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Porcine reproductive and respiratory syndrome virus (PRRSV) in pig meat
Philippe Raymond, Christian Bellehumeur, Malliga Nagarajan, Diane Longtin, Alexandra Ferland, Peter Müller, Rachel Bissonnette, Carole Simard

Abstract
Porcine reproductive and respiratory syndrome, caused by the porcine reproductive and respiratory syndrome virus (PRRSV), is an economically important disease in the swine industry. Previous studies demonstrated the presence of the virus in pig meat and its transmissibility by oral consumption. This study further analyzed the infectivity of PRRSV in commercial pig meat. Fresh bottom meat pieces (n = 1500) randomly selected over a period of 2 y from a pork ham boning plant located in Quebec, Canada, were tested by reverse transcriptase polymerase chain reaction (RT-PCR). Each trimmed meat was stored in the plant freezer, subsampled weekly for up to 15 wk, and tested with quantitative RT-PCR to determine the viral load. Meat infectivity was evaluated using specific pathogen-free piglets, each fed with approximately 500 g of meat at the end of the storage time. Genotype-specific RT-PCR confirmed the presence of PRRSV mainly during cold weather in 0.73% of the fresh meat pieces. Wild and vaccine strains of genotype 2 were detected. Porcine reproductive and respiratory syndrome virus nucleic acid was stable in meat stored at around −20°C during the 15 wk. Serological and molecular analysis showed the transmission of infection by a majority of PRRSV positive meat pieces (5/9) fed orally to naïve recipients. The results confirmed a low prevalence of PRRSV in market’s pig meat, and virus transmissibility by oral consumption to naïve recipients even after several weeks of storage in a commercial freezer. It occurred mainly with meat harboring the highest PRRSV RNA copies, in the range of 10^9 copies per 500 g of meat, with both wild type and vaccine-related strains.

Introduction
Porcine reproductive and respiratory syndrome (PRRS) is among the most economically significant swine infectious diseases (1,2). The role of pig meat and swill feeding in PRRS virus (PRRSV) transmission is questioned as international trade in pork expands (3). The enveloped virus is between 50 and 65 nm in diameter and is classified in the Arteriviridae family within the order Nidovirales. It contains a single-stranded positive-sense RNA of approximately 15 kb in length that encodes at least 9 open reading frames (ORF) (4). There are 2 recognized PRRSV genotypes: the North American (genotype 2, VR-2332 prototype) and the European (genotype 1, Lelystad prototype). Both have similar genomic organizations, but are genetically and antigenically distinct (5). In Canada, the North American genotype has been reported in lineages typical of vaccines (Ingelvac PRRS ATP, Ingelvac PRRS MLV) and of the MN-184 wild types (6,7). Porcine reproductive and respiratory syndrome is mainly characterized by reproductive failure in sows and respiratory...
illness in pigs of all ages. These clinical signs vary markedly between herds and depend on the virulence of the infecting strain. In eastern Europe and China, new highly pathogenic subtypes of PRRSV have been reported (8–10).

Porcine reproductive and respiratory syndrome virus is highly contagious in pig herds. It can be transmitted by direct contact or by infectious aerosols. Vaccines have been used to limit the propagation of the disease, but are often found inefficient (11). The virus can also persist for months in the target cells of the monocyte and macrophage lineages of an infected animal, despite the immune protection (11,12). Transient detection of PRRSV in pig meat following experimental transmission has been observed (12). The virus was isolated in vitro from 6 out of 1049 sample pools of fresh meat (13), while others failed to detect PRRSV in 472 pig carcasses tested by PCR (14).

In countries currently free of PRRS, the risk of importing the PRRS virus in fresh pork is a concern. Some early import risk analyses for chilled or frozen meats have concluded that virally infected pig meat could represent a source for the introduction of the virus in PRRS-free countries (15,16). These analyses were notably based on a study from Lelystad, the Netherlands, showing the transmission of PRRSV through oral uptake of infected porcine muscular tissues by naïve recipients (17,18). In 2003, Magar and Larochelle (6) led a study to investigate whether pig meat could harbor PRRSV and if so, whether viruses in positive meats could infect the animals when fed to SPF pigs. In their study, analyses and bioassays were performed with meat conserved at ultra-low temperatures. It was argued that these experiments were performed in optimal conditions for virus survival in meat (19–21). For instance, van der Linden et al (18) showed that freezing meat at −23°C for 10 d and then thawing it decreased the virus titers in the majority of PRRSV infected muscle samples. The present study was undertaken to reproduce conditions in commercial settings under which meat would be cut, packaged, and stored in a frozen state for many weeks, similar to overseas import or export conditions. It provides additional knowledge on PRRSV prevalence, survival in pig meat, meat viral load, and transmission from meat to naïve piglets.

Materials and methods

Pork samples

Pork carcasses were from market pigs killed at 3 abattoirs from the province of Quebec, Canada. Carcasses were transported in refrigerated trucks and received at a pork ham boning plant (PHBP) located in St. Hyacinthe, Quebec, 2 to 5 d after slaughter. Between 5 and 17 fresh bottom meat pieces of around 900 to 1100 g each were selected randomly from the cutting and trimming lines 2 to 3 d a week (117 visits). The meat pieces could not be associated with a specific abattoir once on the cutting and trimming line. Each meat piece was identified using a unique identification number (ID). Approximately 2 g of muscle tissue was subsampled from each meat piece with a sterile scalpel at 3 distant locations and pooled in a 50 mL sterile tube. These subsamples corresponded to week 0 (Tmax). Meat subsamples were kept on ice until their arrival at the CFIA Saint Hyacinthe Laboratory for RNA extraction a few hours later. At the PHBP, each of the selected fresh bottom meat pieces was put in a labeled plastic bag and stored immediately in cardboard boxes among commercial meats in the company’s freezer, maintained between −24°C and −21°C. Based on the reverse transcriptase polymerase chain reaction (RT-PCR) assays, 1 PRRSV non-confirmed, 9 PRRSV positive, and 2 RT-PCR PRRSV negative frozen meat pieces were selected for further analysis. In storage follow-up experiments, they were subsampled weekly from the PHBP from T1, up to 15 consecutive weeks (Tmax). Subsamples were collected in each frozen meat piece near the first sampling locations using a clean and sterilized bit and a drill. Approximately 2 g of frozen meat were subsampled and kept on ice until processed at the lab a few hours later. The bottom frozen meat pieces were immediately put back in their boxes in the company’s freezer, among commercial meats. At the last sampling time (Tmax), leftover frozen meats were transported in a frozen state and stored at −70°C, until used in viral transmission bioassays.

Viral transmission bioassays

All animals were treated according to the policy and guidelines of the Canadian Council on Animal Care (CCAC) and the Animal Care Committee of our laboratory. For the feeding trials, 9 PRRSV RT-PCR confirmed positive and one non-confirmed pig meat sample were fed to the piglets. These bioassays were adapted from Magar and Larochelle (6) using the same facilities, biosecurity measures, and animal adaption protocol. Each meat sample was used to feed 2 specific pathogen-free (SPF) piglets 5 to 6 wk of age. Each pig pair was housed in a separate cubicule. These piglets were provided by the CFIA Ottawa Laboratory (Fallowfield, Ontario). The meat samples were thawed at 4°C overnight, weighed, cut into small pieces (around 2 cm³) with sterile scalpels, and divided in 4 equal portions of approximately 250 g each. Piglets were fed a portion on 2 consecutive days. The portion used to feed the pigs on the second day was kept at 4°C overnight. In an alternative protocol, 3 meat samples (ID 1311, 1332, and 1424) were also divided into 4 equal portions of around 250 g. While 2 portions were stored at 4°C overnight, the other 2 were stored at room temperature (RT, between 18°C to 23°C). A pig was fed on 2 consecutive days with the meat sample stored at 4°C while the paired animal, located in a separate cubicule, received the other part of the same meat sample stored at RT. Each trial included either 1 or 2 control pigs maintained on a standard pig diet with no thawed meat throughout the experiments.

Enzyme-linked immunosorbent assay (ELISA)

Blood samples were collected on a regular basis from the jugular vein of each piglet with a 20-G needle. Throat mucus samples were collected with minitip flocked swabs (Millipore; Billerica, Massachusetts, USA) to follow PRRSV infection in piglets. Both were collected at arrival and at 0, 7, 14, 21, and 28 d after feeding. Only the throat samples from the first 3 bioassays were tested. Serum and throat samples were used to detect PRRSV by molecular assays. Serum samples were tested for the presence of antibodies to PRRSV in piglets via ELISA (HerdCheck; IDEXX Laboratories, Westbrook, Maine, USA) as recommended by the manufacturer.

RNA extraction

All meat subsamples from the PHBP were homogenized on the same collecting day as previously described (22). Supernatant
aliquots were stored at −70°C until nucleic acid extraction. RNA was extracted from 100 μL of the meat homogenate using the RNeasy Mini Kit according to the manufacturer’s protocol (Qiagen, Hilden, Germany). Total RNA was eluted with 40 μL of RNase-free water. A Nanodrop-1000 (Thermo Scientific, Wilmington, Delaware, USA) was used to quantify total RNA in each sample (OD 260 nM). For the meat-fed piglets, RNA was extracted from 140 μL of serum sample using the QIAamp Viral RNA Mini Kit (Qiagen). RNA was also extracted from throat swab samples using RNeasy Mini Kit with 0.6 mL RLT buffer. All RNA extracts were stored at −70°C prior to the analyses. The OR7 RT-PCR analyses were conducted within a week while samples from decay were combined on the same plate and tested by OR7 qRT-PCR at the end of the storage period. Phylogeny analyses were conducted on RNA extract stored up to 3 y.

**RT-PCR**

The RT-PCR for the detection of the ORF-7 gene of both American and European strains was performed on 1500 fresh meat samples collected randomly as a screen test (T_0). A 3 and European strains was performed on 1500 fresh meat samples collected randomly as a screen test (T_0). A 3 and European strains was performed on 1500 fresh meat samples collected randomly as a screen test (T_0). A 3 and European strains was performed on 1500 fresh meat samples collected randomly as a screen test (T_0).

**SYBR Green quantitative Real-Time RT-PCR (qRT-PCR)**

To assess viral load before the transmission experiment at T_{in} or T_{max} in meats and to evaluate viral decay over time from T_1 to T_{max}, viral RNA copies were estimated by one-step SYBR Green qRT-PCR assay (ORF-7 qRT-PCR) using the QuantiTect SYBR Green RT-PCR Kit (Qiagen) an the RNA transcript standard. The PR15M and PR16M primers and the MX4000 or MX3005p real-time thermocyclers (Agilent Technologies, Santa Clara, California, USA) were used for these assays. The qRT-PCR included a reverse transcription step at 50°C for 30 min and a PCR amplification step with enzyme activation at 95°C for 15 min. These were followed by 40 cycles of 95°C for 15 s, 57°C for 30 s, and 72°C for 30 s, and finally a dissociation step at 95°C for 30 s, and a dissociation step at 95°C for 30 s.

**Porcine circovirus (PCV) detection**

For the detection of PCV nucleic acid in meat extract, DNA was extracted using the DNeasy Tissue Kit (Qiagen) and recovered with 50 μL of elution buffer. A volume of 3 μL was tested using the Platinum Tiq DNA Polymerase Kit (Invitrogen), 1.5 mM MgCl2, and 0.2 μM of each VCP5F (AGTGACGCGGGAAAATGCA) and VCP6R (CACACAGTCTCAGTAGATCATCC) primers. This primer set amplified a region that corresponds to bp position 14969 to 15116 of NVSL (AY545985.1). To detect genotype 1, P4 and P2 primers were used (23). Each presumptive positive sample was retested with distinct ORF-7 RT-PCR using similar RT-PCR mix and amplification conditions, but different primer sets. These results were indicative of a simultaneous non-specific amplification and were detected only below 100 viral RNA copies/μL, which represents the limit of quantification (LOQ). No specific amplification was detected more than 95% of the time below the limit of detection (LOD) of 10 viral RNA copies/μL. The number of viral RNA copies per mg of each quantifiable subsample tested by qRT-PCR was calculated according to the standard curve multiplied by the RNA extract volume (40 μL), divided by the extraction efficiency factor (7%) and by the weight of the meat homogenate sample (20 mg per extract), and normalized using the total RNA extracted.

**Virus decay**

To estimate virus decay over time, a ratio of viral RNA copy (C_v/10^7) was calculated for each quantifiable subsample of a storage follow-up series, where C_v represents the copy numbers/μL for a specific subsample from week 2 to week 10 (T_j), and C_1 represents the value obtained at week one (T_1). The degradation rate “k” was calculated using the following equation:

\[ C_{t} = C_{0} \times e^{-kt} \]

where \( C_{t} \) is the copy number at time t, \( C_{0} \) is the copy number at time 0, and k is the degradation rate constant.
determined by plotting the natural logarithm transformation of the ratio of viral RNA copies (Ct/C0) versus time (weeks). Virus half-life was then calculated using the equation virus half-life = (ln2)/k. Statistical analyses were performed on the linear regression model by ANOVA using the F statistic (Minitab release 16 software). The T0 and T1 values of a same series were also compared using a paired t-test, with P < 0.05 being statistically significant.

**Meat viral load**

The meat viral load was estimated at Tt0 or Tmax by multiplying the normalized PRRSV RNA copies per mg by the total meat quantity given to 1 pig for the bioassay (around 500 g).

**Phylogeny analysis**

Phylogeny analysis was done using the nucleotide sequence of an amplified PRRSV ORF-5 region of NVSL. The RT-PCR was done using primer set P420 and P640 (24) and the One-Step RT-PCR Kit as described. The nested PCR was done using primer set 5FN and 5DN (25) and Platinum Taq DNA Polymerase Kit (Invitrogen, Carlsbad, California, USA). The ORF-5 cDNA sequences were aligned using the neighbor-joining method with Clone Manager (Scientific & Educational Software, Cary, North Carolina, USA). An ORF-5 sequence of 544 bp was used for the alignment, which corresponded to bp positions 13811 to 14354 of NVSL. Genbank reference strains used for the alignment were: 17198-6 (EF442276.1), 2000-54471A (EU556182.1), 34075-NE (U66380.1), 98-6470-1 (AF339493.1), CH-1a (AY032626.1), F1-1 (AY881994.1), HB-1 sh/2002 (DQ642048.1), IA-27 (EU758940.1), IAF-EXP1 (L40898.1), IAF-Klop (U64928.1), Ingelvac ATP MLV (DQ988080.1), Ingelvac RespPRRS MLV (AF066183.4), Lelystad (M96262.2), Lena (F802085.1), MD-001 (AF121131.1), MN184 (EF442777.1), NADC-8 (AF396835.1), PA8 (AF176348.2), PrimePAC (AF066384.1), PRRSV0003749 (DQ477778.1), SDSU73 (EF442775.1), and VR-2332 (AY150564.1).

**Results**

**PRRSV in pig meat**

Overall, 1500 fresh meat samples were collected randomly at the PHBP over a 2-year period. All meat samples were screened for the presence of PRRSV using the RT-PCR targeting both American and European strains. Among meat samples, 16 (1.1%) were found presumptive positive for PRRSV with a typical amplified fragment size of around 303 bp, a very faint band for some samples. Presumptive positives were tested with distinct ORF-7 RT-PCR to confirm the positive results and to define the PRRSV genotypes. Out of 16 presumptive PRRSV positive cases, 11 were confirmed positive with at least a second set of ORF-7 primers. Those confirmed positives represented 0.73% of the overall meat tested. All confirmed positives were typical of the North American strains. From these, 9 were selected to further estimate the viral decay and to determine the infectivity of the stored meat at Tmax by feeding piglets during bioassays. Two samples (ID 245 and 587) were not selected because they were positive for PCV type 2 in RT-PCR (data not shown). A non-confirmed and 2 RT-PCR negative meat samples were also kept for the viral decay study. During the study, 3 confirmed PRRSV positive meats were detected in April (3/98), 2 in September (2/120), 2 in October (2/225), 1 for each January (1/91), February (1/123), May (1/112), and November (1/97), and none during the months of March (0/86), June (0/120), July (0/194), August (0/184), and December (0/50).

**Estimation of PRRSV load and viral decay in pig meat**

To estimate viral decay, qRT-PCR was performed on meat subsamples maintained in the PHBP freezer and collected weekly for up to 15 wk (Table I). During this follow-up series, only 4 meat samples had an average PRRSV RNA concentration above the LOQ. For meats ID 715 and 1311, the number of viral RNA copies in almost all the subsamples could not be estimated (< LOQ) although specific RT-PCR products were detected for PRRSV (10/10 and 9/11, respectively) confirming their positive status. For sample 1424, only 3 out of 10 subsamples were positive and values were below the LOQ. No follow-up subsamples were positive for ID 574 and ID 597 from Tt to Tmax. Sample ID 340 and both negative controls remained negative throughout the storage period.

To estimate the variation of virus decay over time, a ratio of normalized viral RNA copies of each quantifiable subsample of a follow-up series was calculated for samples ID 84, 115, 319, and 1332 (Table I). No significant decay in viral RNA concentrations of PRRSV was observed during the storage period. Subsamples from ID 84 were tested in 3 separate experiments to estimate the inter-assay coefficient of variation (CV%) of the meat extract viral RNA copies/μL. It was estimated at 42%, showing significant variation from one qRT-PCR test to another for the same sample.

The meat viral load was estimated at Tt0 or Tmax based on the viral RNA copies detected per mg of meat (Table I). The extraction efficacy factor was found to be extremely low for the internal extraction control that was used. Only 7% of the input virus in the homogenized meat subsamples was recovered after RNA extraction of the muscle tissues. Recovery rates between 1% and 12% were also reported in previous studies using process control viruses with mouse norovirus, mengo, and hepatitis E virus (26). Numbers vary according to the method, the matrix, and the control virus. Pork meat usually has a high fat content that can interfere with nucleic acid extraction and inhibit the subsequent detection step. Nevertheless, low recovery yields are associated with high inter-assay variations. Based on these results, each piglet consumed approximately 109.2 to 109.8 viral RNA copies/mL. It was estimated to be 42%, showing significant variation from one qRT-PCR test to another for the same sample.

The meat viral load was estimated at Tt0 or Tmax based on the viral RNA copies detected per mg of meat (Table I). The extraction efficacy factor was found to be extremely low for the internal extraction control that was used. Only 7% of the input virus in the homogenized meat subsamples was recovered after RNA extraction of the muscle tissues. Recovery rates between 1% and 12% were also reported in previous studies using process control viruses with mouse norovirus, mengo, and hepatitis E virus (26). Numbers vary according to the method, the matrix, and the control virus. Pork meat usually has a high fat content that can interfere with nucleic acid extraction and inhibit the subsequent detection step. Nevertheless, low recovery yields are associated with high inter-assay variations. Based on these results, each piglet consumed approximately 109.2 to 109.8 viral RNA copies/mL after extraction.

**Experimental transmission**

Results of PRRSV and antibody detection from pigs fed with PRRSV positive meat samples are summarized in Table II. Overall, a total of 18 piglets were fed in 5 different bioassays using 2, 3, 1, 2, and 1 positive PRRSV meat samples, respectively. Two piglets were fed with a non-confirmed PRRSV meat sample. A total of 9 control piglets were included in the bioassays. Meat samples were generally readily consumed after much chewing. Usually, all meat pieces were eaten by the pigs within 30 min to 2 h. At one occasion, the sample was smaller and the pigs receiving the sample were fed...
with a total of 386 g instead of ~500 g (ID 715, Table II). During the 28-day observation period, all pigs appeared healthy. Positive transmission was observed with 5 confirmed PRRSV positive meat samples (5/9, 55.6%). Transmission in PRRSV confirmed positive cases was detected at 7 d post-exposure (DPE) by PCR analysis of the serum and throat samples and at 14 DPE by serology. None of the control piglets were shown to be infected. The meat viral load was an important factor for transmission. Oral transmission was detected with all meat samples (4/4) with quantifiable viral load. The pair of piglets fed with meat sample ID 1332 was found positive with both meat pieces stored at RT and at 4°C. Positive transmission was also detected with only 1 of the 2 piglets exposed to sample ID 1424. This sample remained non-quantifiable throughout the follow-up in the freezer and only a limited set of subsamples at T_y. T_y and T_g were found to be qRT-PCR positive (< LOQ). For this animal, viral transmission occurred only with the sample that was thawed at 4°C. The pair of piglets that was fed with meat sample ID 1311 was also apparently exposed to a lower virus load and was not infected. Average PRRSV RNA copies in meat sample ID 1311 was below the limit of quantification except at T_g and T_y. The pair of piglets that consumed the non-confirmed positive meat sample ID 340 was not infected either.

**Homology of strains**

Strains from positive transmission bioassays were successfully sequenced and matched both before and after transmission (5/5). However, not all 11 PRRSV cases confirmed to be positive were successfully sequenced. The ORF-5 targeted sequence of 544 bp was obtained for 6 isolates only. Two isolates were only partially sequenced (ID 245 and 715). The quality of ID 587 ORF-5 virus sequence remains low. Two viruses could not be amplified by nested RT-PCR despite several attempts (ID 597 and 574). These difficulties could have been attributed to the low concentration of PRRSV RNA in meat samples (i.e., < LOQ) or strain variability. Based on the PRRSV ORF-5 sequence homology, 2 of the positive PRRSV transmissible meats (ID 319 and 1332) showed a close relationship (> 97%) with modified live virus Ingelvac RespPRRS MVL vaccine and the strains of lineage 5 over 544 bp (Figure 1) (27). The meat sample ID 715, confirmed positive but negative in the transmission bioassay, was also closely related to RespPRRS MVL vaccine type (98%) and was 100% homologous to meat sample ID 1332 over a smaller sequence stretch of 457 bp. Four confirmed positive PRRSV meats (ID 84, 115, 1311, and 1424) were related (~ 90%) to wild-type strains of lineage 1 such as MN184 and IAF-Klop (Figure 1). The meat ID 245 was also closely related to the lineage 1 genotype. The meat ID 245 ORF-5 sequence was closely related to meat 84 (100% over 1424 bp). The strain control used during the RT-PCR (NVSL) did not show a high homology with the detected sequences (< 90%).

**Discussion**

The percentage of presumptive RT-PCR positive PRRSV meat samples was very similar to the ones reported previously in meat samples from Quebec (1.07% versus 1.2%) (6). However, only 11/16 were confirmed. In contrast, others have not found PRRSV by

---

Table I. Variations of porcine reproductive and respiratory syndrome virus (PRRSV) concentrations in selected meat samples stored at −20°C over time

<table>
<thead>
<tr>
<th>Meat ID</th>
<th>Weeks at −20°C</th>
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<th>T_3</th>
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<td>+</td>
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<td>0</td>
<td>0</td>
<td>+</td>
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*a* PRRSV negative control (*).  
*b* The total period of time the meat was stored at −20°C (T_max).  
*c* The average PRRS viral RNA copy per mg calculated from meat subsample collected from week 1 (T_1) to the end of the storage period (T_max) and normalized based on the RNA extract concentration.  
+ — Positive samples that are detected but are below the limit of quantification in quantitative RT-PCR; < LOQ — Average value below the limit of quantification of 10^3.5 RNA copies per mg; ID — meat identification number; NT — samples not tested.
Table II. Porcine reproductive and respiratory syndrome virus (PRRSV) detection in pigs experimentally fed with PRRSV positive meat samples

<table>
<thead>
<tr>
<th>Pig</th>
<th>Meat ID</th>
<th>Meat weight (g)</th>
<th>Estimated meat viral load (PRRSV copy)</th>
<th>Bioassay (trial #)</th>
<th>°C</th>
<th>RT-PCR/ELISAa (Days post exposure)</th>
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<td>--/-- +/+- +/+- +/+- +/+- +/+- +/+-</td>
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</table>

a The meat was stored at 4°C or room temperature (RT) for 24 and 48 h after storage at the PHBP.

b Each meat sample was divided in 2 equal parts The quantity of meat sample given on both days to each piglet.

c The total PRRS viral load in equivalent viral copies calculated using the ORF7 qRT-PCR results from aliquots of the meat homogenate stored at −20°C at Tmax. Value calculated using concentration at T10 (*). Confirmed positive samples that are below the limit of quantification in quantitative RT-PCR(+).

d Serum test results from ORF7-RT-PCR genotype 2 assays and HerdCheck ELISA results either positive (+) or negative (−).

ID — meat identification number; NT — not tested.

RT-PCR in carcasses at slaughter (14). The prevalence, the meat sampling changes and the sensitivity of the RT-PCR assays could explain the PRRSV detection variation observed between studies. In addition, meat is a heterogeneous matrix composed of various elements such as fat, muscles, nerves, and vessels. In actively infected animals, PRRSV is not expected to be evenly distributed in meat but rather, located in residual blood (12,28). It is thus expected to be unequally distributed among subsamples collected to conduct the studies, leading to significant variations. In the current study, the levels of viral RNA were relatively low in general and close to the limit of detection of the molecular assays. The impact of assay sensitivity and low recovery yields on prevalence estimates is not negligible in such cases (19). The aging of the screening ORF7 primers is probably not a major issue based on their homologies with the reference PRRSV strains of the 2008 circulating lineages (27). Other factors associated with the sampling scheme could have influenced prevalence estimates, such as the type of meats, the handling of the carcasses before packaging, and the seasons during which the samplings were performed. Despite having taken the necessary precautions, samples collected at the PHBP could have been cross-contaminated in the processing line or during sample analysis. The confirmation steps used herein reduce the probability of overestimating the presence of the virus in pig meat and underestimating their transmission in the bioassays.
No PRRSV positive meat was detected for 3 consecutive months during summer, although 33% of the meat samples were collected during those months. Although more sampling would be needed to confirm this trend, those results suggest that fewer pigs at the age of slaughter are infected with PRRSV or the level of infection is lower during summer. A study conducted by Larochelle et al. (29) in Quebec reported that about 75% of field cases of PRRSV between 1998 and 2002 were submitted from November to April, suggesting a higher prevalence of disease during colder months. Other studies have also shown more frequent mechanical transmissions of PRRSV in cold weather than in warm weather (30,31). Considering that the stability of PRRS virions decreases when the temperature increases (12,32–34), these trends seem to confirm the lower prevalence of PRRSV during the summer months, suggesting a lower risk in oral transmission.

Similarly, the standard meat storage temperature at the packaging center might be too low to affect PRRS viral decay in frozen meat within the study time frame. The 15-week follow-up study was designed to provide insight on the virus integrity in meat throughout the period of time needed for transportation and storage during exportation overseas. No significant decay of viral RNA was detected in our study, but that could be the result of the subsamples...
heterogeneity, the variations in inter-assays, and the poor extraction efficiency. It could also be the result of the study time frame and the RNA stability. Indeed, the data are compatible with the reported absence of PRRSV titer variation after 10 wk at −20°C in cell culture media (12), while others have reported a decrease in viral infectivity over time with storage temperature increase (−20°C to 30°C) in absence of viral RNA concentration variation (34,35).

Several studies have analyzed the oral transmission of PRRSV to pigs in order to assess the risk associated with swill feeding (36). This study is the first to report on the PRRSV RNA concentrations in pig meat collected at the PHBP, that are associated with positive transmission after storage in commercial conditions and thawing. For meat viral loads above 10^{9} total PRRSV RNA copies/500 g of meat, the transmission rate was high (100%) but these samples were still relatively rare in the survey. The transmission rate of positive PRRSV meat samples was slightly lower than the one reported by Magar and Larochelle (6) (5/9 versus 7/11) in the same region. These results are surprisingly similar considering the differences between both studies, including the meat cuts, the storage temperature, and the variation in viral load.

The transmission rates observed with frozen packaged meat from the PHBP were relatively low when taking into account the prevalence of confirmed positive PRRSV meat products. The product of the prevalence and the transmission rate in this study (0.4% for 500 g) was in the same range as a model developed to describe the probability that meat imported from a country where PRRSV is present will contain an infectious dose of PRRSV after shipping (0.18% for 1 kg) (21). Several parameters, which could have influenced the transmission of PRRSV, were not considered in our study and require further investigation. For instance, scrap sizes (500 g versus 10 g) or disposal conditions (prolonged RT), which were not estimated or barely explored, should have an impact on the meat viral load and the transmission rates (37). Legislations, regulations, and training on swill feeding, must also be considered but were outside of the scope of this study. For example, swill feeding is not allowed in Canada. In addition, although the reversion of live attenuated vaccine strains to virulence under field conditions occurs (38), the inclusion of vaccine strains or vaccine strain derivatives in risk assessments requires balancing their benefits. In the current study, close to a third of the sequenced strains were related to vaccine types. These numbers are similar to the ones reported previously in Canada (6,7). The results indicate that the likelihood of meat from the PHBP contains wild strains of PRRSV and is infectious, is low. On the other hand, the elimination of vaccine strains from herds could require an equal amount of effort as the elimination of the wild type strains. The viral and clinical outcomes in piglets orally infected with pig meat and maintained in natural conditions, are still unknown.

In summary, low residual quantities of PRRSV are found in a small percentage of pig meat collected at the pork ham boning plant. The values observed were very similar to and/or slightly lower than the ones reported previously for the province of Quebec. No statistically significant degradation of the PRRSV RNA was observed at −20°C during 15 wk. Previously frozen PRRSV-positive meat was able to infect naïve pigs by oral exposure. Transmission was more efficient for the meat samples containing a quantifiable viral load, in the range of 10^{9} genomic equivalents. However, the prevalence of these meat samples with a high PRRSV viral load remained low, especially when considering field strains only.

Acknowledgments
This research was made possible through the financial support of the Canadian Food Inspection Agency, the Canadian Pork Council, and Olymel. We would like to acknowledge Dr. Bianca Morel and her team for their assistance in the serological assays and sampling, Drs. Fatima Belayat and Yves Robinson for their assistance with autopsies, M. René Mineau for his support in the animal facilities, Ms Sylvianne Paul for her technical assistance, the CFIA Ottawa Fallowfield Laboratory and M. Steve Smith for providing SPF piglets, Dr. Carl Gagnon for consultation, and M. André Perron for revision of the manuscript.

References
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Development of porcine circovirus 2 (PCV2) open reading frame 2 DNA vaccine with different adjuvants and comparison with commercial PCV2 subunit vaccine in an experimental challenge

Changhoon Park, Jiwoon Jeong, Kyuhyung Choi, Su-Jin Park, Ikjae Kang, Chanhee Chae

Abstract

The objective of this study was to compare the protection against challenge with porcine circovirus 2 (PCV2) induced by an experimental vaccine based on open reading frame (ORF) 2 of PCV2 DNA plus an adjuvant (aluminum hydroxide, cobalt oxide, or liposome) and a commercial PCV2 subunit vaccine. A total of 35 colostrum-fed, cross-bred, conventional piglets were randomly divided into 7 groups. The commercial vaccine was more efficacious against PCV2 challenge than the 4 experimental vaccines according to immunologic, virologic, and pathological outcomes. The pigs inoculated with the experimental vaccine containing the liposome adjuvant had significantly higher levels ($P < 0.05$) of neutralizing antibodies and interferon-$\gamma$-secreting cells, and significantly lower levels ($P < 0.05$) of PCV2 viremia than the pigs inoculated with the other experimental vaccines. The pigs inoculated with the experimental vaccines containing either the liposome adjuvant or the cobalt oxide adjuvant had significantly lower lymphoid lesion scores ($P < 0.05$) than the pigs in the group inoculated with the PCV2 DNA vaccine dissolved in phosphate-buffered saline. Liposome proved to be a potent adjuvant that efficiently enhanced both humoral and cellular immune responses induced by the PCV2 DNA vaccine.

