) decreased MACB by 56.9% ± 4.6%. Saline did not significantly (P = 0.898) change MACB. Time to determine MACB and MACT was not significantly...
The administration of ketamine + lidocaine was associated with a 57% decrease in the MAC$_{SEVO}$ and this is consistent with the reported effects of the combination on MAC$_{SEVO}$ in dogs (9).

In a previous study in alpacas (2), an infusion of ketamine at 40 μg/kg BW per minute decreased the MAC$_{SEVO}$ by 37%, but plasma concentrations of ketamine were not reported. In goats (6), a similar infusion rate of ketamine decreased the MAC of isoflurane by 28.7% at a plasma concentration of 952 ng/mL, which is less than the mean value of 782 ng/mL achieved herein. Thus, it is likely that in the present study, the contribution of ketamine to MAC reduction was greater than 28.7%. In dogs, ketamine infusion of 40 μg/kg BW per minute resulted in a plasma concentration of 824 ± 196 ng/mL with an isoflurane MAC decrease of 33%. Yet, in cats (3), a ketamine infusion of 23 μg/kg BW per minute resulted in mean plasma ketamine concentrations of 1750 ng/mL and was associated with an isoflurane MAC decrease of 45% ± 17%. A study in dogs (22) reported how ketamine pharmacokinetics can be greatly variable even within same group of species. Therefore, the discrepancies in the plasma ketamine concentrations among studies cited here are probably due to differences in the ketamine’s pharmacokinetics among species.

Lidocaine infusions of 50 μg/kg BW per minute are associated with decreases in MAC$_{SEVO}$ between 22% in dogs (9) and 27% in horses (16) at plasma concentrations of approximately 1500 ng/mL and 2200 ng/mL, respectively. In goats, an infusion of 100 μg/kg BW per minute resulted in a plasma concentration of 1900 ng/mL to the present study, and was associated with a decrease in the isoflurane MAC of 18% (7). Based on these studies, it is unlikely that lidocaine was associated with more than a 25% decrease in the MAC$_{SEVO}$ herein. Lidocaine metabolism occurs mainly by oxidative dealkylation via cytochrome P-450 enzymes. Dealkylation produces MEGX and GX, the 2 main metabolites of lidocaine, with the MEGX being further metabolized to GX (23).

After an IV administration of lidocaine, plasma concentrations of MEGX and, especially, GX increase with time while lidocaine rapidly decreases (24). Therefore, the difference in the GX plasma concentrations between MAC$_{T1}$ and MAC$_{T2}$ could be expected according to the time elapsing for the post-treatment MAC determinations.

A weakness in our study was that each drug’s effect on MAC was not assessed separately and therefore, the individual contribution of each drug to the decrease in MAC is unknown.

The interaction between ketamine and lidocaine on volatile anesthetic-induced immobility in other species was considered to be additive (7,9). However, it is difficult to compare studies because of differences in species and drug infusion rates; and, in addition, plasma concentrations of the drugs differed even when similar infusion rates were used (4,7,9,12,16). For example, higher infusion rates (100 μg/kg BW per minute) of ketamine and lidocaine in dogs decreased the MAC$_{SEVO}$ by approximately 63% (9), which is comparable to the decrease of 57% in the MAC$_{SEVO}$ in the present study. In goats (7), an infusion of ketamine at 50 μg/kg BW per minute and lidocaine at 100 μg/kg BW per minute decreased the MAC of isoflurane by 69%. In a clinical study in horses (1), ketamine at 50 μg/kg BW per minute combined with lidocaine at 50 μg/kg BW per minute reduced the MAC of isoflurane by 40%. On the contrary, lower infusion rates of ketamine at 10 μg/kg BW per minute combined with lidocaine at 20 μg/kg BW per minute in a clinical

### Table II. Plasma concentration of ketamine, lidocaine, and lidocaine metabolites, monoethylglycinexilidide (MEGX), and glycinexylidide (GX) at the time of post-treatment MAC determination (MAC$_{T1}$ and MAC$_{T2}$) in 8 alpacas given ketamine (LD: 0.5 mg/kg BW IV; CRI: 25 μg/kg BW per minute) combined with lidocaine (LD: 2 mg/kg BW IV; CRI: 50 μg/kg BW per minute), expressed as mean ± SEM

<table>
<thead>
<tr>
<th>Drug</th>
<th>MAC$_{T1}$</th>
<th>MAC$_{T2}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>GX</td>
<td>204 ± 69$^a$</td>
<td>229 ± 69$^a$</td>
</tr>
<tr>
<td>MEGX</td>
<td>884 ± 213$^a$</td>
<td>971 ± 213$^a$</td>
</tr>
<tr>
<td>Lidocaine</td>
<td>1803 ± 239$^a$</td>
<td>1791 ± 241$^a$</td>
</tr>
<tr>
<td>Ketamine</td>
<td>788 ± 303$^a$</td>
<td>708 ± 325$^a$</td>
</tr>
</tbody>
</table>

$^a$ Values in the same row with different letters are significantly different ($P < 0.05$).

different between the treatments (Table I). Plasma concentrations (ng/mL) of lidocaine and its main metabolites, GX and MEGX, and ketamine are reported in the Table II. The mean plasma concentration was 1797 ng/mL for lidocaine, 748 ng/mL for ketamine, 217 ng/mL for GX, and 927 ng/mL for MEGX. A significant ($P = 0.005$) increase in GX plasma concentration was observed from MAC$_{T1}$ to MAC$_{T2}$ determination. Although no other significant difference was observed in plasma concentration of ketamine ($P = 0.860$), lidocaine ($P = 0.905$), and the MEGX metabolite ($P = 0.093$) between MAC$_{T1}$ and MAC$_{T2}$. The mean induction time was 11.7 min and mean arterial blood pressure in all alpacas was greater than 70 mmHg at all times. The time-to-extubation ($P = 0.522$) and time-to-standing ($P = 0.737$) did not significantly differ between treatment groups. The mean time-to-extubation was 18.13 ± 3.22 min for saline and 21.13 ± 3.22 min for ketamine + lidocaine. The mean time-to-standing was 45.25 ± 4.90 min for saline and 42.88 ± 4.90 min for ketamine + lidocaine. The median recovery score was 1 for saline treatment and 1.5 for K + L treatment. There was no significant difference ($P = 0.494$) between treatments for quality of recovery, although one alpaca in the K + L group received a score of 3.

### Discussion

The MAC method used in this study consisted of bracketing SEVO concentrations up and down by 0.1%. In the present study, the mean baseline MAC$_{SEVO}$ was 1.89%, which is less than the MAC$_{SEVO}$ (2.33%) reported in another study in alpacas (18). A variation in MAC values of up to 20% within species is well-accepted in MAC studies (19). Larger variations in MAC values due to different experimental designs (differences in type and intensity of noxious stimulus and the subjectivity of purposeful movement assessment), have been reported (20). Interestingly, in a study on dogs (21), it was speculated that a low number of subjects (low study power) could contribute to MAC variation among different species. The difference in MAC$_{SEVO}$ values for alpacas between the present study and the study done by Grubb et al (18), could be explained by the different of type of noxious stimuli used (clamping a claw versus electrical stimulus, respectively) and the subjective assessment of the purposeful movement (different investigators). One could also speculate if the power of these studies contributed to the MAC variation (8 alpacas versus 6 alpacas, respectively).
study in sheep (25) reduced the requirement for isoflurane by 23%. The doses of ketamine and lidocaine used in the present study were based on studies in alpacas (2) and goats (6,7).

In conclusion, the infusion of ketamine combined with lidocaine, at the doses used in this study, decreased the MAC_{SEVO} by 57% in alpacas, and did not affect the quality of recovery from anesthesia. Based on these results, IV administration of ketamine combined with lidocaine, during SEVO anesthesia, may be a safe source to achieve a balanced anesthesia in alpacas.

**Acknowledgment**

The authors thank John Ladner (RVT) for technical assistance.

**References**


Introduction

Breast cancer is one of the most common cancers in women and a frequent cause of death from cancer (1–2). Classification of breast cancer helps in understanding and treating this condition. Molecular phenotyping to determine the expression pattern of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor-2 (HER-2) is widespread and routinely evaluated in women with breast cancer (3–4). Receptor expression is closely associated with the development and progression pathway of breast cancer (5) and contributes significantly to the treatment outcome and prognosis (6–8). For example, ER-positive and HER-2-positive breast cancers can be treated with tamoxifen and trastuzumab, respectively, by means of blocking the specific receptor pathway (9).

Triple-negative breast cancer is a type of breast cancer that does not express the genes for estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor-2 (HER-2). It is an important and clinically relevant condition as it has a poor prognosis and is difficult to treat. Basal-like triple-negative cancer is highly prevalent in both African-Americans and adolescents. We therefore examined whether such a cancer likewise occurs in specific breeds and age groups in dogs, focusing on basal-like triple-negative cancer in particular. In this study, 181 samples from dogs with malignant mammary carcinoma from the 5 most common breeds and 2 age groups in Korea were analyzed. Histological classification and molecular subtyping, including assessment of immunohistochemical findings, were carried out. Twenty-five of 28 (89.3%) triple-negative carcinomas were identified as basal-like triple-negative carcinomas. Analysis of associations of classified factors revealed that the shih tzu breed (9/25, 36.0%) and advanced-age (19/25, 76.0%) groups were characterized by higher prevalence of basal-like triple-negative tumors with diverse histological types and of a higher grade. These results suggest that breed- and age-related differences can be identified in canine mammary carcinoma and, notably, in the shih tzu breed and at older ages. Further investigation of these distinguishing characteristics of the shih tzu breed is warranted.

Abstract

Triple-negative breast cancer is a type of breast cancer that does not express the genes for estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor-2 (HER-2). Basal-like triple-negative cancer is highly prevalent in both African-Americans and adolescents. We therefore examined whether such a cancer likewise occurs in specific breeds and age groups in dogs, focusing on basal-like triple-negative cancer in particular. In this study, 181 samples from dogs with malignant mammary carcinoma from the 5 most common breeds and 2 age groups in Korea were analyzed. Histological classification and molecular subtyping, including assessment of immunohistochemical findings, were carried out. Twenty-five of 28 (89.3%) triple-negative carcinomas were identified as basal-like triple-negative carcinomas. Analysis of associations of classified factors revealed that the shih tzu breed (9/25, 36.0%) and advanced-age (19/25, 76.0%) groups were characterized by higher prevalence of basal-like triple-negative tumors with diverse histological types and of a higher grade. These results suggest that breed- and age-related differences can be identified in canine mammary carcinoma and, notably, in the shih tzu breed and at older ages. Further investigation of these distinguishing characteristics of the shih tzu breed is warranted.
such as CK5/6, CK14, CK17, and EGFR (11). Because of a particularly poor prognosis, basal-like triple-negative tumors warrant thorough investigation (10,11,13).

In humans, the occurrence of breast cancer shows race- and age-related differences (14,15). The discovery that African-American female patients with breast cancer tended to have a lower survival rate than Caucasian female patients triggered studies of racial differences in breast cancer (14,15). Different aspects of breast cancer related to race and age have since been reported (16). The total number of breast cancer patients was not remarkable in African-American women or younger subjects, but breast cancers with worse prognosis, particularly the triple-negative cancers, tended to occur more often in African-American female patients and younger patients (17–21). These findings are presumed to be related to genetic differences among races.

Recently, basal-like triple-negative tumors have also been identified in dogs (22,23). The dog may be a reliable animal model for human breast cancer (23,24). Therefore, establishing breed-related and age-related differences in mammary tumors in dogs would be meaningful for both human and canine studies. This study aimed to determine the differences in expressions of histological types, molecular types, and other applicable factors in canine mammary cancer and to determine the characteristics of canine mammary carcinoma, with particular reference to the race- and age-related differences in expressions of basal-like triple-negative cancer.

Materials and methods

Sample selection

Entire samples used to diagnose canine mammary tumors were obtained from the Department of Veterinary Pathology, Konkuk University Animal Teaching Hospital, Seoul, Korea. Primary selection was limited to canine malignant mammary carcinoma diagnosed from 2003 to 2013 and consisted of 672 samples. All 672 samples were analyzed according to breed and the 5 most frequent breeds (Maltese, 148; Yorkshire terrier, 99; shih tzu, 72; poodle, 63; and Cocker spaniel, 53) were divided by age into subjects below and above 10 y of age, with 20 samples extracted randomly for each subgroup (10 and 10 y). The final study population consisted of 20 dogs in each age subgroup for each of the 5 breed groups, i.e., 40 dogs in each breed group for a total of 200 samples. As samples excluded after initial screening were not artificially replaced, 181 samples were analyzed.