Résumé

L’objectif de la présente étude était de comparer la protection contre une infection défi avec le circovirus porcin de type (CVP2) induite par un vaccin expérimental à base du cadre de lecture ouvert (ORF) 2 de l’ADN de CVP2 plus un adjuvant (hydroxyde d’aluminium, oxyde de cobalt, ou liposome) et un vaccin CVP2 sous-unitaire commercial. Un total de 35 porcelets croisés, conventionnels et nourris au colostrum ont été séparés de manière aléatoire en sept groupes. Le vaccin commercial était plus efficace contre l’infection par CVP2 que les quatre vaccins expérimentaux en fonction des résultats immunologiques, virologiques, et pathologiques. Les porcs inoculés avec le vaccin expérimental contenant l’adjuvant liposome avaient des titres significativement ($P < 0.05$) plus élevés d’anticorps neutralisants et un nombre significativement ($P < 0.05$) moindre de cellules secrétant de l’interféron-$\gamma$ et une virémie à CVP2 que les porcs inoculés avec les autres vaccins expérimentaux. Les porcs inoculés avec les vaccins expérimentaux contenant l’adjuvant liposome ou oxyde de cobalt avaient des scores de lésions lymphoïdes significativement ($P < 0.05$) plus faibles que les porcs dans le groupe inoculé avec le vaccin ADN CVP2 dissout dans de la saline tamponnée. Les liposomes se sont avérés être un adjuvant puissant qui a augmenté de manière efficace autant la réponse immunitaire humorale que cellulaire induite par le vaccin à ADN CVP2.

(Traduit par Docteur Serge Messier)
reducing PCV2 viremia in mouse models of PCV2 infection (11–15). In contrast, a PCV2 DNA vaccine was not able to reduce PCV2 viremia efficiently in pigs (16), and protective immunity has not been investigated.

Commercially available PCV2 subunit vaccines are still effective in protecting against concurrent PCV2b and the emerging PCV2b mutant strain (9,17). However, it is necessary to develop a vaccine that is more cost-effective than the current PCV2 vaccines. Perhaps a DNA vaccine is the best candidate. Such vaccines have often been disappointing when tested in pigs because of the relatively poor induction of protective immunity (18). One possible approach to improve the immune responses is the use of different adjuvants. Therefore, the objective of this study was to compare the protection against PCV2 challenge induced by a commercial PCV2 vaccine and an experimental PCV2 ORF2 DNA vaccine containing an adjuvant: aluminum hydroxide, cobalt oxide, or liposome.

**Materials and methods**

**Preparation of an ORF-2-based plasmid and DNA vaccine**

The entire ORF2 sequence was amplified from the genomic DNA of PCV2b SNUVR000463 (GenBank) Bethesda, Maryland, USA accession no. KF871068) with the use of specific primers as previously described (14) and subcloned into the mammalian expression vector pCI-neo (Promega, Madison, Wisconsin, USA) to construct the recombinant expression plasmid for ORF2 (pORF2). To ascertain Cap protein expression in porcine cells, PCV-free porcine kidney (PK)-15 cells were transfected with pORF2 with the use of the reagent Lipofectamine 2000 (Invitrogen, Carlsbad, California, USA) according to the manufacturer’s instructions. After a 48-hour incubation the cells were fixed and examined by the indirect immunofluorescent antibody (IFA) technique with the use of a mouse monoclonal antibody against porcine PCV2 ORF2 (Rural Technologies, Brookings, South Dakota, USA) and goat IgG against mouse IgG conjugated with fluorescein isothiocyanate (Kirkegraa and Perry Laboratories, Gaithersburg, Maryland, USA) as the primary and secondary antibodies, respectively. Finally, pORF2 was purified with an EndoFree Plasmid Giga Kit (Qiagen, Valencia, California, USA) for use as a DNA vaccine.

**Preparation of the various forms of experimental vaccine**

Equal volumes of phosphate-buffered saline (PBS; 10 mM, pH 7.4) and pORF2 DNA (2 mg/mL in 2 × PBS) were mixed on a magnetic plate stirred at 45°C for 24 h, and the resultant vaccine was administered to pigs within 4 h of formulation.

Equal volumes of aluminum hydroxide gel (Rehydragel LV; General Chemical, Berkeley Heights, New Jersey, USA) and pORF2 DNA (2 mg/mL in 2 × PBS) were mixed on a magnetic plate stirred at 45°C for 24 h, and the resultant vaccine was administered to pigs within 4 h of formulation.

To add cobalt oxide as an adjuvant, we used a slight modification of previously described methods (19,20). Briefly, cobalt oxide (Co₃O₄) nanoparticles in double-ionized water (500 μg/mL) were disrupted with a probe sonicator (220 to 240 V, 10.3A; Philip Harris Scientific, Hyde, England) at 40°C power for 1 min to break up aggregation. Then equal volumes of pORF2 DNA (2 mg/mL in 2 × PBS) were added and mixed by vortexing for 5 min. The hydrodynamic size and zeta potential were measured with a Zetasizer. For the preparation of liposome nanoparticles to be used as an adjuvant, 1,2-dimyristoyl-sn-glycero-3-phosphoethanolamine (Avanti Polar Lipids, Alabaster, Alabama, USA), dioleoyl phosphatidylcholine (Sigma-Aldrich, St. Louis, Missouri, USA), and cholesterol (Avanti Polar Lipids) were mixed at a ratio of 3:1.5:0.5:1.5:0.5 (w/w/w) according to techniques previously described (21). Briefly, lipid films were created in a glass vial by evaporating the organic solvent [a mixture of chloroform and methanol at a ratio of 6:5:3 (v/v)] under a steady stream of nitrogen gas. Traces of organic solvent were removed by keeping the films in a vacuum desiccator overnight. The lipid films were then hydrated for 12 h by adding pORF2 DNA (1 mg/mL in PBS). Next, the suspensions were sonicated in a bath-type sonicator (Branson Ultrasonics Corporation, Danbury, Connecticut, USA) for 10 min and then extruded through 400-, 200-, and 100-nm membrane filters (Hamilton Company, Reno, Nevada, USA) and stored at 4°C before use. The hydrodynamic size and zeta potential were measured with a Zetasizer.

**In-vitro transfection with the PCV2 molecular DNA clone**

To test the infectivity of the clone in vitro, PCV-free PK-15 cells were grown in 8-well chamber slides as previously described (22). Briefly, when the PK-15 cells were about 85% confluent they were transfected with the clone alone or with different adjuvants (1:1 mixture) as described. Mock-transfected cells with empty pCI-neo vector were included as controls. Three days after transfection the cells were fixed with a solution containing 80% acetone and 20% methanol at 4°C for 20 min, and an IFA assay using a PCV2-specific rabbit polyclonal antiserum (Veterinary Diagnostic Laboratory, Iowa State University, Ames, Iowa, USA) was done to determine the in-vitro infectivity of the molecular DNA clone.

**Animals**

A total of 56 colostrum-fed, cross-bred, conventional piglets born to PCV2-unvaccinated sows were purchased at 5 d of age from a commercial farm. All the piglets were seronegative for PCV2, porcine reproductive and respiratory syndrome virus (PRRSV), and *Mycoplasma hyopneumoniae* and did not have PCV2 or PRRSV viremia according to real-time polymerase chain reaction (PCR). Twenty-one pigs were used for a negative-control study, and the remaining 35 were used as experimental subjects. All of the methods were approved by the Seoul National University Institutional Animal Care and Use Committee.

**Negative-control study**

The 21 pigs were randomly divided into 7 groups (3 pigs/group) by means of the random-number generation function of Excel.
Table I. Experimental design and scores\(^a\) for lymphoid lesions and porcine circovirus 2 (PCV2) antigen in the different groups of piglets

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<th>PCV2 antigen score</th>
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<td>DNA vaccine–liposome</td>
<td>Yes</td>
<td>0.43 ± 0.49(^b,e)</td>
</tr>
<tr>
<td>5</td>
<td>Commercial vaccine</td>
<td>Yes</td>
<td>0.29 ± 0.49(^e)</td>
</tr>
<tr>
<td>6</td>
<td>No</td>
<td>Yes</td>
<td>2.00 ± 0.53(^e)</td>
</tr>
<tr>
<td>7</td>
<td>No</td>
<td>No</td>
<td>0.11 ± 0.26(^e)</td>
</tr>
</tbody>
</table>

\(^a\) Lymphoid lesions were scored as follows: 0 — no lymphoid depletion or granulomatous replacement; 1 — mild lymphoid depletion; 2 — moderate lymphoid depletion; and 3 — severe lymphoid depletion and granulomatous replacement. The PCV2 antigen score was determined by means of the Image J 1.45s Program of the US National Institutes of Health, Bethesda, Maryland, USA (http://imagej.nih.gov/ij/); the number of PCV2-positive cells per unit area (0.25 mm\(^2\)) was counted. Different letters in superscript indicate statistically significant differences (\(P < 0.05\)) among groups.

\(^b\) DNA vaccine dissolved in phosphate-buffered saline (PBS) or prepared with an adjuvant; the commercial vaccine was CircoFLEX (Boehringer Ingelheim Vetmedica, St. Joseph, Missouri, USA).

Experimental design

This study used a randomized, blinded, controlled design. The 35 experimental pigs were randomly divided into 7 groups (5 pigs/group) by means of the random-number generation function of Excel (Table I). Groups 1 to 4 received the PCV2 ORF2 DNA vaccine dissolved in PBS or the PCV2 ORF2 DNA vaccine with aluminum hydroxide, cobalt oxide, or liposome, or null plasmid plus an adjuvant. The negative-control vaccine was prepared the same as the PCV2 DNA vaccine but with a null plasmid instead of the ORF2-based plasmid DNA. The pigs in each group were housed in the same pen within the same room. Blood samples were collected at −42 (7 d of age), −28 (21 d of age), 0 (49 d of age), 7, 14, 21, 42, and 70 (119 d of age) d after challenge. All the pigs were euthanized for necropsy at 70 d after challenge, and superficial inguinal lymph nodes were collected for histopathological and immunohistochemical study.

Quantification of PCV2 DNA in blood

DNA was extracted from serum by means of the QIAamp DNA mini kit (Qiagen, Crawley, England) and used to determine the PCV2 genomic DNA copy numbers by real-time PCR with PCV2b-specific primers as previously described (23).

Serum antibody assay

The serum samples were assayed by a serum virus neutralization test (24). Neutralizing antibody titers were expressed as the reciprocal of the highest serum dilution that completely blocked infection of the PCV-free PK-15 cells with the challenge PCV2b strain.

Enzyme-linked immunospot (EliSpot) assay

The numbers of PCV2-specific IFN-\(\gamma\)-SCs stimulated by the challenge PCV2b strain were determined in peripheral blood mononuclear cells (PBMCs) as previously described (25) with slight modifications. Briefly, 100 \(\mu\)L containing \(5 \times 10^6\) PBMCs in RPMI 1640 medium supplemented with 10% fetal bovine serum (HyClone Laboratories, SelectScience, Bath, England) was seeded onto plates that had been coated with IFN-\(\gamma\) monoclonal antibody against porcine antigen (5 \(\mu\)g/mL; MABTECH, Mariemont, Ohio, USA) and incubated with the PCV2b challenge isolate at a multiplicity of infection (MOI) of 100. The plates were then incubated for 24 h, and the plates were washed to remove the non-adherent cells. The plates were then fixed and permeabilized with 4% paraformaldehyde and 0.5% Triton X-100 for 15 min. The plates were then incubated with a polyclonal antibody against PCV2b (1:1000) and then with a monoclonal antibody against IFN-\(\gamma\) (1:1000) for 1 h. The plates were then washed again and incubated with a chemiluminescent substrate (ECL; GE Healthcare, Buckinghamshire, England) for 5 min. The plates were then washed again and exposed to X-ray film. The numbers of IFN-\(\gamma\)-SCs were determined by counting the number of spots in each well.
of infection (ratio of the number of virions added per cell to the number of cells) of 0.05, phytohemagglutinin (10 μg/mL; Roche Diagnostics GmbH, Mannheim, Germany) as a positive control, or PBS as a negative control for 20 h at 37°C in a 5% humidified CO2 atmosphere. The wells were then washed 5 times with PBS (200 μL per well). Thereafter the procedure was conducted with the commercial EliSpot Assay Kit (MABTECH) according to the manufacturer’s instructions. Spots on the membranes were read by the automated AID EliSpot Reader (AID GmbH, Strassberg, Germany). The results were expressed as the numbers of IFN-γ-SCs per million PBMCs.

**Histopathological and immunohistochemical study**

For morphometric analysis of histopathological lesions and determination of the PCV2 antigen score in lymph nodes, sections of 3 superficial inguinal lymph nodes were examined blindly (22,26). The lymphoid lesion scores ranged from 0 to 3: 0 — no lymphoid depletion or granulomatous replacement; 1 — mild lymphoid depletion; 2 — moderate lymphoid depletion; and 3 — severe lymphoid depletion and granulomatous replacement. The PCV2 antigen scores were determined by means of the Image J 1.45s Program of the US National Institutes of Health, Bethesda, Maryland, USA (http://imagej.nih.gov/ij/); the number of PCV2-positive cells per unit area (0.25 mm2) was counted.

**Statistical analysis**

Before statistical analysis the real-time PCR and neutralizing antibody values were transformed to log10 and log2 values, respectively. The normality of the distribution of the data for the examined variables was evaluated by the Shapiro–Wilk test. Continuous data (for PCV2 DNA, PCV2 serologic findings, and PCV2-specific IFN-γ-SCs) were analyzed with 1-way analysis of variance (ANOVA) followed by Tukey’s multiple-comparison test at each time point. Repeated-measures ANOVA followed by a post-hoc test for least significant differences was used to assess the mean difference in continuous data over time within the same group. Discrete data (lymphoid lesion score and PCV2 antigen score) were analyzed by Mann–Whitney tests. A value of P < 0.05 was considered to be significant.

**Results**

The IFA assay with PCV2-specific antibody confirmed that the molecular DNA clone alone and with different adjuvants was infectious in vitro: about 10% to 15% of the PK-15 cells were transfected.

From ~42 to 0 d after challenge PCV2 DNA was not detected in any of the serum samples from the 7 groups. From 0 to 7 d after challenge the PCV2 genomic copy numbers in the serum increased significantly (P < 0.05) in the 5 vaccinated groups as well as the unvaccinated, challenged group. From 21 to 42 d after challenge the copy numbers decreased significantly (P < 0.05) in the pigs vaccinated with CircoFLEX. From 14 to 70 d after challenge the copy numbers were significantly lower (P < 0.05) in the vaccinated groups than in the unvaccinated, challenged group. At 7, 42, and 70 d after challenge the pigs vaccinated with CircoFLEX had significantly lower (P < 0.05) copy numbers than the pigs vaccinated with PCV2 DNA. At 7, 14, 21, and 42 d after challenge the pigs receiving the vaccine with liposome adjuvant had significantly lower (P < 0.05) copy numbers than the pigs receiving the other variations of the PCV2 DNA vaccine. At 21 d after challenge the pigs receiving the vaccine with aluminum hydroxide adjuvant had significantly lower (P < 0.05) copy numbers than the pigs receiving the vaccine with PBS (Figure 1). No genomic copies of PCV2 were detected in the serum from the unvaccinated, unchallenged pigs throughout the experiment or in the pigs receiving only the null plasmid, only an adjuvant, or the null plasmid plus an adjuvant in the negative-control study.

There was a significant difference (P < 0.05) in the log2-transformed neutralizing antibody titers (Figure 2A) among the groups: the titers increased significantly in the pigs receiving the PCV2 DNA vaccine with liposome or CircoFLEX from ~28 to 0 d after challenge, as well as in all the vaccinated pigs and the unvaccinated, challenged pigs from 0 to 7 d after challenge. Significantly higher titers were induced in the pigs receiving the PCV2 DNA vaccine with liposome or CircoFLEX than in all the other challenged groups at 0, 7, and 14 d after challenge. At 21, 42, and 70 d after challenge the titers were significantly higher in pigs vaccinated with CircoFLEX than in pigs receiving the PCV2 DNA vaccine and the unvaccinated, challenged pigs. At 21 d after challenge the titers were significantly higher in the pigs receiving the PCV2 DNA vaccine with liposome than in the pigs receiving the other PCV2 DNA vaccines and the unvaccinated, challenged pigs. No neutralizing antibodies were detected in the unvaccinated, unchallenged pigs throughout the experiment or in any of the pigs in the negative-control study.

The numbers of PCV2-specific IFN-γ-SCs (Figure 2B) increased significantly (P < 0.05) in the pigs receiving the PCV2 DNA vaccine with adjuvant or CircoFLEX from ~28 to 0 d after challenge, as well as in the pigs receiving the PCV2 DNA vaccine with PBS and the unvaccinated, challenged pigs from 0 to 7 d after challenge. The numbers decreased significantly (P < 0.05) in all the challenged groups from 42 to 70 d after challenge. The numbers were significantly higher (P < 0.05) in the pigs receiving the PCV2 DNA–liposome and CircoFLEX vaccines than in the pigs in the other vaccine groups as well as the unvaccinated, challenged group at 0, 7, and 14 d after challenge. At 0 and 14 d after challenge the numbers were significantly higher (P < 0.05) in the pigs receiving the PCV2 DNA–aluminum hydroxide and PCV2 DNA–cobalt oxide vaccines than in the pigs receiving the PCV2 DNA–PBS vaccine and the unvaccinated, challenged pigs. At 0 d after challenge the numbers were significantly higher (P < 0.05) in the pigs receiving the PCV2 DNA–PBS vaccine than in the pigs in the unvaccinated, challenged group. At 7 d after challenge the numbers were significantly higher (P < 0.05) in the pigs receiving the PCV2 DNA vaccine–cobalt oxide than in the pigs receiving the PCV2 DNA vaccine–PBS and the unvaccinated, challenged pigs. No PCV2-specific IFN-γ-SCs were detected in the unvaccinated, unchallenged pigs throughout the experiment. In addition, no PCV2-specific IFN-γ-SCs were detected in any of the pigs in the negative-control study.

The scores for lymphoid lesions and PCV2 antigen (Table I) were significantly lower (P < 0.05) in the vaccinated groups than in the unvaccinated, challenged group. The pigs in the CircoFLEX group had significantly lower (P < 0.05) lymphoid lesion scores than the pigs in the PCV2 DNA vaccine groups and the unvaccinated, challenged group. The pigs in the PCV2 DNA vaccine–cobalt oxide
and PCV2 DNA vaccine–liposome groups had significantly lower \((P < 0.05)\) lymphoid lesion scores than the pigs in the PCV2 DNA vaccine–PBS group. In addition, no lymphoid lesions or PCV2 antigens were detected in any lymph nodes from the pigs in the negative-control study.

**Discussion**

In this study PCV2-specific neutralizing antibodies and IFN-\(\gamma\)-SCs were induced only in pigs vaccinated with the PCV2 ORF2 DNA vaccines with adjuvant and not in the pigs vaccinated with only null plasmid, only an adjuvant, or null plasmid and an adjuvant. Transfection efficiency of the plasmid is critical for the immunogenicity of a DNA vaccine. In the present study the adjuvants (aluminum hydroxide gel, cobalt oxide nanoparticles, or liposome), receiving the commercial vaccine CircoFLEX (Boehringer Ingelheim Vetmedica, St. Joseph, Missouri, USA), as well as in the serum of unvaccinated, unchallenged pigs \((\bullet)\). Different superscript letters indicate statistically significant differences \((P < 0.05)\) among groups.

Liposome is a potent adjuvant that could efficiently enhance the neutralizing-antibody immune responses induced by a PCV2 DNA vaccine. Aluminum hydroxide remains the primary adjuvant used in veterinary vaccines. As expected, in the present study the vaccine with aluminum hydroxide gel induced the lowest levels of PCV2-specific IFN-\(\gamma\)-SCs compared with the other vaccines with adjuvant. In addition, the vaccine with cobalt oxide nanoparticles was not able to induce higher levels of humoral and cell-mediated immune responses than the vaccine with the liposome adjuvant. These results indicate that liposome may be a good candidate for use as an adjuvant for a PCV2 DNA vaccine.

Induction of humoral and cell-mediated immunity is likely to contribute to a reduction of PCV2 viremia \((5,6,8,27)\), which plays a key role in the development of PCVAD \((8,27)\). In this study the humoral immunity induced by the PCV2 DNA vaccine with liposome contributed to a reduction in PCV2 viremia, as has been reported for commercial vaccines in pigs challenged with PCV2 \((25,28,29)\). The PCV2 DNA vaccine with liposome induced higher levels of neutralizing antibodies and PCV2-specific IFN-\(\gamma\)-SCs than the DNA vaccine alone or with other adjuvants and thus greater protective immunity and a greater reduction in PCV2 viremia. Therefore, the liposome adjuvant is particularly promising for PCV2 DNA vaccination.

As expected, induction of protective immunity and reduction of PCV2 viremia by the commercial PCV2 subunit vaccine based on ORF2 was even better than with the ORF2-based DNA vaccine. These results agree with previous findings in which a prototypic PCV2 subunit vaccine was more efficient in reducing PCV2 viremia in pigs than a PCV2 DNA vaccine \((16)\). However, these results are in contrast with a murine model in which protective humoral immunity induced by a PCV2 DNA vaccine was better than that induced by a PCV2 subunit vaccine \((14)\). We have no clear explanation for this discrepancy, but it may be due to a difference between mice and pigs in activation of dendritic cells by a PCV2 DNA vaccine, as has previously been reported \((18)\).

The PCV2 DNA vaccine used in this study was administered twice to the experimental subjects, at 7 and 21 d of age, since the first vaccination did not provoke sufficient immune response, but the commercial vaccine was administered only once, at 21 d of age, according to the manufacturer’s recommendation. It is not too early to vaccinate at 1 wk, because a previous study showed immune responses to PCV2 vaccine in 5-day-old pigs \((30)\).

Although DNA vaccines have generally been regarded as effective in smaller animals such as mice, but less efficient in larger species...
such as pigs and humans (18), the contrary has been shown as well (31,32). DNA vaccination can stimulate both humoral and cellular immune responses in pig models (14), and the potential utility of plasmid DNA as a component of a PCV2 vaccine is currently an area of active investigation. An additional possible approach to improve immune responses is by the use of different adjuvants. In the present study the pigs vaccinated with the 1-dose commercial PCV2 vaccine exhibited significantly lower levels of PCV2 viremia than the pigs vaccinated with the 2-dose experimental PCV2 DNA vaccine in this study. In the field, swine producers prefer to use a 1-dose PCV2 vaccine because it requires less labor and reduces stress in the animals. Further work is needed to develop a 1-dose experimental PCV2 DNA vaccine that is comparable in efficacy to the 1-dose commercial PCV2 vaccine.

Acknowledgments

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Retrospective study of the relationship of Torque teno sus virus 1a and Torque teno sus virus 1b with porcine circovirus associated disease

Alejandro Vargas-Ruiz, Hugo Ramírez-Álvarez, José I. Sánchez-Betancourt, Víctor Quintero-Ramírez, Ignacio C. Rangel-Rodríguez, Joel A. Vázquez-Perez, Lucia A. García-Camacho

Abstract

Genus Iotatorquevirus consists of 2 species, Torque teno sus virus 1a and Torque teno sus virus 1b, which are ubiquitous in swine populations, and are widely reported in association with porcine circovirus associated disease (PCVAD). To evaluate the relationship with PCVAD, 100 formalin-fixed paraffin-embedded tissue samples were used to detect both Iotatorquevirus species by nested PCR and sequencing. Sixty-eight PCVAD cases were selected as well as 32 porcine circovirus type 2 (PCV2) non-affected cases. Overall, 33 of the 100 cases were positive for Torque teno sus virus 1a and 8 of 100 were positive for Torque teno sus virus 1b. Only 24 of 68 (35%) PCVAD cases were positive for Torque teno sus virus 1a; 39% (9/23) of post-weaning multisystemic wasting syndrome, and 33% (15/45) of PCV2-associated reproductive failure cases. Among PCV2 non-affected cases, 28% were positive for Torque teno sus virus 1a and 6% were positive for Torque teno sus virus 1b. Torque teno sus virus 1b was not detected in PCV2-associated reproductive failure cases. Regardless of the PCV2-status, a lower frequency of both Iotatorquevirus species was found than depicted in other reports and there was no statistical relationship with PCVAD (χ² < 0.01). Given the worldwide genomic variability of Iotatorquevirus species, it is feasible that species prevalent in Mexico share a lower nucleotide sequence identity, leading to different pathogenic potential.

Résumé

Le genre Iotatorquevirus consiste en deux espèces, le virus Torque teno sus 1a et le virus Torque teno sus 1b, qui sont ubiquitaires dans la population porcine, et couramment rapportés en association avec la maladie associée au circovirus porcin (MACVP). Afin d’évaluer la relation avec MACVP, 100 échantillons de tissus fixés dans la formaline et enrobés de paraffine ont été utilisés pour détecter les deux espèces de Iotatorquevirus par réaction d’amplification en chaîne par la polymérase nichée et séquençage. Soixante-huit cas de MACVP ont été sélectionnés ainsi que 32 cas non-affecteds d’infection par le circovirus porcin de type (CVP2). Globalement, 33 des 100 cas étaient positifs pour le virus Torque teno sus 1a et 8 des 100 étaient positifs pour le virus Torque teno sus 1b. Seulement 24 des 68 (35 %) cas de MACVP étaient positifs pour le virus Torque teno sus 1a; 39% (9/23) du syndrome de dépérissement post-sevrage, et 33% (15/45) des cas de problèmes reproducteurs associés au CVP2. Parmi les cas non-affecteds de CVP2, 28 % étaient positifs pour le virus Torque teno sus 1a et 6 % étaient positifs pour le virus Torque teno sus 1b. Le virus Torque teno sus 1b n’a pas été détecté dans les cas de problèmes reproducteurs associés au CVP2. Indépendamment du statut vis-à-vis le CVP2, une fréquence plus basse des deux espèces d’Iotatorquevirus fut trouvée comparativement à ce qui est décrit dans d’autres études et il n’y avait pas de relation statistiquement significative avec MACVP (χ² < 0.01). Étant donné la variabilité génomique mondiale des espèces d’Iotatorquevirus il est possible que les espèces prévalentes au Mexique partagent une plus faible identité de séquences nucléotidiques, entrainant ainsi un potentiel pathogène différent.

(Traduit par Docteur Serge Messier)

Introduction

Torque teno virus (TTV) is a circular single-stranded negative sense DNA virus that enclosed 4 open reading frames (ORF); ORF1, ORF2, ORF 1/1, and ORF 2/2 (former ORF3), and a GC-rich region within an un-translated region (UTR), which was first discovered in human samples with post-transfusion hepatitis of unknown etiology in 1997 (1). Currently, based on the International Committee on Taxonomy of Viruses (2), all human and animal TTV belong to Anelloviridae family. Genus Iotatorquevirus comprises of 2 species, TTSuV1a (former TTSuV1) and TTSuV1b (former TTSuV2). The phylogenetic relationship of incomplete sequences has proposed 4 biotypes (a, b, c, and d) of TTSuV1a, and 2 biotypes (a and b) of TTSuV1b (3,4). Genus Kappatorquevirus, however, includes only one species: Torque teno sus virus k2 (2). High prevalence of co-infection between TTSuV1a and TTSuV1b has been documented worldwide.
Iotat torquevirus species are ubiquitous in domestic and wild pigs, and have been identified in Europe (Hungary, Italy, France, and Spain), Asia (China, Korea, Japan, and Thailand), and North America (Canada and USA).

Transmission among pigs is horizontal and mainly via fecal-oral, but transmission through other routes may be important (3). It is unknown whether TTSuV1a and TTSuV1b infection promotes a specific disease as a primary agent or in co-infection with other pathogens (5). However, it has been suggested that in co-infections with other viruses, TTSuV1a might promote increased disease severity or virulence and TTSuV1b might be associated with reproductive failure (1). In this scenario, several studies have suggested an involvement of both species with porcine circovirus associated disease (PCVAD) since cases of post-weaning multisystemic wasting syndrome (PMWS) have shown a high prevalence of Iotat torquevirus species (6,7). Moreover, clinical manifestations and characteristic lesions of porcine dermatitis and nephropathy syndrome (PDNS) have been reproduced by TTSuV1a inoculation in gnotobiotic pigs (8). In fact, TTSuV1a has been proposed as the additional factor (X-factor) for the development of PCVAD (9). Porcine circovirus associated disease is economically important to the swine industry since it has an impact on production and reproduction parameters. Although all clinical presentations of PCVAD (PMWS, PDNS, porcine circovirus 2-associated reproductive failure (PCV2-RF), and granulomatous enteritis) in Mexico have been confirmed by in situ hybridization (10), the prevalence of Iotat torquevirus species or their possible association with PCVAD has not been recorded. The aim of the present work was to identify TTSuV1a and/or TTSuV1b from well-documented cases of PCVAD in order to assess their potential relationship with the occurrence of PCVAD in an unvaccinated population.

**Materials and methods**

**Case selection**

Archived formalin-fixed paraffin-embedded porcine tissues (lymph node, spleen, tonsils, and fetal hearts) from 2001 to 2009 were selected from 100 swine cases. Sixty-eight cases were from non-vaccinated swine with confirmed PCVAD on the basis of clinical signs, characteristic microscopic lesions, and in situ hybridization (11). The cases were subdivided as follows: 23 PMWS-affected tissues (lymph nodes and spleen), depicting severe lymphoid depletion and granulomatous inflammation, and a diffuse pattern of PCV2-positive in situ hybridization (ISH) and 45 cases of PCV2-RF, consisting of fetal hearts with non-suppurative myocarditis as well as a random ISH pattern positive for PCV2. The PCVAD-positive cases were submitted from 13 states of the Mexican Republic with a high-density swine population (Figure 1). Additionally, 32 PCV2 non-affected cases were evaluated and consisted of 12 tissues (lymph node and tonsil) negative for PCV2 by ISH from age-matched, clinically normal, non-vaccinated pigs from a PCV2 non-affected farm according to clinical criteria (12) and 20 hearts from PCVAD non-infected aborted fetuses submitted for diagnosis. Each tissue was tested individually.

**Primer design**

Due to genomic variability among available sequences, degenerate primers that target ORF1 of TTSuV1a and TTSuV1b were designed using computer software (Primer3 input program (v.3.0.0; Institute for Biomedical Research, Boston, Massachusetts, USA) (13) and Bioedit software program (v7.2.5; Ibis Bioscience, Carlsbad, California, USA) (14)). Ten TTSuV1a sequences from different
countries available in the NCBI database were used to design the primers (GenBank accession numbers: HM633249, HM633253, HM633258, AY823990, HM633257, AB076001, GU188045, GU456383, GU456384, GQ120664). For TTSuV1b, 12 sequences available in the NCBI database from different countries were used to design the primers (GenBank accession numbers: HM633230, JX173484, HQ204188, GU376737, KM461227, JQ782385, HM633218, GU456386, GU188046, GU570207, AY823991, NC014092). First-round reverse primer contains only 1 degenerate base. Primers were synthesized commercially (IDT Integrated DNA Technologies, Coralville, Iowa, USA).