Immunohistochemistry

Four-micrometer sections of formalin-fixed paraffin-embedded tissues were fixed on slides and deparaffinized by xylene, followed by serial dehydration by graded ethanol. Slides were washed 3 times with phosphate-buffered saline (PBS). A 3% hydrogen peroxide solution diluted with PBS was used to block endogenous peroxidase activity. Antigen retrieval was then carried out by the microwave retrieval method or enzyme retrieval method, selected according to the primary antibody.

Microwave retrieval (750W, 60Hz, 15 min) in pH 9.0 Tris-ethylenediamine tetra-acetic acid (EDTA) buffer was applied to the sample for anti-ER (clone: ER88; BioGenex, Fremont, California, USA), PR (PR10A9; Immunotech SAS, Marseille, France), HER-2/neu (CB11; BioGenex), CK5/6 immunostaining (D5/16/B4; Dako, Glostrup, Denmark), and CK14 (LL002; Abcam, Cambridge, UK); 0.05% proteinase K (37°C, 30 min) enzyme digestion was applied for staining with anti-EGFR antibody (31G7; Abcam).

After PBS washing, slides were covered with 5% normal goat serum for 30 min for anti-ER staining to block nonspecific binding. Primary antibody staining was conducted. Incubation for 3 h at room temperature was applied for anti-ER (1:60), HER-2/neu (1:100), and CK14 (1:300) and overnight at 4°C for anti-PR (1:500), CK5/6 (1:100), and EGFR (1:50) antibody staining. PBS washing was carried out, followed by 2-step immunolabeling with secondary antibody-HRP conjugation for 40 min. Visualization was achieved using DAB+ chromogen (REAL EnVision Kit; Dako). Finally, slides were washed with distilled water and counterstained with Gill's hematoxylin and coverslips were applied.

Tumor classification

Histological subtyping was carried out using HE-stained slides according to previously proposed criteria (25). Histological subtypes included carcinoma arising in a mixed tumor, carcinoma-complex type, simple carcinoma, intraductal papillary carcinoma, solid carcinoma, ductal carcinoma, carcinoma-anaplastic, carcinoma-micropapillary invasive, comedocarcinoma, and squamous cell carcinoma.

In the molecular classification of tumors, types of tumors that are positive for hormone receptor expression are called luminal types. Tumors with positive expression for ER or PR and negative expression for HER-2 were classified as luminal A and tumors with positive expression for ER or PR and negative expression for HER-2 were classified as luminal B. Types of tumors that were hormone-receptor negative, but were positive for HER-2 receptor expression, were classified as HER-2-overexpressing type and tumors that were negative for the expression of all 3 receptors were classified as triple-negative type. After identifying triple-negative cancer samples, basal-marker expression was sequentially evaluated for subtyping basal-like triple-negative cancers. Triple-negative cancers expressing at least 1 basal marker were considered basal-like triple-negative cancers.

Grading was done by evaluating the sum of the scores of tubule formation, mitoses, and nuclear pleomorphism (26). Grade 1 indicated well differentiated tumors, Grade 2 indicated moderately differentiated tumors, and Grade 3 indicated poorly differentiated tumors.

Immunohistochemical analysis

Anti-ER or anti-PR staining was considered positive if there was more than 10% expression of clear nuclear staining. Evaluation of anti-HER-2 staining was based on the Hercep test and more than 10% of complete plasma membrane expression was considered positive. CK5/6, CK14, and EGFR were used as basal markers and areas of interest with more than 5% of membrane expression were considered basal-positive.

Statistical analysis

The Statistical Package for Social Science 17.0 software (SPSS, Chicago, Illinois, USA) was used for statistical analysis. Analysis of frequency, Pearson’s Chi-square test, and Fisher’s exact test were applied and P-values less than 0.05 (P < 0.05) were considered significant.
Figure 2. A — Positive expression of estrogen receptor in simple carcinoma. Immunohistochemistry (IHC), mammary gland, canine. Bar = 35 μm.
Table I. Breed-based statistical analysis of malignant mammary carcinoma of canines

<table>
<thead>
<tr>
<th>Breed</th>
<th>Cocker spaniel (n = 38)</th>
<th>Maltese (n = 36)</th>
<th>Poodle (n = 36)</th>
<th>Shih tzu (n = 34)</th>
<th>Yorkshire terrier (n = 35)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Histological type</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Carcinoma — anaplastic (n = 1)</td>
<td>1 (100.0)</td>
<td>0 (0.0)</td>
<td>0 (0.0)</td>
<td>0 (0.0)</td>
<td>0 (0.0)</td>
<td>0.115</td>
</tr>
<tr>
<td>Carcinoma — complex type (n = 47)</td>
<td>12 (25.5)</td>
<td>13 (27.7)</td>
<td>8 (17.0)</td>
<td>5 (10.6)</td>
<td>9 (19.1)</td>
<td></td>
</tr>
<tr>
<td>Carcinoma — microcystic invasive (n = 1)</td>
<td>0 (0.0)</td>
<td>0 (0.0)</td>
<td>0 (0.0)</td>
<td>0 (0.0)</td>
<td>0 (0.0)</td>
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</tr>
<tr>
<td>Simple carcinoma (n = 36)</td>
<td>11 (30.6)</td>
<td>7 (19.4)</td>
<td>7 (19.4)</td>
<td>5 (13.9)</td>
<td>6 (16.7)</td>
<td></td>
</tr>
<tr>
<td>Solid carcinoma (n = 7)</td>
<td>1 (14.3)</td>
<td>1 (14.3)</td>
<td>1 (14.3)</td>
<td>3 (42.9)</td>
<td>1 (14.3)</td>
<td></td>
</tr>
<tr>
<td>Carcinoma arising in a mixed tumor (n = 71)</td>
<td>9 (12.7)</td>
<td>15 (21.1)</td>
<td>18 (25.4)</td>
<td>13 (18.3)</td>
<td>16 (22.5)</td>
<td></td>
</tr>
<tr>
<td>Comedocarcinoma (n = 1)</td>
<td>0 (0.0)</td>
<td>0 (0.0)</td>
<td>0 (0.0)</td>
<td>1 (100.0)</td>
<td>0 (0.0)</td>
<td></td>
</tr>
<tr>
<td>Ductal carcinoma (n = 5)</td>
<td>1 (20.0)</td>
<td>1 (20.0)</td>
<td>0 (0.0)</td>
<td>1 (20.0)</td>
<td>2 (40.0)</td>
<td></td>
</tr>
<tr>
<td>Intraductal papillary carcinoma (n = 8)</td>
<td>3 (37.5)</td>
<td>0 (0.0)</td>
<td>2 (25.0)</td>
<td>2 (25.0)</td>
<td>1 (12.5)</td>
<td></td>
</tr>
<tr>
<td>Squamous cell carcinoma (n = 4)</td>
<td>0 (0.0)</td>
<td>0 (0.0)</td>
<td>0 (0.0)</td>
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</tr>
<tr>
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</tr>
<tr>
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<td>12 (18.5)</td>
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<td>10 (15.4)</td>
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</tr>
<tr>
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<td>3 (10.7)</td>
<td>4 (14.3)</td>
<td>6 (21.4)</td>
<td>10 (35.7)</td>
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<tr>
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<td>34 (22.2)</td>
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<td>5 (17.9)</td>
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<td><strong>Grade</strong></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 (n = 118)</td>
<td>29 (24.6)</td>
<td>25 (21.2)</td>
<td>23 (19.5)</td>
<td>19 (16.1)</td>
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<tr>
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<td>2 (18.2)</td>
<td>7 (63.6)</td>
<td>1 (9.1)</td>
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</tr>
</tbody>
</table>

a ER—/PR—/HER-2+.
b ER+ or PR+/HER-2—.
c ER+ or PR+/HER-2+.
d ER—/PR—/HER-2—.
P < 0.05.

Results

Clinical data

The 181 dogs with malignant mammary carcinoma ranged in age from 3 to 17 y (mean: 9.4 ± 2.72 y). Ninety-two dogs (50.8%) were < 10 y and 89 dogs (49.2%) were ≥ 10 y. The 181 dogs were divided into 5 groups based on breeds with the highest prevalence for canine malignant mammary carcinoma as follows: 38 Cocker spaniels (20 < 10 y, 18 ≥ 10 y), 36 Maltese (18 < 10 y, 17 ≥ 10 y), 36 poodles (18 < 10 y, 18 ≥ 10 y), 34 shih tzus (18 < 10 y, 16 ≥ 10 y), and 35 Yorkshire terriers (18 < 10 y, 17 ≥ 10 y).

Histological classification and molecular phenotype

The malignant canine mammary carcinomas were categorized into histological types according to the classification proposed by Goldschmidt et al (25) based on hematoxylin and eosin (HE) staining (Figure 1). The 181 samples were divided into the following histological types: 71 (39.2%) carcinoma arising in a mixed tumor; 47 (26.0%) carcinoma-complex types; 36 (19.9%) simple carcinomas; 8 (4.4%) intraductal papillary carcinomas; 7 (3.9%) solid carcinomas; 5 (2.8%) ductal carcinomas; 4 (2.2%) squamous cell carcinomas; 1 (0.6%) carcinoma-microcystic invasive; 1 (0.6%) comedocarcinoma; and 1 (0.6%) carcinoma-anaplastic.

Two major types of tumor, carcinoma arising in a mixed tumor and complex type carcinoma, accounted for more than half (65%) of the samples. According to molecular phenotype, the luminal A type was the most prevalent at 42.5%, followed by the luminal B and triple-negative types (15.5%), with the HER-2-overexpressing type being the least common (6.1%). Overall, hormonal receptor positive type (ER positive or PR positive) mammary tumors accounted for 78% of the samples and the triple-negative type, indicating negative expression for anti-ER, anti-PR, and anti-HER-2 antibodies, accounted for 15.5% (Figure 2).

Characteristics of tumor according to breed

Correlations between breeds and characteristics of malignant canine mammary carcinoma are listed in Table I. Statistically significant associations were not found between the breed and the...
histological classification and molecular phenotype. Histological grade revealed decreasing prevalence from Grade 1 to Grade 3 for all the breeds ($P<0.05$). Grade 1 was most common (65.2%), followed by Grade 2 (28.7%), and Grade 3 (6.0%). The prevalence of Grade 3 was higher in the shih tzu breed, however, than in the other breeds ($P<0.05$).

Characteristics of tumor according to age

Data from the 2 age groups are summarized in Table II. Luminal A (44/77, 57.1%) and luminal B type (38/65, 58.5%) tumors were associated with the group of younger dogs (under 10 y), whereas HER-2-overexpressing (8/11, 72.7%) and triple-negative (21/28, 75.0%) types were related to the older group (10 y and over) ($P<0.05$). Grade 1 accounted for 65.2% of all subjects, followed by Grade 2 at 28.7%, and Grade 3 at 6.1%. Of note, Grade 3 was seen more in the older group ($\geq 10$ y) (10/11, 90.9%) than in the younger group ($< 10$ y) (1/11, 9.1%).

Analysis of tumor characteristics according to grade

There were significant correlations ($P<0.05$) among grade and dog breed, age group, histological type, and molecular phenotype. The results of the Chi-square test based on grade are given in Table III. Complex-type carcinoma (27/47, 57.4%), simple carcinoma (28/36, 77.8%), carcinoma arising in a mixed tumor (54/71, 76.1%), ductal carcinoma (4/5, 80.0%), and intraductal papillary carcinoma (5/8, 62.5%) were more predominant in the lower grades. There was a tendency for higher grade expression to be in the solid carcinoma (4/7, 57.1%) and squamous cell carcinoma (3/4, 75.0%). Although there was only 1 case of each, the anaplastic carcinoma (1/1, 100%) and comedocarcinoma (1/1, 100%) were both Grade 3. Grade 1 tended to dominate in all molecular phenotypes, but the triple-negative subtype accounted for 45.4% (5/11) of Grade 3.