### Nested polymerase chain reaction (PCR)

DNA extraction from all tissues was done separately using commercial kits according to the manufacturer’s instructions (QIAamp DNAFFPE Tissue kit; Qiagen, Germany). Briefly, DNA was eluted in a volume of 200 μL molecular grade water, and stored at −20°C. The lowest limit of detection was determined by serial dilution (1:2) and was of 12.5 ng/μL. Nested 50-μL polymerase chain reaction (PCR) were done using the sets of degenerate primers (Table I) in a thermocycler (Eppendorf, Hamburg, Germany) containing 2.5 U Taq DNA polymerase (GoTaq Flexi DNA polymerase; Promega Corporation, Madison, Wisconsin, USA) PCR buffer 1×, magnesium chloride 2.25 mM (TTSuV1a)/1.5 mM (TTSuV1b), 0.2 mM of each deoxy-nucleotide (dNTP), 100 pmol of each primer, and 20 ng of template. The following thermal cycle was as follows: the initial activation step at 94°C for 3 min followed by 40 cycles of 1 min at 94°C, 1 min at 56°C (TTSuV1a) or 53°C (TTSuV1b) and 1 min at 72°C, finally last extension step of 10 min at 72°C. The PCR products were electrophoretically separated in a 1.5% agarose gel stained with ethidium bromide. The gel was visualized under ultraviolet light (Apollo Instrumentation, Claremont, California, USA) and photodocumented (Doc-It System; UVP BioImaging Systems, Cambridge, UK).

#### Sequencing

Two amplified products from each species were randomly selected and purified from agarose gel using a commercial kit (Min Elute Gel Extraction kit; Qiagen) following the manufacturer’s instructions. The sequencing of the purified PCR products was done using high fidelity, processing, and specificity enzyme kits (Taq Platinum Polymerase; High Fidelity, Carlsbad, California, USA).

### Table I. Sequences of the primers utilized for nested polymerase chain reaction (PCR)

<table>
<thead>
<tr>
<th>Species</th>
<th>Sequence Primer</th>
<th>Length</th>
</tr>
</thead>
<tbody>
<tr>
<td>TTSuV1a</td>
<td>5'-AACTGGCAGGACCACCTATG-3' Forward 481 bp</td>
<td></td>
</tr>
<tr>
<td>TTSuV1a</td>
<td>5'-AGGTGTAACCTCCACCTACGACCT-3' Reverse 255 bp</td>
<td></td>
</tr>
<tr>
<td>TTSuV1b</td>
<td>5'-ATGCGTTACAGACGGCTATC-3' Forward 605 bp</td>
<td></td>
</tr>
<tr>
<td>TTSuV1b</td>
<td>5'-GGGTTATTTTGGGCTATC-3' Reverse 211 bp</td>
<td></td>
</tr>
</tbody>
</table>

Sequences of the primers utilized the detection to the TTSuV1a and TTSuV1b for nested PCR. Bold letters indicate degenerate base. All primers were synthesized by another source (Integrated DNA Technologies, Coralville, Iowa, USA).

![Image](image_url)
USA). Internal primers were used to sequence partial sequences of ORF1, using a sequencer (Model 3100; Applied Biosystems, Foster, California, USA).

Data analysis

Nucleotide sequences were edited, aligned, and analyzed using computer software (Bioedit software v7.2.0) (13). Phylogenetic analysis were done using molecular evolutionary genetics analysis version 6 (MEGA6) (15). The maximum likelihood tree was computed using Tamura-Neg Gamma distance. The test of phylogeny was carried out through bootstrap method with 1000 number of replication and the gaps/missing data treated with pairwise deletion. Two by four contingency frames were made based on PCR results to evaluate the relationship of TTSuV1a and TTSuV1b with PCVAD using a Chi-squared test at a trust interval of 99% (P, 0.01).

Results

The PCR protocols using specific degenerate primers to amplify TTSuV1a and TTSuV1b were optimized to obtain of 255 bp (Figure 2A), and 211 bp (Figure 2B), respectively, from PCVAD cases. The sequences of 2 nested products of each virus species proved to be specific (GenBank accession numbers: KU891810 and KU891811 TTSuV1a, KU891808 TTSuV1b and KU891809 TTSuVx2). At alignment, the amplified regions of each species were highly conserved among available sequences. The topography of the phylogenetic tree of TTSuV1a (Figure 3) revealed that the amplified sequences belong to the species 1a, conforming a well-defined cluster (Mexican, German, and Chinese sequences) standing supported with bootstrap values of 89. Conversely, the phylogenetic tree of TTSuV1b (Figure 4) showed that one Mexican sequence clustered with American and Chinese sequences, but another sequence was located in a different clade, revealing a major phylogenetic relationship with TTSuVx2 Brazilian sequences (GenBank accession numbers: AY823991, NC014092).

In the current retrospective study, 100 cases were used (68 PCVAD-affected, and 32 PCVAD-non affected). Overall, 33% (33/100) were positive to TTSuV1a (TTSuV1a1) and 8% (8/100) were positive to TTSuV1b (TTSuV1b1). From all the PCVAD cases, 35% (24/68) were TTSuV1a1 and 9% (6/68) were TTSuV1b1, whereas 28% (9/32) and 6% (2/32) of the non-affected cases were positive to TTSuV1a and TTSuV1b, respectively (Table II). The frequency of PMWS-affected cases compared to age-matched clinically normal piglets is shown in Table III. Thirty-one percent (11/35) of total cases were TTSuV1a1 and 22.8% (8/35) were TTSuV1b1+. From all the PCVAD cases, 35% (24/68) were TTSuV1a+ and 9% (6/68) were TTSuV1b+, whereas 28% (9/32) and 6% (2/32) of the non-affected cases were positive to TTSuV1a and TTSuV1b, respectively. Co-infection was found only in PMWS cases, 3% of the total cases, and 9% (3/35) of PMWS cases. Concerning cases of reproductive failure in sows...
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Table II. Overall nested polymerase chain reaction (PCR) results for open reading frame (ORF1) region of TTSuV1a and TTSuV1b (n = 100)

<table>
<thead>
<tr>
<th>TTSuV1a+</th>
<th>TTSuV1a+</th>
<th>TTSuV1a−</th>
<th>TTSuV1b−</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCVAD affected</td>
<td>3</td>
<td>21</td>
<td>3</td>
<td>41</td>
</tr>
<tr>
<td>PCVAD non-affected</td>
<td>0</td>
<td>9</td>
<td>2</td>
<td>21</td>
</tr>
<tr>
<td>Totala</td>
<td>3</td>
<td>30</td>
<td>5</td>
<td>62</td>
</tr>
</tbody>
</table>

a Not statistically different between porcine circovirus-associated disease (PCVAD)-affected and PCVAD non-affected at P-value 0.01 ($X^2$ test).

Table III. Post-weaning multisystemic wasting syndrome (PMWS) nested polymerase chain reaction (PCR) results for open reading frame (ORF1) region of TTSuV1a and TTSuV1b

<table>
<thead>
<tr>
<th>TTSuV1a+</th>
<th>TTSuV1a+</th>
<th>TTSuV1a−</th>
<th>TTSuV1b−</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>PMWS-affected</td>
<td>3b</td>
<td>6</td>
<td>3</td>
<td>11</td>
</tr>
<tr>
<td>Age-matched clinically normal piglets</td>
<td>0</td>
<td>2</td>
<td>2</td>
<td>8</td>
</tr>
<tr>
<td>Totala</td>
<td>3</td>
<td>8</td>
<td>5</td>
<td>19</td>
</tr>
</tbody>
</table>

a Not statistically different between PMWS-affected and age matched healthy piglets at P-value 0.01 ($X^2$ test).
b Co-infection only in PMWS-affected.

Table IV. Reproductive failure nested polymerase chain reaction (PCR) results for open reading frame (ORF1) region of TTSuV1a and TTSuV1b

<table>
<thead>
<tr>
<th>TTSuV1a+</th>
<th>TTSuV1a+</th>
<th>TTSuV1a−</th>
<th>TTSuV1b−</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCV2-RF</td>
<td>0</td>
<td>15</td>
<td>0</td>
<td>30</td>
</tr>
<tr>
<td>non PCV2-RF</td>
<td>0</td>
<td>7</td>
<td>0</td>
<td>13</td>
</tr>
<tr>
<td>Totala</td>
<td>0</td>
<td>22</td>
<td>0b</td>
<td>43</td>
</tr>
</tbody>
</table>

a Not statistically different between porcine circovirus type 2-associated reproductive failure (PCV2-RF) and non-PCV2-RF at P-value 0.01 ($X^2$ test).
b Not TTSuV1b positive cases.

Discussion

Several reports have suggested that co-infection of *Ioatotorquevirus* species with other viruses might increase severity of disease as a result of synergy (1,5,16). Consequently, both species have been the target of research for the study of multifactorial diseases, such as porcine respiratory disease complex (17–20) and PCVAD (6–8,21–23).

In natural cases of PMWS, a high frequency of co-infection between TTSuV1a and PCV2 has been described (6). In gnotobiotic pig models, TTSuV1a was proposed to act as an aggravating factor of PMWS (7) and characteristic PDNS lesions were reproduced in PCV2-negative pigs after inoculation with TTSuV1a and porcine reproductive and respiratory syndrome virus (8). In PMWS-affected pigs, higher prevalence and increase viral load have been found with TTSuV1b than with TTSuV1a (6,22). In addition, a possible association of TTSuV1b with reproductive failure in sows has been proposed (1,24). Taken altogether, current information not only reveals high worldwide prevalence of *Ioatotorquevirus* species but also a close association of its occurrence in cases of PCVAD. However, a relationship of both TTSuV1a and TTSuV1b with development of PCVAD is still unclear.

In the present study, the degenerate nested PCR proved to amplify TTSuV1a and TTSuV1b ORF1 specific sequences. The nested PCR results revealed that TTVSuV1a and TTSuV1b are widely distributed in Mexican states with a high cluster of pig population, as it

Table IV, 34% (22/65) of cases and 33% (15/45) of PCV2-RF cases were TTSuV1a+. Similarly, 35% (7/20) of the reproductive failure cases not associated with PCV2 were positive for TTSuV1a+. No case of reproductive failure was positive for TTSuV1b. There was no statistical relationship between the manifestation of PCVAD and the presence of TTSuV1a or TTSuV1b by $j^2$ test.
is described for PCV2 (25). Also, both species were amplified from cases of PCVAD. In the current scenario, only 35% of total cases were positive to TTSuV1a. Similar findings were obtained regardless of presentation, 39% (9/23) of PMWS cases and 33% (15/45) of PCV2-RF cases (Table II). The global frequencies of the present work are far lower than reported in other countries, such as Spain (90%), Korea (85%), and China (80%), but comparable to frequencies in Thailand and the United States, which are reported to be 40% and 33%, respectively (26). In the same case series, frequencies in Canada were found to be highly variable (46% to 100%); it was suggested that differences in pig density might influence TTSuV1a prevalence (26). However, we observed a low prevalence of TTSuV1a from Mexican states with the highest proportion of swine farms (37). With regard to TTSuV1b, the gathered data of the present work showed more disparity since European countries have reported higher frequencies (6,21,22,28).

Detection of TTSuV1a and/or TTSuV1b has been strongly associated with cases of PMWS, particularly in Europe. Among PMWS-affected pigs, TTSuV1a seroprevalence of 66% to 76% has been reported in Spain (6,22,23,28). Likewise, TTSuV1a frequencies of 77% and 71.4% were reported in Sweden (21) and Slovakia (29), respectively. Whereas TTSuV1a detection was 41% and 58% in Great Britain from fresh tissues and sera, respectively (30). Frequency of TTSuV1b among PMWS-affected cases, however, showed even more discrepancy since European countries, such as Spain, have reported frequencies of 91% (6,28) and 100% (22) that are consistent with the Sweden prevalence of 94% (21). Moreover, TTSuV1b occurrence of 71% and 64.3% were found in Great Britain (30) and Slovakia (29), respectively.

Altogether, PMWS-affected findings of the present work are not consistent with prevalence in Europe, but are comparable to TTSuV1a prevalence of 48%, 40%, and 30% reported in Brazil (31), Cuba (16), and Japan (18), respectively. However, the latter 2 studies were performed on emaciated pigs without further laboratory confirmation of PCVAD status in affected tissues by ISH or immunohistochemistry. An explanation might be linked to geographic relationship within Asia and North America, but TTSuV1b prevalence of 94.3% found in Brazil (30) from PCV2-affected pigs is not in agreement with that hypothesis. TTSuV1b prevalence frequencies of 37.5% and 31% reported in Cuba (16) and Japan (18), respectively, are still closer to our findings.

Differences in lotatorquevirus species prevalence rates among studies might be related to target tissue. For instance, lower frequencies were found (41% for TTSuV1a and 79% for TTSuV1b) using pools of fresh lung, liver, kidney, spleen, and lymph node, but higher prevalence of both species (77% for TTSuV1a and 94% for TTSuV1b) was obtained from fresh lymph nodes (21). Such differences are most likely associated with PCV2-target tissues because lymph nodes and spleen are regarded as the main target of PMWS-affected pigs, displaying higher PCV2 loads (32). Therefore, testing fresh lymph node samples alone might increase the likelihood of detecting TTSuV species (6,22). In the current study, results were considerably lower compared to most reports despite the fact that severely and diffusely PCV2-affected lymphoid tissues were used. Consequently, the prevalence of TTSuV1a and TTSuV1b from PMWS-affected pigs appears to be low in Mexico.

Prevalence of lotatorquevirus species in cases of PMWS is reported as higher than that of clinically normal pigs (1,21). Results indicate that the prevalence is noticeably lower with PCVAD, however, though the same trend was noted, it is not statistically significant. Co-infection of both species in PMWS cases was much lower than that reported in Spain (76%) (22), but similar to that reported in Japan (10%) (18). However, in the current work, co-infection of age-matched clinically normal pigs was not observed (1,22,24).

Imune suppression caused by PCV2 infection has been suggested as a predisposing factor for the finding of TTSuV1b in PMWS cases because an increased viral load of TTSuV1b was seen in PMWS-affected pigs compared to the viral load of clinically normal pigs. Thus, clinically normal pigs might restrain infection with both lotatorquevirus species and PCV2 (28). Such statements are in agreement with the data presented herein since co-infection, though in low proportion, was only found in PMWS-affected cases.

The role of lotatorquevirus in reproductive failure has been proposed. To the authors’ knowledge, there are no reports of TTSuV1a nor TTSuV1b in cases of PCV2-RF. A higher seroprevalence of TTSuV1a (60% to 75%), compared to TTSuV1b (30% to 34%), has been detected in healthy sows (24,33). Litters from clinically normal sows infected with TTSuV1a or TTSuV1b showed that 43% and 19% of piglets were TTSuV1a+ and TTSuV1b+, respectively (34). Likewise, 50% of stillbirths were TTSuV1a+ but 7% of stillbirths were TTSuV1b+, suggesting that both species may cause in utero infection (33). Viremia in sows is a prerequisite for vertical transmission, with the heart being a frequently affected tissue (37). Histopathology of fetal hearts from cases of reproductive failure in sows showed both non supplicative myocarditis and PCV2-specific ISH (35) in 39% of PCV2-RF and 47% of transplacental transmission. These tissues were included in the present work, revealing a low frequency of TTSuV1a and an absence of TTSuV1b with no significant statistical relation between the occurrence of PCV2-RF and the presence of TTSuV1a.

Using tissue pools from aborted fetuses, the frequency rates of TTSuV1a and TTSuV1b were 17% and 30%, respectively. Heart tissue was part of this tissue pool, but the precise site of infection could not be identified (36). Fetal hearts were one of the individual tissues evaluated in the current study, but other additional tissues that could potentially harbor lotatorquevirus species were not evaluated, thus lotatorquevirus species could not be definitively ruled out in cases of PCV2-RF. Nevertheless, since the heart is the main target organ for vertical transmission, preliminary findings suggest that participation of TTSuV1b regarding PCV2-RF is unlikely.

The current retrospective work is noteworthy because a non-vaccinated porcine population was evaluated from a time period prior to the start of through and worldwide immunization programs. Therefore, it may elucidate distinct viral dynamics as influenced by changing immune status and add to the understanding of the evolving nature of viruses. In addition, a TTSuVx2 sequence was amplified based on a new taxonomy (2). Thus, further classification of TTSuV1b is essential to separate it from TTSuVx2 species.

Taken together, the data reported herein are in agreement with a lack of relationship between lotatorquevirus species and occurrence of PCVAD. Currently, a broad phylogenetic study is being done to ascertain prevailing swine Anelloviridae species in Mexico as well as its genomic variability that might account for a distinctive pathogenic potential.
Acknowledgments

The authors thank Karina Enriquez-Ramírez MSc. for her excellent technical contribution to PCV2-in situ hybridization. The authors are grateful to the CONACYT scholarship program for its commitment to support graduate students. This work was financially supported by a grant from DGAPA-UNAM (PAPIIT IN203309).

References


Identification of the linear ligand epitope on classical swine fever virus that interacts with porcine kidney 15 cells

Yin Mei, Feng Yue, Hong-mei Ning, Juan-juan Zhou, Xuan-nian Wang

Abstract

Binding of the viral ligand to a specific receptor is the first step of virus entry into target cells. The envelope proteins E\textsuperscript{ms}, E1, and E2 of classical swine fever virus (CSFV) are involved in the interaction with host cell receptors to mediate CSFV infection. The aim of this investigation was to identify epitopes that bind to porcine kidney (PK)-15 cells to prevent CSFV infection. Ten peptides representing E\textsuperscript{ms}, E1, and E2 were synthesized. Immunohistochemical study showed that the SE24 peptide, which is derived from the E2 amino acid sequence, could effectively bind to PK-15 cells. Similarly, a flow cytometry assay demonstrated that SE24 binding to PK-15 cells could be blocked by CSFV. The binding of SE24 with PK-15 cells leads to decreased CSFV infection of PK-15 cells in a dose-dependent manner. These results suggest a potential new strategy for the prevention and control of CSFV infection that requires further investigation.

Materials and methods

Peptide synthesis

The bioinformatics software DNASIS MAX (Version 2.5; Informer Technologies, Madrid, Spain) was used to analyze the amino acid sequence of the viral ligand responsible for receptor binding. DNX Technologies (Version 2.5; Informer Technologies, Madrid, Spain) was used to design peptides. The peptides were synthesized by the solid-phase method using the Boc procedure and purified by reverse-phase high-performance liquid chromatography. The purity of the final product was above 98.5%.

Introduction

A viral ligand generally is an epitope molecule that binds to a host cell receptor; namely, the virus attachment protein (1). This receptor is a molecule on the surface of the host cell that recognizes the viral ligand and interacts with it to mediate viral invasion into the host cell (2,3). Binding of the viral ligand to its receptor is the decisive factor in virus–host specificity and tissue tropism. Therefore, full understanding of this interaction is critical to clarifying the pathogenic mechanisms of virus infection. Previous studies have reported on some classical swine fever virus (CSFV) receptors, such as heparin sulfate, mannose-6-phosphate, \( \beta \)-actin, low-density lipoprotein, 50-kDa membrane protein, and 60-kDa actin-binding membrane protein, that are involved in the process of CSFV infection (4–8).

Research on CSFV ligands has confirmed that envelope glycoprotein Erns is a disulfide-linked homodimer known to possess ribonuclease activity. It lacks a typical transmembrane region and is found both on the surface of infected cells and in the culture medium. The interaction of Erns with cell surface glycoaminoglycans contributes in part to binding of the virus to susceptible cells (9–11). The envelope glycoproteins E1 and E2 form a heterodimer that mediates invasion of CSFV into host cells (12,13). The structural protein E2 is a key determinant and major immunogen for viral entry and immunity. Surprisingly, the cellular membrane protein annexin 2 as the host protein can bind to CSFV E2 and promote viral growth in porcine kidney (PK)-15 cells (14).

Blocking the interaction of viral ligand and receptor could inhibit virus infection. However, the amino acid sequence of the viral ligand responsible for receptor binding is unknown. The purpose of this study was to provide a theoretical basis for further revealing the mechanism of CSFV infection, as well as for screening antiviral drugs and designing new vaccines from the perspective of the interaction between viral ligand and receptor.
sequences of CSFV proteins E<sub>rns</sub>, E1, and E2. With standard operating procedures 10 peptides to cover parts of these sequences were synthesized by the solid-phase peptide synthesis method with the 9-fluorenylmethyloxycarbonyl (Fmoc) amino acids attached to Wang resin (Gill Biochemical, Shanghai China). The Cys residue was added at its N terminal to couple the bifunctional reagent sulfosuccinimidyl-4-(N-maleimidomethyl) cyclohexane-1-carboxylate (SMCC). Each synthesized peptide was cleaved from the resin with trifluoroacetic acid, precipitated with cold ether, and recovered by centrifugation. After a wash with cold diethyl ether (at 4°C) to remove impurities, the peptide was dissolved in acetonitrile–water [1:1 (v/v)], which contained 1% acetic acid, and freeze dried. The characteristics of the 10 preliminarily purified synthetic peptides are provided in Table I.

**Cells and viruses**

Porcine kidney-15 cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM; Gibco ThermoFisher Scientific) supplemented with 10% fetal bovine serum (FBS) and incubated at 37°C.
These cells are often used to propagate CSFV in vitro. The CSFV used was the Shimen strain, the standard virulent strain isolated from China in 1945 and grown in PK-15 cells.

**Biotin labeling of the peptides**

Sulfo-NHS (hydroxysulfosuccinimide)–biotin (ThermoFisher Scientific) was dissolved in double distilled water for a concentration of 10 mmol/L. The peptides were dissolved in dimethylsulfoxide (Aladdin Company, Shanghai, China) for a concentration of 40 mg/mL. A peptide–biotin solution was prepared by mixing 80 μL of sulfo-NHS–biotin and 50 μL of the peptide at room temperature for 30 min. After overnight incubation at 4°C the solution was adjusted to a concentration of 1 mg/mL with DMEM containing 10% FBS and frozen for storage.

**Binding assay**

The PK-15 cells were incubated in 96-well plates at 37°C in 5% CO₂, until 100% confluent and then incubated at 4°C for 1 h with the biotin-labeled peptides at concentrations of 0.2, 0.1, 0.05, and 0.025 mmol/L in aliquots of 100 μL per well. Three replicates of each peptide at each dilution ensured full binding of the peptide with the PK-15 cells. Biotin-labeled bovine serum albumin (BSA) was used as a negative control, and DMEM without biotin–peptide was used as a blank control. Unbound peptide was washed away with precooled 0.01 M phosphate-buffered saline (PBS; 0.02% KH₂PO₄, 0.29% NH₄PO₄, 0.02% KCl, and 0.8% NaCl), pH 7.4, containing 0.05% Tween 20 (PBST) (Aladdin). The PK-15 cells were fixed by 0.3% H₂O₂ in methanol (Sinopharm Chemical Reagent Company, Ningbo, China), 50 μL/well, for 10 min at 4°C, then were washed 3 times with PBST and blocked with 10% nonfat milk (Yili Industrial Group Company, Hohhot, China) in PBST, 200 μL/well, for 1 h at 37°C. The cells were again rinsed in PBST 3 times, and avidin labeled with horseradish peroxidase (Boster Biological Engineering, Wuhan, China) in 10% nonfat milk in PBST (1:500), 50 μL/well, was added. After incubation for 1 h at 37°C the supernatant was decanted and the plate washed with PBST 3 times. The AEC staining kit (Sigma-Aldrich, St. Louis, Missouri, USA) was used to detect binding of peptide to cell: 50 μL of substrate was applied to each well for 10 min at room temperature, and the cells were then observed through a microscope.

**Peptide-binding and virus-blocking assays**

Three groups were set up. In group A, biotin-labeled peptide, 0.2 mmol/L, was used to bind to PK-15 cells. In group B, biotin-labeled BSA was used as a negative control with PK-15 cells. In group C, the biotin-labeled peptide was used to bind to PK-15 cells after CSFV adsorption to the PK-15 cells. The biotin-labeled peptides, biotin-labeled BSA, and CSFV, 100 × the median tissue culture infectious dose (TCID₅₀), were incubated with 1 × 10⁶ PK-15 cells for 1 h on ice. Three replicates were done in each group. In all 3 groups, 100 μL of avidin labeled with fluorescein isothiocyanate (1:500) was added and the mixture incubated for 30 min on ice. The supernatant was decanted, and the PK-15 cells were resuspended with 0.5 mL of prechilled PBS. After being mixed, the samples were assayed by
Figure 2. A — binding of biotin-labeled peptide SE24 to PK-15 cells. B — binding of biotin-labeled BSA to PK-15 cells. C — binding of biotin-labeled SE24 to PK-15 cells pretreated with classical swine fever virus (CSFV). Avidin labeled with fluorescein isothiocyanate was applied and flow cytometry used to detect the binding. See Materials and methods for details. The mean proportion of positive cells (± standard deviation) decreased from 50.86% ± 3.30% to 39.43% ± 1.35% after CSFV pretreatment, indicating that the virus can block the binding of peptide SE24 to PK-15 cells.

Figure 3. Rate of CSFV infection of PK-15 cells incubated with peptide SE24 at concentrations of 0.2 (A), 0.1 (B), 0.05 (C), 0.025 (D), 0.0125 (E), and 0.00625 (F) mmol/L. G — BSA (unrelated protein control). H — negative serum (isotype control). See Materials and methods for details. With a decrease in peptide concentration, the rate of CSFV infection gradually increased.
Table II. Inhibition by peptide SE24 of infection of porcine kidney 15 cells by classical swine fever virus

<table>
<thead>
<tr>
<th>Peptide concentration (mmol/L)</th>
<th>Infected cells; mean ± standard deviation</th>
<th>Number</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.2</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>0.1</td>
<td>260.00 ± 9.00</td>
<td>260.00</td>
<td>3.71 ± 0.12</td>
</tr>
<tr>
<td>0.05</td>
<td>286.00 ± 10.53</td>
<td>286.00</td>
<td>4.08 ± 0.15</td>
</tr>
<tr>
<td>0.025</td>
<td>566.33 ± 22.30</td>
<td>566.33</td>
<td>8.09 ± 0.32</td>
</tr>
<tr>
<td>0.0125</td>
<td>684.33 ± 14.01</td>
<td>684.33</td>
<td>9.78 ± 0.20</td>
</tr>
<tr>
<td>0.00625</td>
<td>874.67 ± 21.39</td>
<td>874.67</td>
<td>12.49 ± 0.30</td>
</tr>
</tbody>
</table>

a Different superscript letters in the same column indicate a significant difference (P < 0.05).
b The total number of cells per well was 7.0 × 10^4.

Attune Acoustic Focusing Cytometer (Applied Biosystems, Waltham, Massachusetts, USA); 1 × 10^4 cells were counted for each sample.

**Infection-inhibition assay**

The PK-15 cells were cultured in 96-well plates until 70% to 80% confluent and then incubated with peptide SE24 at concentrations of 0.2, 0.1, 0.05, 0.025, 0.0125, and 0.00625 mmol/L, for 100 µL/well, in 10% DMEM. Three replicates were done. For controls, BSA was used as an unrelated protein and negative serum was used as an isotype. After incubation at 37°C for 1 h the supernatant was decanted, and 10 × TCID50 of CSFV was added to infect the cells. After 3 washes with 10% FBS in DMEM and refreshment with 5% FBS in DMEM, the cells were cultured at 37°C for 24 to 48 h.

For immunohistochemical staining the cells were rinsed in PBST 3 times for 5 min each time, then fixed by being incubated with 4% paraformaldehyde for 10 min at 4°C, and then rinsed again in PBST 3 times for 5 min each time. Nonspecific binding sites were then blocked by incubation of the cells with 10% skimmed milk powder for 1 h at 37°C followed by rinsing in PBST 3 times for 5 min each time. Next, rabbit anti-CSFV serum (1/200) was added and the mixture incubated for 30 min at 37°C and then rinsed in PBST 3 times for 5 min each time. Then goat IgG against rabbit antigen (1/40) was added and the mixture incubated for 30 min at 37°C and then rinsed in PBST 3 times for 5 min each time. Finally, the AEC kit was used again, with substrate applied for 10 min at room temperature. The number of infected cells was counted through a microscope.

**Statistical analysis**

Data were analyzed with the paired-samples t-test by means of IBM SPSS Statistics for Windows, Version 19.0 (IBM Corporation, Armonk, New York, USA) and are presented as mean ± standard deviation. P-values of less than 0.05 were considered statistically significant.

**Results**

The binding-assay results showed that peptide SE24 could interact with PK-15 cells at all the tested concentrations. Data for the other peptides are not shown. Positive cells stained brownish red (Figure 1, panels A to D). Neither the biotin-labeled BSA [negative control (Figure 1, panel E)] nor the DMEM [blank control (Figure 1, panel F)] showed binding to the PK-15 cells. Peptide SE24, whose amino acid sequence is shown in Table I, is located on the E2 protein of CSFV and has a molecular weight of 5.73 kDa. Its binding occurred in a dose-dependent manner and was mainly on the cell membrane.

The ability of CSFV to block the binding of peptide SE24 to PK-15 cells was measured by flow cytometry (Figure 2). The mean proportion of positive cells and the mean fluorescence intensity of the binding were 50.86% ± 3.30% and 23.52% ± 1.24%, respectively, but when the PK-15 cells were pretreated with CSFV the means were significantly lower (P < 0.05), at 39.43% ± 1.35% and 20.26% ± 0.58%, respectively. The means for the negative-control group were significantly lower still (P < 0.05), at 14.50% ± 1.21% and 17.47% ± 0.71%, respectively. The infection-inhibition assay showed that peptide SE24, 0.2 mmol/L, could completely inhibit CSFV infection of PK-15 cells (Figure 3, panel A). With a decreasing concentration of the peptide, the number of infected cells increased (Figure 3, panels B to F), as did the infection rate (Table II). The infection of PK-15 cells by CSFV was not inhibited by BSA, the negative control, at any concentration, and the cells stained reddish brown (Figure 3, panel G). With the blank control, no PK-15 cells stained reddish brown (Figure 3, panel H). With the SE24 concentration as the independent variable and the inhibition rate as the dependent variable, and log transformation of the data, Figure 4 shows graphically that with a decrease in peptide concentration, the infection rate increased gradually.

**Discussion**

According to previous studies, the envelope glycoproteins of CSFV — namely, E^envelope, E1, and E2 — participate in virus adsorption and invasion (9–13). On this basis, 10 peptides were synthesized to
cover parts of the amino acid sequences of these glycoproteins. The infection-inhibition assay confirmed the inhibitory effect of peptide SE24 on CSFV infection of the target cells, which was consistent with the blocking by CSFV of peptide SE24 binding to PK-15 cells. Surprisingly, our study showed that peptide SE24 and CSFV competitively bind to the same receptor on the membrane of PK-15 cells. The binding of peptide SE24 to PK-15 cells can block CSFV adsorption but also suppress PK-15 cells already infected with CSFV. These findings suggest that peptide SE24 is a potential drug target for the prevention and treatment of classical swine fever.

The highest concentration of peptide SE24 used in the infection-inhibition assay, 0.2 mmol/L, almost entirely inhibited CSFV infection of PK-15 cells. Water solubility and hydrophilicity can affect peptide activity. Thus, improving the solubility and hydrophilicity of the peptide would be very important in development of a peptide drug. Currently, the biologic activity and function of the polypeptide, and shortening of its length, are being studied in our laboratory. Now that we have shown in vitro that peptide SE24 effectively binds to target cells and inhibits infection of those cells by CSFV, further study is needed to verify the binding function and infection-inhibition activity of the peptide in vivo.

The peptide SE24 consists of 50 amino acids located on the CSFV E2 protein (amino acids 955 to 1004). Many experimental features of SE24 are consistent with E2 function. For instance, E2 is a determinant of cell culture tropism, and the E1 and E2 proteins formed a heterodimer that mediated invasion of host cells by CSFV (9,12,13). The E2 glycoprotein is likely to be involved in the host specificity of pestiviruses at their point of uptake into cells (15), and the E2 protein of CSFV can interact with swine host factors by the yeast 2-hybrid system (16). According to the prediction of E2 structure (17), peptide SE24 is located close to the transmembrane region rather than the antigen region. In theory, the viral ligand should be in the antigen region or the extracellular domain, which facilitates binding with the viral receptor. Whether peptide SE24 can be used as a viral ligand is unknown. The process by which viruses invade cells is complex. This study has suggested a novel approach for the prevention and treatment of viral diseases: inhibiting viral infection of host cells by blocking the virus receptor–ligand interaction.