### Table II. Age-based statistical analysis of malignant mammary carcinoma of canines

<table>
<thead>
<tr>
<th>Histological type</th>
<th>(&lt; 10) y</th>
<th>(\geq 10) y</th>
<th>(P)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carcinoma — anaplastic (n = 1)</td>
<td>0 (0.0)</td>
<td>1 (100.0)</td>
<td>0.061*</td>
</tr>
<tr>
<td>Carcinoma — complex type (n = 47)</td>
<td>32 (68.1)</td>
<td>15 (31.9)</td>
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</tr>
<tr>
<td>Carcinoma — micropapillary invasive (n = 1)</td>
<td>1 (100.0)</td>
<td>0 (0.0)</td>
<td></td>
</tr>
<tr>
<td>Simple carcinoma (n = 36)</td>
<td>18 (50.0)</td>
<td>18 (50.0)</td>
<td></td>
</tr>
<tr>
<td>Solid carcinoma (n = 7)</td>
<td>3 (42.9)</td>
<td>4 (57.1)</td>
<td></td>
</tr>
<tr>
<td>Carcinoma arising in a mixed tumor (n = 71)</td>
<td>32 (45.1)</td>
<td>39 (54.9)</td>
<td></td>
</tr>
<tr>
<td>Comedocarcinoma (n = 1)</td>
<td>0 (0.0)</td>
<td>1 (100.0)</td>
<td></td>
</tr>
<tr>
<td>Ductal carcinoma (n = 5)</td>
<td>3 (60.0)</td>
<td>2 (40.0)</td>
<td></td>
</tr>
<tr>
<td>Intraductal papillary carcinoma (n = 8)</td>
<td>3 (37.5)</td>
<td>5 (62.5)</td>
<td></td>
</tr>
<tr>
<td>Squamous cell carcinoma (n = 4)</td>
<td>0 (0.0)</td>
<td>4 (100.0)</td>
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</table>

<table>
<thead>
<tr>
<th>Molecular type</th>
<th>(&lt; 10) y</th>
<th>(\geq 10) y</th>
<th>(P)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HER-2 overexpressing (n = 11)</td>
<td>3 (27.3)</td>
<td>8 (72.7)</td>
<td>0.005</td>
</tr>
<tr>
<td>Luminal A (n = 77)</td>
<td>44 (57.1)</td>
<td>33 (42.9)</td>
<td></td>
</tr>
<tr>
<td>Luminal B (n = 75)</td>
<td>38 (58.5)</td>
<td>27 (41.5)</td>
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</tr>
<tr>
<td>Triple negative (n = 28)</td>
<td>7 (25.0)</td>
<td>21 (75.0)</td>
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</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Triple negative</th>
<th>(&lt; 10) y</th>
<th>(\geq 10) y</th>
<th>(P)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-triple negative (n = 153)</td>
<td>85 (55.6)</td>
<td>68 (44.4)</td>
<td>0.003</td>
</tr>
<tr>
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<td>7 (25.0)</td>
<td>21 (75.0)</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Grade</th>
<th>(&lt; 10) y</th>
<th>(\geq 10) y</th>
<th>(P)</th>
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<tbody>
<tr>
<td>1 (n = 118)</td>
<td>61 (51.7)</td>
<td>57 (48.3)</td>
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<td>30 (57.7)</td>
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<tr>
<td>3 (n = 11)</td>
<td>1 (9.1)</td>
<td>10 (90.9)</td>
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</tbody>
</table>

* ER−/PR−/HER-2+.
* ER+ or PR+/-HER-2−.
* ER+ or PR+/HER-2+.
* ER−/PR−/-HER-2−.
* $P<0.05$.
* Fisher’s exact test.
Characteristics of basal-like triple-negative mammary cancer

Twenty-five of the tumors were basal-like triple-negative tumors (Figure 3), which represented 89.3% of the triple-negative type cancers. The most frequent histological type was carcinoma arising in a mixed tumor (12/25, 48%) and the most common breed was the shih tzu (9/25, 36%) (Table IV). Grade 1 (16/25, 64%) was the predominant grade within the basal-like triple-negative tumors and Grade 3 was solely expressed by the shih tzu breed, except for 1 Poodle. The shih tzu breed had the broadest spectrum of histological types. By age, 76% (19/25) of the total cases were ≥ 10 y of age and 36.8% (7/19) of these dogs were shih tzus.

Discussion

Determining and categorizing the characteristics of breast cancer may help to treat this disease and to establish the causative factors of the cancer (27). A molecular-based classification scheme has been proposed (28), in which triple-negative breast cancer and basal-like breast cancer are problematic in terms of having comparatively poor prognoses and therapeutic challenges (19).

Many factors have been implicated in the pathogenesis of breast cancer, including hormonal influences, environmental and hereditary factors, and age (18,29). Investigations into the characteristics of tumor development yielded higher incidences of triple-negative breast cancer in young patients and African-American women (12,14). These predilections of race and age appear to be related to the hereditary factors causing breast cancer.

The dog is one of the most common species that develops mammary tumors and can therefore serve as an acceptable model for human breast cancer. Basal-like triple-negative tumors have also been identified in dogs (22,23). In this study, we investigated associations between types and characteristics of tumors. We especially focused on breed and age group by using previously reported criteria. Among the tumor types, particular emphasis was placed on the basal-like triple-negative tumor. Characteristics of basal-like triple-negative cancers were analyzed by frequency and relations between certain clinicopathological parameters were evaluated.

While the types of tumor expression did not clearly differ according to breed, the shih tzu breed exhibited some distinguishing characteristics. In analyzing histological type, types of tumors known to have worse prognoses were the most outstanding characteristics of the shih tzu.

Table III. Statistical analysis of malignant canine mammary carcinoma based on grade

<table>
<thead>
<tr>
<th>Histological type</th>
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<th>P</th>
</tr>
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<tbody>
<tr>
<td>Carcinoma — anaplastic (n = 1)</td>
<td>1 (100.0)</td>
<td>0.000</td>
</tr>
<tr>
<td>Carcinoma — complex type (n = 47)</td>
<td>20 (42.6)</td>
<td>0.000</td>
</tr>
<tr>
<td>Carcinoma — micropapillary invasive (n = 1)</td>
<td>1 (100.0)</td>
<td>0.000</td>
</tr>
<tr>
<td>Simple carcinoma (n = 36)</td>
<td>8 (22.2)</td>
<td>0.000</td>
</tr>
<tr>
<td>Solid carcinoma (n = 7)</td>
<td>3 (42.9)</td>
<td>0.000</td>
</tr>
<tr>
<td>Carcinoma arising in a mixed tumor (n = 71)</td>
<td>21 (52.8)</td>
<td>0.000</td>
</tr>
<tr>
<td>Squamous cell carcinoma (n = 4)</td>
<td>3 (75.0)</td>
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<table>
<thead>
<tr>
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<tr>
<td>HER-2 overexpressingab (n = 11)</td>
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<td>0.010*</td>
</tr>
<tr>
<td>Luminal Aab (n = 77)</td>
<td>28 (36.4)</td>
<td>0.010*</td>
</tr>
<tr>
<td>Luminal Bbc (n = 65)</td>
<td>18 (27.7)</td>
<td>0.010*</td>
</tr>
<tr>
<td>Triple negativeab (n = 28)</td>
<td>6 (21.4)</td>
<td>0.010*</td>
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<tr>
<td>45 (58.4)</td>
<td>28 (36.4)</td>
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<td>46 (70.8)</td>
<td>18 (27.7)</td>
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<td>17 (60.7)</td>
<td>6 (21.4)</td>
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<tr>
<td>Luminal Aab (n = 77)</td>
<td>28 (36.4)</td>
<td>0.000</td>
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<tr>
<td>Luminal Bbc (n = 65)</td>
<td>18 (27.7)</td>
<td>0.000</td>
</tr>
<tr>
<td>Triple negativeab (n = 28)</td>
<td>6 (21.4)</td>
<td>0.000</td>
</tr>
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</table>

P < 0.05.
ab Fisher’s exact test.
all of the other types of tumors. In molecular phenotype analysis, triple-negative type tumors were more common in the shih tzu than in other breeds. Overall, the number of subjects decreased from Grade 1 to Grade 3, but the shih tzu group had the highest percentage of Grade 3 subjects. Of the 25 basal-like triple-negative tumors in 28 triple-negative mammary tumors, 36% (9/25) were from shih tzu dogs. The shih tzu group had a broader distribution of histological types, whereas histological types of tumors in other breeds tended towards better prognostic types, such as carcinoma-complex type and carcinoma arising in a mixed tumor (Figure 3). Although expressions of grade in other groups were primarily Grade 1 or Grade 2, a higher proportion of Grade 3 was a distinguishing characteristic of the shih tzu breed.

When analyzing the characteristics of tumors according to age groups, we ascertained that histological types of mammary tumors with the worse prognosis tended to develop in older dogs ($\geq 10$ y), particularly in the case of basal-like triple-negative tumors. Regarding molecular phenotype, both luminal A and luminal B types of tumors were more likely to be expressed in the younger age group, while HER-2-overexpressing types and triple-negative types of tumors tended to be expressed in the older age group. Analysis by Fisher’s exact test revealed that the histological types with better prognoses, such as complex carcinoma, carcinoma arising in a mixed tumor, and simple-type carcinoma, were significantly associated with younger age.

In contrast, triple-negative tumors, which are known to have a worse prognosis in human studies and poor response to treatment, were more common in the older group of dogs. Basal-like triple-negative tumors were expressed more frequently in the older group (19 of 25 cases (76.0%)), with the shih tzu being the most common breed among these 19 subjects (36.8% (7/19)).

Additional analysis was carried out according to tumor grade. An association was identified between tumor types with worse prognosis and higher grades and relations between triple-negative tumors and Grade 3. Some histological types of tumors known to have worse prognoses showed higher grades ($P < 0.05$). Molecular phenotype analysis that showed grade 3 tumors had the highest percentage of triple-negative type tumors.

To summarize the results, breeds did not show statistical significance by tumor molecular phenotypes, but the shih tzu breed most frequently developed basal-like triple-negative tumors and was significantly associated with Grade 3. In age, the older group of $\geq 10$ y was associated with basal-like triple-negative and Grade 3 tumors.

Breed-related differences in the development of basal-like triple-negative tumors in dogs are similar to the race-related characteristics of African-Americans in the development of human breast cancer. The shih tzu breed was not the most common breed to develop mammary carcinoma, but it showed a higher prevalence of basal-like triple-negative mammary tumors than all the other breeds, just as African-Americans have the highest prevalence of human triple-negative breast cancer.

Basal-like triple-negative carcinomas were associated with both older non-shih tzu subjects and older shih tzu breed subjects. Differences were revealed between humans and dogs in developing basal-like triple-negative tumors because there was a higher prevalence of these tumors in the older dogs ($>10$ y). In humans, younger patients are usually more likely to develop these tumors.

Menopause is one of the important standards in classifying when breast cancer develops in humans. When breast cancer develops during the postmenopausal period, it does not develop under conditions of typical cyclic hormonal influence. As there is no physiological

---

menopausal period in dogs, they do not experience cessation of ovarian hormone release and the accompanying lobular involution of the mammary tissue. To correctly determine the age-related differences between dogs and humans, further studies will be needed to clarify and exclude the effects of a change in hormone status, accompanied by the effects of aging on tumor development or to provide correspondent factors in the dog, such as an ovariohysterectomy.

Although the results were not identical to those in human cases, there were considerable breed- and age-related differences in canine tumor characteristics. There were enough disparities in the expression of tumor types by breed to consider that certain hereditary factors may influence cancer development. We can therefore infer that this prevalence is based on genetic inequality.

Associations between triple-negative tumors and the BRCA1 mutation have recently been investigated in humans (30–33). The BRCA1 mutation is a hereditary factor and representative mutation of genes that can cause breast cancers. In the present study, associations of the BRCA mutation with canine mammary tumors have been investigated. In addition, associations of the BRCA mutation with canine mammary tumors have been investigated in many other studies (34–35). One study found noticeable characteristics of the shih tzu breed in overexpression of BRCA1 (36). Because the antibody used in this study was limited to exon 11 mutations of the human BRCA1 gene, these results cannot fully reflect the various BRCA1 mutations in dogs. Considering that 84% of the human and canine BRCA1 genes are homologous (37), however, these common characteristics of the shih tzu breed are noteworthy.

Previous studies suggest that there are racial differences in triple-negative breast cancer (29), that BRCA mutation locus varies among races (38–40), and that there is an association between the occurrence of triple-negative tumors and BRCA mutation (6,33). Further studies must be conducted on canine breeds, however, to better understand breed-related hereditary factors that can influence the type of tumor that develops, basa-like triple-negative cancers of specific breeds, and the specific locus of BRCA1 gene mutation, as well as to establish associations between certain breeds and basal-like triple-negative cancers with BRCA1 mutations. When considering those common characteristics in the shih tzu breed, triple-negative tumors and BRCA1 mutation seem to be affected by certain hereditary influences in tumor development. There may be an association between basal-like triple-negative cancers and BRCA1 mutation as well as other genetic factors in tumor development. Further study to investigate and understand canine mammary cancers may be useful for canine mammary cancer to serve as an effective model in human studies.

Acknowledgments

The authors thank Rae-Hwa Jang for her excellent technical assistance. This article is part of the PhD thesis of Hyun-Woo Kim. This research was supported by the Basic Science Research Program of the National Research Foundation of Korea (NRF) and funded by the Ministry of Science, Information/Communication Technology (ICT) and Future Planning (2014R1A2A2A01003470).

Table IV. Characteristics of distributions of basal-like triple-negative mammary cancer

<table>
<thead>
<tr>
<th>Breed</th>
<th>Cocker spaniel</th>
<th>Maltese</th>
<th>Poodle</th>
<th>Shih tzu</th>
<th>Yorkshire terrier</th>
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<tbody>
<tr>
<td><strong>Frequency</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Carcinoma — complex type (n = 4)</td>
<td>1 (25.0)</td>
<td>1 (25.0)</td>
<td>0 (0.0)</td>
<td>1 (25.0)</td>
<td>1 (25.0)</td>
</tr>
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<td>Simple carcinoma (n = 1)</td>
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<td>0 (0.0)</td>
<td>0 (0.0)</td>
<td>0 (0.0)</td>
<td>100 (100.0)</td>
</tr>
<tr>
<td>Solid carcinoma (n = 4)</td>
<td>0 (0.0)</td>
<td>1 (25.0)</td>
<td>1 (25.0)</td>
<td>2 (50.0)</td>
<td>0 (0.0)</td>
</tr>
<tr>
<td>Carcinoma arising in a mixed tumor (n = 12)</td>
<td>1 (8.3)</td>
<td>1 (8.3)</td>
<td>4 (33.3)</td>
<td>2 (16.7)</td>
<td>4 (33.3)</td>
</tr>
<tr>
<td>Intraductal papillary carcinoma (n = 2)</td>
<td>0 (0.0)</td>
<td>0 (0.0)</td>
<td>1 (50.0)</td>
<td>1 (50.0)</td>
<td>0 (0.0)</td>
</tr>
<tr>
<td>Squamous cell carcinoma (n = 2)</td>
<td>0 (0.0)</td>
<td>0 (0.0)</td>
<td>0 (0.0)</td>
<td>0 (0.0)</td>
<td>0 (0.0)</td>
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<tr>
<td><strong>Grade</strong></td>
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<td>2 (12.5)</td>
<td>2 (12.5)</td>
<td>4 (25.0)</td>
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<td>3 (n = 5)</td>
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<td>0 (0.0)</td>
<td>1 (20.0)</td>
<td>4 (80.0)</td>
<td>0 (0.0)</td>
</tr>
</tbody>
</table>

References

7. Sorenko K, Rasotto R, Zappulli V, Goldschmidt MH. Development, anatomy, histology, lymphatic drainage, clinical
The effects of intravenous alfaxalone with and without premedication on intraocular pressure in healthy dogs

Bianca S. Bauer, Barbara Ambros

Abstract

The objective of this study was to investigate the effects of intravenous alfaxalone with and without premedication on intraocular pressure (IOP) in healthy dogs. Thirty-three dogs were randomized to receive 1 of 3 treatments: acepromazine [0.03 mg/kg body weight (BW)] with butorphanol (0.2 mg/kg BW) intramuscularly (IM), followed by intravenous (IV) alfaxalone (1.5 mg/kg BW); dexmedetomidine (0.002 mg/kg BW) with hydromorphone (0.1 mg/kg BW) IM, followed by alfaxalone (1 mg/kg BW) IV; and saline 0.9% (0.02 mL/kg BW) IM, followed by alfaxalone (3 mg/kg BW) IV. Intraocular pressure (IOP) was measured at baseline, 15 min, and 30 min after premedication, after pre-oxygenation, after administration of alfaxalone, and after intubation. After induction and after intubation, the IOP was significantly increased in all groups compared to baseline. While premedication with acepromazine/butorphanol or dexmedetomidine/hydromorphone did not cause a significant increase in IOP, the risk of vomiting and the associated peak in IOP after dexmedetomidine/hydrromorphone should be considered when selecting an anesthetic protocol for dogs with poor tolerance for transient increases in IOP.

Résumé

L’objectif de la présente étude était d’examiner les effets de l’administration intraveineuse (IV) d’alfaxalone avec et sans prémédication sur la pression intraoculaire (PIO) chez des chiens en santé. Trente-trois chiens ont été randomisés pour recevoir un des trois traitements : acépromazine [0,03 mg/kg de poids corporel (PC)] avec du butorphanol (0,2 mg PC) par voie intramusculaire (IM), suit d’alfaxalone (1,5 mg/kg PC) IV; dexmedetomidine (0,002 mg/kg PC) avec de l’hydromorphone (0,1 mg/kg PC) IM, suit d’alfaxalone (1 mg/kg PC) IV; et saline 0,9 % (0,02 mL/kg PC) IM, suit d’alfaxalone (3 mg/kg PC) IV. La PIO a été mesurée au départ, 15 min et 30 min après la prémédication, après pré-oxygénation, après administration d’alfaxalone, et après intubation. Suite à l’induction et après intubation, la PIO était augmentée de manière significative dans tous les groupes comparativement à la valeur de base. Bien que la prémédication avec les combinaisons acépromazine/butorphanol ou dexmedetomidine/hydrromorphone n’a pas causée d’augmentation significative de la PIO, le risque de vomissements et le pic associé de la PIO suite à l’administration de dexmedetomidine/hydromorphone devraient être pris en considération lors de la sélection d’un protocole d’anesthésie pour des chiens avec une faible tolérance à une augmentation transitoire de la PIO.

(Traduit par Docteur Serge Messier)

Introduction

Intraocular pressure (IOP) is maintained by a balance between the production of aqueous humor and its outflow (1). Anesthetic agents can alter intraocular pressure by changing the rate of aqueous production or outflow or by increasing extraocular muscle tone (2). When choosing an anesthetic protocol for intraocular surgeries, the effect of the protocol on intraocular pressure must be taken into account (2,3). Abrupt increases in IOP throughout anesthesia of patients undergoing ophthalmic surgery can have significant effects. For example, minimal increases in IOP (30 to 35 mmHg) in glaucomatous animals can significantly lower axoplasmic flow within the optic nerve, resulting in further nerve injury (4). Inadvertent perforation of a deep corneal ulcer before or during surgery due to IOP elevations can complicate the surgical procedure and worsen the postoperative prognosis (5,6).

Alfaxalone is a synthetic neuroactive steroidal anesthetic that is injectable and acts as an agonist at gamma-aminobutyric acid A (GABA_A) receptors within the central nervous system (CNS) (7). Alfaxalone was first introduced into veterinary medicine in 1971 (8) and is currently licensed as Alfaxan (Jurox, Kansas City, Missouri, USA) for use in several countries, including Canada, Australia, and most of Europe. Anesthesia can be induced and maintained with alfaxalone, producing rapid and smooth induction with excellent muscle relaxation (9–11). The cardiopulmonary effects of alfaxalone have been well-investigated in dogs (9–12). The reported effects of alfaxalone on IOP in dogs are varied (13,14). In 1 study without pre-anesthetic medication, a single bolus of alfaxalone induced a transient nonsignificant increase in IOP, followed by a significant reduction in IOP (14). In another study, a significant increase in IOP was observed when alfaxalone was administered after premedication with acepromazine and hydromorphone (13).
Pre-anesthetic medication is commonly used to calm the patient, reduce anesthetic requirements, promote smooth induction and recovery from anesthesia, and provide analgesia (15). Combinations of acepromazine and butorphanol or dexmedetomidine and hydromorphone are commonly used as pre-anesthetic medications in dogs (15,16). Although the effect of combined acepromazine and butorphanol on IOP has been evaluated in 1 study (17), the combination of dexmedetomidine and hydromorphone on IOP has not yet been studied. The objective of this study was to determine the effects on IOP in healthy dogs after the administration of alfaxalone alone compared with alfaxalone administered after pre-anesthetic medication with acepromazine-butorphanol and dexmedetomidine-hydromorphone.

**Materials and methods**

Thirty-three healthy shelter and client-owned dogs undergoing elective surgery procedures were used for this study. Informed owner consent was obtained and the protocol was approved by the University of Saskatchewan’s Animal Research Ethics Board and followed the guidelines provided by the Canadian Council on Animal Care. Breed, gender, and body weight were recorded. Age was not recorded as definitive age could not be established for most patients, but all dogs were considered adults on the basis of physical and dental examination. Pre-anesthetic physical examination and blood work consisting of packed cell volume (PCV), total protein (TP), blood glucose, and blood urea nitrogen (BUN) were conducted in all cases and dogs were classified according to the American Society of Anesthesiologists (ASA) classification.

Before entering the study, all dogs underwent a complete ophthalmic examination (Schirmer tear tests, intraocular pressure measurement with rebound tonometry, slit lamp biomicroscopy, and indirect ophthalmoscopy) by a veterinary ophthalmologist (BB) who was blinded to the treatment group. Dogs that were deemed unhealthy or had ophthalmological abnormalities were excluded from the study. Brachycephalic breeds and aggressive dogs that were difficult to restrain were also excluded from the study.

All IOP measurements were taken by a veterinary ophthalmologist (BB) using rebound tonometry (Tonovet; Icare Finland Oy, Helsinki, Finland) and both eyes (oculus uterque [OU]) were measured in all subjects at all time points. Each IOP obtained was an average of 6 readings and only measurements with < 2.5% variance were used. All animals were restrained in sternal position with the head raised, avoiding pressure against the globe, jugular veins, or eyelids.

Dogs were allocated into 1 of 3 treatment groups by random drawing, using an envelope containing the assignment to a treatment. The main investigators (BA and BB) were unaware of which premedication combination had been administered. The 3 treatment groups were: Group ABA — acepromazine [0.03 mg/kg body weight (BW)] (Atravet; Ayerst Laboratories, Montreal, Quebec) with butorphanol [0.2 mg/kg BW] (Turbogesic; Wyeth Animal Health, Guelph, Ontario) intramuscularly (IM), followed by intravenous (IV) alfaxalone [1.5 mg/kg BW] (Alfaxan; Abbott Laboratories, Montreal, Quebec); Group SA — saline 0.9% (0.02 mL/kg BW) (0.9% Sodium Chloride; Hospira, Montreal, Quebec) IM, followed by alfaxalone (3 mg/kg BW) IV.

After pre-medication, an intravenous catheter was placed in a cephalic vein and dogs were pre-oxygenated with a fitting face mask for 3 min. If dogs did not tolerate the application of the face mask, i.e., became agitated and struggled, pre-oxygenation was discontinued and procedures were paused for 3 min. Anesthesia was then induced with intravenous alfaxalone. The prepared dose of alfaxalone was administered by hand injection over 60 s, after which jaw tone was assessed by a board-certified anesthesiologist (BA). If jaw tone was not lost after 20 s, further boluses of 0.5 mg/kg BW of alfaxalone were given over 20 s until jaw tone was absent such that intubation could be achieved. After induction was complete, post-induction intraocular pressures were obtained right away, followed immediately by intubation by a board-certified anesthesiologist (BA). The number of attempts and any difficulty in orotracheal intubation were recorded. Intraocular pressures were measured at the following 6 time points: baseline at initial ophthalmic examination (BL); 15 min after premedication (15); 30 min after premedication (30); after pre-oxygenation and before induction (Post O2); immediately after administration of alfaxalone (Post A); and after intubation, before connection to the anesthetic breathing system (Post INT).

Quality of sedation at time point 30, anesthesia induction, and intubation was scored by a board-certified anesthesiologist (BA) blinded to the treatment (Appendix 1). Behavior after premedication was observed and any incidences of vomiting or retching were recorded. After the final IOP measurement, dogs continued on to the planned surgical procedure. All procedures were carried out by the same 2 people (BA and BB) between 0800 and 1400 h.

**Statistical analysis**

A commercially available software package (Graph Pad Prism 6 for MAC OS X; Graph Pad Software, San Diego, California, USA) was used for statistical analyses. Normality was tested by the Kolmogorov-Smirnov test. A paired t-test was done to compare IOP between right and left eye at each time point. A 1-way analysis of variance (ANOVA) with Tukey's post-hoc test was used to evaluate within-group changes in IOP. Between-treatment effects in IOP were analyzed with 2-way repeated measures ANOVA with Tukey’s post-hoc test. Data are reported as mean ± standard deviation (SD). Sedation, induction, and intubation scores were compared with a Mann-Whitney test and data are presented as a median. Population and drug data were compared among groups with independent t-tests. A value of $P < 0.05$ was considered significant.