**Acknowledgments**

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**References**


Direct repeat unit (dru) typing and antimicrobial resistance of methicillin-resistant Staphylococcus pseudintermedius isolated from dogs in Atlantic Canada

Matthew E. Saab, J. Scott Weese, J.T. McClure

Abstract

There are few reports investigating the characterization of methicillin-resistant Staphylococcus pseudintermedius (MRSP) in dogs in Canada and none from Atlantic Canada. The objectives of this study were to strain type MRSP isolates cultured at a regional diagnostic laboratory using direct repeat unit (dru) typing and to describe their antimicrobial resistance profiles. Ninety-four isolates recovered from dogs between 2010 and 2012 had dru typing, cluster analysis, and antimicrobial susceptibility testing done. The majority of isolates belonged to type dt11a (30.9%), dt10h (24.5%), dt9a (18.1%), and dt11af (10.6%) with the remaining 15.9% of isolates distributed among 13 dru types. The predominant dru types identified were similar in Ontario; however, cluster 9a appears to be less common in Atlantic Canada. A significant difference in the distribution of clusters among Atlantic provinces was detected ($P = 0.01$). Resistance to $\geq 2$ non-β-lactam antimicrobials was observed in 71.4% of the isolates. The MRSP isolates from this study were notably less resistant than those reported in the literature. A more comprehensive study of the MRSP dru types could help further elucidate the distribution of this pathogen in Canada.

Introduction

Over the last decade, methicillin-resistant Staphylococcus pseudintermedius (MRSP) has emerged as a major opportunistic pathogen in dogs, and as the leading cause of skin disease, otitis, surgical site infections, and wound infections (1,2). Methicillin-resistance in MRSP is mediated by the mecA gene, which codes for the expression of a modified penicillin-binding protein, PBP2', and resistance to most β-lactam antimicrobials (2). Increasing antimicrobial resistance to antimicrobials other than β-lactams, has been reported in MRSP isolates, leaving few therapeutic options available in many instances (1–3). Methicillin-resistant Staphylococcus pseudintermedius has been implicated in hospital-acquired infections within tertiary hospitals, and the potential of hospital-wide outbreaks should not be underestimated (1,4). Not only has MRSP been reported in the canine population, but also there have been several reports of humans infected or colonized with MRSP (5–11). Understanding the diversity and dissemination of MRSP is essential for developing mitigation and control strategies and for future epidemiologic studies. Currently, there is no study characterizing MRSP isolates in Atlantic Canada.

A number of molecular tools have been reported to genetically discriminate among staphylococci. Most of these tools were first developed and standardized for methicillin-resistant Staphylococcus aureus (MRSA), but few have been further refined for discriminating MRSP (1,12–16). While pulsed-field gel electrophoresis (PFGE) remains the preferred method for typing MRSA, it is not as efficient in typing MRSP. Newer methods such as direct repeat unit (dru) typing and to describe their antimicrobial resistance profiles.
gold standard for MRSA outbreak investigations, it has been shown to be less useful for MRSP investigations (12,14,17,18). Historically, multi-locus sequence typing (MLST) has been the primary tool for investigating the population genetic structure of MRSP, and 2 major sequence types (ST) have been identified (1,16,19). Both PFGE and MLST are laborious, costly, and require the use of reference strains, thus making them impractical tools for implementation in smaller diagnostic laboratories.

The use of a single-locus marker for isolate discrimination has also been explored for MRSA, and provides a less expensive and less laborious approach to strain typing (20,21). Sequence analysis of the staphylococcal protein A region (spa typing) has been shown to have greater discriminatory power than MLST and PFGE for MRSA, but it is less useful for strain typing all MRSP isolates (14,20–22). The mec-associated direct repeat unit (dru) typing was first demonstrated to be useful in discriminating highly-clonal MRSA isolates in Scotland (13), and has more recently been successfully applied to MRSP isolates from Canada, the United States (US), Europe, and Australia (22–24). In a previous study, significant associations between dru cluster and MLST were established, in which cluster 9a was associated with ST71 (the “International clone”) and cluster 11a was associated with ST68 (the “North American clone”) (1,22). This single-locus sequence-based method is rapid, standardized, and cost-effective, making it an ideal candidate for use in small-scale laboratories (22,23).

In a previous study, significant differences have been reported in the distribution of the 2 main dru clusters, 9a and 11a, between Canada, the US, and Europe (22). The report also inferred that variation within a country may exist, as some dru types were detected in some US states, but not the others included in the study. To date, there has been only one study investigating dru types in Canada, which was limited to Ontario; therefore, genetic information on MRSP isolates from Canadian regions other than Ontario is needed. Thus, the primary objective of this current study was to explore the strain type diversity of MRSP isolates from dogs using the dru typing method from submissions to a regional diagnostic laboratory in Atlantic Canada. A secondary objective was to describe the antimicrobial resistance (AMR) pattern of these MRSP isolates.

### Materials and methods

#### Isolate screening and collection

Isolates were recovered from canine specimens submitted to the Atlantic Veterinary College (AVC) Diagnostic Services Bacteriology Laboratory for routine culture and susceptibility testing. Staphylococci were identified by colony morphology, including hemolysis, a positive tube coagulase test and matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry. Antimicrobial susceptibilities to the following drugs were determined using the Kirby-Bauer disk diffusion method, following Clinical Laboratory Standards Institute (CLSI) standard M31-A3 (25): ampicillin (10 μg), amikacin (30 μg), amoxicillin-clavulanic acid (30 μg), cephalexin (30 μg), cefovecin (30 μg), chloramphenicol (30 μg), clindamycin (30 μg), doxycycline (30 μg), enrofloxacin (5 μg), erythromycin (15 μg), gentamicin (10 μg), penicillin (10 μg), and trimethoprim-sulfamethoxazole (SXT; 25 μg). Methicillin resistance was detected by oxacillin (1 μg) disk diffusion, and confirmed by mecA expression using the PBP2’ latex agglutination test (PBP2’ Latex Agglutination Test; Oxoid Company, Nepean, Ontario). All isolates identified presumptively as MRSP were placed in a medium containing 15% glycerol and frozen at −80°C.

Patient province of residence, submitting clinic, anatomical source/site of specimen, and antimicrobial susceptibility data were extracted from the laboratory data management system and descriptive statistics were computed. Only 1 isolate from each patient was included for analysis in most patients. If multiple MRSP isolates on repeated lab submissions were recovered from a patient but had the same dru type, only the first isolate was included for analysis. If different dru types were isolated from the same patient on different lab submissions, then both isolates were retained for analysis.

#### Molecular identification and typing

Genomic material was extracted using a resin matrix (InstaGene Matrix; Bio-Rad Laboratories, Montreal, Quebec). Manufacturer’s guidelines were followed except a large loopful of bacterial colonies were suspended in 1.0 mL of polymerase chain reaction (PCR) water, and the incubation time at 56°C was increased from 30 min to 1 h. Isolated DNA was frozen at −20°C until use. A multiplex PCR reaction was used to identify coagulase-positive staphylococci to the species level based on partial amplification of the mec locus (26). *Staphylococcus pseudintermedius* isolates were characterized using the dru typing method, as previously described (17). A 40-μL reaction was prepared containing 4 μL of template DNA, 0.8 μL of DNA polymerase, buffer mixture containing 1.5 mM MgCl2 and 200 μM of each dNTP (KAPA2G Fast Hot Start DNA polymerase; KAPA Biosystems, Boston, Massachusetts, USA), additional 0.3 mM MgCl2 and 0.8 μM of the forward primer druGF (5’-GTTAGCATATTACCTCTCTTGGC-3’) and the reverse primer druGR (5’-GCCGATTGTGCCTGATGAG-3’). Reaction mixtures were thermally cycled for an initial denaturation step of 94°C for 2 min followed by 30 cycles at 94°C for 1 min, 52°C for 1 min, and 72°C for 1 min (17). The PCR products were purified using a kit (QIAquick PCR Purification Kit; Qiagen, Mississauga, Ontario) prior to sequencing.

The dru repeats and types were assigned using a website (www.dru-typing.org) following the previously described nomenclature (17). Cluster analysis was used to compare the relatedness among dru types, and a minimum spanning tree (MST) was generated. Distance intervals (or similarity values) were created using the TRST plug-in tool (BioNumerics version 6.6; Applied Maths, Austin, Texas, USA), as described previously for MRSP dru typing (22–24,27).

#### Statistical analysis

Multi-drug resistant (MDR) MRSP were defined as being resistant to ≥ 2 antimicrobials classes in addition to β-lactams. Dru clusters were used for comparisons to decrease the number of tested groups.
Unconditional associations between dru cluster and patient data and dru cluster and resistance to the non-β-lactam antimicrobials were assessed using Chi-squared or Fisher’s exact tests, where appropriate, with significance set at $P \leq 0.05$. Subtables were explored for any significant association to determine where the differences were. All statistical computations were done using computer software (Stata/IC 13.1 for Mac; StataCorp, College Station, Texas, USA).

### Results

#### Isolate collection

The diagnostic laboratory collected 129 isolates from 90 dogs between January 2010 and December 2012. Of those, 98 isolates were retained for analysis. Twenty-three patients had multiple submissions. Isolates were collected from various specimens ($n = 98$): skin ($n = 52, 53.1\%$), ears ($n = 18, 18.4\%$), wounds ($n = 8, 8.2\%$), surgical sites ($n = 6, 6.1\%$), urine ($n = 5, 5.1\%$), abscesses ($n = 2, 2.0\%$), and other ($n = 7, 7.1\%$). Most of the patients were seen by private veterinary clinics in the region (71.4%) compared to being seen by the AVC Veterinary Teaching Hospital (AVC-VTH) (28.6%).

#### Antimicrobial resistance patterns

Only 8.2% ($n = 8$) of isolates were susceptible to all tested drugs with the exception of β-lactams (Table I). Another 20.4% ($n = 20$) of isolates were resistant to 1 non-β-lactam antimicrobial class, while 71.4% ($n = 70$) were resistant to ≥ 2 non-β-lactam antimicrobial classes. The most common resistance was to SXT (74.5%), followed by erythromycin (68.4%), clindamycin (55.1%), enrofloxacin (46.9%), gentamicin (34.7%), chloramphenicol (23.5%), doxycycline (15.3%), and fusidic acid (3.1%). In isolates that displayed multi-drug resistance ($n = 70$), 68.6% of isolates were resistant to SXT, erythromycin, and clindamycin, while 55.7% ($n = 70$) of those were also resistant to enrofloxacin. Amikacin resistance was not detected in any of the isolates.

#### Direct repeat unit typing

The dru types were determined for 94/98 isolates, because 4 isolates were not available for typing. From the 94 isolates, 18 dru types were recovered with 3 predominant dru types contributing more than 70% of the distribution: dt11a (30.9%), dt10h (24.5%), and dt9a (18.1%). The frequency of each dru type can be found in Table II. Nine of the dru types were previously unidentified at the time of analysis and are represented by one isolate each: dt5k, dt6t, dt8ag, dt9bd, dt10cc, dt10cj, dt11ca, and dt11cm.

### Table I. Number (and percent) of antimicrobial resistant methicillin-resistant Staphylococcus pseudintermedius (MRSP) isolates overall and by dru cluster, following Clinical Laboratory Standards Institute (CLSI) guidelines. Multidrug resistance (MDR) in each cluster is also reported

<table>
<thead>
<tr>
<th>Antimicrobial</th>
<th>Overall (n = 94)</th>
<th>9a (n = 18)</th>
<th>10h (n = 27)</th>
<th>11a (n = 45)</th>
<th>No cluster (n = 4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amikacin</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>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>23 (24.5%)</td>
<td>3 (16.7%)</td>
<td>0 (0%)</td>
<td>19 (42.2%)</td>
<td>1 (25%)</td>
</tr>
<tr>
<td>Clindamycin</td>
<td>54 (57.5%)</td>
<td>16 (88.9%)</td>
<td>7 (25.9%)</td>
<td>29 (64.4%)</td>
<td>2 (50%)</td>
</tr>
<tr>
<td>Doxycycline</td>
<td>14 (14.9%)</td>
<td>1 (5.6%)</td>
<td>7 (25.9%)</td>
<td>4 (8.9%)</td>
<td>2 (50%)</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>63 (67.0%)</td>
<td>17 (94.4%)</td>
<td>11 (40.7%)</td>
<td>32 (71.1%)</td>
<td>3 (75%)</td>
</tr>
<tr>
<td>Enrofloxacin</td>
<td>46 (48.9%)</td>
<td>16 (88.9%)</td>
<td>0 (0%)</td>
<td>29 (64.4%)</td>
<td>1 (25%)</td>
</tr>
<tr>
<td>Fusidic acid</td>
<td>2 (2.1%)</td>
<td>1 (5.6%)</td>
<td>1 (3.7%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>34 (36.2%)</td>
<td>10 (55.6%)</td>
<td>2 (7.4%)</td>
<td>20 (44.4%)</td>
<td>2 (50%)</td>
</tr>
<tr>
<td>SXT</td>
<td>70 (74.5%)</td>
<td>18 (100%)</td>
<td>23 (85.2%)</td>
<td>27 (60.0%)</td>
<td>2 (50%)</td>
</tr>
<tr>
<td>MDR</td>
<td>66 (70.2%)</td>
<td>17 (94.4%)</td>
<td>15 (55.6%)</td>
<td>31 (68.9%)</td>
<td>3 (75%)</td>
</tr>
</tbody>
</table>
| MDR — trimethoprim-sulfamethoxazole.

### Table II. Frequency of methicillin-resistant Staphylococcus pseudintermedius dru types from 94 isolates collected at a regional diagnostic laboratory between 2010 and 2012

<table>
<thead>
<tr>
<th>dru type</th>
<th>Frequency (n = 94)</th>
<th>Cluster</th>
</tr>
</thead>
<tbody>
<tr>
<td>dt11a</td>
<td>29 (30.9%)</td>
<td>11a</td>
</tr>
<tr>
<td>dt10h</td>
<td>23 (24.5%)</td>
<td>10h</td>
</tr>
<tr>
<td>dt9a</td>
<td>17 (18.1%)</td>
<td>9a</td>
</tr>
<tr>
<td>dt11af</td>
<td>10 (10.6%)</td>
<td>11a</td>
</tr>
<tr>
<td>dt10as</td>
<td>3 (3.2%)</td>
<td>10h</td>
</tr>
<tr>
<td>dt10cc</td>
<td>1 (1.1%)</td>
<td>10h</td>
</tr>
<tr>
<td>dt10cj</td>
<td>1 (1.1%)</td>
<td>11a</td>
</tr>
<tr>
<td>dt11bn</td>
<td>1 (1.1%)</td>
<td>11a</td>
</tr>
<tr>
<td>dt11ca</td>
<td>1 (1.1%)</td>
<td>11a</td>
</tr>
<tr>
<td>dt11v</td>
<td>1 (1.1%)</td>
<td>11a</td>
</tr>
<tr>
<td>dt11y</td>
<td>1 (1.1%)</td>
<td>11a</td>
</tr>
<tr>
<td>dt9b</td>
<td>1 (1.1%)</td>
<td>—</td>
</tr>
<tr>
<td>dt9d</td>
<td>1 (1.1%)</td>
<td>—</td>
</tr>
<tr>
<td>dt11cm</td>
<td>1 (1.1%)</td>
<td>11a</td>
</tr>
</tbody>
</table>

* Novel dru types identified in this study.
A minimum spanning tree (MST) was constructed to identify relatedness among 

dru types, and to determine the predominate clusters (Figure 1). The cluster analysis identified 3 main 
dru clusters that included 90/94 typed isolates (Figure 1). Cluster 11a contained 
the most diversity, comprising 9 
dru types and 47.9% of the typed 
isolates. Cluster 10h contained 3 
dru types and 28.7% of the isolates, 
while cluster 9a contained 2 
dru types and 19.2% of the isolates. 

Table II contains the 
dru type and cluster association. 

Associations among dru clusters, patient demographics, and AMR 

Significant differences were determined among the distributions of dru clusters in the 4 Canadian Atlantic provinces (P = 0.01; 
Table III). In Newfoundland and Labrador (NL), cluster 9a was over-represented with 5/7 strains belonging to dt9a. Cluster 10h was present in Nova Scotia (NS; 15/52) and New Brunswick (NB; 5/23), but not NL, and was the predominant cluster from Prince Edward Island (PEI; 7/12) isolates. Both mainland provinces, NB and NS, shared the same predominant cluster, 11a, which comprised over 
50% of the isolates from each province. 

The dru cluster was not significantly different among the AVC-VTH and private practice (P = 0.18) or specimen types (P = 0.91). 

Significant associations among AMR and dru clusters were detected for all antimicrobials except fusidic acid (P = 0.31; Table I). For clindamycin, erythromycin, enrofloxacin, and SXT, cluster 9a had a significantly higher proportion of resistant isolates (P ≤ 0.01). Cluster 10h had significantly higher proportion of isolates resistant to doxycycline (P = 0.04), while cluster 11a had a significantly higher proportion of chloramphenicol resistance (P < 0.001). The proportion of MDR isolates was significantly different among dru clusters (P = 0.03), with cluster 9a having the highest proportion of MDR (94.4%), followed by 10h (55.6%) and 11a (68.8%). 

Discussion 

The majority of isolates (73.5%) collected as part of this convenience sampling at the AVC Diagnostic Services Bacteriology Laboratory were represented by dru types dt11a, dt10h, and dt9a, which is similar to the findings in a previous multi-national study (22). However, in the current study, clusters 11a and 10h comprised
more than 75% of the isolates collected, whereas in the Ontario study, cluster 11a (including dt10h) and 9a were evenly distributed (22). The distribution of MRSP dru clusters in Atlantic Canada was more similar to what has been reported in California, Illinois, North Carolina, Tennessee, and Texas (22). Differences among geographic regions within the US were also noted in the previous study (22).

Significant differences in the diversity of strains were detected among provinces of Atlantic Canada (Table III). It should be noted that this study was not designed for prevalence estimation because of the convenience sampling approach. Although it may appear that the incidence of MRSP is higher in certain provinces, these numbers are reflective of the sample submission demographics from these regions (i.e., the laboratory receives approximately 45% of their canine samples from NS, but only 5% are from NL). These provincial differences in the distribution of dru types could be attributed to geographic separation, since differences among Newfoundland and Labrador and the Maritime Provinces (NB, NS, and PEI) exist, as well as population density, since provinces with larger populations have greater dru type diversity. The small sample size of isolates from Newfoundland and Labrador may bias its estimate. It is possible that the true diversity of the isolates from some provinces is underestimated, since each province except PEI has its own animal health microbiology laboratory, decreasing submissions from these provinces to the AVC diagnostic laboratory.

This study detected 9 dru types that had not been previously identified (Table II), and required entry into the dru database. These new dru types are results of a random single-nucleotide polymorphism in one of the 40bp dru repeat sequences, subsequently creating a new dru repeat and thus new dru type. Novel dru types have also been identified in a previous study (22). Although there were no significant differences in the distribution of the new dru types, most of the novel dru types were isolated from Nova Scotia (5/9), and from skin samples (5/9).

More than 70% of the isolates recovered in this study were resistant to ≥ 2 non-β-lactam antimicrobial classes, highlighting the clinical concerns regarding management of MRSP infections. Multi-drug resistance has been frequently reported in MRSP isolated from canine specimens (1,28). The isolates recovered in this study were from submissions to a regional diagnostic bacteriology laboratory that also is the diagnostic laboratory for the AVC-VTH, thus a selection bias is possible, based on submission of specimens from complicated referral cases from the AVC-VTH. However, most samples in this study were from primary care practices. Information about prior antimicrobial exposure was not always available, but submissions to a diagnostic laboratory for bacterial culture and susceptibility testing can be from cases proving difficult to treat, with the primary reason being non-response to antimicrobial therapy. It is likely that diagnostic laboratory submissions are biased to be more resistant.

Interestingly, cluster 9a isolates had the highest proportion of MDR at 94.4%, and also had a significantly higher proportion of resistant isolates for each antimicrobial except fusidic acid, chloramphenicol, and doxycycline. Strong associations between cluster 9a and ST71 have been shown (22), and reports have also shown ST71 to have an increased antimicrobial resistance compared with other STs (1,29). MLST was not done on the isolates in this study, but the cluster 9a isolates in this study likely belong to ST71. Although this study was not designed for prevalence estimation, the ST71 clone associated dru cluster 9a seems to have not disseminated into Atlantic Canada (18.1%), with the possible exception of Newfoundland, to the degree that it has in Europe (90%), the US (66%), and Ontario, Canada (47%) (22).

Resistance of the MRSP isolates to the non-β-lactam antimicrobials in this study was lower than in previous reports of multi-drug resistance in MRSP, which could be explained by the low prevalence of cluster 9a isolates. However, caution should be taken when comparing studies because of differences in testing methodology. Specifically, it was reported that 62% (n = 107) of study isolates were resistant to doxycycline in Ontario (24), while in our study doxycycline resistance of isolates was 15%. One study in Australia and another in the United Kingdom (UK) found similarly high tetracycline resistance at over 50% and 35%, respectively (23,30). Since the time of this study, canine specific doxycycline breakpoints for S. pseudintermedius were proposed and implemented by CLSI (31,32). This change in zone diameter breakpoints would likely increase the number of doxycycline resistant isolates in both this study and the Ontario study. Similar trends can be observed where the proportion of resistant isolates in Atlantic Canada is much lower than those reported in Australia and the UK. A recent study completed at a Texas (USA) Veterinary Medical Teaching hospital reported amikacin resistance in 36% of their MRSP isolates from dogs, whereas in our study amikacin resistance was not detected (33). A systematic literature review of antimicrobial resistance in MRSP isolates reported individual antimicrobial resistance ranging between 0% and 100%, with most studies reporting AMR estimates > 50% for most antimicrobials, except for chloramphenicol and amikacin (28). Thus, it can be inferred that the MRSP isolates in Atlantic Canada are typically less resistant, specifically to doxycycline and amikacin, than their counterparts in some other regions, even though multi-drug resistance is still common. A possible explanation for this low-level resistance could be the low population density of the region as a

<table>
<thead>
<tr>
<th>Table III. Number (and percent) of methicillin-resistant Staphylococcus pseudintermedius (MRSP) isolates in each province overall and by dru cluster</th>
</tr>
</thead>
<tbody>
<tr>
<td>dru cluster</td>
</tr>
<tr>
<td>Overall (n = 94)</td>
</tr>
<tr>
<td>9a (n = 18)</td>
</tr>
<tr>
<td>10h (n = 27)</td>
</tr>
<tr>
<td>11a (n = 45)</td>
</tr>
<tr>
<td>No cluster (n = 4)</td>
</tr>
</tbody>
</table>

NB — New Brunswick; NL — Newfoundland and Labrador; NS — Nova Scotia; PEI — Prince Edward Island.
whole, when compared with larger, more densely populated regions, which could mean less exposure to AMR organisms and less total antimicrobial use.

The overall picture of MRSP in Atlantic Canada is similar to reports elsewhere in Canada and the world. The distribution of the dru types reported in this study is similar to other reports from North America; however, the distribution is more similar to what was observed in the US versus Ontario, Canada. The international MRSP clone, ST71, which is disseminated throughout Europe and made up almost half the isolates in Ontario, Canada, was less common in Atlantic Canada. This confirms that dru type distributions can vary significantly across the same country, and a larger, more comprehensive study of the dru types in Canada could help further clarify the dissemination of this pathogen. Multi-drug resistance in these isolates is common, especially within cluster 9a isolates, but resistance to the non-β-lactam antimicrobials is still considerably lower than has been previously reported.

**Acknowledgments**

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**References**


The impact of carboplatin and toceranib phosphate on serum vascular endothelial growth factor (VEGF) and metalloproteinase-9 (MMP-9) levels and survival in canine osteosarcoma

Tracy L. Gieger, Julie Nettifee-Osborne, Briana Hallman, Chad Johannes, Dawn Clarke, Michael W. Nolan, Laurel E. Williams

Abstract

In this pilot study, 10 dogs with osteosarcoma (OSA) were treated with amputation and subsequent carboplatin chemotherapy (300 mg/m² IV q3wk × 4 doses) followed by toceranib phosphate (2.75 mg/kg PO q48h starting at day 14 post carboplatin). Monthly clinical monitoring and serum measurements of vascular endothelial growth factor (VEGF) and matrix metalloproteinase-9 (MMP-9) were acquired. No dogs were removed from the study due to toxicity. Levels of VEGF and MMP-9 did not change over time. Seven dogs died related to local recurrence and/or pulmonary or bone metastasis and the remainder died of other causes. Median OSA-free survival was 238 d with 34% 1-year progression-free survival. Median overall survival was 253 d with 30% alive at 1.5 y and 10% alive at 2 y. Although this regimen was well-tolerated, survival times did not exceed previously published data from dogs treated with chemotherapy alone.

Résumé

Dans cette étude pilote, 10 chiens avec un ostéosarcome (OSA) ont été traités par amputation et chimiothérapie subséquente avec du carboplatin (300 mg/m² IV q3sem × 4 doses) suivi de phosphate de toceranib (2,75 mg/kg PO q48h débutant au jour 14 suivant le carboplatin). Un suivi clinique mensuel et une mesure des taux sériques du facteur de croissance de l’endothélium vasculaire (FCEV) et de la matrice de la métalloprotéinase-9 (MMP-9) ont été obtenus. Aucun chien ne fut retiré de l’étude pour cause de toxicité. Les niveaux de FCEV et MMP-9 n’ont pas changé dans le temps. Sept chiens sont décédés due à une rechute locale et/ou des métastases osseuses et les autres sont morts d’autres causes. La durée médiane de survie libre d’OSA était de 238 j avec 34 % de survie sans progression de la condition après 1 an. La durée de survie médiane était de 253 j avec 30 % en vie après 1,5 an et 10 % en vie après 2 ans. Bien que ce traitement ait été bien toléré, les temps de survie n’ont pas excédé les résultats publiés antérieurement pour des chiens traités par amputation et chimiothérapie uniquement.

(Traduit par Docteur Serge Messier)

Introduction

An estimated 10 000 cases of canine osteosarcoma (OSA) are diagnosed in pet dogs annually in the United States (1). It is a locally invasive and highly metastatic disease, with micrometastasis present in approximately 75% to 95% of dogs at the time of diagnosis, based on studies of survival following amputation (2,3). While surgery is indicated to remove the painful and functionally limiting primary tumor, amputation is considered palliative therapy and does not result in prolonged survival. Median survival times following amputation alone range from 102 to 168 d, with 1- and 2-year survival rates of 11% to 21% and 2% to 4%, respectively. The administration of adjuvant chemotherapy in an effort to delay or prevent metastasis and improve the outcome, is the current standard of care in veterinary medicine. Three drugs have been identified as having significant activity against osteosarcoma: cisplatin, carboplatin, and doxorubicin (2–4). Median survival times ranging from 290 to 324 d and 1-year survival rates of 45.5% have been reported with the administration of adjuvant cisplatin chemotherapy (3,4). Carboplatin chemotherapy is comparable, with a median survival of 321 d and 1-year survival of 35.4% (5). Similarly, the median survival time following a course of doxorubicin chemotherapy is 366 d with 1- and 2-year survival rates estimated at 50.5% and 9.7%, respectively (6).

A variety of strategies have been attempted in an effort to improve outcomes with chemotherapy. These include altering the time at which chemotherapy is initiated and using cisplatin or carboplatin in combination with doxorubicin (7,8). Unfortunately, these efforts have failed to demonstrate significant improvements in median survival times for dogs treated with chemotherapy alone.
survival times over those seen with conventional single-agent treatment protocols. Novel therapeutic approaches are needed to control metastatic disease and improve survival.

Angiogenesis is the process by which tumors induce new blood vessel formation and is essential for continued tumor growth and metastasis. In the absence of angiogenesis, tumors are restricted to sizes ranging from several microns up to 1 to 2 mm (9). Endogenous angiogenesis inhibitors have been detected in the urine of OSA tumor-bearing dogs and these factors demonstrated potent inhibition of endothelial cell proliferation (10). These endogenous factors were absent in urine collected from the same dogs 1 to 12 wk after surgical amputation of the limb bearing the primary tumor. The pro-angiogenic shift that occurs after primary tumor removal suggests that a strategy designed to pharmacologically control angiogenesis should be evaluated for its capacity to suppress the rapid progression of microscopic metastases to life-threatening metastatic disease. The use of angiogenesis inhibitors has emerged as a clinically valid therapeutic approach in human oncology. This strategy may also have applicability to veterinary oncology, and is particularly appealing for OSA where death is the result of progressive enlargement of metastatic foci.

Anti-angiogenic treatment strategies fall into 2 categories: i) those that are directly cytotoxic to endothelial cells, and ii) those acting indirectly by eliminating critical pro-angiogenic factors [e.g., vascular endothelial growth factor (VEGF)] and promoting anti-angiogenic factors. Vascular endothelial growth factor is a 45-kDa homodimer with pluripotent effects in angiogenesis — stimulating the proliferation, invasion, and migration of endothelial cells. In addition, VEGF is a key target of several angiogenic factors and proteases such as matrix metalloproteinases (MMPs), with roles in tumor metastasis (11). Matrix metalloproteinases are enzymes that degrade structural elements outside of cells and play a critical role in tissue remodeling during tumor invasion, angiogenesis, and metastasis. Among MMPs, MMP-9 is of particular interest. Matrix metalloproteinase-9 is induced via VEGF receptor (VEGF-R) activation and levels of this downstream factor may be useful as a surrogate marker of VEGF-R activity (12); however, there have been no studies directly correlating serum MMP-9 levels with VEGF-R activity in serum or tissues of OSA-bearing dogs.

Vascular endothelial growth factor is expressed by a variety of tumors, and therapies designed to decrease production or otherwise block VEGF activity are being explored as a means of inhibiting tumor proliferation. In an evaluation of 24 dogs with OSA, pretreatment platelet-corrected serum VEGF levels correlated significantly with disease-free interval (13). Results of this study and similar human studies support an important role for VEGF in the development and progression of metastatic disease in OSA. We hypothesize that the inhibition of VEGF activity will delay the progression of OSA micrometastases.

Toceranib phosphate [TP (Palladia; Zoetis, Durham, North Carolina, USA)] is an FDA-approved drug for use in dogs, is a selective inhibitor of several members of the receptor tyrosine kinase family, including vascular endothelial growth factor receptor (VEGF-R), KIT, FLT-3, and platelet-derived growth factor receptor (PDGFR-R). c-KIT wild type receptor overexpression was identified in a study of 74 human pediatric high-grade OSA, indicating a potential role of the inhibition of KIT signaling in OSA (14). While initially developed for its activity against KIT — a receptor mutated in approximately 30% to 50% of canine mast cell tumors (MCT) — the activity of TP against other members of this receptor tyrosine kinase family, such as VEGF-R, suggests a potentially wider spectrum of activity. Anti-tumor responses are reported in MCT lacking a KIT mutation, providing support for this theory and suggesting that other inhibited receptors, such as VEGF-R, play an important role in tumor progression and regression (15). Toceranib phosphate exerts antiproliferative effects on endothelial cells in vitro, suggesting a role in angiogenesis, and produces clinically meaningful disease stabilization in vivo in dogs with osteosarcoma (16–18). As part of a larger study of dogs with solid tumors being treated with TP, 11/23 (48%) dogs with metastatic OSA had stabilization of their disease (18). The oral dosing of TP on an alternate-day schedule ideally lends itself to chronic inhibition of tumor-promoted angiogenesis to prevent the progression of OSA micrometastases.