**Results**

Thirty-three dogs were initially included in the study, but 2 dogs in the saline group were excluded from data processing due to significant differences between the IOP in the right and left eye. As there were no significant differences between the right and left eye in the remaining 31 dogs at any time point, the data from both eyes were pooled for subsequent analysis. Of the 31 dogs included in the data...
analysis, there were 17 mixed breed, 3 border collies, 3 Weimaraners, 2 Siberian huskies, 2 Australian shepherds, 1 Labrador retriever, 1 Staffordshire terrier, 1 Rottweiler, and 1 Portuguese water dog.

Body weight, sedation, induction and intubation scores, baseline IOP, and the number of dogs requiring alfaxalone top-ups, and alfaxalone top-up dose are provided in Table I. There were no significant differences in body weight and baseline IOP among groups. Sedation scores were significantly different between the ABA group (median 3 (2 to 4)) and SA groups (median 1) (P = 0.0001) and between the DHA group (median 2 (1 to 3)) and SA groups (median 1) (P = 0.0002), but not between the ABA and DHA groups (P = 0.09).

Two dogs in the DHA group vomited, but no other side effects were observed. All anesthetic regimes were successful in eliminating laryngeal reflex and preventing cough; 4 dogs required alfaxalone top ups (Table I). There were no difficulties in carrying out orotracheal intubation. All dogs were successfully intubated on the first attempt and all subjects were intubated within 2 min of the beginning of the alfaxalone injection.

When comparing IOP among groups, a significant difference was observed between the SA (15.4 ± 3.7 mmHg) and ABA (19.0 ± 2.6 mmHg) groups (P < 0.01) at 15 min (Table II). No other significant differences were observed among treatment groups (P > 0.05, Table II). In the ABA group, IOP was significantly different among BL (15.9 ± 2.1 mmHg) and post O₂ (14.5 ± 3.4 mmHg) (P < 0.01). In the DHA group, IOP was significantly different among all time points (P < 0.05, Table II). In the SA group, IOP was significantly different among BL (15.4 ± 3.5 mmHg), post O₂ (15.4 ± 3.5 mmHg), and post A (21.8 ± 3.8 mmHg) (P < 0.01).

### Table I. Baseline characteristics for dogs administered ABA (acepromazine, butorphanol, alfaxalone) (n = 11), DHA (dexmedetomidine, hydromorphone, alfaxalone) (n = 11), and SA (saline, alfaxalone) (n = 9)

<table>
<thead>
<tr>
<th>Variable</th>
<th>ABA group</th>
<th>DHA group</th>
<th>SA group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (kg)</td>
<td>20.1 ± 10.4</td>
<td>18.4 ± 6.0</td>
<td>17.8 ± 7.3</td>
</tr>
<tr>
<td>Baseline IOP (mmHg)</td>
<td>15.9 ± 2.1</td>
<td>16.0 ± 2.9</td>
<td>16.9 ± 3.4</td>
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<tr>
<td>Sedation score (1 to 5)</td>
<td>3 (2 to 4)</td>
<td>2 (1 to 3)</td>
<td>1</td>
</tr>
<tr>
<td>Induction score (1 to 3)</td>
<td>1</td>
<td>1 (1 to 2)</td>
<td>1 (1 to 2)</td>
</tr>
<tr>
<td>Intubation score (1 to 4)</td>
<td>1 (1 to 2)</td>
<td>1 (1 to 2)</td>
<td>1 (1 to 2)</td>
</tr>
<tr>
<td>Number of dogs requiring alfaxalone top up</td>
<td>2</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Alfaxalone top-up dose (mg/kg)</td>
<td>1 ± 0.5</td>
<td>1</td>
<td>0.5</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± SD or median (range). Treatment groups were compared with unpaired t-test (body weight), 2-way analysis of variance (ANOVA) for repeated measures (intraocular pressure [IOP]), and Mann-Whitney test (sedation scores, induction, and intubation scores).

No significant differences were observed among treatments, except for sedation scores. * Sedation scores between SA and ABA and between SA and DHA were significantly different. P-values < 0.05 were considered significant.

### Table II. Mean ± SD values for intraocular pressure (mmHg) recorded at different time points in healthy dogs receiving either ABA (acepromazine, butorphanol, alfaxalone) (n = 11), DHA (dexmedetomidine, hydromorphone, alfaxalone) (n = 11), or SA (saline, alfaxalone) (n = 9)

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Time points</th>
<th>SA</th>
<th>ABA</th>
<th>DHA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BL</td>
<td>16.9 ± 3.4</td>
<td>15.9 ± 2.1</td>
<td>16.0 ± 2.9</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>15.4 ± 3.7 *</td>
<td>19.0 ± 2.6</td>
<td>16.3 ± 3.4</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>15.4 ± 3.5</td>
<td>15.3 ± 4.6 P</td>
<td>16.2 ± 4.3</td>
</tr>
<tr>
<td></td>
<td>Post O₂</td>
<td>15.9 ± 3.1</td>
<td>14.5 ± 3.4 P</td>
<td>15.3 ± 4.4</td>
</tr>
<tr>
<td></td>
<td>Post A</td>
<td>21.8 ± 3.8 aaaa,bbbbb,cccccc,dddd</td>
<td>20.3 ± 3.9 aaaa,cccccc,dddd</td>
<td>19.6 ± 3.5 aaaa,cccccc,dddd</td>
</tr>
<tr>
<td></td>
<td>Post INT</td>
<td>22.4 ± 3.8 aaaa,bbbbb,cccccc,dddd</td>
<td>22.3 ± 4.0 aaaa,cccccc,dddd</td>
<td>22.2 ± 3.5 aaaa,bbbb,cccccc,dddd</td>
</tr>
</tbody>
</table>

P-values < 0.05 were considered significant.

BL — Baseline; 15 to 15 min after premedication; 30 to 30 min after premedication; Post O₂ — Post pre-oxygenation; Post A — Post alfaxalone administration; and Post INT — Post intubation.

* Values are significantly different between group ABA and SA (P < 0.01).

a Significantly different from baseline value for the respective treatment.

b Significantly different from (15) value for the respective treatment.

c Significantly different from (30) value for the respective treatment.

d Significantly different from post O₂ value for the respective treatment.

(*) P < 0.05; (aa) P < 0.01; (aaa) P < 0.001; (aaaa) P < 0.0001. 
(14.5 ± 3.4 mmHg, \( P < 0.0001 \)) among 30 and post A (\( P < 0.0001 \)) and post INT (\( P < 0.0001 \)), among post O and post A (\( P < 0.0001 \)) and post INT (\( P < 0.0001 \)). In the DHA group, IOP did not differ among BL (16.0 ± 2.9 mmHg) and 15 min (16.3 ± 3.4 mmHg) or 30 min (16.2 ± 4.3 mmHg) after premedication (\( P > 0.05 \)). There was, however, a significant difference among BL and post A (19.6 ± 3.5 mmHg, \( P < 0.01 \)) and post INT (22.2 ± 3.5 mmHg, \( P < 0.0001 \)); among post O (15.3 ± 4.4 mmHg) and post A (\( P < 0.01 \)) and post INT (\( P < 0.0001 \)); between 15 and post INT (\( P < 0.0001 \)); and between 30 and post INT (\( P < 0.0001 \)). For the SA group, IOP was significantly different between BL (16.9 ± 3.4 mmHg) and post A (21.8 ± 3.9 mmHg, \( P < 0.001 \)) and post INT (22.4 ± 3.9 mmHg, \( P < 0.0001 \)); between 15 (15.4 ± 3.7 mmHg) and post A and post INT (\( P < 0.0001 \)); between 30 (15.4 ± 3.5 mmHg) and post A and post INT (\( P < 0.0001 \)); between post O (15.9 ± 3.1 mmHg) and post A and post INT (\( P < 0.0001 \)).

**Discussion**

This was a clinical study that investigated the effects of 2 common pre-anesthetic medication protocols, followed by induction with alfaxalone, on IOP in healthy dogs scheduled for routine surgery. The drug combinations and doses used in this study were those routinely used to achieve a level of sedation and induction necessary for intubation. The baseline IOP values obtained in this study before treatment are in agreement with the generally accepted normal range for IOP in dogs (15 to 18 mmHg) (18).

It has previously been reported that IOP does not significantly increase after IM administration of combined acepromazine/butorphanol (17) or acepromazine/hydromorphone (13,19). Our results using combined acepromazine/butorphanol show a significant difference in IOP at 15 min. Although statistically significant, we do not believe that this is clinically significant. Our results also demonstrate that no significant elevation in IOP occurs with the IM administration of dexmedetomidine in combination with hydromorphone.

To the authors’ knowledge, there are no previous reports evaluating the effects on IOP of this particular drug protocol. One study, however, demonstrated an elevated IOP in dogs in lateral recumbency after IV administration of dexmedetomidine and butorphanol (20). This study compared IV medetomidine (0.3 mg/m²) with butorphanol (6 mg/m²) to IV dexmedetomidine (0.3 mg/m²) with butorphanol (6 mg/m²) and, although both groups demonstrated significant increases in IOP relative to baseline, the dexmedetomidine/butorphanol group was significantly higher than the medetomidine/butorphanol group (20). It was proposed that the significant differences between the groups were due to the potency of the dexmedetomidine dose chosen for the dexmedetomidine/butorphanol group. The IOP elevation noted by Rauser et al (20) in both groups was deemed to be due to elevations in systemic vascular resistance and blood pressure indirectly influencing IOP. These IOP increases were not determined to be clinically significant, however, as the IOPs did not exceed 20 mmHg. In contrast, Artigas et al (21) determined that IV dexmedetomidine (0.005 mg/kg BW) given alone did not significantly increase IOP of healthy dogs in sternal recumbency (21). While low doses of dexmedetomidine should not increase systemic vascular resistance enough to increase IOP, the dose used by Rauser et al (20) (approximately 0.01 mg/kg BW, IV) was sufficient to cause an increase in IOP in dogs.

Our study demonstrated that there is no increase in IOP at a 0.002 mg/kg BW dose of dexmedetomidine. The overall effect of dexmedetomidine combined with hydromorphone on IOP is minimal at the time points at which IOP was measured, which suggests that IM sedation with dexmedetomidine/hydromorphone at these dosages is a satisfactory option for surgical premedication when an increase in IOP is undesirable. However, the adverse effects of this combination need to be considered. Two out of 11 dogs in our study vomited shortly after sedation with dexmedetomidine and hydromorphone. Coughing, retching, and vomiting and any maneuver that increases central venous pressure may induce a dramatic increase in IOP (2). This elevation in central venous pressure results in a steep increase in choroidal blood volume and IOP. Emesis and the associated increase in IOP is a possible side-effect of administering systemic \( \mu \)-opioid receptor agonist (22) and alpha-2 adrenergic receptor agonist (23). The risk of vomiting and the associated elevation in IOP after administration of dexmedetomidine and hydromorphone should be considered when selecting an anesthetic protocol for a patient at risk of globe rupture.

When comparing IOP among all 3 treatment groups, a statistically significant difference was observed between SA (15.4 ± 3.7 mmHg) and ABA (19.0 ± 2.6 mmHg) at 15 min after premedication. Given that IOP changes within the respective groups were not statistically significant compared to baseline values and remained within the normal range at the measured time points, this finding was deemed not clinically relevant. A significant increase in IOP has been demonstrated after induction of anesthesia in healthy dogs with propofol (13,24,25), alfaxalone (13), and a combination of ketamine, diazepam, and ketamine/diazepam (26). Our study findings are similar as IV induction with alfaxalone resulted in a statistically significant increase in IOP relative to baseline and 30 min post-premedication in all study groups. These results are consistent with the findings of Hasiuk et al (13), in which 1.5 mg/kg BW of IV alfaxalone was used for induction following premedication with IV acepromazine and hydromorphone in dogs and IOPs were measured in sitting or sternal recumbency.

In contrast to these findings, a more recent study observed a transient nonsignificant increase in IOP, followed by a significant reduction in IOP after a single bolus of 3 mg/kg BW of alfaxalone was administered to healthy non-premedicated dogs (14). There are several possible explanations for this difference in IOP after alfaxalone. Dogs in our study and in the study by Hasiuk et al (13) were sedated with acepromazine/butorphanol or dexmedetomidine/hydromorphone and acepromazine-hydromorphone, respectively. The simultaneous use of pre-anesthetic medication could have induced pharmacologic or pharmacokinetic interactions, which promoted an increase in IOP. Another more likely explanation is the different duration of the 3 studies. Costa et al (14) monitored IOP for 30 min after alfaxalone administration, whereas measurements of IOP pressure were stopped after orotracheal intubation in our dogs as well as in the study by Hasiuk et al (13). It is possible that a reduction in IOP at a later time point was missed due to the shorter monitoring window after alfaxalone administration.
Another difference between studies is orotracheal intubation following alfaxalone administration. Dogs in the study by Costa et al (14) were not intubated, while dogs in our study and in the study by Hasiuk et al (13) were intubated. Although intubation criteria were met for all the dogs herein and no obvious response to orotracheal intubation, such as gagging or coughing, was observed, IOP increased further in all 3 treatment groups post-intubation. This observation is consistent with the findings of Hasiuk et al (13). An increase in IOP of 5 mmHg secondary to laryngoscopy and intubation has been described in humans (27). Hofmeister et al (25) reported a significant increase in IOP in dogs after intubation. This increase in IOP is most likely related to the cardiovascular response to intubation. Laryngoscopy and tracheal intubation can cause tachycardia and hypertension due to sympathetic discharge caused by stimulation of the upper respiratory tract (28). While brief elevation of IOP during intubation is normally of little consequence, it can be harmful to patients with penetrating globe injuries.

Intraocular pressure during anesthesia can be affected in several ways. Anesthetic agents can alter intraocular pressures by changing the rate of aqueous production or outflow or by increasing extracocular muscle tone and scleral rigidity (2). It has been suggested that extracocular muscle tone has a minimal effect on IOP during induction of anesthesia in healthy dogs when propofol or alfaxalone are used (13). Aqueous humor production and outflow can be affected by changes in central nervous system (CNS) output, blood pressure (BP), central venous pressure (CVP), partial pressure of oxygen in arterial blood (PaO₂), and partial pressure of carbon dioxide in arterial blood (PaCO₂) (2). Hofmeister et al (24,29) demonstrated that in unpremedicated dogs, hypercapnea did not play a role in IOP changes with propofol. Although BP, CVP, PaO₂, and PaCO₂ were not specifically evaluated in this study, Ambros et al (10) determined that, in dogs premedicated with acepromazine and hydromorphone, PaCO₂ increases significantly post-induction with alfaxalone or propofol and that PaCO₂ is unaltered. A limitation of the present study is that PaO₂ and PaCO₂ were not measured and it is therefore possible that hypoventilation and hypercapnea may have played a role in the IOP alterations noted in our dogs.

Changes in CNS control of the production and outflow of aqueous humor may also play a role in alfaxalone induction in dogs similar to propofol induction in humans (30). As alfaxalone induces significant respiratory depression (10), it is possible that hypoxemia or elevated CVP during induction could also contribute to the increase in IOP observed in our study. All dogs were given oxygen for at least 3 min before induction, however, and were intubated within 2 min of the beginning of the alfaxalone injection. In our study, the pre-oxygenation had no significant effects on IOP as expected and hypoxemia would not be expected to develop within 2 min of the onset of apnea after pre-oxygenation (31). With regard to CVP, to avoid affecting ocular venous drainage, no pressure was placed on either external jugular vein during handling of the patients.

In conclusion, the results of this study show a statistically significant increase in IOP after induction with alfaxalone, with or without pre-anesthetic medication. Premedication with acepromazine/butorphanol or dexmedetomidine/hydromorphone did not cause a significant increase in IOP and are satisfactory pre-anesthetic combinations to use alone or before anesthesia induction in dogs.

The risk of vomiting and the associated elevation in IOP after dexmedetomidine and hydromorphone are administered should be considered when selecting an anesthetic protocol for a patient with limited tolerance for short-lived increases in IOP. Additional studies are needed to evaluate the effect of alfaxalone on the eyes of patients with ocular diseases, such as glaucoma.

### Appendix

<table>
<thead>
<tr>
<th>Scores for Sedation, Induction, and Intubation</th>
<th>[from Maddern et al (32)]</th>
</tr>
</thead>
</table>

#### Sedation Score

I. No discernable effect
II. Mild sedation: appears sleepy
III. Moderate sedation: very sleepy may be recumbent, but could be aroused
IV. Heavy sedation: recumbent, difficulty rousing
V. Profound sedation: lateral recumbency, not arousable

#### Anesthesia Induction Score

I. Smooth uneventful induction
II. Some mild paddling, twitching, excitement
III. Poor induction, pronounced paddling, twitching, excitement

#### Tracheal Intubation Score

I. Smooth intubation
II. Some mild coughing
III. Pronounced coughing
IV. Swallowing, coughing, gagging — failed attempt

### Acknowledgment

This study was supported by the Companion Animal Health Fund, Western College of Veterinary Medicine.

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**Evaluation of the effect of 4 types of knots on the mechanical properties of 4 types of suture material used in small animal practice**

Xytilis Avoine, Bertrand Lussier, Vladimir Brailovski, Karine Inaekyan, Guy Beauchamp

**Abstract**

The influence of the type of material used, knot configuration, and use of an additional throw on the tensile force at failure, the elongation, and the mode of failure of different configurations of linear sutures and knotted suture loops was evaluated in this *in-vitro* mechanical study. We hypothesized that all types of knots would significantly influence the initial force and elongation of suture materials and would influence the force and elongation at which the knotted loops break, but not their mode of failure. A total of 432 samples of 4 types of size 3-0 suture material (polydioxanone, polyglecaprone 25, polyglactin 910, and nylon), representing 9 configurations, were tested in a tensiometer. The configurations were 1 linear suture without a knot and the following loops: square (SQ) knot; surgeon’s (SU) knot; granny (GR) knot; and sliding half-hitch (SHH) knot using either 4 and 5 or 3 and 4 throws, depending on the material. For polydioxanone, SQ and SU knots did not decrease the initial force at failure of the suture. Granny (GR) and SHH knots decreased the tensile force at failure and elongation by premature failure of the loop. For polyglecaprone 25, all knots decreased the initial force at failure of the suture, with SHH being weaker than the other knots. For coated polyglactin 910, all knots decreased the initial force at failure of the suture and slippage increased significantly compared with the other 3 sutures. The use of SQ knots with 3 throws did not result in a safe knot. For nylon, knots did not alter the original mechanics of the suture. In conclusion, all knots and types of suture material do not necessarily have the same effect on the initial tensile force at failure of suture materials.

**Résumé**

L’objectif de la présente étude était de déterminer, dans des conditions expérimentales contrôlées, l’influence de quatre types de nœud, du nombre de croisements et du type de matériel de suture utilisés sur le mode de rupture, l’elongation et la résistance de différentes configurations de boucles de suture. Un total de 432 échantillons de quatre types de fil de suture 3-0 (polydioxanone, polyglecaprone 25, polyglactin 910, nylon), représentant neuf configurations, ont été évalués à l’aide d’un tensiomètre. Plusieurs paramètres ont été analysés : la force atteinte lors de la rupture, l’élongation maximale de la boucle et son mode de rupture. Les différentes configurations sont : suture linéaire, nœud plat (NP), de chirurgien (NC), de vache (NV) et le nœud coulissant barré (NCB) en réalisant 4 et 5 croisements pour les monofilaments et 3 et 4 croisements pour le multifilament. Les différents matériaux et nœuds utilisés n’affectent pas tous de la même manière la force initiale du matériel de suture. Pour le polydioxanone, le NP et NC ne diminuent pas la force initiale du fil de suture. Le NV et le NCB diminuent la force initiale du fil de suture et diminuent son elongation par un bris prématuré du fil de suture. Pour le polyglecaprone 25, tous les nœuds réduisent la force initiale de la suture ; le NCB est celui qui influence le plus le matériel de suture. Pour le polyglactin 910 enrobé, tous les nœuds réduisent la force initiale de la suture ; le glissement est augmenté de manière significative comparé aux trois autres types de matériels de sutures, une elongation significative de la boucle de suture a été observée avec la présence d’un NP avec trois croisements. Pour le nylon, les nœuds n’ont pas modifié les propriétés mécaniques d’origine du fil de suture. En conclusion, un NP et NC avec un polydioxanone 3-0 ne diminue pas la force initiale du fil de suture. Le polyglactin 910 enrobé est significativement associé à un glissement accru. Il n’y a pas de difference statistiquement significative à l’ajout d’un croisement à une boucle de suture 3-0.

**(Traduit par les auteurs)**

**Introduction**

Surgery is an essential part of veterinary medicine, regardless of the area of practice. Sutures play a crucial role in surgery. The knot is the weakest part of a suture and is generally the site of failure (1–3). Of the many types of knots, the following 4 are commonly used in small animal practice: square knot, surgeon’s knot, granny knot (technical error), and the sliding half-hitch.

The ideal knot should be quick and easy to make, while remaining safe and secure (4). A “safe knot” has been defined as a knot that breaks rather than unties due to slippage when tested to failure (5). Additionally, the knot should have as little affect as possible on the...
suture material. Many factors influence the safety of a suture, including the type of material, the type of knot, the number of throws, and the surgeon’s experience. Various studies have been conducted on the configuration of suture loops, evaluating the knot configuration (4,6), the number of throws (7,8), and the type of material (1,4,9,10). To our knowledge, there are no reports in the literature that evaluate, under the same experimental conditions, the behavior of 3-0 knotted loops of suture material frequently used in small animal surgery.

The aim of this study was to evaluate, under controlled experimental conditions, the influence of the type of material used, knot configuration, and use of an additional throw on the tensile force at failure, the elongation, and the mode of failure of different configurations of linear sutures and knotted suture loops. We hypothesized that all types of knots would significantly influence the initial force and elongation of each of the linear sutures. Our second hypothesis was that all types of knots would significantly influence the force and elongation at which the knotted loops break, but not their mode of failure.

**Materials and methods**

**Suture material**

Four types of size 3-0 suture materials were evaluated: polydioxanone (PDSII), polyglecaprone 25 (Monocryl), coated polyglactin 910 (Vicryl), and nylon (Ethilon) [Ethicon (Johnson & Johnson), Somerville, New Jersey, USA].

**Suture configuration**

Each type of suture material was evaluated in 9 configurations: 1 linear strand of suture without a knot (control group) and 8 loop configurations. All knots were individually tied by the same operator (BL) using 2-handed hand ties wrapped around the combination of a 20-mL syringe (Terumo, Somerset, New Jersey, USA) and an adjacent metallic pin (3-mm diameter) so that the suture loops could easily be removed from the syringe after the metallic pin is withdrawn, thus minimizing friction-related microtrauma to the suture loops. The knot ears were cut to a standardized length of 5 mm (1,3). The syringe was fixed in a vice on a bench top to ensure consistent tension when tightening the knots and all the knots were inspected for slippage before cutting the ears.

The 8 loop configurations were: square (SQ) knot; surgeon’s (SU) knot; granny (GR) knot; and the sliding half-hitch (SHH) knot, with a standard number of throws (4 for monofilament or 3 for multifilament) and another series was tied with a standard number of throws plus 1 (5 for monofilament or 4 for multifilament). For all configurations, the supplemental throws made over the initial throw consisted of square knots. The number of throws for monofilament (4) versus multifilament suture (3) was based on recommendations published earlier (11). For each configuration, 12 samples were tied (based on a pilot study). All knots were tied in an alternate sequential order according to the type of knot; square, surgeon’s, granny, sliding half-hitch: square +1, surgeon’s +1, granny +1, sliding half-hitch +1. The work was divided into 4 sessions to prevent surgeon fatigue and creating a potential bias. A total of 432 samples was obtained.

**Mechanical testing**

All samples were tested using a materials-testing machine (Electroforce 3200; Bose, Eden Prairie, Minnesota, USA). All samples were tested in the following sequential order for each type of suture: polydioxanone, polyglecaprone 25, coated polyglactin 910, and nylon. When placed in the materials-testing machine, each loop of tied suture was wrapped around 2 rods that were able to rotate, thus reducing constraints due to friction (Figure 1). The linear sutures were placed between 2 clamps using a previously described method (12).

Samples were subjected to an initial tension of 5 Newtons (N) for 3 min and an elongation rate of 5 mm per min (to avoid the influence of the strain rate on the behavior of the suture material) until failure occurred. Tensile force and elongation were recorded continuously with dedicated software (WinTest 4.0; Bose). The following parameters were analyzed: (a) tensile force (N) measured at the failure of the loop; (b) elongation of the loop (mm) measured at the failure of the loop; and (c) mode of failure, defined as failure at the knot, failure of the suture excluding the knot, and slippage of the knot.