The goals of this study are: i) to evaluate the impact of maintenance antiangiogenic therapy using TP as a sole agent following amputation and carboplatin chemotherapy as a means of improving outcome for dogs with OSA, and ii) to measure circulating levels of endogenous pro-angiogenic factors before and during treatment with TP, which may serve as biomarkers for survival and disease progression. We hypothesize that the administration of TP following amputation and carboplatin chemotherapy will inhibit the progression of metastasis through disruption of receptor tyrosine kinases with subsequent inhibition of angiogenesis. This will lead to prolonged remission duration and survival in dogs with appendicular OSA compared to rates previously achieved in a group of dogs treated with amputation and carboplatin alone (5). We hypothesize that the administration of TP following amputation and carboplatin chemotherapy will decrease circulating levels of pro-angiogenic MMP-9, a downstream factor and potential surrogate marker of VEGF-R inhibition in dogs with appendicular OSA.

**Materials and methods**

Ten dogs with histologically confirmed appendicular OSA with no radiographic evidence of pulmonary metastasis at the time of enrollment and for whom owner consent was given, were prospectively enrolled. Institutional IACUC approval was obtained. All dogs were evaluated with a physical examination, complete blood (cell) count (CBC), serum biochemistry panel, urinalysis, 3-view thoracic radiographs, and a quality-of-life (QOL) survey completed by the owner prior to treatment. Two weeks following amputation, carboplatin (Paraplatin; Bristol Myers Squibb, New York, New York, USA) chemotherapy was administered intravenously at a dosage of 300 mg/m² at 3-week intervals for a total of 4 treatments. A CBC was performed immediately before each carboplatin treatment and 7 d and 14 d following the first 2 treatment cycles to monitor for potential hematologic toxicity. A 25% dosage reduction was prescribed for any patient developing grade 3 or higher bone marrow or gastrointestinal toxicity as defined by the Veterinary Co-operative Oncology Group Common Terminology Criteria for Adverse Events (VCOG-CTCAE) v1.0 (19). Three-view thoracic radiographs were repeated prior to the third carboplatin chemotherapy treatment and
immediately before initiation of TP. Two weeks following the final carboplatin chemotherapy treatment, patients began TP therapy at a dosage of 2.75 mg/kg body weight (BW), PO, q48h. This drug was to be continued indefinitely unless evidence of toxicity, development of concurrent co-morbidities, death, or owner withdrawal of the pet from the study, occurred. Dogs that developed local recurrence or metastasis were censored from statistical analysis but were allowed to continue to receive TP if the owner elected to do so; owners were also counseled about all other potential treatment options outside of the study. To minimize the likelihood of toxicity, all dogs received omeprozole (Prilosec 0.5 to 1.0 mg/kg BW, PO, q24h; AstraZeneca Pharmaceuticals, Stockholm, Sweden) or famotidine (Pepcid 0.5 to 1.0 mg/kg BW, PO, q12-24h; McNeil Consumer Pharmaceuticals, Fort Washington, Pennsylvania, USA) and, if needed, metoclopramide (0.2 to 0.4 mg/kg BW, PO, q8h) and/or maropitant (Cerenia 2 mg/kg BW, PO, q24h × consecutive 5 d each week; Zoetis, Parsippany-Troy Hills, New Jersey, USA). To avoid confounding variables, NSAIDs were discontinued for the duration of the study period. Patients requiring ongoing analgesia received tramadol (1 to 4 mg/kg BW, PO, q6-12h), amantadine (3 to 5 mg/kg BW, PO, q24h), and/or gabapentin (5 to 10 mg/kg BW, PO, q8-12h).

**Sample collection**

Serum samples for enzyme-linked immunosorbent assay (ELISA) evaluation of VEGF and MMP-9 were collected for analysis: i) immediately before initiation of TP; ii) immediately prior to initiation of TP; and iii) 3 and 6 wk after starting TP; and iv) every 8 wk thereafter for 18 mo or until the time of disease progression if this occurred at less than 18 mo.

**Patient monitoring**

Disease status was monitored by a physical examination and 3-view thoracic radiographs at 8-week intervals until disease progression was documented. To monitor for potential TP-related toxicity, CBC and body weight were evaluated weekly for the first 6 wk and a chemistry panel and urinalysis were also performed at weeks 1, 3, and 6. Subsequently, a CBC, body weight, chemistry panel, and urinalysis were performed at 8-week intervals. Quality-of-life surveys were also submitted by pet owners at each of these time points. Toxicity was recorded at each time point. A 1-week cessation of TP therapy followed by incremental dosage reductions (0.5 mg/kg BW increments to a minimum of 2.2 mg/kg BW) and/or decreased TP administration frequency (from q48h to Monday, Wednesday, and Friday schedule) was instituted for any dog experiencing > grade 2 adverse event(s).

**Sample analysis**

Approximately 7 to 10 mL of whole blood was collected into serum and plasma separator tubes by jugular venipuncture. Immediately after collection, blood samples were centrifuged and the serum frozen at −70°C until assayed. Commercially available ELISA test kits were used for VEGF (CAVEOO Canine VEGF Quantikine ELISA Kit; R&D Systems, Minneapolis, Minnesota, USA) and MMP-9 (SEA553CA ELISA Kit for MMP9 Organism species; Cedarlane Labs) and tests were performed according to the manufacturers’ directions. To minimize the impact that VEGF released from platelets contributed to serum VEGF levels, serum VEGF levels were corrected for platelet counts as follows: serum VEGF (pg/mL)/platelet count (× 10⁶/μL) (20,21).

**Statistical analysis**

A sample size of 10 dogs provides a power of 80% and a 50% level of significance to detect a 47% increase in 1-year survival (i.e., increase the probability of survival at 1 yr, from 34.5% to 50.7%) in dogs with appendicular OSA treated with carboplatin chemotherapy followed by TP following amputation. The historical control consisted of a previously reported group of 48 dogs treated with amputation and 4 cycles of carboplatin chemotherapy with a median survival of 321 d and 1-year survival of 34.5% (5). Osteosarcoma-free survival time was defined as the date of amputation to the date of documentation of local recurrence or OSA metastasis. Overall survival (OS) was defined as the date of amputation until death from any cause. A commercially available statistical program was used (GraphPad Prism 6; San Diego, California, USA). The Kaplan-Meier method was used to calculate OSA-free survival and overall survival (OS). A Friedman’s test was used to compare VEGF and MMP values at baseline, pre-TP, and end of study. A Kruskal-Wallis test of repeated measures was used to compare platelet-corrected VEGF values at baseline, pre-TP, and end of study. P < 0.1 was considered significant. A P-value = 0.1 was selected due to the small sample size to try to avoid type II error.

**Results**

Median age was 9.5 y (range: 5 to 11 y). Median body weight was 31 kg (range: 19 to 48 kg). Seven were spayed females and 3 were castrated males. There were 3 golden retrievers, 1 mixed breed dog, and 1 of each of the following breeds: Belgian tervuren, great Dane, border collie, Rottweiler, Labrador retriever, and St. Bernard. Tumor locations included the distal radius (n = 5), distal femur (n = 2), distal tibia (n = 2), and proximal humerus (n = 1). No patients had CBC abnormalities prior to treatment. Serum biochemical abnormalities included increased alkaline phosphatase in 2 dogs [726 and 164 IU/L (normal < 140)] and in one dog, increased BUN [67 mg/dL (normal < 26)], creatinine [3 mg/dL (normal < 1.5 mg/dL)], phosphorous [6.1 mg/dL (normal < 5.6 mg/dL)], and potassium [5.8 mmol/L (normal < 5.6 mmol/L)] (in this dog, the urine specific gravity was 1.018). Seven dogs were treated with NSAIDs prior to being enrolled in the study for a variable duration of time; these drugs were discontinued on the first day of chemotherapy in 3 dogs and 3 to 35 d prior to initiation of chemotherapy in the remaining dogs.

**Chemotherapy administration**

Carboplatin chemotherapy was started a median of 16 d post-amputation (range: 11 to 30 d). Eight dogs received the first dose of carboplatin at 300 mg/m²; 1 dog received 239 mg/m² (unknown reason; given at RDVM) and the dog with renal insufficiency received 150 mg/m² (arbitrary dose reduction due to renal insufficiency). Four dogs required dose reductions of carboplatin due to neutropenia; no dogs required hospitalization for supportive care. The carboplatin dose was reduced from 300 mg/m² to 225 mg/m²...
after the first dose in 3 dogs and after the second dose in 1 dog (who also had a 1-week treatment delay due to prolonged neutropenia).

**Toceranib phosphate administration**

Eight dogs received TP at doses ranging from 2.2 to 2.9 mg/kg (median dose: 2.7 mg/kg). After starting TP, 1 dog had two 1-week drug holidays related to grade I diarrhea and 1 dog had dose reductions and an extended dosing interval due to persistent, non-progressive grade I neutropenia.

**Toxicity outcomes**

No dogs were removed from the study due to toxicity or declining QOL. Quality-of-life surveys were subjectively scored, and scores remained stable throughout the study (scores did not decline from baseline after starting carboplatin or TP) until the patient experienced a clinical decline typically related to disease progression.

**Tumor-related outcomes**

Seven dogs developed metastasis as the first event. Pulmonary metastasis was suspected based on thoracic radiographs in 6 dogs 60 to 240 d post-amputation (median 180 d) and osseous metastasis to the vertebrae and ribs was suspected in one dog based on radiographs at 575 d after amputation. One dog that developed pulmonary metastasis also concurrently developed an amputation-site stump recurrence 180 d post-amputation. Two of 10 (20%) dogs developed pulmonary metastasis while receiving carboplatin chemotherapy and did not continue on to the TP phase of the study. These 2 dogs were included in outcome analysis on an intent-to-treat basis. Of the 8 dogs that received TP, 3 died of metastasis (pulmonary in 2 dogs, osseous in 1 dog), 1 died of concurrent pulmonary metastasis and stump recurrence, 3 died of other causes [lymphoma that was diagnosed during the study while the dog was receiving TP (n = 1), metastatic hemangiosarcoma that developed while the dog was receiving TP (n = 1), and progressive spinal pain with no definitive diagnosis despite and extensive workup (n = 1)], and 1 dog was withdrawn from the study per the owner’s request with no evidence of OSA (n = 1). Toceranib phosphate was continued in 3 dogs at the owners’ request after metastasis was detected (dogs were censored from analysis on the date metastasis was diagnosed) and these dogs remained on the drug until death. An additional dog received treatment with doxorubicin after pulmonary metastasis was detected. All dogs were followed until the time of their death and all are deceased. Only 1 dog underwent necropsy.

**Survival data**

The median OSA-free survival was 238 d (range: 50 to 575 d) with 34.3% progression-free survival at 1 y (Figure 1). Overall survival was 253 d with 30% alive at 1.5 y and 10% alive at 2 y (Figure 2).

**VEGF and MMP-9 data**

Pre-treatment serum VEGF levels ranged from 0.8 to 20.4 (median: 6.5) and MMP-9 levels ranged from 8.9 to 25.2 (median: 12), with wide variations in intra- and inter-patient variability. There was no significant difference in MMP (Figures 3 and 5) or VEGF [absolute (Figures 4 and 6) or platelet-corrected] values from baseline and pre-TP, baseline and TP discontinuation, or pre-TP to TP discontinuation.

**Discussion**

In this study, continuous administration of TP was well-tolerated in dogs with OSA treated with amputation and adjuvant carboplatin chemotherapy, although a survival benefit was not shown. In the present study, OS was 253 d with 30% alive at 1.5 y post-amputation, which is comparable to a historical group treated with amputation and adjuvant carboplatin chemotherapy in which OS was 321 d with 35% alive at 1 y (5). To date, 2 previously published studies of dogs with OSA treated with carboplatin chemotherapy and metronomic chemotherapy (MC) have failed to demonstrate improved survival times versus historical publications of dogs treated with carboplatin alone. In one publication, as part of a larger study of dogs with OSA treated with chemotherapy + MC [piroxicam and oral cyclophosphamide (10 to 12 mg/m² per day)], 14 dogs were treated with carboplatin concurrent with MC (22). Three dogs were removed from the study due to toxicity (gastrointestinal). The overall
The lack of improvement with post-chemotherapy MC (including TP in some dogs) was also confirmed in a recent, larger study (23). One hundred twenty-six dogs with appendicular OSA were treated with amputation followed by 4 doses of carboplatin chemotherapy and then randomized to receive MC [piroxicam/cyclophosphamide (10 mg/m² q48h)] with or without addition of TP [MC + TP (2.75 mg/kg PO q48h)]. Thirty-two dogs developed metastasis before the onset of MC or MC + TP, and a total of 35 received MC and 46 received MC + TP. Toxicities were greater in the MC + TP group, but most were mild and resolved with supportive care. The median DFI was 215 and 233 d and the OS was 242 and 318 d, respectively, for the MC versus MC + TP groups, which did not differ significantly. The 1-y survival rate was 35% and 38% for the MC versus MC + TP groups, respectively.

Although MC administration has not yet been shown to improve outcomes in dogs with OSA when used in combination with traditional maximally tolerated dose (MTD) chemotherapy, its potential usefulness in highly metastatic diseases such as OSA, continues to be explored. In an attempt to further elucidate the tolerability and mechanism of action of MC, a recent study evaluated toxicity and lymphocyte profiles in tumor-bearing dogs treated with MTD doxorubicin (MTD-dox) only and those treated with MTD-dox alone and in combination with metronomic cyclophosphamide (MCTX) (24). A phase I study was performed and established that the standard doxorubicin dose of 30 mg/m² (IV q21d) and a previously published MC dose of cyclophosphamide of 15 mg/m² per day was tolerable when administered concurrently. Dogs were then randomized to receive MTD-dox (n = 8) or MTD-dox + MCTX (n = 8). Both treatment groups showed declines in both total and
T-regulatory lymphocytes (Tregs) by day 7 after MTD-dox, and there was no statistically significant difference between the groups in either total lymphocyte count or Tregs. In contrast to previous studies demonstrating that Tregs were selectively inhibited by CTX (25), there was no selective depletion of Tregs. The authors theorized that concurrent administration of MTD-dox and MC may not be beneficial if the MTD chemotherapy dampens the inhibition of the selective Treg depletion seen with MCTX alone. Although this study used doxorubicin and the current study as well as a previously published larger study used carboplatin, this theory could explain why MTD carboplatin + MC did not yield prolonged survival times in dogs with OSA. Other potential mechanisms of efficacy of MC include other immunomodulatory effects (e.g., restoring NK effector function), anti-angiogenic effects [including decreased tumor microvessel density and decreased mobilization of circulating endothelial precursors (CEPs)], inhibition of normal stromal cells that support growing cancer cells, and possibly direct anti-tumor effects. The use of NSAIDs in MC can serve as a confounding factor when comparing studies since they inhibit COX-2, which may result in an inability of CEPs to survive and proliferate within the tumor microenvironment (24); therefore, the contribution of metronomic chemotherapy drugs such as cyclophosphamide to the outcome can be difficult to determine when NSAIDs are used concurrently. It is likely that the full potential of MC in addition to MTD chemotherapy for OSA will only be realized with clinical trials that compare use of standardized MTD chemotherapy and MC regimens both concurrently and sequentially.

One aspect of the present study that is unique, is the attempt to quantify the degree of antiangiogenesis via measurement of serum MMP-9, VEGF, and platelet-corrected VEGF levels at baseline (post-amputation), pre-TP (after 4 cycles of carboplatin), over time during TP administration, and at the time when TP was discontinued. We anticipated changes in VEGF and MMP-9 levels concomitant with changes in disease status. If changes were repeatable and consistent among the study dogs, these serum markers may have served as surrogate markers for the remission status of OSA. In a study of dogs with OSA treated with amputation, pre-amputation platelet-corrected VEGF was significantly correlated with DFI (13). In a study of dogs treated with metronomic chemotherapy (NSAID + cyclophosphamide) (26) for various metastatic tumors, VEGF levels were measured before (but not during) treatment, and dogs with lower VEGF levels had longer survival times and were more likely to respond to treatment than dogs with levels above the median. Unfortunately, in the current group of dogs, the MMP-9 and VEGF (absolute or platelet-corrected) values varied widely both inter- and intra-patient and there was no statistical correlation of the values at any time point. A limitation to this study is the lack of serum to test these factors before amputation because most dogs were referred for treatment and subsequent enrollment in the study after amputation had been performed. In addition to obtaining pre-treatment serum samples, ideally, all of the tumors would have been reviewed and graded by a single pathologist. This was a pilot study, and as a result, conclusions are inherently limited due to small sample size and lack of a contemporaneous control group. Failure to reliably assay angiogenesis biomarkers in this study show that other measures are needed to determine subclinical implications of anti-angiogenic therapy in dogs. To our knowledge, this is the first study evaluating the use of single-agent TP (without other concurrent MC drugs such as NSAIDs and/or cyclophosphamide) after amputation and carboplatin chemotherapy in dogs with OSA. Although there was no improvement in outcome compared to historical controls, further work evaluating the use of TP in dogs with OSA should be conducted. Since it has been shown that dogs with gross metastasis from OSA can have stable disease and a clinical benefit from treatment with TP (18), it is likely that using TP in a microscopic disease setting before the onset of visible metastasis may be beneficial.

In conclusion, the combination of carboplatin followed by TP was well-tolerated by dogs with appendicular OSA. There was inter- and intra-patient variability of VEGF and MMP-9 levels at all time points and survival times did not differ from previously published data from dogs treated with amputation and adjuvant chemotherapy. Further larger studies to devise more effective therapeutic options for this disease are warranted.

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References


Oxidative stress and food supplementation with antioxidants in therapy dogs

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Abstract

The objective of this study was to evaluate the ability of a long-term antioxidant-supplemented diet to regulate the oxidative stress and general health status of dogs involved in animal-assisted intervention (AAI) programs. Oxidative stress is a consequence of the accumulation of reactive oxygen species (ROS). Exercise-induced oxidative stress can increase muscle fatigue and fiber damage and eventually leads to impairment of the immune system. A randomized, placebo-controlled, crossover clinical evaluation was conducted with 11 healthy therapy dogs: 6 females and 5 males of different breeds and with a mean age of 2.7 ± 0.8 y (mean ± SEM). The dogs were divided into 2 groups, 1 fed a high quality commercial diet without antioxidants (CD) and the other a high quality commercial diet supplemented with antioxidants (SD) for 18 wk. After the first 18 wk, metabolic parameters, reactive oxygen metabolite-derivatives (d-ROMs), and biological antioxidant potential (BAP) levels were monitored and showed a significant reduction of d-ROMs, triglycerides, and creatinine values in the SD group (P < 0.05) and a significant increase in amylase values in the CD group (P < 0.01). At the end of this period, groups were crossed over and fed for another 18 wk. A significant decrease in amylase and glutamate pyruvate transaminase (GPT) values was observed in the CD and SD group, respectively (P < 0.05). In conclusion, a controlled, balanced antioxidant diet may be a valid approach to restoring good cell metabolism and neutralizing excess free radicals in therapy dogs.

Résumé

L'objectif de la présente étude était d'évaluer la capacité d'une diète long-terme supplémentée en antioxidant à réguler le stress oxydatif et l'état de santé général de chiens impliqués dans des programmes d'intervention avec assistance animale (IAA). Le stress oxydatif est une conséquence de l'accumulation d'espèces oxygène réactive (EOR). Le stress oxydatif induit par l'exercice peut augmenter la fatigue musculaire et les dommages aux fibres et éventuellement mener à un mauvais fonctionnement du système immunitaire. Une évaluation clinique croisée, randomisée, et avec groupe témoin-placebo a été menée avec 11 chiens d’assistance en santé: 6 femelles et 5 mâles de races différentes et d’un âge moyen de 2.7 ± 0.8 ans (moyenne ± écart-type). Les chiens ont été divisés en deux groupes, un premier groupe nourri avec une diète commerciale de haute qualité sans antioxidant (DC) et l’autre groupe avec une diète commerciale de haute qualité supplémentée avec des antioxydants (DS) pour 18 semaines. Après les premières 18 semaines, les paramètres métaboliques, les métabolites dérivés d’oxygène réactive (MDOR), et les niveaux de potentiel antioxydant biologique (PAB) ont été surveillés et ont montré une réduction significative des valeurs des MDOR, des triglycérides et de la créatinine dans le groupe DS (P < 0.05) et une augmentation significative des valeurs de l'amylase dans le groupe DC (P < 0.01). À la fin de cette période, les groupes ont été croisés et nourris pour 18 semaines supplémentaires. Une diminution significative des valeurs de l'amylase et de la glutamate pyruvate transaminase (GPT) a été obtenue dans les groupes DC et DS, respectivement (P < 0.05). En conclusion, une diète contrôlée, équilibrée en antioxidant pourrait être une approche valide pour restaurer un bon métabolisme cellulaire et neutraliser les radicaux libres excédentaires chez les chiens d’assistance.

Introduction

Oxidative stress is a consequence of the accumulation of reactive oxygen species (ROS), reactive nitrogen species (RNS), such as hydroxyl radicals (OH·), superoxide anion radicals (O2·), lipid per-oxyl radicals (LOO·), and nitrate radicals (NO·) (1). Factors involved in the production of free radicals and reactive metabolites include physical exercise, characterized by an increase in the body’s oxygen consumption and correlated to an increase in ROS production, which causes oxidative stress (2-4). It has been shown that exercise-induced oxidative stress contributes to increased muscle fatigue, muscle fiber damage (5), and eventually leads to immune system impairment (6). Both muscles and blood are endowed with antioxidant enzymes, such as superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase (CAT), and non-enzymatic antioxidants, such as vitamins A, C, and E, to counteract damage caused by free radicals (2,7,8).

Oxidative stress induced by physical exercise has been reported in horses (9-11) and dogs (12,13). One study investigated the...
oxidative status of 9 dogs after 20 min and 4 h of aerobic exercise, after 30 d of rest (14). Results showed a significant oxidative status due to the high level of reactive oxygen metabolite-derivatives (d-ROMs) and the low level of biological antioxidant potential (BAP) in relation to the values before starting the exercises ($P < 0.01$) (14).

Ethane exhaled in the breath may be considered a marker of oxidative stress as it is a product of free radical-mediated oxidation of cell membrane lipids (15). The authors evaluated oxidative stress in terms of exhaled ethane in 8 human, 12 dog, and 11 equine athletes after a physical performance (15). A significant increase in exhaled ethane was observed in all species after training and was associated with oxidative stress.

Evaluation of oxidative status with specific parameters could become an interesting and practical method of monitoring the activity of working dogs. Indeed, it is difficult to quantify ROS in practice because of their very short half-life and it requires complex techniques over a long period of time. Due to their high reactivity, ROS react with practically every organic molecule they meet, producing reactive oxygen metabolites (ROMs), which are more stable than the ROS and are therefore easier to quantify. Conversely, BAP matches the total antioxidant capability of plasma and includes either exogenous (ascorbate, tocopherols, carotenoids) or endogenous (protein, GPx, SOD, CAT) components that can oppose the oxidant action of reactive species (16).

In conditions of oxidative stress, a controlled, balanced antioxidant diet may be a valid approach to restoring good cell metabolism and neutralizing excess free radicals. Two studies in dogs and rabbits demonstrated an increase in tissue oxidative stability by supplementing with a diet enriched with different antioxidants (17,13). The effect on exercise-induced oxidative stress of supplementing with oral vitamin E (11 to 14 mg) for 10 wk was studied in 8 dogs and induced a significant reduction in paroxonase-1 activity, erythrocyte membrane fluidity, and vitamin E and a significant increase in plasma malondialdehyde levels (18). After 10 d of vitamin E supplementation, these modifications were significant in the placebo group, but not in the supplemented group.

The objective of this study was to ascertain the ability of a long-term antioxidant-supplemented diet to regulate the oxidative stress and general health status of working dogs involved in animal-assisted intervention (AAI) programs.

### Materials and methods

Operative procedures and animal care were carried out in compliance with national and international regulations (Italian regulation D.L. vo 116/1992 and European Union regulation 86/609/EC). The recommendations of the Consolidated Standards of Reporting (CONSORT) 2010 statement on randomized controlled trials were also consulted and considered (19).

### Subjects

Eleven therapy dogs (6 females and 5 males) of different breeds (1 Ciniacco dell’Etna, 6 Labrador retrievers, 3 mixed breed, and 1 pit-bull), aged 2.7 ± 0.8 y and with a body weight of 22.12 ± 8.43 kg (mean ± SEM), were enrolled in this clinical evaluation. Before the recruitment, laboratory examinations (hemogram, biochemical profile, urine analysis, and serum protein electrophoresis) and serological tests (Ehrlichia canis, Rickettsia spp., Leishmania infantum, Anaplasma phagocytophilum, Bartonella spp., Toxoplasma gondii, Neospora caninum, and Borrelia burgdorferi) were conducted on all dogs to confirm their good health status. Owner consent was obtained for each dog.

All dogs were enrolled in 5 weekly (30 min each) animal-assisted intervention (AAI) sessions in the rehabilitation or social care of humans. All sessions were held in the same place and at similar temperature and humidity conditions. Patients involved in AAI sessions consisted of 55 children with pervasive developmental disorders. Each 30-minute session consisted of 15 min of active playing, such as ball throwing or running, 10 min of collaborative activities, such as the child and the dog looking for a hidden object together, and 5 min of dog-care activities, such as feeding and brushing the dog.

### Diets

The dogs were fed 2 varieties of dry dog food: Natural Trainer Adult Medium and Personal Trainer Long Life Adult Medium, which is supplemented with antioxidants (both from Nova Foods, Vicenza, Italy). The Natural Trainer Adult Medium diet consisted of fresh chicken and turkey meat, corn, rice, dehydrated chicken and turkey meat, lard, dehydrated pork meat, flax seed, beet, corn oil, dehydrated fish flour, chicory extract, fructooligosaccharides, brewer’s yeast, pea fiber, Spirulina algae (*Arthrospira platensis*), ribonucleotides extract from yeast, green-lipped mussels (*Perna canaliculus*), apple dried extract, black raspberry extract, salt, chloride-choline, methionine, vitamin E, vitamin A (18 000 UI/kg), vitamin D3 (1.350 UI/kg), vitamin E (265 mg/kg), and copper (20 mg/kg).

The Personal Trainer Long Life Adult Medium dog food consisted of corn, dehydrated chicken and turkey meat, fresh chicken and turkey meat, rice, corn oil, lard, hydrolyzed proteins of chicken, beef, corn gluten, flax seeds, dehydrated fish flour, brewer’s yeast (0.50%), sodium chloride, pea fiber, wood cellulose, calcium carbonate, fruit oligosaccharides, broccoli dried extract, green-lipped mussels (*Perna canaliculus*) (0.05%), Vitaberry Plus (grape seed extract, quercetin, blueberry dried extract, resveratrol, and strawberry and blackberry dried extracts), and silicon dioxide (0.025%).

### Experimental design

Dogs were randomly divided into 2 groups using a dedicated software (Research Randomizer) (20). The first group ($n = 5$) received a high quality commercial control diet (CD) without antioxidant (Natural Trainer Adult Medium, Nova Foods), while the second group ($n = 6$) received a high quality commercial diet (SD) supplemented with antioxidants (Personal Trainer Long Life Adult Medium, Nova Foods) for 18 wk. At the end of this period, a crossover design of the same duration was conducted.

### Sample collection

Blood samples were collected from each dog at the beginning of the evaluation and at the end of the first experimental period after 18 wk. The same procedure was carried out before and at the end of the crossover period. Blood samples were collected from the cephalic vein and stored in 2 tubes, one with heparin and the other without anticoagulant. Heparinized plasma samples and serum samples were obtained by blood centrifugation at 6000 $\times$ g for 1.5 min at 37°C.
Metabolic profile evaluation

Ten milliliters of blood were collected from the cephalic vein for the oxidative stress assessment. After blood centrifugation in heparinized test tubes, the plasma was separated from the serum and analyzed. Oxidative stress was measured with a spectro-photometric point-of-care assay (Free Radical Analytical System FRAS 4; Evolvo, Langhirano, Italy). The dROMs test and the BAP test were completed. In the dROMs test, reactive oxygen metabolites (primarily hydroperoxides) of the sample generated alkoxyl and peroxyl radicals, in the presence of iron released from plasma proteins by an acidic buffer, according to the Fenton reaction. Such radicals then oxidized an alkyl-substituted aromatic amine (N,N-dietylparaphenyldiamine), thus producing a pink-colored derivative, which is photometrically quantified at 505 nm. The concentration of the dROMs is directly proportional to the color intensity and is expressed as U CARR (Carratelli units). One U CARR corresponds to 0.08 mg/dL hydrogen peroxide. In normal dogs, dROM values range from 50 to 90 U CARR.

In the BAP test, the plasma samples were mixed with a colored solution obtained by mixing a ferric chloride solution with a thiocyanate derivative solution that causes a discoloration, the intensity of which is measured photometrically at 505 nm and is proportional to the ability of the plasma to reduce ferric ion (16). The results are expressed as μmol/L of reduced ferric ions. In normal dogs, BAP values range from 2400 to 2200 μmol/L. The reference values of dROMs and BAP test were validated for canine species (14,21) and are summarized in Table I.

A metabolic profile was also investigated for each dog, including albumin, alkaline phosphatase (ALP), total bilirubin, calcium, cholesterol, creatine phosphokinase (CPK), creatinine, phosphorus, gamma-glutamyl transferase (GGT), glucose, aspartate aminotransferase (GOT), glutamate pyruvate transaminase (GPT), total proteins, triglycerides, and urea (Dimension RxL Max Integrated Chemistry System; Siemens Healthcare Diagnostics, Milan, Italy).

Statistical analysis

Data were analyzed using GraphPad Prism 6 software (GraphPad Software, La Jolla, California, USA). All data are presented as the means ± SEM and were first checked for normality using the D’Agostino-Pearson normality test. Differences in dROMs, BAP, and metabolic profile parameters between the 2 diets before (T0) and at the end of the crossover period were analyzed using a 2-way analysis of variance (ANOVA), followed by Sidak’s multiple comparisons test.

| Table I. Reference values of dROMs (1 U Carr = 0.08 mg H2O2/dL) and BAP test |
|-----------------------------------------------|-----------------------------------------------|
| dROMs reference values | BAP reference values |
| Normal values | 50 to 90 U CARR | Optimal values | 2400 to 2200 μmol/L |
| Threshold borderline | 92 to 95 U CARR | Threshold borderline | 2200 to 2000 μmol/L |
| Mild oxidative stress | 100 to 120 U CARR | Slight deficiency | 2000 to 1800 μmol/L |
| Oxidative stress | 140 to 200 U CARR | Deficiency | 1800 to 1600 μmol/L |
| Strong oxidative stress | 220 to 300 U CARR | Strong deficiency | 1600 to 1400 μmol/L |
| Strong oxidative stress | Over 300 U CARR | Very strong deficiency | < 1400 μmol/L |

Both diets were similar in chemical composition and fatty acid profile. The predominant fatty acids of the lipid fraction in both diets were palmitic acid (C16:0) and oleic acid (C18:1 c9), which were present in similar proportions (22% for palmitic acid and 35% for oleic acid). The amount of linolenic acid (C18:3 n3) was higher (+28%) in the experimental than in the control diet.

The metabolic profile trend of the CD and SD group during the evaluation period is shown in Figure 1. Amylase concentration significantly increased from a T0 value of 778 ± 32.10 U/L to 1059 ± 82.75 U/L at T1 in the CD group (P < 0.01) (Figure 1C). Conversely, creatinine and triglycerides decreased significantly in the SD group from a T0 value of 1.22 ± 0.06 mg/dL to 1.00 ± 0.06 mg/dL at T1 and from a T0 value of 164.6 ± 55.73 mg/dL to 60.20 ± 5.91 mg/dL at T1, respectively (P < 0.05) (Figures 1H to P).

The concentration of dROMs and BAP decreased significantly from a T0 value of 142.6 ± 13.86 U CARR to 111.4 ± 7.97 U CARR at T1 in the SD group and from a T0 value of 3332 ± 177.5 μmol/L to 2954 ± 58.14 μmol/L at T1 in the CD group, respectively (P < 0.05) (Figure 2).

At the end of this period, groups were crossed over and fed for another 18 wk and the metabolic profile was reassessed (Figure 3). Amylase and GPT concentration significantly decreased from a T0 value of 1059 ± 82.75 U/L to 859.2 ± 61.02 U/L at T1 in the CD group and from a T0 value of 70.20 ± 10.83 U/L to 50.60 ± 5.38 U/L at T1 in the SD group, respectively (P < 0.05) (Figures 3C to I). Although no significant variation of dROMs was observed, a decrease in the CD group and an increase in the SD group was observed at the end of the crossover period, probably due to the antioxidant activity of the diet (Figure 4A). Similar to what was observed in the first evaluation, a decrease in BAP concentration was observed in the CD group (Figure 4B).

Discussion

The objective of this study was to evaluate the potential ability of a long-term antioxidant-supplemented diet to control the oxidative stress and general health status of therapy dogs involved in AAI programs. Environmental stress, such as temperature, working, and dog competitions may induce psychological and physical stress, which could also influence the food intake of dogs, whether it is excessive or not enough.

An unbalanced diet lacking in essential nutrients weighs on the general health status of dogs and may represent a risk factor for...
Figure 1. The metabolic profile trend of the commercial diet (CD) and supplemental diet (SD) groups during the evaluation period (continues on next page).
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metabolic and degenerative diseases. Among the mechanisms that can influence health status, the balance of oxidative stress plays a relevant role. Metabolic oxidative reactions constantly take place in animals in order to balance the production of free radicals with antioxidant molecules.

The significant reduction ($P < 0.05$) of dROMs and BAP values observed in dogs fed the SD diet during the first experimental period clearly demonstrated the ability of the diet to reduce oxidative stress, thus indicating a positive trend towards the normal values observed for healthy dogs (14). Conversely, the significant reduction observed in the CD-fed group confirmed the lack of any antioxidant effect related to the diet. It is worth noting that, while a long period of washout was not observed, the overall trend of BAP and dROMs improved after SD supplementation, although slightly influenced by the previous diet assumption.

On the other hand, the reduction of the amylase enzyme after the crossover could be related to the inhibitory activity of antioxidant molecules on key enzymes linked to type-2 diabetes ($\alpha$-amylase and $\alpha$-glycosidase) as previously observed in in-vitro studies by using flavonoid, anthocyanin, and other polyphenol compounds (22–25). The decrease in GPT level after the crossover suggests that the SD diet is endowed with liver-protective properties as reported in studies where different polyphenol extracts were tested in laboratory animals (26,27). As a result, the beneficial effects of antioxidant supplementation on liver and pancreatic metabolism were demonstrated.

The antioxidant formulation used in this study was based on grape seed extract, quercetin, blueberry, resveratrol, and strawberry and blackberry dried extracts (Vitaberry Plus). All these compounds contain anthocyanins and polyphenols, which have antioxidant effects. The total proanthocyanidin content of grape seed extract also included catechins and epicatechins and possesses antioxidant properties and protects the vascular system (28). The present study clearly showed a decrease in dROM species after the administration of a specific diet enriched with antioxidants. This scavenger activity was in agreement with other studies about the antioxidant effects of single active ingredients included in antioxidant supplements. For example, it has been demonstrated that supplementing with grape seed extract decreased the activity of the oxidative stress-responsive transcription factors NF-$\kappa$B and Nrf2 in the duodenal mucosa of pigs (29).

In a similar study, grape seed extract significantly reduced (by 60%) the formation of azoxymethane-induced aberrant crypt foci, probably due to its activity in reducing the levels of cyclin D1, COX-2, and iNOS involved in the foci genesis (30). While grape seed and blueberry extract are endowed with antioxidant activity, quercetin supplementation is known to prevent age-related diseases in dogs (31). Moreover, the anthocyanins and polyphenols in blueberry extract have been shown to exert antioxidant effects in...
Figure 3. Graphical representation of metabolic profile of dogs. A significant decrease in GPT concentration was observed after SD diet supplementation (*P < 0.05). (A) albumin, (B) alkaline phosphatase, (E) calcium, (G) creatine phosphokinase, (I) Gamma-glutamyl transpeptidase, (J) glucose, (K) Glutamic-oxaloacetic transaminase, (L) Glutamic — pyruvic transaminase, (O) total protein (continues on next page).
Figure 3. Graphical representation of metabolic profile of dogs. A significant decrease in GPT concentration was observed after SD diet supplementation (3L, *P < 0.05). (A) albumin, (B) alkaline phosphatase, (E) calcium, (G) creatine phosphokinase, (I) Gamma-glutamyl transpeptidase, (J) glucose, (K) Glutamic-oxaloacetic transaminase, (L) Glutamic — pyruvic transaminase, (O) total protein (continues on next page).
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Figure 4. Graphical representation of oxidative stress parameters concentration of dogs. A — dROMs values and B — BAP before (T0) and after 18 weeks (T1) of diets supplementation.

some pathological conditions, such as hypobaric hypoxia, due to the inhibition of lipid peroxidation by interfering with glutathione activity (32,33). The antioxidant levels of sled dogs supplemented with blueberries were studied and blood creatine kinase levels and oxidative status were evaluated 48 h after exercise (34). Although the exercise protocol did not influence concentrations of creatine kinase, supplementation with blueberries significantly increased the total antioxidant status after exercise. In addition to being an antioxidant, blueberry extract has also demonstrated genoprotective and behavioral effects (35). Specifically, 30 mice received a supplementation of blueberry extract for 30 d. After that period, all treated animals had a higher significant memory retention in all test sessions after 7 and 30 d of supplementation, while in-vitro examination revealed significantly reduced DNA damage in hippocampal tissues than in the control group.

Resveratrol (3,5,4’-trihydroxystilbene), a polyphenol naturally occurring in grapes, berries, peanuts, and other vegetables, has also been shown to have antioxidant, anti-inflammatory, and anti-proliferative properties (36,37). Although there is a lack of scientific studies on resveratrol supplementation in pets, it has been demonstrated that resveratrol was able to protect from oxidative stress in hepatic steatosis conditions, significantly increasing GSH and GPx levels in liver tissues and significantly decreasing ROS levels in 30 mice (38).

Antioxidant and anti-inflammatory activity have been ascribed to strawberry extract due to its high content of flavonoids (39). For example, a daily intake of 500 g of strawberries for 9 d increased non-urate plasma 2,2-diphenyl-1-picrylhydrazyl radical decomposition, thus possibly decreasing the risk of systemic oxidants over activity (excessive activity) (40).

Antioxidant and anti-inflammatory activities were also investigated for a polyphenolic blackberry extract by means of the oxygen radical absorbance capacity assay (ORAC) (41). The ORAC value of the blackberry extract was higher than the ORAC of quercetin and ellagic acid. Moreover, the blackberry extract inhibited superoxide production by NADPH oxidase (nicotinamide adenine dinucleotide phosphate-oxidase) in THP-1 cells and nitrite production in J774A.1 cells after 4 h of incubation, showing a scavenger activity, while a reduction of nitrites was observed after 24 h of incubation.

A dog’s diet may be qualitatively and quantitatively appropriate to the type and intensity of the activity as well as to its age, breed, and gender. Balanced nutrition could therefore be crucial to restoring good overall health status. This study confirms that long-term supplementation with a specific diet enriched with antioxidants is able to regulate the antioxidant status and general health status of working dogs involved in animal-assisted intervention (AAI) programs and prevent damage due to oxidative stress before and after their work.
Acknowledgment

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References

Evaluation of transmission infrared spectroscopy and digital and optical refractometers to identify low immunoglobulin G concentrations in alpaca serum

Ibrahim Elsohaby, Jennifer J. Burns, Christopher B. Riley, J. Trenton McClure

Abstract

This study aimed to evaluate the digital Brix and optical serum total protein (STP) refractometers for measuring concentrations of serum immunoglobulin G (IgG) in alpacas and compare them to IgG concentrations measured by the reference method of radial immunodiffusion (RID) assay. The appropriate cutoff point for Brix and STP refractometers and the transmission infrared (TIR) spectroscopy method was determined for low IgG concentrations (< 10 g/L). Serum samples were collected from alpacas (N = 169) and tested by both refractometers. The correlation between Brix % and STP was high [correlation coefficient (r) = 0.99]. However, the correlation coefficients between Brix % and STP with serum RID-IgG concentrations were only 0.56 and 0.55, respectively. Twenty-one (12.4%) of 169 alpaca serum samples had IgG concentrations of < 10 g/L. Using receiver operator characteristic curve (ROC) analysis, the optimal cutoff points for the TIR assay, digital Brix, and optical STP refractometers for assessing low IgG (RID < 10 g/L) were 13 g/L, 8.8%, and 50 g/L, respectively. The TIR assay showed higher sensitivity (Se = 95.2%) and specificity (Sp = 96.8%) than either the digital Brix (Se = 90.5% and Sp = 65.5%) or optical STP (Se = 81% and Sp = 73.7%) refractometers for assessing alpacas with low IgG. In conclusion, the Brix and STP refractometers lack accuracy in measuring alpaca IgG concentrations, but may be useful for screening animals for low serum IgG. However, the TIR assay with a cutoff point of 13 g/L was more appropriate for identifying low IgG than either refractometer. Another study that focuses on neonatal crias is recommended in order to evaluate the usefulness of these assays for field diagnosing of failure of transfer of passive immunity (FTPI).

Résumé

Cette étude visait à évaluer un réfractomètre Brix digital et un réfractomètre optique pour protéines sériques totales (PST) pour mesurer les concentrations sériques d’immunoglobulines G (IgG) chez les alpagas et de les comparer aux concentrations d’IgG mesurées par la méthode de référence d’immunodiffusion radiale (IDR). La valeur seuil appropriée pour les réfractomètres Brix et PST ainsi que pour la méthode de spectroscopie infrarouge à transmission (SIT) fut déterminée pour des faibles concentrations d’IgG (< 10 g/L). Des échantillons de sérum ont été prélevés d’alpagas (N = 169) et testés avec les deux réfractomètres. La corrélation entre le % Brix et PST était élevée [coefficient de corrélation (r) = 0.99]. Toutefois, les coefficients de corrélation entre % Brix et le PST et les concentrations d’IgG déterminées par IDR étaient seulement de 0,56 et 0,55, respectivement. Vingt-et-un (12,4 %) des 169 échantillons de sérum d’alpagas avaient des concentrations d’IgG < 10 g/L. En utilisant l’analyse de la courbe de la fonction d’efficacité du récepteur, les valeurs seuils optimales pour l’épreuve SIT, et les réfractomètres Brix digital, et PST optique pour évaluer un faible niveau d’IgG (RID < 10 g/L) étaient de 13 g/L, 8,8 % et 50 g/L, respectivement. L’épreuve SIT a montré une sensibilité (Se = 95,2 %) et une spécificité (Sp = 96,8 %) plus élevée que le réfractomètre Brix digital (Se = 90,5 % et Sp = 65,5 %) ou le réfractomètre PST optique (Se = 81 % et Sp = 73,7) pour évaluer les alpagas avec de faibles concentrations d’IgG. En conclusion, les réfractomètres Brix et PST manquent de précision pour mesurer les concentrations d’IgG d’alpagas, mais peuvent être utiles pour vérifier les animaux pour des faibles concentrations d’IgG sériques. Toutefois, l’épreuve SIT avec une valeur seuil de 13 g/L était plus appropriée que les deux réfractomètres pour identifier des valeurs faibles d’IgG. Une autre étude qui s’attardait aux crias nouveau-nés est recommandée afin d’évaluer l’utilité de ces épreuves pour le diagnostic des déficiences de transfert d’immunité passive.

(Traduit par Docteur Serge Messier)

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Camelids are born hypogammaglobulinemic as the diffuse epitheliocorial structure of their placentation does not allow immunoglobulins to pass from dam to fetus (1). Newborn camelids therefore rely on the transfer of maternal immunoglobulins through intake of the dam’s colostrum in order to acquire passive immunity (2). Failure of transfer of passive immunity (FTPI) occurs when newborn camelids fail to ingest or absorb a sufficient amount (< 10 g/L) of colostral IgG within 48 h of birth (1,3,4). There is a recognized association between FTPI and increased infectious disease, including septicemia, diarrhea, pneumonia, and septic arthritis, in neonatal camelids (5–7). Early diagnosis of FTPI is therefore a vitally important part of neonatal camelid husbandry and is critical in decreasing neonatal morbidity and mortality rates (8).

Several assays are available to measure IgG concentrations directly or indirectly and to assist in assessing conditions characterized by low IgG, such as FTPI in camelids (1,9). Direct methods of assessment include the radial immunodiffusion (RID) assay (10), immunoturbidimetric (IT) assay (11), and transmission infrared (TIR) spectroscopy (12). Indirect methods include zinc sulfate turbidity (13), glutaraldehyde coagulation assay, serum gamma-glutamyl transferase (GGT) activity (14), and total serum protein and globulin concentration measurements (4,13). The RID assay is the historical gold standard method for direct quantification of IgG level in camelids (15). Radial immunodiffusion (RID) has significant disadvantages, however, including the time it takes to obtain results (18 to 24 h), higher costs than other methods, and a lack of agreement about which commercially available RID kits should be used to measure IgG concentration in camelids (10,11).

While infrared spectroscopy has recently been used to measure alpaca serum IgG (12), refractometry is the most common indirect method for estimating serum IgG and assessing FTPI on-farm (13). Previous studies have evaluated optical serum total protein (STP) refractometry for assessing FTPI in neonatal camelids (13,16). To the authors’ knowledge, however, no study has been published that describes the use of digital Brix refractometry for measuring serum IgG concentrations and assessing IgG of < 10 g/L, which is the cutoff point for FTPI in alpacas.

The objectives of this study were to i) evaluate the digital Brix and optical STP refractometers for measuring serum IgG concentrations in alpacas and compare values with those measured by the reference method of radial immunodiffusion (RID) assay; and ii) determine the optimal cutoff points for assessing low IgG concentrations (< 10 g/L) in alpacas using digital Brix and optical STP refractometers and the transmission infrared (TIR) spectroscopy method.

### IgG direct measurement methods

Results of serum RID and TIR IgG (g/L) measurement for the same samples from a related previous study were used (12). In the earlier study, a commercial alpaca RID IgG assay (Triple J Farms, Bellingham, Washington, USA) and transmission infrared (TIR) spectroscopy (Tensor 37; Bruker Optics, Coventry, UK) were used. Radial immunodiffusion (RID) IgG assay was used as a reference method to determine each alpaca’s serum IgG concentration. Each serum sample and assay standard was tested in replicates of 5 to generate an average IgG concentration value. For TIR spectroscopy, each sample was tested in replicates of 6 as described in a previous study (12). Sample spectra were collected over the wavenumber range of 4000 to 400 cm⁻¹, with a nominal resolution of 4 cm⁻¹, with 512 scans collected for data acquisition. Data for the measured TIR IgG values were used to calculate an optimal cutoff point to identify low IgG (< 10 g/L) for this study.

### IgG indirect measurement methods

Only 169 of the original 175 serum samples were available for testing by refractometers, as there was not enough volume in the remaining 6 samples to allow for further testing. Samples were vortexed for 10 s and serum Brix (%) and STP (g/L) levels were determined using a digital Brix refractometer (PAL-1; Atago, Bellevue, Washington, USA) and an optical handheld refractometer (Westover RHC 200ATC handheld refractometer; Woodinville, Washington, USA), respectively. The same person conducted both refractometer

### Study population and sampling

Banked sera from a previous study stored at −80°C were used for this study (12). The study was approved by the University of Prince Edward Island (UPEI) Animal Care Committee and the Animal Ethics Committee of the University of Adelaide. Alpacas (N = 175) enrolled in this study between 2009 and 2011 were from 6 farms in Ontario and New Brunswick, Canada (n = 82) and 3 farms in the Adelaide Hills region of South Australia (n = 93). All alpacas appeared clinically healthy and were not visibly dehydrated. Age and gender data were only available for 129 of the 175 alpacas. Of these 129 alpacas, 81 were female and 48 were male. Four of these alpacas were < 2 mo old, 5 were 2 to 3 mo old, 15 were 3 to 6 mo old, 12 were 6 mo to 1 y old, and 93 were > 1 y old.

In Canada, blood samples were collected from alpacas by jugular venipuncture into a sterile, plastic vacutainer tube without anticoagulant, then centrifuged at 1500 x g for 10 min at room temperature. Serum was collected into a cryovial and frozen at −20°C until transport to the Atlantic Veterinary College (AVC). Blood samples collected from alpacas in Australia were taken for hematologic and biochemical analysis as part of a separate study. The remaining serum was stored at −80°C before being shipped frozen to AVC, UPEI, Canada where it was stored at −80°C for later batch analysis.

### Materials and methods

#### Study population and sampling

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<table>
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RID — radial immunodiffusion assay; TIR — transmission infrared spectroscopy; Digital — Brix refractometry; Optical — serum total protein (STP) refractometry.

#### Table I. Descriptive statistics of immunoglobulin G (IgG) concentrations, Brix percentage (%) and serum total protein (STP) measurements in 169 alpaca serum samples
Radial immunodiffusion (RID) assay for 169 alpaca serum samples. Digital Brix refractometry and immunoglobulin G (IgG) determined by TIR spectroscopy, Brix, and STP refractometers to determine the relationship among measurements by STP refractometers. Spearman rank coefficient of correlation was calculated to determine the relationship among measurements by STP refractometers. Descriptive statistics for IgG measurements were calculated by RID, TIR spectroscopy, digital Brix, and optical STP refractometers. Spearman rank coefficient of correlation was calculated to determine the relationship among measurements by RID, Brix, and STP refractometers.

Alpacas with serum RID IgG concentrations of < 10 g/L were considered to have low IgG, which is consistent with the definition of FTPI in camelid neonates (1,3,4). A receiver operating characteristic (ROC) analysis was conducted on data obtained with TIR spectroscopy and Brix and STP refractometers to determine the optimal cutoff point based on the Youden index. Sensitivity (Se), specificity (Sp), accuracy, Youden index (J), and area under the curve (AUC) were used to compare the diagnostic performance of each method. Accuracy, which was defined as the percentage of correctly classified samples, was calculated as follows: \[ Se \times p + [Sp] \times (1 - p) ]\), where \( p\) represents the prevalence of samples with low IgG (< 10 g/L) observed in the studied population. Finally, kappa statistic was used to assess the level of agreement among the results of TIR spectroscopy, Brix, and STP refractometers versus the reference RID assay, as well as between results of digital Brix and optical STP refractometers.

Statistical analysis

Statistical analyses were carried out using statistical software (Stata Version 14.0; Stata Corporation, College Station, Texas, USA and MedCalc for Windows Version 15.8.0; MedCalc Software, Mariakerke, Belgium). A value of \( P < 0.05\) was considered statistically significant. The data were evaluated for normality by applying the Shapiro-Wilk test. Descriptive statistics for IgG measurements were calculated by RID, TIR spectroscopy, digital Brix, and optical STP refractometers. Spearman rank coefficient of correlation was calculated to determine the relationship among measurements by RID, Brix, and STP refractometers.

The Spearman rank correlation coefficient between IgG levels from TIR were normally distributed (\( P = 0.321\)). Descriptive statistics (median, minimum, and maximum) of IgG concentrations in alpaca serum obtained by Brix and STP measurement are shown in Table I. The median IgG concentration measured by RID assay (24.8 g/L) in the present study was marginally higher than that previously published, which ranged from 17.2 g/L to 23.4 g/L (3,4,11,14). The range of IgG concentrations in these studies was more likely to be higher, however, because they were not limited to healthy alpacas.

The RID and TIR assays were conducted at an earlier time than the refractometers, with the serum stored in a freezer at –80°C between testing. Degradation of IgG between testing periods is unlikely as it has been previously reported that freezing at this temperature over a period of years has no significant effect on IgG concentration (17,18). Only 21 of the 169 alpacas had an IgG concentration of < 10 g/L, the most common cutoff point used to determine FTPI (1), which resulted in a true prevalence of 12.4%. This value falls within the range of FTPI prevalence (9% to 20.5%) previously reported in the literature (4,15). The differences in the prevalence of low IgG concentrations (< 10 g/L) in the present and previous studies may be related to the age of alpacas enrolled in these earlier studies, as well as to management practices on the farms, including biosecurity measures, nutrition, vaccination programs, geographic location, and climate (19). Since alpacas in this study population were of various ages and included crias and adults, we are limited in assessing the clinical usefulness of these assays for diagnosing FTPI. A future study focusing on neonatal crias is therefore recommended in order to better evaluate the usefulness of TIR spectroscopy (12), Brix, and STP refractometers for diagnosing FTPI.

Correlations

The Spearman rank correlation coefficient between IgG levels was 0.56, as estimated by digital Brix refractometry and RID assay (Figure 1). The STP-based serum IgG measurements were correlated with those assessed by RID assay (\( r = 0.55\); Figure 2). These findings were lower than correlations reported between immunoturbidimetric (IT) (11) or infrared spectroscopy methods (12) and RID-based

Results and discussion

While the IgG values from RID, Brix %, and STP assays were not normally distributed (\( P < 0.001\), respectively), the values for IgG concentrations were normally distributed (\( P = 0.321\)). Descriptive statistics (median, minimum, and maximum) of IgG concentrations in alpaca serum obtained by Brix and STP measurement are shown in Table I. The median IgG concentration measured by RID assay (24.8 g/L) in the present study was marginally higher than that previously published, which ranged from 17.2 g/L to 23.4 g/L (3,4,11,14). The range of IgG concentrations in these studies was more likely to be higher, however, because they were not limited to healthy alpacas.

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measurements of IgG concentrations. Furthermore, the correlations between the digital Brix or the optical STP refractometers and RID assay were lower than those reported for bovine (20–22) and lamb sera (23). There was a high correlation between digital Brix and optical STP refractometers values ($r = 0.99$; Figure 3), which indicates that there was no advantage of one method over the other. Although a similarly high level of correlation between Brix and STP refractometers has previously been reported (24), correlation coefficients from 0.73 to 0.91 have been reported for other species (20–22).

**Test characteristics for detection of low IgG**

Test characteristics of the TIR spectroscopy, digital Brix, and optical STP refractometers for assessing alpacas with low IgG concentration ($<10$ g/L) were calculated using optimal cutoff points determined by ROCs (Table II). The optimal combination of Se (95.2%; 95% CI: 90.9 to 98.2) and Sp (96.8%; 95% CI: 92.3 to 98.9) for the TIR spectroscopic diagnosis of low IgG was achieved at $\leq 13$ g/L (Figure 4). The calculated Se and Sp for the digital Brix refractometer using $\leq 8.8\%$ as the optimal cutoff point were 90.5% (95% CI: 69.6 to 98.8) and 65.5% (95% CI: 57.3 to 73.2), respectively (Figure 5). This compares to the best combination of Se (81%; 95% CI: 58.1 to 94.6) and Sp (73.7%; 95% CI: 65.8 to 80.5) for the optical STP refractometer at a cutoff point of $\leq 50$ g/L (Figure 6).

The optimal Se and Sp values for the TIR spectroscopic approach were higher than those of the Brix and STP refractometers. Also, the Se (95.2%) of TIR spectroscopy at a cutoff point of $\leq 13$ g/L was higher than the Se (81%) from the original study when a cutoff point of 10 g/L was used for the same samples (12). The objectives of the original study (12), however, were to develop an algorithm that best predicted serum IgG concentration values over the TIR spectrum and looked at how well the TIR spectroscopic method performed against the RID using the same cutoff value of $\leq 10$ g/L for each assay. In this study, we used ROC analysis to determine the TIR IgG value that best correlates with serum IgG of $\leq 10$ g/L as determined by the reference RID assay. The Se and Sp of the TIR spectroscopy using a cutoff IgG of $\leq 13$ g/L value was similar or higher when compared with the reported Se and Sp for other available methods to assess low serum IgG in alpacas (11,13,25). The digital Brix refractometer showed higher Se and slightly lower Sp than the optical STP refractometer at a cutoff point of $\leq 8.8\%$, which indicates that digital Brix may be preferred as a screening test compared to optical STP refractometer. The Se and Sp of the digital Brix refractometer at a cutoff point of $\leq 50$ g/L were close to the Se (71%) and Sp (80%) previously reported with the same cutoff point (13). When a cutoff point of 51.5 g/L was used, however, a higher Se (87.5%) and Sp (87.9%) for assessment of low serum IgG were reported (25). The difference in the optimal cutoff points between studies may be due to variations between instruments or differences in animal populations (22).

**Agreement among tests**

The agreement among results of TIR spectroscopy and Brix and STP refractometers for assessing alpaca serum with low IgG concentrations ($<10$ g/L) compared to the reference RID assay is presented.

**Table II. Test characteristics for detecting low serum IgG ($<10$ g/L) in 169 alpaca serum samples by transmission infrared (TIR) spectroscopy and Brix and optical serum total protein (STP) refractometers as calculated using the radial immunodiffusion (RID) assay as the reference.**

<table>
<thead>
<tr>
<th>Test characteristics</th>
<th>TIR</th>
<th>Brix</th>
<th>STP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cutoff point</td>
<td>$\leq 13$ g/L</td>
<td>$\leq 8.8%$</td>
<td>$\leq 50$ g/L</td>
</tr>
<tr>
<td>Sensitivity (Se)</td>
<td>95.2</td>
<td>90.5</td>
<td>81</td>
</tr>
<tr>
<td>Specificity (Sp)</td>
<td>96.8</td>
<td>65.5</td>
<td>73.7</td>
</tr>
<tr>
<td>Accuracy</td>
<td>96.6</td>
<td>68.5</td>
<td>74.6</td>
</tr>
<tr>
<td>Youden index ($J$)</td>
<td>0.92</td>
<td>0.56</td>
<td>0.55</td>
</tr>
<tr>
<td>Area under the curve (AUC)</td>
<td>0.99</td>
<td>0.81</td>
<td>0.81</td>
</tr>
<tr>
<td>Kappa</td>
<td>0.82</td>
<td>0.30</td>
<td>0.33</td>
</tr>
</tbody>
</table>

Cutoff point — optimal criteria determined by receiver operating characteristic curves (ROCs).
in Table II. The overall percentages of agreement among results of TIR spectroscopy, Brix, and STP refractometers to the reference RID assay, i.e., accuracy, with a corresponding kappa value are also listed in Table II. The level of agreement between the results of the 2 refractometers was 85.2%, however, with a kappa value of 0.66. This finding shows that TIR spectroscopy had a substantial agreement with the RID assay that was better than that observed for both refractometers. The analytic advantage of TIR is that the serum IgG content creates a unique molecular absorption (fingerprint) in the infrared spectrum, which can be used for qualitative and quantitative measurement (26). However, refractometers measure STP or Brix %, which indirectly related to serum IgG concentration as a portion of total solids (13). The level of agreement found between the 2 refractometers (85.2%) was similar to the level of agreement (85%) reported between 2 refractometers for assessing FTPI in dairy calves (20).

In conclusion, this study found that the TIR spectroscopic method using the calculated optimal cutoff point of 13 g/L provided better sensitivity, specificity, and accuracy for diagnosing low IgG (< 10 g/L) than digital Brix or optical STP refractometers at their optimal cutoff points of 8.8% and 50 g/L, respectively. The digital Brix and optical STP refractometers were poor predictors of serum IgG concentrations, and although they may be useful for screening animals for low IgG (< 10 g/L), further confirmation testing would be required due to the limited specificity of the assays. Another study focusing on neonatal crias is recommended to further evaluate the specific usefulness of TIR spectroscopy for diagnosing FTPI.

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References

Cystic duct pressures after ligation with a novel absorbable device in an ex vivo caprine cholecystectomy model

Andrea J. Sundholm Tepper, Odd V. Höglund, Bonnie G. Campbell, Chi-Ya Chen, Boel A. Fransson

Abstract

Laparoscopic cholecystectomy is the standard of care in human medicine for gall bladder disease. Although infrequently reported in veterinary literature, laparoscopic cholecystectomy is an option for uncomplicated gall bladder disease in canine patients. Due to the risk of cystic duct ligature slippage or clip dislodgement, we wanted to explore the use of a LigaTie; a novel absorbable medical device modeled after a cable tie. Our object was to describe the use of the LigaTie in a caprine cadaveric study of cholecystectomies as a model for canine patients and demonstrate the leak pressure of the cystic duct compared with cholecystectomies performed with 2 large endoscopic hemoclips. Samples of caprine gall bladder, liver, and cystic duct were collected. The cystic duct was ligated with either 2 large endoscopic hemoclips or a LigaTie. Maximum cystic duct pressure was recorded. Results showed that there was no statistically significant difference in the maximum cystic duct pressure achieved for cystic ducts ligated with 2 large endoscopic hemoclips or the LigaTie (P = 0.865). No leakage was observed from the cystic duct, hemoclip, or LigaTie site in either group. Supraphysiologic pressures were achieved in both groups and high pressure occlusion of the infusion pump determined the maximum intraluminal pressure achieved. Based on these results, the LigaTie may provide advantages in minimally invasive surgery, especially when considering ligation of a friable or thickened cystic duct during laparoscopic cholecystectomy. Future in vivo studies are warranted to determine minimally invasive maneuverability, tissue interaction, complications, and outcomes.

Résumé

La cholécystectomie par laparoscopie est le standard de soin en médecine humaine pour les maladies de la vésicule biliaire. Bien que rapportée peu fréquemment dans la littérature vétérinaire, la cholécystectomie par laparoscopie est une option pour les maladies non-compliquées de la vésicule biliaire chez les patients canins. Due au risque de glissement de la ligature du canal cholédoque ou au déplacement de l’agrafe, nous avons voulu explorer l’utilisation de LigaTie; un nouveau dispositif médical résorbable modélisé d’après une attache de câble. Notre objectif était de décrire l’utilisation de LigaTie dans une étude sur des cadavres de chèvres de cholécystectomies comme modèle pour des patients canins et de démontrer la pression de fuite du canal cholédoque comparativement à des cholécystectomies réalisées avec deux larges agrafes hémostatiques endoscopiques. Des échantillons de vésicules biliaires, de foies et de canal cholédoques caprins ont été prélevés. Le canal cholédoque était ligaturé avec soit deux larges agrafes hémostatiques endoscopiques ou du LigaTie. La pression maximale dans le canal cholédoque fut enregistrée. Les résultats ont montré qu’il n’y avait pas de différence statistiquement significative dans la pression maximale atteinte dans le canal cholédoque par les deux méthodes de ligature (P = 0,865). Aucune fuite ne fut observée des sites de ligature du canal cholédoque que ce soit du groupe avec agrafes ou celui avec LigaTie. Des pressions supra-physiologiques ont été atteintes dans les deux groupes et l’occlusion de la pompe à infusion due à la haute pression a déterminé la pression intraluminaire maximale atteinte. En fonction de ces résultats, le LigaTie est avantageux comme méthode chirurgicale minimalement invasive, surtout si l’on considère la ligature d’un canal cholédoque friable ou épaissi durant une cholécystectomie par laparoscopie. Des études in vivo ultérieures sont nécessaires afin de déterminer la manœuvrabilité invasive minimale, l’interaction tissulaire, les complications et les résultats.
Introduction

The laparoscopic revolution started in the late 1980s and early 1990s, with minimally invasive surgery (MIS) for cholecystectomy and adrenalectomy in humans (1). By the early 2000s, cholecystectomy via laparoscopic approach had become the procedure of choice, with more than 75% of cholecystectomies in North America being performed laparoscopically (2), a number rising to exceed 90% within the last decade (3). The procedure is typically conducted on an outpatient basis with conversion to open surgery occurring in only 5% to 10% of cases (2). Advantages of laparoscopic procedures are well-documented in human medicine and it is known that laparoscopic cholecystectomy results in decreased post-operative pain, more rapid return to normal activity, and improved cosmesis (4). Likewise, studies in small animal patients have shown reduced pain (5,6) and more rapid return to normal activity (7,8). Laparoscopic cholecystectomy in canine patients is infrequently reported in veterinary literature but is gaining popularity as a minimally invasive option. Mayhew et al (9) described laparoscopic cholecystectomy for uncomplicated mucocoeles in 6 dogs. All dogs had successful laparoscopic cholecystectomy without conversion to an open approach and clinical signs improved or resolved in all cases. More recently, short-term outcome of 16 dogs with laparoscopic cholecystectomy was determined to be good, with 6/16 (38%) being converted due to necrotic or ruptured gall bladder (10). In addition, possible complications of laparoscopic cholecystectomy in dogs include cystic duct rupture and bile spillage from cystic duct ligation (11).