The mode of failure was determined by analyzing the “load-displacement” curves and visually inspecting the knotted loops. Video recordings were made to document all trials and were reviewed when needed to validate the mode of failure. For data analysis, failure forces and elongations at failure were considered for all the samples. Samples were excluded from the force data analysis only if the displacement limit of the testing machine was reached, but no failure occurred or if knot slippage resulted in the loop opening during testing (Table I). For elongation, however, loops that did not rupture at the end of the displacement were considered
for statistical analyses. For the mode of failure, all samples were considered for descriptive analyses.

**Statistical analyses**

A linear model was used to compare the tensile force and elongation at failure. A descriptive analysis was used for the failure mode. In each dataset, $P < 0.05$ was considered statistically significant. SAS version 9.3 software (SAS Institute, Cary, North Carolina, USA) was used to conduct the statistical analysis. *A posteriori* statistical analyses using logistic regression were used, with the occurrence of slippage as the dependent variable and the type of knot and number of throws as independent variables. For each type of knot, an exact Chi-square test was carried out to determine whether suture type was associated with the prevalence of slippage. This was followed by pairwise comparisons among suture types.

### Table 1. Results of mechanical sample testing for tensile force and elongation at failure

<table>
<thead>
<tr>
<th>Suture material</th>
<th>Knot configuration</th>
<th>n</th>
<th>Throws</th>
<th>Tensile force at failure (N)</th>
<th>Elongation at failure (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Mean</td>
<td>SD</td>
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<td>44.59*</td>
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<td>55.19*</td>
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<td>3.52</td>
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<tr>
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<td>62.24*</td>
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<td>54.06*</td>
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<td>21.72*</td>
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<td>22.26*</td>
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<td>39.92*</td>
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<td>4</td>
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<td>33.99*</td>
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<td>37.84</td>
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<td>5</td>
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<td>5</td>
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<td>2.46</td>
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<tr>
<td>Linear suture (without knot)</td>
<td></td>
<td>12</td>
<td>N/A</td>
<td>40.74</td>
<td>2.68</td>
</tr>
</tbody>
</table>

** Machine displacement limit was reached.

** Erroreneous data acquisition.

* Significant result ($P < 0.05$).

N/A — Not applicable.
Results

A total of 432 samples were submitted for evaluation in the materials-testing machine. Seventeen specimens were excluded from data analysis of tensile force, 14 because the machine displacement limit was reached, but no failure occurred and 3 because of an erroneous data acquisition. The results of mechanical testing of the samples for tensile force and elongation are summarized in Table I.

Tensile force at failure

Influence of knot versus linear suture on force at failure — As shown in Figure 2, for polydioxanone, the presence of a knot significantly decreased the tensile force at failure of the loops for granny and sliding half-hitch knots, but not for square and surgeon’s knots. For polyglecaprone 25 and coated polyglactin 910, all knots significantly decreased the force at failure of the loops and for nylon, only the surgeon’s knot with an additional throw significantly decreased the force at failure of the loops.

Influence of types of knots on force at failure — As shown in Figure 3, for polydioxanone, the presence of a granny knot and a sliding half-hitch significantly decreased tensile force at failure of loops compared to square or surgeon’s knots. For polyglecaprone 25, the presence of a sliding half-hitch significantly decreased tensile force at failure compared to a square knot. For coated polyglactin 910, the presence of a granny knot or a sliding half-hitch significantly decreased tensile force at failure compared to a surgeon’s knot and for the nylon, there was no significant difference among the 4 types of knots.

Addition of supplemental throw — There was no statistically significant difference in the tensile force at failure of suture loops

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![Figure 2. Effect of 8 loop configurations on the tensile force Newton (N) at failure of 4 suture materials. All loop configurations are compared to linear suture for each type of suture material. Significant values are indicated by symbol: x.](image-url)
associated with the addition of a supplemental throw for each type of the 4 suture materials.

**Elongation at failure**

**Influence of knot versus linear suture on elongation at failure** — As shown in Figure 4, for polydioxanone, the presence of a sliding half-hitch significantly decreased the elongation of the loops at failure, which means the loop breaks earlier. For polyglecaprone 25 and nylon, there was no significant difference in loop elongation at failure. For coated polyglactin 910, significant loop elongation was observed with the presence of a square knot with 3 throws and with the granny and the sliding half-hitch knots.

**Influence of types of knots on elongation at failure** — As shown in Figure 5, for polydioxanone, the presence of a granny and a sliding half-hitch knot significantly decreased elongation at failure compared to square or surgeon’s knots. For polyglecaprone 25, the presence of a sliding half-hitch significantly decreased elongation at failure compared to a square knot. For coated polyglactin 910 and nylon, the presence of the knot did not significantly influence the elongation at failure of the loop.

**Addition of a supplemental throw** — There was no statistically significant difference in the loop elongation associated with the addition of a supplemental throw for each type of suture material.

**Mode of failure**

**Polydioxanone** — Of the 96 samples, 2 were discarded due to erroneous data acquisition. Of the 94 samples, 89 (94.6%) broke at the knot and 5 did not break (reached displacement limit).

**Polyglecaprone 25** — Of the 96 samples, 1 was discarded due to erroneous data acquisition. Of the 95 samples 92 (96.8%) broke at the knot, 2 broke outside the knot, none slipped, and 1 did not break (reached displacement limit).

**Coated polyglactin 910** — Of the 96 samples, 48 (50%) broke at the knot, 45 (46.8%) slipped, 1 broke outside the knot, and 2 did not break (reached displacement limit). Our *a posteriori* analyses showed that the prevalence of slippage is significantly increased when using coated polyglactin 910 compared to the other types of suture material. Furthermore, the type of knot (granny and sliding half-hitch) significantly increased slippage, but the number of throws did not.

**Nylon** — Of 96 samples, 89 (92.7%) broke at the knot, 1 broke outside the knot, none slipped, and 6 did not break (reached displacement limit).

**Discussion**

Suture failure can lead to both minor complications (delayed healing, inappropriate scarring, or superficial infection) and major complications (wound separation, wound dehiscence, or deep infection) that subsequently lead to catastrophic events, such as intestinal leakage, evisceration, and peritonitis (1–4,7). It is therefore important to objectively select the type of suture material used based on its intrinsic properties (strength, elongation, and failure mode) and the safety of the knot used. Moreover, depending on the type of suture material used, each type of knot will influence the tensile force and elongation at failure.

Our first hypothesis was that all types of knots would significantly influence the force and elongation at failure of each of the linear sutures. Our hypothesis with regard to the tensile force (Figure 2) was partially correct for polydioxanone, correct for polyglecaprone 25 and coated polyglactin 910, and incorrect for nylon, with the exception of the surgeon’s knot with 5 throws, which could be the result of a type-I error. Our hypothesis with regard to elongation (Figure 4) was partially correct for polydioxanone and coated polyglactin 910 and incorrect for polyglecaprone 25 and nylon.
Interestingly, in disagreement with several textbooks (13–15), we found that not all suture materials and knots affect the initial force at failure of linear suture materials and not to the same extent. For polydioxanone, polyglecaprone 25, coated polyglactin 910, and nylon, we documented a variation of the force at failure of the linear suture when using a knotted loop with a square knot of, respectively 5.6%, 14.6%, 48.3%, and 0.9%. When using a surgeon’s knot, the variations were: 1.4%, 22.4%, 38.2%, and 7.1%, respectively.

It has been reported that the knot is the weakest point in a suture line or a ligature, as tying a knot results in friction between 2 strands, which weakens the suture (13,15). Naleway et al (16) have shown that a single knot decreased the force at failure of nylon by 62%. It has also been previously reported that shear stresses at the point between the loop and the first throw of the loop weaken the suture material (4). In our study, we observed this behavior for polydioxanone when using a sliding half-hitch knot; loop elongation decreased significantly at failure due to premature breakage of the loop compared to the linear suture. Furthermore, when comparing the knotted loops, the use of a granny knot or sliding half-hitch knot significantly decreased loop elongation at failure compared to the square or surgeon’s knots. This finding is also associated with premature breakage of the loops.

Our second hypothesis was that all types of knots would significantly influence the force and elongation at which the knotted loop breaks, but not their mode of failure. Our hypothesis with regard to tensile force (Figure 3) was correct for polydioxanone, partially correct for polyglecaprone 25 and coated polyglactin 910, and incorrect for nylon. Our hypothesis with regard to elongation (Figure 5) was

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**Figure 4. Effect of 8 loop configurations on the elongation (mm) at failure of 4 suture materials. All knot configurations are compared to linear suture for each type of suture material. Significant values are indicated by symbol:**

![Graphs showing elongation at failure for each knot configuration](image-url)
correct for polydioxanone, partially correct for polyglecaprone 25, and incorrect for coated polyglactin 910 and nylon. Furthermore, our a posteriori statistical analyses have shown that, for all knots, the use of coated polyglactin 910 significantly increased slippage versus breakage compared to polyglecaprone 25, polydioxanone, and nylon.

Regarding elongation in our study, each loop measured 2 cm in diameter, which corresponds to 62.8 mm. As the maximal elongation observed in our study was 12 mm, this correlates to 19% of elongation. In the healing process, this value is within physiological tolerance to deformation (100% for granulation tissue) (14). We propose that elongation of suture loops as evaluated in our study has no clinical relevance.

The square knot and the surgeon’s knot are the most common knots used in surgical procedures (8). It is not uncommon for the novice surgeon to create granny knots than square knots (8, 17, 18). The impact of this technical error has been outlined in the present study (premature failure, influence on elongation at failure). A square knot can easily become a sliding half-hitch when tied by a novice or even an experienced surgeon. It has been reported that 80% of square knots can slip into a sliding half-hitch depending on the suturing material, the configuration of the knot, and the surgeon’s tying skills (1, 6, 7, 17, 18).

Marturello et al (1) showed that the surgeon’s experience and training were significant factors affecting knot security. We emphasized that a modification in the knot due to suboptimal technique could significantly affect the tensile force of a suture. Furthermore, it is important to recognize the consequences of tying a granny or sliding half-hitch knot by mistake, as these knots significantly decreased the tensile force and elongation at failure of the knotted loops because of the premature failure. We also noted that adding a supplemental throw did not significantly change our results. Marturello et al (1) also reported that 3 throws were as secure as 6 throws and, although the tensile failure load was greater with 6 throws, there was no significant difference in knot security with more than 4 throws for each suture material tested. It has also been reported by Van Rijssel et al (19) that the size of the suture material is more important than the number of throws for knot security.

When selecting polyglactin 910 for our study, we considered it as a true multifilament because there was no mention on the packaging (box and suture envelopes) that it was coated. After analyzing our data, it was difficult to explain the results obtained. When looking closer at the monograph inside the box, we realized that polyglactin 910 was coated, even though this was not specified on the packaging. This may explain why this suture did not exhibit the behavior of a multifilament but behaved instead as a coated suture. Interestingly, as shown in Figure 6, the square knot with 4 throws and the surgeon’s knot exhibited the most constant behavior.

We have found that the mechanical properties of knotted loops using polyglactin 910 are similar to those reported in the literature (3, 7, 17, 20, 21). Polyglactin 910 seems to exhibit more slippage and its safety is uncertain, as the use of additional throws does not seem to be correlated with the safety of the knotted loop. In a study by Ching et al (8), when tensile strengths were evaluated for knots that failed by breakage and those that failed by slippage, the number of throws did not significantly affect the tensile strength of the polyglactin 910 (size 2-0) (8). Behm et al (20) reported that the failure rates for sizes 0 and 2-0 of polyglactin 910 were still fairly high (40%) even with 5 throws. As reported by Rodeheaver et al (21), when using polyglactin 910, knots were made secure by using 1 more throw than with coated polyglycolic acid sutures. Our findings are also consistent with the observations of Rosin et al (5) who evaluated the knot security of size 2-0 knotted sutures and observed that a surgeon’s knot was more secure than a square knot when using polyglactin 910. Finally, it has also been determined that, for size 0 polyglactin 910, knot security continued to improve until 5 throws were used (17).