Options for laparoscopic cystic duct ligation include extracorporeally or intracorporeally tied sutures, and hemoclips (9). Cystic duct ligation with a vessel sealant device has also been described in a cadaveric study with normal hepatobiliary systems (12). Concern for hemoclip slippage, especially in thickened or friable cystic duct tissue, and challenges encountered with intra/extracorporeally suture ligation have encouraged research into alternative methods for cystic duct ligation (13).

The LigaTie (Resorbable Devices AB, Uppsala, Sweden) is an absorbable self-locking device that was manufactured based on the principle of a cable tie. It is currently not commercially available in the USA. Initially, the device was made of polydioxanone (14); however, because of concerns of pliability, it is now a co-polymer of glycolide and trimethylene carbonate (15). The original intent of the LigaTie was vessel ligation due to the excellent tissue grip and ability to close to a loop end diameter of zero (14). Höglund et al (15) described its use for ligation of the spermatic cord in dogs, with specific interest in the feasibility as well as biocompatibility. The study showed that no complications were found related to the device and that the subcutaneous tissues were capable of resorbing the polyglycolic based co-polymer, indicating appropriate biocompatibility. The same device was also used for ligation of the canine ovarian pedicle and its performance was compared to traditional suture ligation (16). This feasibility study showed that the ligation time of the mesovarium was significantly shortened by using the self-locking implant versus a single traditional ligature. A study by Aminlashedgari et al (17) tested the mechanical performance of the device and its degradation over time in vitro as well as short-term tests in vivo in pigs. Results of this study suggested that sufficient strength of the LigaTie was retained during healing time of the blood vessels. In contrast, non-absorbable cable ties, previously described in veterinary medicine for ovariohysterectomy, have resulted in adverse complications such as fistulous draining tracts, retroperitoneal abscesses, and granuloma formation (18–20); their use in surgery is generally considered not appropriate.

Due to the movement towards minimally invasive surgery and need for a secure and reliable device for cystic duct ligation, we wanted to explore the use of the LigaTie. The objective of this study was to describe the use of the LigaTie in an ex vivo caprine cadaveric cholecystectomy model and demonstrate the leak pressure of the cystic duct compared with cholecystectomies performed with 2 large endoscopic hemoclips. Our null hypothesis was that the leak pressure of the cystic duct occluded with a LigaTie and 2 large endoscopic hemoclips in an ex vivo cadaveric model would not be different.

Materials and methods

This study was approved by the Washington State Institutional Animal Care and Use Committee. Twelve goats were used in the study. A caprine model was chosen due to anatomical similarities to dogs, and the limited availability of fresh canine cadavers. There were 4 males, 2 females, and 6 wethers. Breeds represented were Lamancha (n = 3), Boer cross (n = 3), alpine cross (n = 2), Saanen (n = 2), and pygmy cross (n = 2). Goats were quarantined for a minimum of 14 d prior to administration of general anesthesia, to ensure appropriate health status. A complete physical examination and packed-cell volume/total protein (PCV/TP) was carried out before induction of general anesthesia for a terminal surgery laboratory. Goats were euthanized for reasons unrelated to this study. Immediately after euthanasia, a right paracostal approach was conducted to access the duodenum, common bile duct, gall bladder, and right liver lobes. In the original study design, a duodenotomy was completed and the common bile duct was cannulated retrograde from the intestinal lumen with a 4 Fr double lumen catheter (Milacath; Mila International, Florence, Kentucky, USA) and secured to the common bile duct with a circumferential suture. A cholecystectomy was carried out through the right paracostal approach with the cystic duct being ligated with either 2 large stainless steel endoscopic hemoclips (Horizon Metal Ligation Systems; Teleflex, Morrisville, North Carolina, USA) or the LigaTie (Resorbable Devices AB, Uppsala, Sweden). A pilot sample elucidated multiple communications between the biliary system and the pancreatic duct in the goat, and infusion of fluid through a retrograde catheter (which resided in the common bile duct) resulted in marked pancreatic edema. Therefore, we were unable to maintain and determine the true pressure within the cystic duct as infused fluid exuded into the pancreas. In the final study design, after the paracostal approach was performed, the gall bladder, and approximately 4 cm of the cystic duct was removed with the surrounding parenchyma of the right medial and quadrate liver lobe by sharp transection. The gall bladder was examined for gross pathology and gently expressed to ensure a patent cystic duct. The cystic duct was dissected from the surrounding liver parenchyma with blunt dissection until a stalk large enough to fit 2 endoscopic hemoclips (Figure 1) or a LigaTie.
Figure 1. Gall bladder catheterized with double lumen catheter (black arrow). Cystic duct ligated with 2 large endoscopic hemoclips (white arrow).

Figure 2. Gall bladder with cystic duct ligated with the LigaTie (white arrow).

Figure 3. Gall bladder catheterized with double lumen catheter (large black arrow). One lumen is connected to a pressure transducer (small black arrow) and the other is connected to the fluid ingress.

(Figure 2) was exposed. The LigaTie was placed in a similar fashion as a cable tie and tension was placed on the LigaTie “tail” until no further closure of the device could be achieved. Ligation method was designated for the first sample based on a coin toss, and thereafter the method was alternating in the subsequent samples. Once either the hemoclips or LigaTie had been placed, the apex of the gall bladder was cannulated using a 4 Fr double lumen catheter (Milacath; Mila International). One port of the double lumen catheter was connected to a 1-liter bag of sterile saline infused with fluorescein dye in order to augment identification of leakage within the system. The other port was connected to an extension set and pressure transducer (Hewlett Packard CMS 24 Omicare; HP, Palo Alto, California, USA) (Figure 3). The transducer was calibrated to atmospheric pressure before testing each gall bladder. No reinforcement or ligation was needed at the catheter-gall bladder interface due to the apparent water tight seal created when fluid infusion was initiated. Fluid was infused through the catheter at a rate of 999 mL/h while the system was observed for leakage. Leak pressure was recorded when fluid was seen extruding from the gall bladder wall, cystic duct, or at the site of the clips or LigaTie. If no leakage was observed, the maximum pressure achieved with the fluid pump was recorded.

Data analysis

Statistical analysis was performed with a commercially available software program (GraphPad Prism 5; GraphPad Software, La Jolla, California, USA). Leak pressure between the 2 groups was normally distributed as determined with a D’Agostino-Pearson omnibus K2 test and the means were analyzed using a Student t-test. Mean weight of the goats was likewise normally distributed and the means were analyzed using a Student t-test. A P-value, 0.05 was considered significant.

Results

Twelve goats of various breeds were included in this study. There were no statistically significant differences between the weight of goats between groups (P = 0.564). Abnormal physical examination findings included mucoid nasal discharge in 3, lice in 1, and stimulus induced myotonia which was clinically irrelevant in 1. Results of preoperative PCV, and total protein were within normal limits in all goats. Goats were treated by the attending clinician for abnormal preoperative findings and were considered healthy before anesthesia and euthanasia. Subjectively, all gall bladders and livers were free of gross disease, and when expressed, normal bile easily passed through the cystic duct, demonstrating patency. All cystic ducts were < 5 mm in diameter and visibly free of pathology.

There were 12 samples included in the study: 6 ligated by hemoclips and 6 ligated by the LigaTie. The mean cystic duct pressure achieved in the hemoclip group was 310.5 mmHg [± 75.4 mmHg standard deviation (SD)]. The mean cystic duct pressure of the LigaTie group was 317.2 mmHg [± 55.9 mmHg]. There were no statistically significant differences between the maximum cystic duct pressures between groups (P = 0.865). No leakage was observed from the cystic duct, hemoclip site, or LigaTie site in either group. However, very small amounts of fluid were observed diffusely exuding from the gall bladder wall in 4 samples at
pressures > 330 mmHg. Also, in 1 sample, a small amount of leakage was observed around the catheter insertion site at the apex of the gall bladder, at a pressure of > 230 mmHg. Gentle digital pressure at the catheter entrance site was enough to allow continued filling of the gall bladder without loss of pressure. The final pressure measures were obtained when the infusion pump stopped due to the high pressure within the system.

**Discussion**

The results of this study indicate that there was no difference between the maximum cystic duct pressure occluded with 2 large endoscopic hemoclips or a LigaTie in an *ex vivo* caprine cadaveric model for cholecystectomies.

Both the large hemoclip and LigaTie group reached supraphysiologic pressures within the gall bladder and cystic duct. Small amounts of fluid that diffusely seeped through the gall bladder wall at pressures > 330 mmHg were observed in 4 gall bladder samples. This did not affect the overall pressure achieved within the gall bladder or cystic duct because the transducer would continue to show increases in pressure. This equivocal leakage was assumed to be secondary to the marked intraluminal increases in hydrostatic pressure and the infusion pump eventually stopped when the pressure became too high. Therefore, it is possible that higher pressures could have been achieved with manual pressure and leakage from the hemoclips or LigaTie site could have been observed. However, the pressures obtained are already supraphysiologic and testing to failure would not be clinically relevant. The normal common bile duct pressure in a dog is 12 mmHg (21). Although the normal canine cystic duct or common bile duct pressure is unknown, it is unlikely to reach pressures close to those noted in this study, indicating that both large hemoclips and the LigaTie may be appropriate for cystic duct ligation in dogs. Additionally, there are no known studies that measure cystic or common bile duct pressures in dogs with hepatobiliary disease. In humans, common bile duct pressures can increase up to 29.4 mmHg with gallstones and suppurative cholangitis (22).

The LigaTie was manufactured to display characteristics of hemostasis and excellent tissue grip making possible applications of the device quite diverse. Initial studies found that a single device placed on the renal artery of pigs showed complete hemostasis in 12 arteries for 5 min (14). To further document resistance to slippage, the ability of the device to withstand a ligature slip-off was tested by applying a force of 10 N (14). The LigaTie has successfully been used in *vivo* on ovarian pedicles (15) and the spermatic cord (23) with histological evidence of vascular occlusion and biocompatibility. Future applications such as lung lobectomies are currently being investigated at the authors’ institutions. In humans, the use of a self-locking non-absorbable inert polymer clip (Hem-O-Lok clip) has been shown to be a secure option during basic laparoscopic procedures including cholecystectomy, appendectomy, splenectomy, and nephrectomy (24). These clips can ligate up to 10-mm of tissue through a 5-mm portal or up to 15-mm of tissue through a 10-mm portal (24). Due to the concern of migration or dislodgement of metallic clips, these locking clips have been preferred for various procedures and share common advantages to the LigaTie.

One main advantage with using the LigaTie on the cystic duct is its ability to occlude and compress tissue without major limitation on duct size or tissue pliability. During cholecystectomies, the cystic duct may be friable and not amendable to extensive dissection or manipulation. Additionally, if the tissue is fibrous or dilated, large hemoclips may not adequately occlude the duct or provide adequate hemostasis of the cystic artery; risking slippage and biliary leakage (9). The LigaTie is flexible and could be used on any size cystic duct with a single, secure ligation with no collateral thermal damage. Laparoscopic cholecystectomy is performed in other species, including wild animals (25), in which the LigaTie may be an alternative for wide cystic ducts with fibrous walls.

The ideal use for the LigaTie is implementing it in minimally invasive surgery, specifically laparoscopic cholecystectomy. However, there is hesitation for complete laparoscopic cholecystectomies in canine patients which is because of the inability to flush the common bile duct to confirm patency. Recent reports have described open cholecystectomies without catheterization of the common bile duct (26). Malek et al (26) also found that 53.5% of common bile ducts were not catheterized before cholecystectomy. No significant association was found in patient outcome between the methods of catheterizing the common bile duct during cholecystectomy and whether or not the common bile duct was catheterized. However, recommendations on the necessity or technique for flushing the common bile duct during surgery could not be made based on their findings (26). A recent study by Scott et al (27) described perioperative complications and outcomes of laparoscopic cholecystectomy in 20 dogs. Six dogs (30%) required conversion to an open cholecystectomy; however, all dogs were discharged from the hospital and no significant difference was found between hospitalization time in laparoscopic *versus* conversion to an open procedure (27). Patients with evidence of extra-hepatic biliary obstruction were not included in the study; therefore, patency of the common bile duct was not confirmed intraoperatively. Based on that study, laparoscopic cholecystectomy can be performed successfully for uncomplicated gall bladder disease with careful case selection. The authors of the current study are not necessarily advocating for laparoscopic cholecystectomy without catheterizing of the common bile duct, rather, understanding that it may be an option and prospective studies are required to help further define the ideal patient.

Limitations of this study include small sample size, using caprine cadavers instead of live or cadaver canine models, and the inability to model a standard cholecystectomy. However, given that this study was intended to be a proof of concept, future studies can be more targeted in both the species and study design to better analyze the clinical uses of LigaTie in traditional open and laparoscopic cholecystectomy. An analysis of the histologic differences of a caprine gall bladder compared to a canine gall bladder was not conducted. Given there were no differences between the groups in this study and the supraphysiologic pressures that were obtained, the difference in histologic appearance would likely not be clinically significant. It would have been ideal to be able to model a clinical canine cholecystectomy in this study’s caprine model. However given the gross anatomic differences from a canine cadaver, this was not possible. Instead, it was decided that the focus would be on mechanical testing, in a consistent and repeatable manner, although the design
set-up is not applicable to in vivo conditions. Additionally, no attempt was made to place the LigaTie in a laparoscopic model; therefore, we cannot comment on the ability to maneuver the device in that setting. Instrument design is underway at our institution to use the LigaTie in a minimally invasive fashion.

In conclusion, no statistically significant differences were found in the maximum cystic duct pressures between 2 large endoscopic hemoclips and a LigaTie in a caprine cholecystectomy model. Future in vivo studies are necessary to characterize its interaction with canine cystic ducts as well as to determine any short- and long-term complications associated with this novel device.

References

Short Communication

Evaluation of serum symmetric dimethylarginine in dogs with heartworm infection

Bom-Sul Choi, Hyeongsun Moon, Sang-IL Suh, Changbaig Hyun

Abstract

This study evaluated the circulating levels of serum symmetric dimethylarginine (SDMA) in 12 dogs with different severities of heartworm disease (HWD) and assessed the biochemical renal markers (blood urea nitrogen, creatinine). Dogs were classified into 2 groups based on the severity of clinical signs. Group A — asymptomatic to mild clinical signs, group B — moderate to severe clinical signs. The serum SDMA levels were higher in dogs in group B. Although the serum SDMA levels in dogs in group A were also higher than those of the control dogs, the difference was not statistically significant. There was a good correlation between renal markers and severity of clinical signs. This study demonstrated that the glomerular filtration rate was significantly decreased in dogs in group A; therefore, earlier detection of renal impairment is required for successful management of dogs with HWD.

Résumé

La présente étude visait à évaluer les niveaux sériques de diméthylarginine symétrique (DMAS) chez 12 chiens atteints de maladie du vers du cœur (MVC) de différentes sévérités et d’examiner les marqueurs biochimiques rénaux (azote uréique sanguin, créatinine). Les chiens ont été classés en deux groupes sur la base de la sévérité des signes cliniques. Le Groupe A — asymptomatique à signes cliniques légers, groupe B — signes cliniques modérés à sévères. Les niveaux de DMAS sériques étaient plus élevés chez les chiens du groupe B. Chez les chiens du groupe A, bien que les niveaux sériques de DMAS étaient également plus élevés que ceux des chiens témoins, la différence n’était pas statistiquement significative. Il y avait une bonne corrélation entre les marqueurs rénaux et la sévérité des signes cliniques. Cette étude a permis de démontrer que le taux de filtration glomérulaire était diminué de manière significative chez les chiens du groupe A; ainsi, une détection précoce de déficience rénale est nécessaire pour la gestion réussie des chiens avec MVC.

(Traduit par Docteur Serge Messier)
examinations (i.e., persistent coughing, severe exercise intolerance, signs related to right-sided congestive heart failure, marked pulmonary infiltration with pulmonary arterial dilation and right ventricular enlargement, echocardiographic evidence of pulmonary hypertension with > 2.8 m/s peak velocity of tricuspid regurgitation or > 2.2 m/s peak velocity of pulmonary regurgitation.

To minimize the influence of feeding, all dogs were fasted for 12 h before the collection of blood samples. Whole blood was withdrawn from either the cephalic or jugular veins for determination of serum level of SDMA. Blood samples were drawn directly into sterile tubes (Vacutainer tubes; BD, Franklin Lakes, New Jersey, USA) and then centrifuged at 1.500 × g for 10 min at 4°C. The SDMA concentrations were determined by a reference laboratory (IDEXX Laboratories). Levels of blood urea nitrogen (BUN) and creatinine were determined using an automated biochemistry analyzer (VetScan VS2, Abaxis, Union City, California, USA).

Statistical analyses were done using commercially available statistical software (SPSS 15.0 for Windows; IBM, New York, USA). Descriptive statistics for quantitative variables between study groups were calculated using analysis of variance (ANOVA) with Tukey’s multiple comparison test. The correlation between renal markers (e.g., BUN, creatinine, and SDMA) was determined by Pearson’s coefficient of bivariate correlation analysis. A probability value less than 0.05 was considered statistically significant in our analysis.

Serum SDMA concentrations were 8 ± 2 µg/dL in controls, 13 ± 5 µg/dL in group A, and 21 ± 4 µg/dL in group B. Serum BUN was 18 ± 7 mg/dL in controls, 26 ± 13 mg/dL in group A, and 41 ± 25 mg/dL in group B. Serum creatinine was 0.7 ± 0.2 mg/dL in controls, 1.0 ± 0.4 mg/dL in group A, and 1.5 ± 0.5 mg/dL in group B. Pair-wise comparisons showed significant differences in serum BUN, creatinine, and SDMA concentrations in control and disease groups (P < 0.05), as well as in HWD groups with different severities of clinical signs (P < 0.05).

Based on previous veterinary references (3,5), upper limits were set as 25 mg/dL for BUN, 1.4 mg/dL for creatinine, and 14 µg/dL for SDMA. The number of dogs with BUN higher than the upper limit was 0/6 in controls, 2/6 in group A, and 3/6 in group B; the number with creatinine higher than the upper limit was 0/6 in controls, 1/6 in group A, and 2/6 in group B. The number of dogs with SDMA above upper limit values was 0/6 in controls, 2/6 in group A, and 6/6 in group B.

Bivariate analysis revealed close correlations among renal markers (i.e., SDMA, creatinine, and BUN) were found. Serum BUN, creatinine, and SDMA concentrations were not correlated with body weight but were closely correlated with the severity of clinical signs (Table I). Serum SDMA concentration was correlated with serum BUN and creatinine concentration (Table I).

Azotemia is one of the most common complications of advanced heart failure caused by HWD and other heart diseases (6,7). Unlike other heart diseases, HWD in dogs can induce azotemia through immune-mediated glomerulonephritis caused by microfilaria and debris of dead adult worms, while lower cardiac output-induced ischemic renal injury in decompensated heart failure is a major risk factor for azotemia (1). Therefore, early detection of renal impairment in HWD would enable clinicians to establish a safe but efficient management plan in dogs with HWD. Current diagnostic tests for renal impairment are limited to dogs with apparent renal disease or dysfunction, although direct measurement of GFR with creatinine or iothexol can detect earlier stages of renal impairment (5). However, indirect methods using BUN and creatinine are less sensitive, and are influenced by age, body mass, and other systemic diseases (5).

Recent veterinary studies found that the level of circulating SDMA was more accurate and useful for early detection of renal dysfunction in dogs, and is not significantly affected by lean body mass and age (3,4). Our study also demonstrated gradual but significant elevation of serum SDMA with progression of HWD, implying that renal impairment might be more obvious with progression of clinical signs in HWD. Unlike creatinine, the serum level of SDMA started to increase in dogs with ~40% loss of nephrons (3). In this study, the serum level of SDMA was higher than the reference range in 2/6 dogs in group A and 6/6 dogs in group B, while the serum level of creatinine was higher in 1/6 dogs in group A and 2/6 dogs in group B. This finding suggests that, in dogs, circulating SDMA can detect renal impairment earlier than does creatinine. Further, one recent canine study of chronic mitral valve disease found that SDMA increased with the severity of mitral insufficiency and indicated renal impairment earlier than did creatinine in dogs (8). The increased serum levels of SDMA in dogs with advanced stages of HWD (i.e., group B) in this study suggested a higher risk of renal impairment in dogs with higher worm burden and severe clinical signs. Interestingly, 2/6 dogs in group A in this study showed serum SDMA levels higher than the reference range, suggesting that many dogs without clinical signs related to HWD could have reduced GFR via impaired renal perfusion or renal injury due to immunological causes, although they generally appeared clinically normal. Therefore, earlier intervention to prevent renal injuries might be beneficial for successful management of HWD in dogs. Routine SDMA testing in dogs with HWD will help clinicians to more properly decide the choice of HW therapeutic protocols (e.g., slow kill versus melarsomine), the timing of initiation of adulticidal therapy and management guideline during adulticidal treatment, based on the renal status of dogs.

There are several limitations in this study. First, the study population was small, which may not provide sufficient data to adequately correlate SDMA with the severity of HWD in dogs. Second, the SDMA levels of all affected dogs in this study population could not be monitored over time owing to refusal by the owners. We

<table>
<thead>
<tr>
<th>Table I. Correlation of clinical severity of heartworm disease (HWD) and symmetric dimethylarginine (SDMA) concentrations with other variables</th>
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</thead>
<tbody>
<tr>
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<tr>
<td></td>
</tr>
<tr>
<td>BW — bodyweight; BUN — blood urea nitrogen; CRE — creatinine; R-correlation coefficient.</td>
</tr>
<tr>
<td>BW</td>
</tr>
<tr>
<td>Clinical severity</td>
</tr>
<tr>
<td>BUN</td>
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<tr>
<td>CRE</td>
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<td>SDMA</td>
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obtained SDMA test results over time for only a few affected dogs (2/6 dogs in group B) after melarsomine therapy and found that the SDMA levels returned to the reference range 2 mo after treatment (unpublished data). Since melarsomine is eliminated by the kidney to an extent, further studies should be aimed at investigating the influence of melarsomine on renal function and SDMA levels in a larger study population. In addition, further study should also be aimed at identifying which therapeutic protocol (e.g., slow kill or melarsomine-based protocol) is more suitable for HWD infected dogs with prolonged kidney damage. Nevertheless, our study demonstrated that the GFR was significantly decreased in dogs with lower worm burden and milder clinical signs; therefore, earlier detection of renal impairment is required for successful management of dogs with HWD. Our study also implied that earlier recognition and stabilization of renal impairment prior to and during adulticidal therapy might be more important for clinicians to achieve therapeutic goals by slow kill or melarsomine.

**References**


Maternal and fetal arterial blood gas data in normotensive, singleton, isoflurane anesthetized sheep at 124–126 days of gestation

Claire M. Loughran, Matthew W. Kemp, Gabrielle C. Musk

Abstract

The aim of this case series was to describe the differences between maternal and fetal blood-gas results during anesthesia. Sixteen singleton adult merino ewes weighing 60.1 ± 5.1 kg at 125.7 d (124 to 126 d) gestation were anesthetized. Maternal (radial) and fetal (umbilical) arterial blood gas samples were collected 79 ± 6 min after the start of anesthesia if maternal mean arterial pressure (MAP) was stable and > 65 mmHg. Fetal pH, partial arterial pressure of oxygen (PaO₂), glucose, arterial hemoglobin oxygen saturation (SaO₂), sodium, and chloride were significantly lower and fetal partial arterial pressure of carbon dioxide (PaCO₂), lactate, hematocrit, total hemoglobin, potassium, and calcium were significantly higher than maternal blood-gas values. Fetal pH, PaO₂, and BE were lower and fetal lactate was higher than fetal umbilical arterial samples previously reported, which may indicate a non-reassuring fetal status. Further refinement of the ovine experimental model is warranted with fetal monitoring during maternal anesthesia.

Résumé

L’objectif de l’étude était de décrire les différences dans les résultats d’analyse des gaz sanguins maternel et fœtal durant l’anesthésie. Seize brebis mérinos primipares pesant 60.1 ± 5.1 kg à 125,7 j (124 à 126 j) de gestation ont été anesthésiées. Des échantillons de sang artériel maternel (radiale) et fœtal (ombilicale) ont été prélevés 79 ± 6 min après le début de l’anesthésie si la pression artérielle moyenne (PAM) maternelle était stable et > 65 mmHg. Pour le sang fœtal, les valeurs de pH, de la pression artérielle partielle en oxygène (PaO₂), du glucose, de la saturation en oxygène de l’hémoglobine fœtale (SaO₂), du sodium, et du chlorure étaient significativement inférieures, et les valeurs de la pression artérielle partielle en dioxyde de carbone (PaCO₂), du lactate, de l’hémocrit, de l’hémoglobine totale, du potassium, et du calcium étaient significativement supérieures que celles du sang maternel. Les valeurs fetales du pH, de la PaO₂, et de BE étaient plus basses et le lactate fœtal étaient plus élevées que les valeurs d’échantillons provenant du sang artériel ombilical fetal rapportées précédemment, ce qui pourrait indiquer un statut fœtal non-rassurant. Des améliorations de ce modèle expérimental ovine sont souhaitées avec un suivi fœtal durant une anesthésie maternelle.

(Traduit par Docteur Serge Messier)
Table I. Mean ± SD of the measured indices from ewes (N = 16) over the duration of anesthesia prior to collection of arterial blood

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart rate (beats/min)</td>
<td>86.7 ± 17.9</td>
</tr>
<tr>
<td>Respiratory rate (breaths/min)</td>
<td>10.1 ± 1.9</td>
</tr>
<tr>
<td>MAP (mmHg)</td>
<td>70.8 ± 5.5</td>
</tr>
<tr>
<td>ETCO₂ (mmHg)</td>
<td>37.6 ± 3.4</td>
</tr>
<tr>
<td>Temperature (°C)</td>
<td>38.2 ± 0.9</td>
</tr>
<tr>
<td>Isoflurane vaporizer setting (%)</td>
<td>1.5 ± 0.5</td>
</tr>
<tr>
<td>Time of blood gas collection after onset of anesthesia (min)</td>
<td>78.8 ± 5.9</td>
</tr>
</tbody>
</table>

MAP — mean arterial pressure; ETCO₂ — end tidal carbon dioxide.

(Compound Sodium Lactate solution; Baxter Healthcare, Toongabbie, New South Wales, Australia) at 10 to 20 mL/kg per hour and succinylated gelatin solution (Gelofusine 4%; Braun Australia, Bella Vista, New South Wales, Australia) at 2 to 10 mL/kg per hour as required to maintain normotension (MAP > 65 mmHg). The physiological changes associated with anesthesia were monitored continuously and displayed on a multivariable monitor (Advisor Vital Signs Surgivet Monitor V9212AR; Smiths Medical, Lincoln Park, New Jersey, USA). These parameters included heart rate, respiratory rate, electrocardiography, pulse oximetry, esophageal temperature, invasive blood pressure, and inspiratory and ETCO₂. The experimental procedure involved laparotomy and hysterotomy to access the fetus. While remaining in utero the fetal trachea was intubated and the fetal lung was ventilated. At the end of the procedure the ewe and fetus were euthanized with intravenous pentobarbitone 160 mg/kg (Valabarb, Pentobarbitone 320 mg/mL; Jurox, Rutherford, New South Wales, Australia).

The paired maternal and fetal arterial blood gas samples were collected 70 to 90 min after the onset of anesthesia and analyzed provided maternal MAP had been stable for 15 min and was greater than 65 mmHg at the time of blood sample collection. Maternal arterial blood samples were collected from the radial arterial catheter and fetal arterial samples were collected from the umbilical artery. The samples were collected into heparinized syringes, any air bubbles were expelled and a cap was placed on the syringe immediately. Samples were analyzed within 15 min of collection using a benchtop arterial blood gas analyzer (RapidLab 1265; Siemens Healthcare Diagnostics). The data collected from these analyses included pH, PaO₂, PaCO₂, BE, lactate, glucose, hematocrit (Hct), tHb, SaO₂, sodium (Na⁺), potassium (K⁺), calcium (Ca²⁺), and chloride (Cl⁻).

Maternal and fetal arterial oxygen content (CaO₂; mL/L) was calculated as follows: CaO₂ (mL/L) = [(Hb] (g/dL⁻¹) × 1.36 × SaO₂ (%) + (0.0031 × PaO₂ (mmHg))] × 10. Normalized blood data were compared with a Student’s t-test: Otherwise a Mann–Whitney Rank Sum test was used. All tests were performed with Prism 6, 2015 (Graphpad Software; La Jolla, California, USA); P < 0.05 was considered to be statistically significant. Normalized blood data are presented as mean ± SD, otherwise data are expressed as median (interquartile range).

Nineteen pairs of maternal and fetal blood gas results were obtained, but 3 pairs of results were excluded from analysis due to maternal hypotension (MAP < 65 mmHg) at the time of arterial blood gas collection. Sixteen sets of arterial blood gas results were subsequently analyzed. Maternal physiologic parameters, ETCO₂, peripheral capillary oxygen saturation measured by the pulse oximeter (SpO₂), and MAP at the time of blood gas collection were within normal limits (Table I). Ten of the sheep had a mean arterial blood pressure between 55 and 65 mmHg for 5 to 15 min at the beginning of anesthesia. This hypotension was managed with fluid therapy (temporary increase in the rate of infusion up to the high end of the aforementioned range for both the crystalloid and the gelofusin) and alteration of the vaporizer setting. Fetal pH, PaO₂, glucose, SaO₂, Na⁺, and Cl⁻ were significantly lower than maternal values while fetal PaCO₂, lactate, Hct, tHb, K⁺, and Ca²⁺ were significantly higher than maternal values (Table II). Maternal CaO₂ was significantly higher than fetal CaO₂.

This study compared paired maternal and fetal arterial blood gas data at a single time point from dorsally recumbent, normotensive isoflurane-anesthetized singleton ewes and their fetuses at a gestational age of 125 d (124 to 126 d). Comparison of these data demonstrated a significantly higher fetal PaCO₂, lactate, Hct, tHb, K⁺, and Ca²⁺ relative to maternal results and a significantly lower fetal umbilical PaO₂, glucose, SaO₂, Na⁺, Cl⁻, and CaO₂ relative to maternal arterial values.