Taking into account all the data evaluating the mechanical properties of 4 types of size 3-0 suture materials from this study, we can conclude that for polydioxanone, the use of a square or a surgeon’s knot did not decrease the tensile force at failure of the linear suture and the use of a granny or sliding half-hitch knot decreased the tensile force at failure and significantly reduced the elongation at failure of the knotted loop because of the premature failure. These 2 knots should therefore be avoided. For polyglecaprone 25, all the

![Figure 5. Effect of 4 loop configurations on the elongation (mm) at failure of 4 suture materials. All knot configurations are compared to each other for each type of suture material. The number of throws is 4 for polydioxanone, polyglecaprone 25, and nylon and 3 for coated polyglactin 910.](image)
knots decreased the tensile force at failure of the linear suture and the sliding half-hitch knot was significantly less resistant than the other knots and should be avoided. For coated polyglactin 910, all knots decreased the tensile force at failure of the linear suture and this material was associated with a significantly high rate of slip-

Figure 6. Load-displacement curves of coated polyglactin 910. Each line represents a tested suture loop: 12 loops were evaluated per knot configuration. Tensile force (N) versus displacement (mm).

page. Using a square knot with 3 throws with coated polyglactin 910 did not seem to result in a secure knot. For nylon, the tensile force and the elongation at failure were not influenced by the presence of a knot. Overall, however, nylon seemed to be the weakest suture material; the initial tensile force was 73.9%, 54.9%, and 61.3%,
respectively, of the force at failure of linear suture with polydioxanone, polyglycaprone 25, and coated polyglactin 910. This study had many limitations, including the difficulty of extrapolating the findings of an in-vitro experimental study to an in-vivo clinical situation. In addition, the knotted loops were not incubated in saline or plasma before mechanical testing, which might have mimicked a more physiological condition. Other suture materials, knot configurations, and numbers of throws could have been evaluated, thus evaluating fatigue, and cyclic loading was not tested. Further studies are warranted in order to evaluate the properties of knotted loops in more physiological conditions. The observations made in this study indicate that, when using different types of size 3-0 suture material, the results from mechanical testing pertaining to tensile force, elongation, and mode of failure cannot be extrapolated among various types of sutures and knots.

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References

Active surveillance of Anaplasma marginale in populations of arthropod vectors (Acari: Ixodidae; Diptera: Tabanidae) during and after an outbreak of bovine anaplasmosis in southern Manitoba, Canada

Matthew E.M. Yunik, Terry D. Galloway, L. Robbin Lindsay

Abstract

Bovine anaplasmosis is the disease caused by the bacterium Anaplasma marginale. It can cause production loss and death in cattle and bison. This was a reportable disease in Canada until April 2014. Before then, infected herds were quarantined and culled, removing infected animals. In North America, A. marginale is biologically vectored by hard ticks (Acari: Ixodidae), Dermacentor variabilis and D. andersoni. Biting flies, particularly horse flies (Diptera: Tabanidae), can also act as mechanical vectors. An outbreak of bovine anaplasmosis, consisting of 14 herds, was detected in southern Manitoba in 2008. This outbreak lasted multiple rounds of testing and culling before eradication in 2011, suggesting local maintenance of the pathogen was occurring. We applied novel approaches to examine the vector ecology of this disease in this region. We did not detect A. marginale by screening of 2056 D. variabilis (2011 and 2012) and 520 horse flies (2011) using polymerase chain reaction (PCR).

Résumé


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program until there were no longer infected animals in the region (10,11). Vaccines are not currently available in North America and no antibiotics are currently licensed for use in Canada. The Canadian Food Inspection Agency (CFIA) conducted a Bovine Serological Survey (BSS) every 3 to 5 y to affirm that the national herd remained free of *A. marginale* as well as other pathogens. In 2007–2008, the BSS revealed that the prevalence of infection in the Canadian herd was higher than the acceptable 0.02% for *A. marginale* (11). A portion of the positive samples was traced to Manitoba. Enhanced surveillance efforts led to the detection of animals infected with *A. marginale* from herds belonging to 13 beef producers and 1 bison producer. A quarantine, test, and cull program was initiated in 2009 and concluded in the summer of 2011 (11). From a Canadian perspective, this outbreak was unique because infected animals were detected during multiple summers, requiring multiple rounds of culling to end local transmission. At the initiation of the program, producers who participated in the study had a cumulative beef cattle herd size of approximately 1278 animals (range of head per farm: 40 to 373), 15% of which were culled after the first round of testing by the CFIA. Prevalence of *A. marginale* infection ranged from 2.7% to 32.0% per herd. In the spring of 2011, 7 additional animals were culled from a herd of 115, while one additional animal was culled in the summer from a herd of approximately 30. These data are based on results from a questionnaire distributed by the CFIA to participating producers in 2011.

The exact role that arthropods, especially ticks, play in the epidemiology of bovine anaplasmosis is understudied (1). Certain strains of *A. marginale* have varying ability to infect vertebrate and arthropod hosts (12–14). Additionally, the behavior, physiology, and life history of the tick vectors can vary throughout their geographic range (15). Only recently have we had the molecular tools to conduct large-scale, active surveillance of arthropod vectors in a pastoral setting, which has been suggested as a key element to understanding the ecology of bovine anaplasmosis. The objective of this study was to assess what role, if any, ticks and horse flies had in the potential maintenance *A. marginale* in the outbreak region in Manitoba.

In April 2011, we contacted CFIA personnel who were the primary responding veterinarians responsible for the control of the anaplasmosis outbreak in southeastern Manitoba. Staff from the CFIA provided essential historical background on the presence of *A. marginale* in the region over previous years and initiated contact with producers who owned the pastures in which infected animals had been detected. Of those contacted, 9 producers participated in the study. Ticks were collected by drag sampling using a piece of white flannel (1.3 × 0.70 m), spread by a plastic spar, dragged by researchers at a leisurely pace through the pastures. Tick dragging was conducted on non-rainy days when the air temperature was above 10°C; after snow had melted in April until August when questing ticks were no longer present. The flannel, along with researchers’ clothes, was examined for ticks approximately every 10 m. Collected ticks were then placed in a 56-mL plastic vial with a perforated snap-top lid. Upon leaving the pasture, the date and location of the collection were recorded, and the vials were placed in a plastic bag with a moistened paper towel, and deposited in an insulated container for transport. The time allotted for drag sampling the over 18 km² of pasture, distributed among the 9 participating producers, was based on suitability of tick habitat, recent herd infection history, including number of animals culled per herd and pasture utilization, and producer interest. At least 100 ticks were collected from land owned by each producer involved in the study.

Once returned to the lab, ticks were identified based on morphological characteristics to be *D. variabilis* before being surface-sterilized in 4 different solutions, and grouped according to collection site. The first solution consisted of one drop of Tween 80 per 10 mL of 0.5% bleach. The second solution was 0.5% benzalkonium chloride. The third solution was 70% ethanol. The final solution was filtered water. All ticks collected from the same pasture on the same day were placed in 50 mL centrifuge tubes and approximately 45 mL of the first solution was added. The tubes were then sealed and slowly and repeatedly inverted for 3 min. The solution was decanted and the next solution added. This process was repeated for all solutions. After decanting the filtered water, the ticks and centrifuge tube were dried with a paper towel; the ticks were replaced into the tube and frozen at −80°C for storage.

Horse flies were collected using a Manitoba Horse Fly Trap (16). In 2011, a trap was operated for 4 days from 9 a.m. to 8 p.m. in the first week of June when fly populations appeared to be at their highest. The trap was placed for the first 2 d on a pasture where fly intensity appeared to be the highest and where previously infected animals had been maintained in 2009–2010. On subsequent days, the trap was placed in close proximity to the herd that contained a cow which had an active *A. marginale* infection approximately one month prior. Once removed from the field, flies were killed by being placed in a freezer at −5°C for approximately 20 min before being transferred to a sterile plastic bag and placed in a freezer at −80°C.

Ticks were removed from storage at −80°C and sorted by location and date collected, as well as gender, and placed into pools of no more than 5. Each tick was then cut in half sagittally using a scalpel on a sterile Petri dish in a biosafety cabinet. The scalpel was disinfected between ticks by washing in 10% chlorine bleach solution followed by a rinse in 90% ethanol. Half of each tick was frozen individually in a 2 mL microtube (Sarstedt AG and CO, Newton, North Carolina, USA), while the other half remained in a designated pool. This ensured that individual positive ticks found in pools which tested positive for the presence of *A. marginale* could be identified. The ticks in each pool were then cut further into smaller pieces using a sterile scalpel to enhance DNA extraction.

Extraction of DNA from the pools was conducted using DNAeasy Blood and Tissue Kits (Qiagen, Austin, Texas, USA) following the blood and tissue protocol. All extracts were then stored in a freezer at −80°C. Extractions were screened for *A. marginale* DNA using real-time polymerase chain reaction (RT-PCR) with primers and a probe that had been successfully used in previous studies targeting the 16S rRNA gene (17). The RT-PCR was conducted using 96-well fast plates on a VIIATM 7 RT-PCR system (Applied Biosystems, Foster City, California, USA). The thermocycling regime consisted of an activation stage lasting for 2 min at 50°C, an initial denaturation lasting 10 min at 95°C, and 40 cycles of 95°C, and 60°C lasting for 15 s and 1 min, respectively. Each reaction contained 12.5 μL of TaqMan Universal Master Mix (2X) (Applied Biosystems), forward and reverse primers at a concentration of 0.667 μM each, a probe at a
Anaplasmosis is classified as an endemic, non-regulated pathogen and potential spatial link for pathogen transmission. These animals may come in close proximity to livestock, providing a ready reservoir for the pathogen. Although the exact role wild reservoirs play in transmission is not fully understood, there is evidence that shows they may be involved in the maintenance of the infection. This is supported by the presence of the pathogen in wild reservoirs, such as O. virginianus and O. capricornatus, which are known to be natural hosts for Anaplasma marginale.

The success of the CFIA's quarantine, test, and cull program that the CFIA implemented in 2000 dramatically reduced the probability that infected, and neighboring herds, were deemed free of anaplasmosis. Although the herd of A. marginale that routinely migrates between Minnesota and the outbreak region in Manitoba has been monitored for A. marginale in the past. On the basis of serological tests, the herd has been considered free of anaplasmosis since at least 2004.

Although the results of this study were negative there are many aspects of this work that are novel and noteworthy. This was the first large-scale study to use field-collected ticks and flies from pastoral settings to examine their potential role in the transmission of A. marginale in North America. This was accomplished through the use of molecular techniques not previously applied to such specimens. The use of fly mouthparts as opposed to the whole fly, demonstrating the potential role of the fly as a mechanical vector, is also very important. In a preliminary study, whole horse fly heads were used for DNA extraction. A component of the fly's large compound eyes inhibited conventional and real time PCR. This is also the first time the Manitoba Horse Fly Trap has been used for surveillance of A. marginale. This trap has been used to evaluate fly population composition and density, and to reduce fly pressure.

There are numerous factors that may have been responsible for our failure to detect A. marginale. The most likely factor was the success of the quarantine, test, and cull program that the CFIA enacted in 2009 and concluded in 2011, when all herds that had been infected, and neighboring herds, were deemed free of anaplasmosis. This success by the CFIA dramatically reduced the probability that A. marginale would be detected in the arthropod populations, particularly the biting flies. Vector competency might have also been an issue. Although Dermacentor ticks collected in Canada have been experimentally shown to transmit A. marginale, some strains are not vectored by ticks (12,15). Finally, the lack of detection could also be caused by the absence of infected wild reservoirs that were previously in the area. Although the exact role wild reservoirs play in the maintenance of bovine anaplasmosis is not well-understood, O. virginianus and C. canadensis are present in southeastern Manitoba and are known to come in close proximity to livestock, providing a potential spatial link for pathogen transmission. These animals may also frequently cross the international border into Minnesota where anaplasmosis is classified as an endemic, non-regulated pathogen.

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References


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Breed- and age-related differences in canine mammary tumors
Hyun-Woo Kim, Ha-Young Lim, Jong-II Shin, Byung-Joon Seung, Jung-Hyung Ju, Jung-Hyang Sur ................. 146

The effects of intravenous alfaxalone with and without premedication on intraocular pressure in healthy dogs
Bianca S. Bauer, Barbara Ambros ................. 156

Evaluation of the effect of 4 types of knots on the mechanical properties of 4 types of suture material used in small animal practice
Xytiris Avoine, Bertrand Lussier, Vladimir Brailovski, Karine Inaekyan, Guy Beauchamp............................. 162

Short Communication/
Communication brève
Active surveillance of Anaplasma marginale in populations of arthropod vectors (Acari: Ixodidae; Diptera: Tabanidae) during and after an outbreak of bovine anaplasmosis in southern Manitoba, Canada
Matthew E.M. Yunik, Terry D. Galloway, L. Robbin Lindsay ......................................................... 171