Simultaneous fetal and maternal arterial blood gas samples in this study revealed maternal PaO₂ within the expected range, but fetal PaO₂ lower than values recorded in previous studies (8). Anesthetized pregnant sheep breathing up to 98% O₂ almost 5 times the oxygen concentration of room air, are expected to have a PaO₂ of approximately 484 mmHg ≈ 21 mmHg according to the alveolar gas equation (9). This value is similar to the fetal PaO₂ reported in this study. Maternal circulating oxygen is transported across the placenta and into the fetal circulation by passive diffusion in response to a pressure gradient. Consequently, fetal PaO₂ should be significantly lower than maternal values, as reported in this study. The fetal PaO₂ from the singleton animals in this study was unexpectedly lower than those reported for twin fetuses from anesthetized ewes (8). This discrepancy may indicate a greater degree of maternal-fetal placental perfusion mismatch in the current study and a reduction in the oxygen transfer between the maternal and fetal blood. Furthermore, and perhaps more importantly, the fetal PaO₂ values in this study were taken at 79 min ± 6 min, whereas the fetal twin arterial samples were collected at 28 min ± 6 min and 56 ± 15 min after the onset of anesthesia for the first and second twin, respectively, indicating that the duration of anesthesia may negatively impact fetal PaO₂.

Despite the low PaO₂ of fetal blood, fetal tissue oxygen requirements may exceed those of the mother. Consequently, the fetus has adapted to lower PaO₂ levels by having a higher Hct and hemoglobin concentration with a greater affinity for oxygen than maternal hemoglobin. Maternal ovine Hct decreases during anesthesia (10). The maternal Hct reported in the current study, however, was lower than values reported previously in anesthetized non-pregnant sheep. This finding is consistent with a previous report that pregnant sheep are anemic during anesthesia (8). The fetal Hct in this study was significantly higher than maternal values and when the CaO₂ in fetal and in maternal blood is compared, it is apparent that although
maternal CaO₂ is significantly higher, the difference is smaller than might be expected. It is unknown whether anemia during anesthesia of pregnant sheep is acute or chronic and this finding warrants further investigation. Pre-operative blood samples were not collected in this study.

Simultaneous fetal and maternal arterial blood gas samples in this study revealed that maternal PaCO₂ was within the target range, but fetal PaCO₂ was higher than values recorded in the fetus of conscious pregnant sheep (73 mmHg versus 52 mmHg) (11). Ventilation in this study was controlled with the aim of maintaining an ETCO₂ of 35 to 45 mmHg. Consequently the maternal PaCO₂ was similar to values reported in conscious pregnant ewes breathing room air at 103 to 104 d of gestation and confirms findings of previous studies which failed to demonstrate a significant difference between the PaCO₂ of conscious pregnant and non-pregnant sheep (12). Fetal PaCO₂ in this study was significantly higher than maternal PaCO₂. These figures are consistent with those in other studies; however, the gradient for the exchange of CO₂ between maternal and fetal blood was higher than the gradient reported in twin pregnant anesthetized ewes (8). The increased CO₂ gradient could indicate a greater fetal-maternal perfusion mismatch in this study. The fetal PaCO₂ in this study was 40% higher than fetal values recorded in singleton conscious ewes, indicating fetal hypercapnia (11). Based on the fetal-maternal PaCO₂ gradient observed in this and other studies it may be warranted to target mild maternal hypocapnia (PaCO₂: 30 to 35 mmHg) during anesthesia to reduce fetal PaCO₂ to a level comparable to fetal measurements in conscious ewes (11). Maternal hypocapnia is normally tolerated for short periods, with few apparent effects (13). Extreme hypocapnia can reduce placental blood flow in sheep, but this physiological state occurs when maternal PaCO₂ values are considerably lower than those being recommended here (14).

Maternal blood glucose concentrations in this study were within the expected range, but fetal glucose concentrations were lower than values recorded in previous studies (8). This discrepancy may relate to the timepoint at which the sample was collected, which was 79 min ± 6 min after anesthesia. The fetus is dependent on maternal circulation to supply glucose so low fetal blood glucose could indicate a failure of placental transfer due to the development of maternal-fetal placental perfusion mismatch over time. Maternal lactate values in this study were within the expected range, but fetal lactate was higher than values recorded in previous studies (8). Lactate production by the fetus is a direct end product of anaerobic metabolism.

Consistent with previous reports, the maternal pH in this study was similar to values recorded in conscious sheep and fetal pH was significantly lower than maternal levels (8,10). However, the fetal pH values in this study were lower than those recorded in fetal samples taken from singleton conscious ewes (11). A specific definition of acidemia in the fetus is difficult to describe as the fetus has adapted to withstand short periods of acidosis. Acute fetal acidosis is almost always respiratory in origin, but quickly progresses to a mixed respiratory metabolic acidosis as seen in this study by the increase in fetal BE compared to fetal samples from anesthetized and conscious sheep (8,10).

The electrolyte concentrations in this study varied between maternal and fetal circulations. Ewes were slightly hypokalemic, hypomagnesemic, normonatremic, and normochloremic (15). Fetal electrolyte levels were significantly lower (Cl⁻ and Na⁺) and higher

| Table II. Mean ± SD or median (interquartile range) of measured and calculated indices of arterial blood gas data from the ewe and fetus during isoflurane anesthesia in singleton sheep at 124 to 126 d of gestation |
|-----------------|-----------------|---------------------------|---------------------|
|                | Maternal | Fetal | Difference between maternal and fetal samples | P-value |
| pH             | 7.4 ± 0.05  | 7.2 ± 0.07  | 0.2 ± 0.09 | < 0.0001 |
| BE (mmol/L)    | 1.4 ± 2.4   | −0.9 ± 3.0  | 2.3 ± 4.3   | < 0.0001 |
| PaCO₂ (mmHg)   | 42.1 ± 5.1  | 73.0 ± 9.1  | −30.9 ± 8.5 | < 0.0001 |
| PaO₂ (mmHg)    | 486.3 ± 51.0| 20.1 ± 4.2  | 461.2 ± 51.0| < 0.0001 |
| Hct (%)        | 19.6 ± 2.6  | 35.6 ± 2.7  | −15.9 ± 3.5 | < 0.0001 |
| tHb (g/dL)     | 6.6 (6.1–7.2)| 12.1 (11.3–12.5)| −5.4 [-6.5~(-4.2)]| < 0.0001 |
| SaO₂ (%)       | 99.9 (99.8–99.9)| 54.7 (44.7–60.7)| 45.2 (39.2–55.2)| < 0.0001 |
| Glucose        | 3.0 ± 0.5   | 1.0 ± 0.2   | 2.2 ± 0.4   | < 0.0001 |
| Lactate (mmol/L)| 1.4 ± 0.5  | 3.63 ± 0.9  | −2.3 ± 1.0  | < 0.0001 |
| Ca²⁺ (mmol/L)  | 1.1 (1.0–1.1)| 1.7 (1.6–1.8)| −0.6 [-0.7~(-0.5)]| < 0.0001 |
| Na⁺ (mmol/L)   | 144.1 ± 1.5 | 137.6 ± 1.7 | 6.4 ± 1.5   | < 0.0001 |
| K⁺ (mmol/L)    | 3.0 (2.8–3.2)| 4.0 (3.7–4.3)| −1.0 [-1.1~(-0.7)]| < 0.0001 |
| Cl⁻ (mmol/L)   | 111.2 ± 3.7 | 100.1 ± 2.0 | 11.06 ± 3.9 | < 0.0001 |
| CaO₂ (mU/L)    | 104.9 ± 12.2| 83.2 ± 18.8 | 20.6 ± 19.6 | 0.0006  |

BE — base excess; PaCO₂ — partial arterial pressure of carbon dioxide; PaO₂ — partial arterial pressure of oxygen; Hct — hematocrit; tHb — total hemoglobin; SaO₂ — percentage hemoglobin saturation with oxygen; Ca²⁺ — calcium; Na⁺ — sodium; K⁺ — potassium; Cl⁻ — chloride; CaO₂ — arterial oxygen content of the blood.
(Ca\(^{2+}\) and K\(^{+}\)) than maternal levels and lower than ranges found in 1- to 3-month-old sheep (16). Reference ranges are not available for fetal lambs and there is no information in the literature regarding electrolyte levels during gestation. The fetal electrolyte levels measured in this study were similar to values in twin fetuses, with the exception of K\(^{+}\), which was lower in this study (8).

Although the fetuses in this study underwent ventilation, the placental circulation was intact. This intervention does not alter fetal blood gas results (17) so based on the results of this study, ovine maternal health as assessed by arterial blood gas analysis and routine physiological monitoring during anesthesia, does not necessarily constitute fetal health. While this study highlights poorer fetal blood gas parameters in anesthetized sheep compared to fetal blood gas parameters from conscious sheep, this finding is not unexpected given the negative impact of anesthesia on the fetal and maternal cardiovascular system, and by extension, placental blood flow, and nutrient and waste product transfer. Improvements in fetal blood gas results (lower PaCO\(_2\), and lactate; higher pH, PaO\(_2\), and BE) in future studies may be possible with refinements of the anesthetic protocol: determination of the optimal approach to the management of hypotension (fluid therapy versus vasopressors or inotropes); sophisticated regional anesthesia; neuromuscular blockade; intraoperative analgesic drug infusion (e.g., fentanyl), and propofol based total intravenous anesthesia (18).

This case series presents the differences between maternal and fetal arterial blood gas samples in dorsally recumbent isoflurane anesthetized singleton pregnant ewes. Despite the ewes being normotensive and normocapnic, the fetus showed signs of acidosis signaling non-reassuring fetal status. Further work is required to determine both the significance of these findings on fetal health beyond delivery, and to optimize the anesthetic regime for pregnant ewes at different gestational ages in a biomedical research setting.

### Acknowledgments

Thanks is also extended to Astrid Armitage, Andrew Wilson, and the Animal Care Services staff at the Large Animal Facility, for their expert care and husbandry of the pregnant ewes.

### References

Towards an improved estimate of antimicrobial use in animals: Adjusting the “population correction unit” calculation

Brian R. Radke

Abstract

International comparisons of animal antimicrobial use (AMU) have typically been based on total national estimates of antimicrobials sales standardized by the national animal biomass calculated as the population correction unit (PCU). The objective of this paper was to compare the currently accepted PCU calculation with that of the adjusted population correction unit (APCU), which re-evaluates the standard animal weights used in the calculation and accounts for animal lifespan. The APCU calculation resulted in substantial changes to the 2009 national biomass estimates for cattle, pigs, and poultry in 8 European countries and Canada. The estimated national biomass for cattle increased 35% to 43%, while the estimated national biomass of pigs and poultry typically decreased by approximately 51% and 87%, respectively. Among the 9 countries, the total national APCU ranged from an increase of 1% to a decrease of 40% relative to PCU, and these differences were statistically significant. Adjusted population correction unit is preferred over PCU in comparing and contrasting AMU among animals with different lifespans because it is more transparently derived and is a reasonable approximation of the animal biomass at risk of antimicrobial treatment.

Résumé

Les comparaisons internationales de l’utilisation d’antimicrobiens chez les animaux (UMA) ont typiquement été basées sur les totaux nationaux estimés de ventes d’antimicrobiens standardisés pour la biomasse animale nationale calculée comme l’unité de correction pour la population (UCP). Les objectifs de cet article étaient de comparer les calculs d’UCP présentement acceptés à ceux de l’unité de correction pour la population ajustée (UCPA), qui réévalue les poids animaux standards utilisés dans les calculs et tient compte de la durée de vie des animaux. Les calculs de l’UCPA ont entraîné des changements substantiels aux estimés nationaux de 2009 de la biomasse pour les bovins, porcs et volailles dans 8 pays européens et le Canada. La biomasse nationale estimée pour les bovins a diminué de 35 % à 43 %, alors que les biomasses nationales estimées pour les porcs et les volailles ont typiquement diminué d’environ 51 % et 87 %, respectivement. Parmi les neuf pays, l’UCPA nationale totale variait d’une augmentation de 1 % à une diminution de 40 % relativement à l’UCP, et ces différences étaient statistiquement significatives. L’UCPA est préférée par rapport à l’UCP pour la comparaison et la mise en contraste de l’UMA chez les animaux avec différentes durées de vie étant donné qu’elle est dérivée de manière plus transparente et qu’elle est une approximation raisonnable de la biomasse animale à risque d’un traitement antimicrobien.

(Traduit par Docteur Serge Messier)
or how it is related to antimicrobial use. Instead, PCU calculations typically reference Monforts (7) and the European Medicines Agency (8), that simply define AWT as the mean body weight for animals raised for slaughter, and the maximum body weight for other animal groups (e.g., breeding animals). Even based on these definitions, some of the currently used AWT values do not appear to accurately reflect animal weights. For example, the average mature cow weights approximately 600 kg (9–12), while a weight of 425 kg is currently used for PCU calculations.

Antimicrobial use in an animal population is affected by the weight of the animals, and their length of life. The opportunity for antimicrobial use increases with increased length of life, and length of life of PCU’s livestock and poultry categories vary considerably. The PCU doesn’t take into account length of life (4) and this is the second concern with the PCU method of estimating animal biomass. The PCU’s failure to incorporate the variable lifespans of the animal categories has potential implications not only for AMU comparisons between species, but also for comparisons of total national usage. This failure potentially results in underestimation of AMU in countries with a preponderance of short-lived animal categories, such as poultry, and overestimation in countries with disproportionately more longer-lived categories such as cattle.

It is important that PCU calculations accurately reflect animal biomass for the animal categories of interest because inaccurate PCU values may lead to erroneous conclusions when comparing and contrasting AMU data. The objective of this paper is to compare the currently accepted PCU biomass calculation with one that re-evaluates AWT (based on current data regarding production animal weights) and accounts for the lifespan of the animal categories in question, using data from 8 European countries and Canada.

Currently, a country’s PCU is calculated as follows:

\[
PCU = \sum c n_c AWT_c - \sum c n_c AWT + \sum c n_c AWT_c
\]  

(Equation 1)

where \( n_c \) is the total number of animals in category \( j \) (i.e., for breeding animals, \( n_j \) is the number of animals present in a year; if \( j \) are slaughter animals, \( n_j \) is the total number of animals slaughtered annually); \( AWT_j \) is the average weight at treatment of an animal in \( j \) (kg); \( c \) is the animal categories raised and slaughtered within the country in question; \( i \) is the animal categories exported to the country; and \( e \) is the animal categories exported from the country.

The proposed equation, adjusted PCU (APCU), is as follows:

\[
APCU = \sum c n_c LAW_c - \sum c n_j LAW + \sum c n_c LAW_c
\]  

(Equation 2)

where \( LAW_j \) is the life adjusted weight of an animal in category \( j \). Life adjusted weight (LAW) is calculated as:

\[
LAW_j = AWT_j \times LL_j
\]  

(Equation 3)

The \( AW_j \) is the adjusted weight of an animal in \( j \) (kg) calculated using Monforts’ (7) and the European Medicines Agency’s (8) definitions of the animal weights (i.e., the mean weight for slaughter animal categories and the maximum weight for all other animal categories). The \( LL_j \) variable is the length of life for category \( j \) animals as measured in years.

For the animal categories most commonly included in PCU calculations, established AWT values were obtained from the European Medicines Agency (6) (Table I).

Adjusted weights (AW) were arrived at in several different ways depending on the data available. For the cattle categories, the slaughter (ending) weights were calculated by dividing the average carcass weight by a live-to-carcass weight conversion factor (13) to determine the average weight of live animals. Average carcass weights for 28 EU countries were calculated by dividing the total animal weight at slaughter for a given animal category, by the number of animals slaughtered for that category (14). Subsequently, using Monforts’ definition, the mean body weight of the cattle slaughter categories was calculated by averaging a birth weight of 45 kg and the final weight at slaughter. The AW of imported and exported cattle for slaughter is the mean weight of slaughter heifers, bullocks, and bulls.

For the remaining animal categories, Eurostat data regarding carcass weights and number of animals slaughtered were not available. For this reason, the international literature was reviewed to provide a contemporary estimate of animal weights in each category. It was determined that these AWT are generally consistent with the (average) body weights defined elsewhere (9–12), including Canadian PCU calculations (Canadian Integrated Program for Antimicrobial Resistance personal communication, 2016). The AWT was therefore used as AW for the non-cattle categories with the exception of pigs imported or exported for fattening. The AW of these pigs is 25 kg. However, Monforts (7) and the European Medicines Agency (8) include weight of piglets up to 25 kg in the sow weight so the AW for exported fattening pigs was set to zero (Table I). Similarly, the 25 kg AW for imported fattening pigs was set to zero as this weight is already recognized as the beginning weight in the slaughter pig’s category (Table I).

An animal category’s length of life (LL) was calculated using the inverse of its number of cycles per year on an average farm. For example, if the typical broiler farm has 9 cycles per year, then the average LL for broiler chickens is 0.11 y (1/9). Data regarding the number of cycles per year for each animal category were obtained from Monforts (7) and the European Medicines Agency (8). Neither reference included the number of cycles for slaughter heifers, bullocks or bulls. A LL of 1.5 y is assigned to slaughter heifers, bullocks, and bulls based on knowledge of these industries.

Using the most recently published values of \( n \) for the 8 European countries (6) and Canadian data (Canadian Integrated Program for Antimicrobial Resistance personal communication, 2016), and AWT (Table I), the 2009 PCU for the 9 countries was reproduced using Equation 1. The APCU for each country was calculated using Equation 2, the same values of \( n \), and LAW from Table I. A two-tailed paired t-test was used to determine whether total PCU and total APCU were significantly different among countries using Stata v.13.1 (StatCorp, College Station, Texas, USA) and APCU as a percentage change from PCU [i.e., (APCU-PCU)/PCU × 100%] was calculated.

The 2009 PCU for the 9 countries is reported in Table II, as is the APCU and the percentage change. For cattle, APCU was 35% to 43% greater than PCU for each of the 9 countries, while for pigs, poultry, sheep, and goats, the APCU was consistently less than the PCU. For example, using APCU, the national poultry biomass decreased by 81% to 89%. The estimated national biomass of horses and fish were
the same for APCU and PCU. The estimated national biomass for slaughtered rabbits decreased for France and Canada.

For each country as a whole, the difference between APCU and PCU was variable. For Finland and Norway, APCU was respectively 11% and 1% greater than PCU. For the remaining 7 countries, APCU was between 2% and 40% less than PCU. The APCU and PCU were statistically significantly different ($P = 0.02$, $t = 3.01$, d.f. = 8) for the 9 countries.

Use of the 2 different animal biomass calculations (APCU versus PCU) resulted in substantially different national values for most animal categories included in this analysis, as well as for most of the countries as a whole. These differences could have substantial

<table>
<thead>
<tr>
<th>Animal category</th>
<th>PCU average weight at treatment (AWT) (kg)</th>
<th>Adjusted weight (AW) (kg)</th>
<th>Length of life (LL) (year)</th>
<th>Life adjusted weight (LAW) (kg year)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cattle</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Slaughter cows</td>
<td>425&lt;sup&gt;a&lt;/sup&gt;</td>
<td>627</td>
<td>1</td>
<td>627</td>
</tr>
<tr>
<td>Slaughter heifers</td>
<td>200&lt;sup&gt;b&lt;/sup&gt;</td>
<td>269 (45, 493)&lt;sup&gt;e&lt;/sup&gt;</td>
<td>1.5</td>
<td>404</td>
</tr>
<tr>
<td>Slaughter bullocks and bulls</td>
<td>425&lt;sup&gt;a&lt;/sup&gt;</td>
<td>329 (45, 612)&lt;sup&gt;e&lt;/sup&gt;</td>
<td>1.5</td>
<td>494</td>
</tr>
<tr>
<td>Slaughter calves and young cattle</td>
<td>140&lt;sup&gt;c&lt;/sup&gt;</td>
<td>169 (45, 293)&lt;sup&gt;e&lt;/sup&gt;</td>
<td>0.56</td>
<td>94</td>
</tr>
<tr>
<td>Imported/exported cattle for slaughter</td>
<td>425&lt;sup&gt;a&lt;/sup&gt;</td>
<td>299</td>
<td>1.5</td>
<td>449</td>
</tr>
<tr>
<td>Imported/exported cattle for fattening</td>
<td>140&lt;sup&gt;c&lt;/sup&gt;</td>
<td>169 (45, 293)&lt;sup&gt;e&lt;/sup&gt;</td>
<td>0.56</td>
<td>94</td>
</tr>
<tr>
<td>Livestock dairy cows</td>
<td>425&lt;sup&gt;a&lt;/sup&gt;</td>
<td>627</td>
<td>1</td>
<td>627</td>
</tr>
<tr>
<td><strong>Pigs</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Slaughter pigs</td>
<td>65&lt;sup&gt;d&lt;/sup&gt; (25, 105)&lt;sup&gt;e&lt;/sup&gt;</td>
<td>65</td>
<td>0.33</td>
<td>22</td>
</tr>
<tr>
<td>Imported/exported pigs for slaughter</td>
<td>65</td>
<td>65</td>
<td>0.33</td>
<td>22</td>
</tr>
<tr>
<td>Imported/exported pigs for fattening</td>
<td>25&lt;sup&gt;f&lt;/sup&gt;</td>
<td>0</td>
<td></td>
<td></td>
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<td>Livestock sows</td>
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<td>1</td>
<td>240</td>
</tr>
<tr>
<td><strong>Poultry</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Slaughter broilers</td>
<td>1</td>
<td>1</td>
<td>0.11</td>
<td>0.11</td>
</tr>
<tr>
<td>Slaughter turkeys</td>
<td>6.5</td>
<td>6.5</td>
<td>0.37</td>
<td>2.4</td>
</tr>
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<td>Imported/exported broilers for slaughter</td>
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<td>1</td>
<td>0.11</td>
<td>0.11</td>
</tr>
<tr>
<td><strong>Sheep and goats</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Slaughter sheep and goats</td>
<td>20 (NA&lt;sup&gt;a&lt;/sup&gt;, 40–45)&lt;sup&gt;e&lt;/sup&gt;</td>
<td>20</td>
<td>0.5</td>
<td>10</td>
</tr>
<tr>
<td>Imported/exported sheep and goats for slaughter</td>
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<td>20</td>
<td>0.5</td>
<td>10</td>
</tr>
<tr>
<td>Livestock sheep</td>
<td>75</td>
<td>75</td>
<td>1</td>
<td>75</td>
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<tr>
<td><strong>Horses</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Living horses</td>
<td>400&lt;sup&gt;i&lt;/sup&gt;</td>
<td>400</td>
<td>1</td>
<td>400</td>
</tr>
<tr>
<td><strong>Fish</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Slaughter fish&lt;sup&gt;h&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Rabbits</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Slaughter rabbits</td>
<td>1.4</td>
<td>1.4</td>
<td>0.15</td>
<td>0.21</td>
</tr>
</tbody>
</table>

<sup>a</sup> Adult cow weight.

<sup>b</sup> 0-1-year-old bovine weight.

<sup>c</sup> Veal calf weight.

<sup>d</sup> Fattening pig (25 to 105 kg).

<sup>e</sup> Beginning weight for animal category, ending weight for animal category.

<sup>f</sup> Weaner pig (to 25 kg).

<sup>g</sup> Weight for sow and piglets until 25 kg.

<sup>h</sup> Not available.

<sup>i</sup> Horses 600 kg, ponies 250 kg.

<sup>j</sup> The mean of a slaughter heifer, bullock, and bull weight (i.e., $269 \div 329/2$).

<sup>k</sup> Eurostat data available only as live-weight at slaughter; information on AWT is unavailable.
effects on international comparisons of AMU as well as national comparisons among animal categories. For example, using PCU as the denominator, 2009 AMU in UK cattle is over 3-fold greater than AMU in pigs and poultry (5). In contrast, 2009 AMU in UK cattle is less than the AMU in pigs and poultry when APCU is used as the denominator (calculations not shown). These differences are primarily attributable to including length of life in the calculation, although for cattle categories and traded fattening pigs, adjusting the weights used in the calculation also had an impact. Given that analyses of AMU rely on accurate estimation of animal biomass to enable comparisons among animal categories and between countries, these results have significant implications on how AMU is calculated.

Amending or replacing the conventional PCU biomass calculations with the APCU calculation presented here should be considered for 2 reasons. First, the APCU uses weight values that are clearly defined and supported by current data regarding animal weights. For example, cattle AW estimates using Canadian data yielded results similar to the Eurostat data. In the future, AW values could be further improved by collecting international contemporary weight data for all animal categories as is currently collected for cattle. The European Medicines Agency has revised the weight for beef and dairy cows upwards to 500 kg in their DDDA calculations (2), but these weights remain less than those suggested by the Eurostat data (Table I).

Second, although PCU is controlling for animal demographics which vary among countries and includes standardizing for differences in animal weights, it does not include controlling for differences in animals’ lifespans (4). Bondt et al (1) objected, in principal, to the PCU approach of adding weights of breeding stock to those of animals slaughtered during the year without accounting for length of life because this approach does not accurately reflect the population at risk for antimicrobial treatment. The DDDAs used in

<table>
<thead>
<tr>
<th>Animal category</th>
<th>Czech Republic</th>
<th>Denmark</th>
<th>Finland</th>
<th>France</th>
<th>Netherlands</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PCU</td>
<td>APCU</td>
<td>% Δ</td>
<td>PCU</td>
<td>APCU</td>
</tr>
<tr>
<td><strong>Cattle</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Slaughtered cows</td>
<td>308</td>
<td>421</td>
<td>37%</td>
<td>403</td>
<td>566</td>
</tr>
<tr>
<td>Slaughtered heifers</td>
<td>52</td>
<td>76</td>
<td>41%</td>
<td>80</td>
<td>119</td>
</tr>
<tr>
<td>Slaughtered bullocks and bulls</td>
<td>5</td>
<td>10</td>
<td>41%</td>
<td>9</td>
<td>18</td>
</tr>
<tr>
<td>Slaughtered calves and young cattle</td>
<td>47</td>
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<td>50%</td>
<td>47</td>
<td>55</td>
</tr>
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<td>1</td>
<td>41%</td>
<td>19</td>
<td>13</td>
</tr>
<tr>
<td>Net exported cattle for fattening</td>
<td>0</td>
<td>0</td>
<td>0%</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><strong>Livestock dairy cows</strong></td>
<td>163</td>
<td>241</td>
<td>22%</td>
<td>244</td>
<td>360</td>
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<td><strong>Pigs</strong></td>
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<td></td>
</tr>
<tr>
<td>Slaughtered pigs</td>
<td>245</td>
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<td>53%</td>
<td>1820</td>
<td>768</td>
</tr>
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<td>Net exported pigs for slaughter</td>
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<td>41%</td>
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</tr>
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<td>0</td>
<td>0%</td>
<td>162</td>
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<td>47</td>
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<td>100%</td>
<td>323</td>
<td>323</td>
</tr>
<tr>
<td><strong>Poultry</strong></td>
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<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Slaughtered broilers</td>
<td>154</td>
<td>17</td>
<td>89%</td>
<td>112</td>
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</tr>
<tr>
<td>Slaughtered turkeys</td>
<td>136</td>
<td>15</td>
<td>10%</td>
<td>100</td>
<td>11</td>
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<tr>
<td>Net exported broilers for slaughter</td>
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<td>7%</td>
<td>12</td>
<td>1</td>
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<tr>
<td><strong>Sheep and goats</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Slaughtered sheep and goats</td>
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<td>15</td>
<td>10%</td>
<td>9</td>
<td>8</td>
</tr>
<tr>
<td>Net exported sheep for slaughter</td>
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<td>0%</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><strong>Livestock sheep</strong></td>
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<td>28</td>
<td>100%</td>
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<td>70</td>
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<td>Live weight fish slaughtered</td>
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<td>20</td>
<td>100%</td>
<td>34</td>
<td>34</td>
</tr>
<tr>
<td><strong>Slaughtered rabbits</strong></td>
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<td>0</td>
<td>0%</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>771</td>
<td>617</td>
<td>20%</td>
<td>1458</td>
<td>40%</td>
</tr>
</tbody>
</table>

Table II. Calculation of population correction unit (PCU), adjusted population correction unit (APCU), and APCU as a percentage change (% Δ) from PCU for 8 European countries and Canada, 2009 (1000 tonnes)
the Netherlands (15) and Denmark (16) account for animal lifespan. The LL used in the APCU calculations are representative of Canadian production practices.

Population correction unit is a purely technical unit of measure and not a real value for the animal population biomass that could potentially be treated with antimicrobial agents (6). By adjusting the animal weights and incorporating length of life, the APCU approach is an improved approximation of the actual animal biomass at risk of antimicrobial treatment.

Using a calculation that better reflects average weights and includes length of life for each animal category resulted in values for total annual animal biomass that were significantly different than those obtained using a traditional PCU calculation. As a result, APCU provides a reasonable approximation of the actual animal biomass at risk of antimicrobial treatment. Consideration should be given to replacing PCU with APCU in AMU calculations comparing and contrasting AMU among animals with different lifespans. The methodology used to transparently derive APCU will increase the credibility of this measure of animal biomass, improve comparisons of AMU data among animal categories and countries, and foster increased acceptance and harmonization of AMU calculations.

### Table II. (continued)

<table>
<thead>
<tr>
<th>Animal category</th>
<th>PCU</th>
<th>APCU</th>
<th>% Δ</th>
<th>PCU</th>
<th>APCU</th>
<th>% Δ</th>
<th>PCU</th>
<th>APCU</th>
<th>% Δ</th>
<th>PCU</th>
<th>APCU</th>
<th>% Δ</th>
</tr>
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<tbody>
<tr>
<td><strong>Cattle</strong></td>
<td></td>
<td></td>
<td></td>
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<td></td>
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</tr>
<tr>
<td>Slaughtered cows</td>
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<td>96</td>
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<td>150</td>
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<td>231</td>
<td>103</td>
<td>–56%</td>
<td>674</td>
<td>306</td>
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<td>793</td>
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</tr>
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<td></td>
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<td>308</td>
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<td>0%</td>
<td>141</td>
<td>141</td>
<td>0%</td>
<td>520</td>
<td>520</td>
<td>0%</td>
<td>417</td>
<td>417</td>
<td>0%</td>
</tr>
<tr>
<td>Live weight fish slaughtered</td>
<td>0</td>
<td>0</td>
<td></td>
<td>0</td>
<td>0</td>
<td></td>
<td>197</td>
<td>197</td>
<td></td>
<td>142</td>
<td>142</td>
<td></td>
</tr>
<tr>
<td>Slaughtered rabbits</td>
<td>0</td>
<td>0</td>
<td></td>
<td>0</td>
<td>0</td>
<td></td>
<td>0</td>
<td>0</td>
<td></td>
<td>1</td>
<td>0</td>
<td>100%</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>440</td>
<td>443</td>
<td>1%</td>
<td>825</td>
<td>752</td>
<td>–9%</td>
<td>5925</td>
<td>5307</td>
<td>–10%</td>
<td>7133</td>
<td>7007</td>
<td>–2%</td>
</tr>
</tbody>
</table>

the Netherlands (15) and Denmark (16) account for animal lifespan. The LL used in the APCU calculations are representative of Canadian production practices.

Population correction unit is a purely technical unit of measure and not a real value for the animal population biomass that could potentially be treated with antimicrobial agents (6). By adjusting the animal weights and incorporating length of life, the APCU approach is an improved approximation of the actual animal biomass at risk of antimicrobial treatment.

Using a calculation that better reflects average weights and includes length of life for each animal category resulted in values for total annual animal biomass that were significantly different than those obtained using a traditional PCU calculation. As a result, APCU provides a reasonable approximation of the actual animal biomass at risk of antimicrobial treatment. Consideration should be given to replacing PCU with APCU in AMU calculations comparing and contrasting AMU among animals with different lifespans. The methodology used to transparently derive APCU will increase the credibility of this measure of animal biomass, improve comparisons of AMU data among animal categories and countries, and foster increased acceptance and harmonization of AMU calculations.

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